Amino Acids (1993) 5: 103–216

Amino
Acids

Abstracts

3rd International Congress on Amino Acids, Peptides, and Analogues

Vienna, August 23–27, 1993

Basic Chemistry

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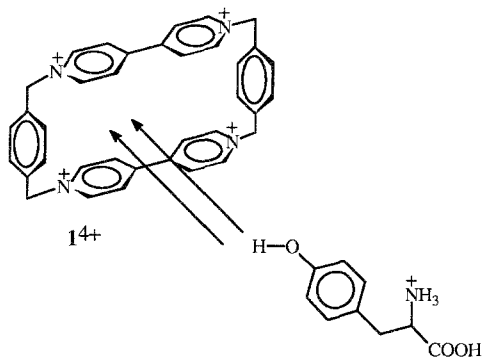
Cyclobis(paraquat-*p*-phenylene): A synthetic receptor for amino acids with electron-rich aromatic moieties

The synthetic tetracationic receptor cyclobis(paraquat-*p*-phenylene) $\mathbf{1}^{4+}$ forms very stable inclusion charge transfer complexes with amino acids possessing electron-rich aromatic subunits. The formation of charge transfer complexes between electron acceptors and amino acids with donor character, such as tryptophan, tyrosine, and phenylalanine is well known. However, these interactions are rather weak, with binding constants in the range $1-10\ M^{-1}$. In contrast, the title compound exhibits a much higher affinity for the same amino acids in aqueous media. The corresponding binding constants are approximately two orders of magnitude higher than those exhibited by simple electron acceptors. The origin of the unusual binding ability of $\mathbf{1}^{4+}$ resides in its conformational rigidity. The title receptor has a box-like structure with a very well defined cavity lined by the two paraquat acceptor subunits (see scheme below). This cavity is thus ideally suited to include donor aromatic rings.



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Lipophilicity of unusual amino acids and nonstandard peptides

Non-proteinogenic amino acids, in particular α,α-disubstituted open-chain or cyclic α-amino acids, were the subject of numerous investigations over recent years. Such uncoded amino acids have the important property to be able to induce or stabilize secondary peptide structures such as α-helices and β-turns. Recently we have synthesized a series of new amino acids with highly promising properties by an efficient and general method leading to pure enantiomers of α,α-disubstituted amino acids. Chiral resolving by chromatographic techniques is routinely done taking advantage of large differences in secondary structure as confirmed by X-ray and NMR.

In the present contribution we report on a more detailed study of the lipophilicities (hydrophobicities) of several selected new amino acids and peptides including these. Prediction and understanding of the lipophilicities of peptides is important for e.g. the separation of mixtures of peptides in biochemical research or in the design of orally active peptides in pharma research. Current fragmental methods for the estimation of lipophilicities of organic compounds do not work well for peptides, due to a number of unparameterized effects, including folding, also called hydrophobic collapse. Therefore a better understanding of these phenomena is a prerequisite for successful predictions of the physicochemical property lipophilicity.

As an extension of our previous studies on peptidomimetic renin inhibitors, we have compared for the present amino acids and peptides the experimentally obtained lipophilicity by RP-HPLC to two different theoretical approaches using addivity schemes of fragments. Furthermore a number of molecular properties such as solvent accessible surface have been calculated for 11 pairs of peptide diastereoisomers and compared to the experimental differences in lipophilicities.

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Acid base and tautomerism equilibria of glycylhistamine and sarcosylhistamine by NMR

The tautomerism of the imidazole ring in its compounds is an important factor in view of their biological action. The knowledge of the basicity of N^1 and N^3 nitrogens of imidazole ring at the molecular level may be of importance, concerning for example the action of histamine or his potential agonist especially on the H_2-receptor or the mechanism of peptide hydrolysis promoted by α-chymotrypsin. In both latter cases, the proton transfer is mediated by the imidazole ring in a concerted mechanism where the proton-acceptor and proton-donor sites are the two nitrogens.

Macroscopic deprotonation constants were determined pH-metrically: $pK_1 = 6.78$, 6.77 and $PK_2 = 8.04$, 8.31 for Glycylhistamine (Glyhist) and Sarcosylhistamine (Sarhist).

Deprotonation of fully protonated ligand LH_2 yields the protonation microspecies LH_a and LH_i, according to either the amino group (k^a) or the imidazole ring (k^i) is involved in the first step. A second deprotonation from either LHi (k^a_i) or LHa (k^i_a) results into the neutral species L. These values were determined from ^1H NMR measurements, since the pH-dependent chemical shifts of neighbouring protons reflect independently the protonation states of both concerning proton-binding sites. The concentration ratio of two tautomers LH_a/LH_i or (k_a/k_i) is 0.148 and 0.071 for Glyhist and Sarhist respectively.

The species LH_i and L are themselves a mixture of two tautomeric forms, according to whether deprotonation of the imidazolium cation occurs on nitrogens N^3 or N^1, respectively. The molar ratios of tautomers N^3–H and N^1–H were calculated on the basis of the ^{14}N titration curves of chemical shifts versus pH for Glyhist, Sarhist. The calibration were etablished with N^1- or N^3-methylhistamine and N-methylimidazole. The influence of the deprotonation of a given nitrogen in N-methyl derivatives results into a + 71.5 ppm shift at that nitrogen and a −7.2 ppm shift at the nitrogen bounded to a methyl group. The observed deprotonation shifts of imidazole nitrogens, e.g. Δ_{N^3} for N^3 nitrogen, may be expressed by:

$$\Delta_{N^3} = 71.5\ p - 7.2\ (1 - p)$$

p is the proportion of the form tautomeric $N^1 - H$.

The ^{14}N NMR-pH profile of imidazole was used to test the validity of the method, in which case equal proportions (at ±1%) of each tautomer were effectively computed. The calculated p values are 0.69, 0.70, 0.72 and 0.68 for Gly-Hist, Sar-Hist respectively.

The full set of microconstants for Gly-Hist (and Sar-Hist) are: $pk^a = 7.65$, (7.94), $pk^{N^1} = 6.98$, (6.95); $pk^{N^3} = 7.33$, (7.32); $pk_a^{N^1} = 7.31$ (7.28); $pk_a^{N^3} = 7.66$, (7.65); $pk^a_{N^1} = pk^a_{N^3} = 7.98$, (8.28).

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Preparation and study of a Cu(II) complex with a carbohydrate α-amino acid

As a part of our studies on metal-carbohydrate α-amino acid complexes, we present here the synthesis and characterization of a Cu(II) complex with the carbohydrate α-amino acid 2-amino-2-deoxy-D-glycero-L-gluco heptonic acid (HGa). Thus, the reaction between HGa, which was obtained by hydrolysis of corresponding 2-amino-2-deoxyheptononitrile, with Ni(II) in aqueous solution affords a solid of composition [$CuGa_2$], which was characterized by means of elemental analysis, diffuse reflectance, ESR, infrared spectroscopy and magnetic study. The thermal behaviour has also been studied.

The IR spectrum shows a value of 239 cm^{-1} for $\Delta(\nu_{asym} - \nu_{sym})$ corresponding to the carboxylate stretches. Besides, the $\delta(NH_3^+)$ band of ligand is not observed in the Cu(II) complex. All the aforementioned provide evidence that HGa probably acts as a bidentate ligand.

The $d - d$ reflectance spectrum shows more clearly the type of coordination. Thus, it exhibits a strong, broad and barely asymmetric band at 16,950 cm^{-1}. This value is in agreement with those given in the literature for Cu(II) compounds with a tetragonal deviation from octahedral geometry. This band has been ascribed to $d \rightarrow d$ transitions in a symmetry near to D_{4h}.

The solid state magnetic susceptibility data indicate a Curie-Weiss law behaviour in the 70–130K temperature range. The magnetic behaviour confirms the distortion of the octahedral symmetry.

The electron paramagnetic resonance spectrum showed two shoulders and a strong signal. The g values, $g_\parallel = 2.24$ and $g_\perp = 2.07$, are consistent with the presence of copper in a distorted elongated tetragonal environment.

The thelmal data of this complex reveal that it is stable up to 190°C, at this temperature starts the pyrolytic degradation of the sample in three steps ending at 450°C. The residual weight is in good agreement with the theoretical value and it was analyzed by IR spectroscopy and X-ray powder diffraction pattern.

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Reactivity of the surface-exposed cysteine residue of HIV-1 protease

The protease encoded by the human immunodeficiency virus (HIV) is essential for the processing of viral polyproteins into mature viral proteins. The 99 residue protease from HIV-1 contains 2 cysteine residues, 1 of which (Cys-67) is located on the solvent-exposed surface. It was shown previously that Cys-67 of the native enzyme reacted with Ellman's reagent (DTNB) at pH 6.2, causing a reversible inactivation of the protease. However, there was no reaction when the protein was denatured in 6M guanidine, implying that the native conformation rendered Cys-67 more reactive. To investigate the structural basis of the lowered pKa in the native protein, we synthesized a 17 reidue peptide matching the sequence of residue 59–75. The reactivity of this synthetic peptide with DTNB mimicked that of the protease, being more reactive when native than when denatured. Either His-69 and Lys-70 could facilitate ionization of the SH group of Cys-67, required for reaction with DTNB. Apparently both residues are important, because increased reactivity of the native peptide was eliminated when either His-69 or Lys-70 were changed to Ala. Replacement of His-69 by Glu reversed the reactivity, so that the native peptide was less reactive than that denatured in guanidine. Thus, the reactivity of Cys-67 is modulated by the charges on residue 69 and 70 in the protein. The

presence of His-69 and Lys-70 renders the native protease especially susceptible to oxidation.

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Membrane permeability to amino acids and modified amino acids: Mechanisms involved in translocation

The permeability of liposomes to various classes of amino acids was determined. The rates of flux of lysine, glycine, serine, phenylalanine and tryptophan were measured in large unilamellar vesicles composed of egg phosphatidylcholine. Variations in pH had only a minor effect on the permeability coefficients observed. The permeability coefficients for the polar and charged amino acids were similar to those previously measured for monovalent cations such as K^+ and Na^+ (in the range of 10^{-12} cm · s^{-1}), while the hydrophobic amino acids were observed to have permeability coefficients of approximately 10^{-10} cm · sec^{-1}. Transient defects in the bilayer barrier have been implicated as the mechanism responsible for the rates of permeation observed for cations such as K^+ and Na^+. The permeation rates for the neutral, polar and charged amino acids may therefore also be controlled by bilayer fluctuations and transient defects that permit ionic solutes to bypass the Born energy barrier. Permeation of hydrophobic amino acids may be enhanced by partitioning into the hydrocarbon region of the bilayer. Partitioning becomes dominant if charges are removed from the solute. For instance, experiments with lysine methyl ester (an amino acid modified to create a weak base) established that it is transported in a manner consistent with the permeation of the neutral (deprotonated) form, with the external pH having a large effect on permeation rates. The permeability coefficient for the neutral form of lysine methyl ester was approximately 10^{-2} cm · sec^{-1}, corresponding to extremely rapid transbilayer movement. Zwitterionic amino acids may also translocate via the neutral form. The small size of the neutral population (theoretically 10^6–10^8 less than the charged forms) could account for the slow permeation rates observed for these amino acids. Both mechanisms (neutral form translocation and transient bilayer defects) of transport may therefore be utilized by amino acids, whereas modified amino acids would permeate predominantly as the neutral form at appropriate pH values. It is suggested that translocation of the neutral species may be pertinent to the mechanism of transbilayer permeation of peptides, such as signal sequences, which exhibit weak base characteristics. (Supported by grants/fellowships from NASA, NASA NSCORT and the Medical Research Council of Canada.)

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2 amino 3-ketobutyrate ligase: *NMR* study of its enzyme substrate complexes

The exchangeable protons of the pyridoxal phosphate cofactor were studied on an AMX 600 NMR spectrometer. The resonance shifts have been used as probes to study the binding of substrates at the active site of the enzyme. The spectral shifts observed when glycine, acetyl CoA and aminomalonate react with the enzyme give evidence for the reaction mechanism not available by other spectral techniques.

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Triglycine sulphate doped with L-alanine: crystal habits and dielectric constant

Single crystals of triglycine sulphate (TGS) are of interest because of their ferroelectric second-order phase transition and their applications as infrared thermal detectors. The chemical formula of TGS corresponds to a bis-glycinium glycine sulphate $(NH_3^+H_2COO^-)(NH_3^+CH_2COOH)_2SO_4^{2-}$. TGS is a water soluble crystal readily grown from solution. The main shortcoming of this material is that its Curie point is relatively low ($T_c = 49°C$) so that TGS ceases to be pyroelectric at temperatures higher than T_c. To avoid this the crystal is doped with some quantities of L-alanine (CH_3CHNH_2COOH). The partial substitution of molecules of L-alanine for glycine in the TGS lattice (LATGS) induces an internal bias field (B) which keeps the crystal permanently polarized.

Crystals of LATGS were grown by gradually lowering the temperature from 48°C to room temperature from a TGS water solution containing different concentrations of L-alanine (6%, 7% and 10% alanine/(alaine + glycine) moles). The cooling rate was in the 0.05 to 0.5 K/day range. The growth device is based on the rotating disc method in which several modifications were introduced. The temperature regulation is controlled laterally and from the lower part of the solution. The vertical gradient of temperature inside the solution is 5×10^{-4} K/cm and the thermal fluctuations are lower than 10^{-3} K. The seed rotates at 13 r.p.m in both senses with another up and down movement of 2 cm/minute. The TGS seeds were 1 cm^2 of section and were mounted in a disc in such a way that only the face perpendicular to the ferroelectric axis was shown to the solution. Crystals of about 10 g in mass were grown in 20 days and showed two crystal habits named as "short" and "tall".

The dielectric constant ε have been measured and it has been found that "short" crystals have lower values of ε than "tall" crystals grown from solutions with the same concentration. As the ferroelectric behaviour of LATGS crystals is equivalent to the behaviour of a pure TGS crystals under an external applied electric field then "short" crystals have higher values of B (what means higher L-alanine content) than "tall" crystals.

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Library of atomic multipole moments for precise modelling of electrostatic properties of aminoacids

Contemporary theoretical models used in describing electrostatic properties of aminoacids in polypeptides rely usually on atomic point charges. Recently noted defects of such models in reproducing protein folding originate from the electrostatic term and its inability to account for local anisotropy of molecular charge distribution. Such defects could be corrected by Cumulative Atomic Multipole Moments (CAMM) derived directly from any high quality quantum chemical wavefunctions (ab initio LCAO MO SCF, MP2, MRD-CI, etc.) In addition, high quality

Point Charge Models (PCM) can be analytically derived from CAMM database.

Examples illustrating various uses of multicenter multipole moment database of protein building blocks in modelling various properties of aminoacids and polypeptides will be presented, including calculation of molecular electrostatic potential maps, interactions between protein residues, estimates of pK_a shifts induced by changes in molcular environment, modelling static and dynamic properties of optimal catalytic environment in mutated enzymes, etc.

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Modelling of protein structures

The modelling of protein structures (isolated, in solution, or involved in recognition processes) will be reviewed at an educational (non-mathematical) level. After outlining the goals and present expectations, this review will deal with the information available and needed (databases and parameters), the theoretical foundations for the modelling procedures (with emphasis on the potential energy functions), the existing simulation techniques and prediction methods, the difficulties still to be faced, and the usage of available software packages.

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The use of theoretical amino acid descriptors in structure-activity relationships

Theoretically derived amino acid descriptors or descriptors of principal property type can be utilized to develop mathematical relationships between structures (e.g. peptides, proteins etc.) and experimentally determined properties such as biological activities. These models, called quantitative structure-activity relationships (QSARs), can then be used to determine which amino acid properties have the greatest influence on the investigated problem to aid the elucidation of underlying mechanisms of action as well as enable the development of new entities.

The development of such descriptors are described and their use exemplified through the analysis of some analogues of parathyroid hormone (PTH) as well as some azurin mutants from Pseudomonas aeruginosa.

The analogues of bovine PTH-(1–34) are monosubstituted at positions 3 and 6 and their biological (adenylyl cyclase) activities determined in UMR 106-HT rat osteosarcoma cells. The relationship between the structures (peptides) and the measured biological property is delineated using the method of partial least squares projections to latent structures (PLS).

Azurin, a small blue copper protein involved in electron transfer, contain a copper type 1 site and is a member in the denitrificatin/respiratory chain of some denitrifying bacteria. The reduction potentials of such proteins, which reflect the differences in stability between an oxidized and reduced copper site, have been measured for some mutants modified in positions 47, 48, 114 and 121. The relationship between the structures of the mutants and the measured property is investigated in a similar manner as previously mentioned for the PTH analogues.

In both examples the final statistical models, QSARs, are derived using a variable selection scheme based on experimental design principles (GOLPE type).

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The comparative thermal chemistry of simple amino acid derivatives

Understanding the thermal chemistry of simple but appropriate derivatives of amino acids may be considered as a useful first step towards assessing relative importance among the diverse processes that take place in the thermal degradation of proteins. Such an assessment has particular relevance in, for example, food and wool processing technologies.

Two recent opportunities to compare reactivities in a series of amino acid amides and esters under common sets of conditions have allowed us to identify the more significant transformations. In particular, we have identified two intrinsic reactions of cystine derivatives whose importance may have been underestimated previously.

The first study compared the lability of the N-acetylmethyl amides of eleven protein amino acids under neutral and basic aqueous conditions, with most attention given to the cystine derivative. While the products formed in alkali reflect those reported to occur in analogous treatment of wool keratin, the extremely facile formation of thiocystine at lower pH affords some new insights.

In the second investigation, the reactions of 31 amino acids in ethane-1,2-diol at 160°C were examined. Most were used for mechanistic and selectivity studies in the condensation reactions leading to cyclic dipeptides, but alternative sidechain reactions account for the major products in 14 cases. Both categories of reaction proceed most commonly *via* ethane diol esters generated by alkyl-oxygen bond formation.

Reactions competing with condensation include dealkylation, deamination, decarboxylation and cyclization but, again, the cystine-related substrates undergo the least familiar transformations and are the most reactive. In fact, the reactions can be properly analysed only under much milder conditions, and were studied using methyl and ethyl esters at lower temperatures. These compounds are then converted almost quantitatively to a diastereomeric mixture of thiazolidine derivatives.

The variety of compounds formed by heating simple derivatives of amino acids is of potential interest in several contexts and some of these will be briefly discussed.

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***Ab-initio* computations on amino acid diamides and the quantification of side-chain/backbone interaction**

The intrinsic stabilites of the different amino acid residue conformations is a fundamental step toward the understanding of protein folding.

In recent *ab-initio* MO calculations, the conformational space of Glycine, Alanine and Valine derivatives of the type $HCONH\text{-}CHR\text{-}CONH_2$ has been explored.

The relative stability of the characterized conformations can be better analyzed by decomposing the computed energy of each of the structures into different energetic contributions.

With the aid of such quantification, the individual role of

those contributions (side-chain/backbone interaction, geometrical distortions) can be analyzed.

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An *Ab-initio* modeling of *cis-trans* isomerization of peptide bonds in various environments

Protein secondary conformation is usually expressed in terms of the values assumed by the more flexible geometrical paramemers of its constituting amino acids: the torsional angles ϕ and ψ.

Since rotation about the peptide bond is known to be hindered by its partial π character, due to the existence of two resonant forms, the third type torsional angle ω is usually assumed to keep the nominally value of 180°, corresponding to the lowest energy conformation *trans*. However, *cis-trans* isomerization may occure under specific conditions.

The influence of the protein environment on such isomerization is modelled by an external electric field acting on an amide simulating a peptidic bond. The variation of the barrier to internal rotation with the magnitude and the orientation of the electric field is studied by quantum chemical *ab-initio* computations.

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Electrochemical oxidation reaction mechanisms of amino acids

An electrochemical investigation has been made on the amino acids glycine, α- and β-alanine, α-, β-, and γ-amninobutyric acid, asparagine and the dipeptide, glycylglycine in phosphate buffer (pH 7.0) in order to obtain information on the kinetics, mechanisms, and surface effects of the electrochemical oxidation reactions at a Pt electrode. Steady-state current potential measurements, cyclic voltammetry, and ac impendance measurements have been used to study 0.45 M aqueous solutions of these amino acids.

The Tafel slopes determined from steady-state polarization experiments gave results consistently in the 350 to 400 mV region for all the amino acids studied. These results are similar to those obtained previously for some of these amino acids under different pH conditions. The mechanisms consistent with these high Tafel values correspond to surface absorption through the carboxyl group accompanied by electron transfer, and following this by decarboxylation which is the rate determining step. A transfer of a second electron occurs either during the hydrolysis reaction of the adsorbed radical at the electrode surface to give an aldehyde or during the formation of a carbonium ion which is subsequently hydrolyzed in solution to an aldehyde.

Surface adsorption behaviour of the amino acids have been studied by cyclic voltammetry and ac impedance. The surface charge density resulting from surface adsorption of the amino acid was compared for the end potential 0.4 V corresponding to normally a monolayer surface coverage of OH. The surface coverage for glycine at pH 7.0 was $\theta = 0.2$, while α-alanine and α-aminobutyric acid each gave $\theta = 0.5$. This difference may have resulted from end on adsorption through the carboxyl groups of the larger amino acids, compared with surface interaction of both carboxyl and amino groups with glycine under these potentials. When the full under potential deposition region was used, a comparison of the surface charge density showed a large increase with increasing pH for all the amino acids, indicative of the strong adsorption of the carboxylate anion. These results are compared with ongoing investigations in our laboratory on the surface adsorption behaviour of proteins at the platinum electrode.

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Study of the solid-liquid state diagrams of the aqueous systems containing amino acids and peptids with a view to develop thermostable freeze-dried biopreparations

Amino acids and peptids are widely used for stabilization of enzymes, viruses, etc. These substances are characterized by a tendency to develop a glassy state at freezing of the aqueous solutions. The solid-liquid state diagrams are used for description of such unequilibrium systems. In the present work solid-liquid state diagrams of the binary and ternary aqueous systems containing amino acids (glycine, lysine), protein hydrolyzates (peptone, hydrolized gelatins with various hydrolysis levels) and sucrose were studed by DTA and X-ray diffraction methods.

The order of phase transitions at cooling of the solutions with 90–60% water content is as follows: primary water crystallization, secondary water + glycine crystallization (for water-glycine system and certain compositions of water-glycine-sucrose system), and vitrification of the concentrated solution remaining after water crystallization. Melting peak of ice and two endothermic steps are registered on DTA heating curves. These steps are identified as a structural glass-transition, Tg' (abrupt change in heat capacity; vitreous phase transforms into the structural-liquid state, however, it preserves the mechanical properties of solid because of high viscosity), and mechanical glass-transition, Tam' (amorphous phase changes to viscous-flow state, this effect is known as "ante-melting"). For systems with glycine some additional transitons are registered. Tg', Tam' are the functions of R (R is the X/sucrose ratio, X is hydrolized gelatins, pepton or glycine) and are independent of the initial water content. The following features are also noted for ternary systems. For water-hydrolized gelatin-sucrose systems there are minimums on the plots Tg', Tam' = f(R) at R = 0.1–0.2. For water-glycine-sucrose system the crystallization order is changed by cooling rate (B) and R. So, secondary crystallization occures either at cooling (B being lower than the critical one, Bcr, B < Bcr), or at heating (B > Bcr), or does not occur at all (R < 0.25).

Two effects, Tg and Tam, are registered on DTA curves for low-moisture compositions (water content varies from 15% to 0%). Tam step is accompanied by collapse (change of the sample form). Also, for water-glycine-sucrose system exothermic peaks of sucrose + glycine crystallization are observed. The state diagram surfaces Tg, Tam = f(R, W) (W-water content) are studied.

Compositions showing promise as stabilizers for thermostable biopreparations are proposed on the basis of the physicochemical analysis results. The fitness of the proposed stabilizers was studied at preparing the thermostable measles vaccine, surface antigen of the B-hepatitis, protein A and IgG conjugates with horsradish peroxidase, HIV antibody positive and negative sera.

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The crystal structures of two Aib- containing tripeptides

Synthetic peptides containing α-aminoisobutyric acid (Aib), a naturally occuring residue in peptide antibiotics of the alamethicin family (peptaibols), have provoked a great deal of interest because they provide excellent models for the analysis of the structural basis of the peptaibol interactions with biological membranes. The properties of peptaibols are influenced by the conformational constraints which the Aib residues impose on the peptide backbone: The conformational space available to Aib residues comprises only a small fraction of the space accessible to standard helices numrous crystallographic, NMR and CD studies of Aib-containine peptides have revealed α-, 3_{10}-helical, mixed α-/3_{10}-helical or mixed helical/extended conformations. Which conformation is adopted depends on several factors, e.g. petide length, potential for intra- or intermolecular hydrogen bond formation, percentage of Aib residues in the sequence etc.

Have we report the X-ray structures analysis of two synthetic, protected peptides Z-Ala-Aib-Pro-Ome(I) and Z-Val-Aib-Pro-Ome(II) (Abbreviation: Z, benzyloxycarbonyl). (I) crystallizes in C2 with a = 19.07 Å, b = 6.19 Å, c = 20.59 Å and β = 110.03° with one molecule in the asymmetric unit; (II) cystallizes in P1 as a twin crystal with two different lattices; the unit cell edges in both lattices are a = 6.26 Å, b = 10.18 Å, c = 20.65 Å; and the unit cell angles are $\alpha_1 = 87.9°$, $\beta_1 = 88.6°$, $\gamma_1 = 72.6°$ and $\alpha_2 = 95.2$, $\beta_2 = 95.6°$, $\gamma_2 = 107.4°$ respectively. For structure determination and refinement all common reflections of the two lattices were left out and the the molecules in the first lattice were investigated. There are two independent molecules in the unit cell. Despite the different crystalline environment, the backbone conformations of the two peptides are very similar. All there (one for I and two for II) conformations adopt φ, ψ angles in the Ala, Val and Pro residues which lie in the extended region, while the torison angles for the Aib residues lie in the region of left handed helices. The molecular arrangements in the crystal and the hydrogen bonding patterns are very similar for both compounds.

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Copper-catalyzed oxidations of amino acids and derivatives

The biological active form of many peptide hormones is obtained through amidation of terminal COOH group; this process implies α-carbon hydroxylation of a glycine-extended precursor followed by cleavage into amidated peptide and glyoxylic acid. The first step is catalyzed by peptidyl-glycine hydroxylase (PGH), a copper-dependent enzyme requiring O_2 and ascorbate, recently shown to be a monooxygenase. Hence, amidation of P–COOH occurs according to:

$$\text{P–CONH–CH}_2\text{–COOH} \xrightarrow{\text{PGH}} \text{P–CONH–CH(OH)–COOH} \longrightarrow \text{P–CONH}_2 + \text{CHO–COOH}$$

We report here a simple chemical model of this enzymatic activity: copper-catalyzed oxidation of N-salicyloyl-glycine **1** leads in a first step to N-salicyloyl-α-hydroxyglycine **2**, relatively stable in its Cu^{II} salt form, then, more slowly, to salicylamide **3** and glyoxylic acid Selective α-hydroxylation is obtained as well with O_2/Cu^{I} system than with copper peroxide or trimethylamine oxide (oxygen donor)/Cu^{II} system. Since all reactions are performed in anhydrous conditions, oxidizing reagents are likely the source of incorporated HO-oxygen atom in **2**; trivalent copper Cu^{III} appears to be the key intermediate in the course of these transformations.

On the other hand, we have shown that similar copper-based systems are able to oxidize amino acids themselves, whereas their Cu^{II} complexes are generally quite stable. In this case, where amino groups are not protected, decarboxylation/dehydration processes lead for instance from alanine to acetonitrile and from diphenyl glycine to benzophenone azine. Mechanisms will be discussed in relation with carboxylic acids and amines properties towards copper-catalyzed oxidizing systems.

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Turn tendency of α,β-unsaturated peptides Ac-Pro-ΔX-NHCH$_3$ studied in solution by NMR, IR and CD spectroscopy

α,β-Dehydroamino acids are considered to promote βII-turn formation. Such turns were shown for peptides with (Z)-ΔPhe and (Z)-ΔLeu in the (*i* + 2) position. To compare turn tendencies of different α,β-dehydroamino acids we have investigated conformations in solution, of a set of model peptides Ac-Pro-ΔX-NHCH$_3$, where ΔX = ΔAla, (Z)-ΔAbu, (*E*)-ΔAbu, ΔVal, (*Z*)-ΔLeu, and (*Z*)-ΔPhe, using ^{1}H NMR, ^{13}C NMR, IR, UV, and CD spectroscopies. The peptides Ac-Pro-ΔX-NHCH$_3$ are capable of forming isomers with *cis* disposition of the Ac-Pro amide bond. These isomers cannot assume any structure with hydrogen bonding 4 → 1 (β-turn) or 3 → 1 (γ-turn) so they can serve in NMR measurements as internal standard of open peptide forms. X-Ray crystal structures of most of the model peptides were solved (see our related poster).

The solvent dependence of N*H* chemical shifts and the dependence of N*H* resonance linewidths on TEMPO concentration in both *cis* and *trans* isomers, as well as IR spectra in the *NH* stretch region show that spectroscopic properties of N*H*(ΔX) are significantly disturbed by the interactions between the C = C and the peptide bond. The properties of N*H*CH$_3$ are disturbed slightly. Hence, the use of the above spectroscopic parameters for a quantitative estimation of β-turns in peptides with unsaturated residue in the (*i* + 2) position requires caution and the definition of γ-turns is hardly possible.

In $CDCl_3/CH_2Cl_2$ all the peptides except Ac-Pro-ΔAla-NHCH$_3$ adopt a βII-turn thereby this conformation is the least populated in Ac-Pro-(*E*)-ΔAbu-NHCH$_3$ and population differences among the others are not significant. In Ac-Pro-ΔAla-NHCH$_3$, the ΔAla moiety assumes the extended conformation and a γ-bend around the Pro residue is quite probable, however, it cannot be undoubtedly confirmed. In polar solvents, peptides are unfolded. Only in Ac-Pro-(*Z*)-ΔPhe-NHCH$_3$ an ordered structure other than the βII-turn seems perceptible. The disposition of (*Z*)-α,β-dehydropeptides to form β-turns has been assumed to result from a steric hindrance introduced by their (*Z*) substituent. Peptide Ac-Pro-(*E*)-ΔAbu-NHCH$_3$ takes the β-turn, though, the extended ΔAla-like conformation should be accessible for (*E*)-ΔAbu residue. Therefore, the β-turn promotion by α,β-dehydroamino acids may have another reason.

S.-I. Ichinose

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Molecular information and hydrogen bonding

A functional protein might well be a *little universe* in which charge, energy and information can be stored and transported. The storage and transport mechanisms would involve keto-enol transition of the hydrogen bonding backbone structure of proteins. In the present study, by employing a simple model we study the effect of an alpha helical protein molecule on the electron transfer between the donor and acceptor molecules. In particular the protein molecule plays a catalytic role to facilitate the electron transport between the donor and acceptor molecules attached to it. After a proton is transferred, leaving behind an enole chain instead of the original keto chain, the protein may back to the keto chain, making the injected electron transfer in the backward direction. With such a resetting mechanism, this is a viable possibility for repeated electron transport along the alpha helical protein molecule.

It is shown experimentally that an electron transferred covers distances of order 30–70 A. It is unlikely that the electron transfer covering such large distances is realized via a simple mechanism of tunneling. In fact, the proton impurity H^+ in the protonated protein can play the role of the *shuttle* (proton shuttle) for the electron which can therefore propagate through the insulating proteins. In conclusion, both free proton impurity H^+ and the charged radical H* are expected to play a fundamental role in the transport properties of hydrogen bonding materials such as proteins.

H. Kozlowski

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Impact of metal ions on chemistry and biology of amino acids and peptides

Many essential metal ions act as the important factor influenceing the structure of natural and synthetic oligopeptides and as the consequence they may have critical impact on their biological activity. Peptides contain a variety of effective donor centres and their complexes can exist in the virtually infinitive conformations.

The most important donor site is usually the N-terminal nitrogen, which is often a primary amino group or secondary nitrogen as it is the case in *Pro* or *Ser* residues. Many metal ions anchored on the N-terminal nitrogen, like Cu(II), Ni(II), Pt(II), Co(II), are able to induce proton dissociation from the adjacent amide nitrogen involving this donor in the metal ion co-ordination. This is a very potent binding mode leading to strong and specific metal-peptide complexes.

Many natural peptides contain a competitive donor atom in the side-chain. The most important among them are pyridine-like imidazole nitrogen of *His*, the sulpfur atom of *Cys* and *Met*, the carboxylate oxygen of *Asp*, phenolic oxygen of *Tyr* and in some cases the nitrogen of *Lys*.

In the case of short peptides with non-co-ordinating side chains such metal ions like Cu(II) start binding at N-terminal nitrogen and then co-ordinate to consecutive amide nitrogens. The effective nitrogen donor atoms as well as the 5-membered chelate rings lead to formation of very stable metal complexes. However, various effects deriving from the specific peptide sequences may change drastically the complex stability.

Peptides with co-ordinating side chains as *His* or *Cys* may compete strongly with N-terminal anchoring site and often metal binding begins at side chain donor atom rather than at N-terminal amino group. *His* residue is often critical for the biological activity of natural peptides and involving it in metal ion co-ordination may drastically change the biological activity of a peptide. As an example one can use *LHRH*, a decapepitde amide, a main mediator in the neuroregulation of the secretion of gonadotrophins, luteinizing hormone (*LH*), and the follicle stimulating hormone (*FSH*). It has been shown that metal ions bound to *LHRH* may drastically influence its biological activity. This and other examples will be given during the lecture. The aqueous solution studies have may not be very relevant to the natural environment of the cell as many of metal-peptide interactions happen close or on the cell membranes. The simple study witg SDS micelles has shown that effect of hydrophobic medium may change dramatically the structure and stabiltity of the formed complex species.

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Spectrophotometric kinetics study of the reaction of α-amino acids with p-benzoquinone

The reaction rates of α-amino acids with p-benzoquinone were investigated as a function of pH of the reaction medium, temperature, basicities, and steric environment of amino group. Taking in our consideration the different equilibria of amino acids species in solution and under the assumption that the neutral and anion species are the most reactive species react with rate constant k_z and K_A respectively the suggested kinetic equation is

$$k_2 = \frac{k_z}{K_D} + \frac{k_A K_2}{[H^+]}$$

Linear relationship between log K_A and pK_2 of tested amino acids was obtained, and a quantitative estimation of the polar and steric parameters on the rates of reaction were calculated using Hammett-Taft free energy relationship. The activation parameters as well as the mechanism of reaction had been already discussed.

N. Raos

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The chemistry of copper(II) chelates with N-alkylated amino acids

N-alkylated α-amino acids are compounds very suitable for studying the sterical effects in chelates with heavy metals. There are very pronounced stereoselective effects (diastereoselectivity and enantioselectivity) in copper chelates with these compounds which is not true of copper chelates with naturally occurring amino acids. The enantioselectivity effect in copper(II) chelates with N-alkylated amino acids has been throughly studied in

order to be applied for resolution of the racemic amino acid mixtures by means of ligand-exchange chromatography.

From another point of view, copper(II) complexes are very interesting compounds for studying the distortion of the copper coordination polyhedron and *cis/trans* isomerism. In order to reproduce the shape of copper(II) coordination polyhedron in these compounds molecular mechanics models with new functions for copper(II)/ligand interactions has been developed in our laboratory.

U. Seebacher and M. Ramek

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Structure-energy relationship in amino acids and related compounds

The potential energy surfaces of ω-amino acids, ω-hydroxy acids, and ω-amino alcohols have been investigated by ab-initio RHF calculations. In the case of β-alanine (21 minima, 33 internal coordinates) structure-energy relationships become obvious when similar conformations are compared with each other. The H-bond influence can be estimated in a straightforward manner from the energy differences between similar conformations. This is, however, not generalizable: in the case of γ-amino butyric acid (62 minima, 42 internal coordinates) no such comparison is possible due to the greater flexibility of the molecule. The same is true for 4-hydroxy butyric acid (63 minima, 39 internal coordinates), 4-amino butanol (110 minima, 48 internals), 3-hydroxy propionic acid (19 minima, 30 internals) and 3-amino propanol (35 minima, 36 internals).

The energy of a molecule is usually described as a function of bond lengths R, bond angles Θ and torsional angles Φ:

$$E_{\text{bonded}} = \sum_{\text{bonds}} K_R(R - R_0)^2 + \sum_{\text{angles}} K_\Theta(\Theta - \Theta_0)^2 + \sum_{\text{dihedrals}} V_n[\cos(n\Phi)]$$

Since there are much more minimum geometries than variables, the parameters K_R, K_Θ and V_n can be optimized in a least-square fit method. Each geometry is excluded successively from the fit. The geometry, whose omission yields the best correlation coefficient, is left out for the next loop. The procedure stops, when enough minima have been sorted out and the correlation becomes better than some predefined threshold.

Applying the energy function to the molecules mentioned above we found that the energy does not depend significantly upon bond lengths and bond angles, but is almost solely a function of the torsional angles. Moreover, it is sufficient to consider only those dihedral angles, which are essential for the description of the molecular backbone. The method works best when we extend the sum over the cosines to a second term:

$$E = \sum_{i}^{\text{essential}} V_{1,i}[\cos(\Phi_i)] + \sum_{i}^{\text{essential}} V_{3,i}[\cos(3\Phi_i)]$$

The difference between the given (ab initio) and the estimated energies are small for minimum geometries which stayed within the set to fit the parameters and are large for those which have been omitted. The energy differences turn out to be in the range of the interaction energy predicted for the H-bond contribution. The method can therefore be used as a semi-quantitative measure of the influence of additional interactions to the conformational energy.

A. Shimada and I. Nakamura

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Depressed recognition of tryptophanase for tryptophan enantiomers in highly concentrated ammonium phosphate solution

Substrate specificity of enzyme has a ambiguity of the order which can react with substrate and its analogous compounds. On the other hand, it is so rigid in the recognition of amino acid optical isomer that most of enzymes can react with L-amino acid only. We assume that such a high level specificity should be acquired through chemical evolution in the age of origins of life in which amino acid world was racemic. The interaction between enzyme and its surroundings would probably make the specificity develop. If this assumption is valid, it may be possible to depress the strict specificity to L-amino acid by controlling the environment.

We have so far studied on both elucidation and molecular evolution of D-tryptophan metabolism in *Escherichia coli*. In the process we have noted tryptophanase. Tryptophanase from *E. coli*, which consists of 4 subunits, is MW 220,000. Coenzyme being pyridoxal 5'-phosphate, activator is both ammonium ion and potassium ion. Each of subunit bonds with a pyridoxal 5'-phosphate and can form holoenzyme. The enzyme can specifically decompose L-tryptophan into indole, pyruvate and ammonia. It, however, cannot react with D-tryptophan for the very high specificity to L isomer at all. On the basis of the above hypothesis, we investigate whether or not the activity to D-tryptophan is appeared by modifying the surroundings around tryptophanase. Although high pressure, temperature, electric field, etc. can be considered as environmental parameters which make the specificity of tryptophanase change, we here study the effect on salt concentration.

We present that only tryptophanase in the solution of concentrated ammonium sulfate and ammonium phosphate can decompose D-tryptophan. The activity to D-tryptophan is merely 4% of that to L-tryptophan. It is our purpose to enhance this very low activity, too. We discuss its evolutionary significance in addition. The mechanism of the D-tryptophan decomposing activity is unknown in the present stage. The results will nevertheless give a interesting hypothesis about enzyme evolution. Primitive enzymes, the molecular weight of which would be very low, could not interacted with their peripheral surroundings and could not strictly take L-amino acid from D-amino acid until they had longer polypeptide chain or higher order structure. Results suggest that the interaction between enzyme and its surroundings would be important in L-amino acid selection in origins of life.

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Microcapsules prepared through interfacial cross-linking of proteins

Microcapsules are microparticles consisting of a solid or liquid core enclosed in a membrane. Among a variety of uses in different fields, microcapsules have interesting biomedical and biotechnological applications, either as microcontainers for con-

trolled release of drugs, or as microreactors containing cells, microorganisms or enzymes. Otherwise, they may behave as efficient scavengers for metal ions or other toxic compounds. This communication presents three methods for the preparation of biocompatible microcapsules from proteins, which illustrate these different aspects.

The first part deals with a microencapsulation process through interfacial cross-linking using acid dichlorides in emulsion systems. Microcapsule properties (morphology, size, biodegradability ...) were shown to depend on the reaction parameters, mostly pH, reaction time and concentration of acylating agent. Structural studies of human serum albumin (HSA) microcapsules were conducted using Fourier transform infrared spectroscopy and chemical determination of microcapsule $-NH_2$ content. A progressive involvement of amino, hydroxy and carboxylate groups of HSA was demonstrated upon increasing each of the reaction parameters, paralleling the observed changes in microcapsule properties.

The second part discloses a simple method for the preparation of chelating microcapsules from proteins, which directly derives from the above cross-linking process. Microcapsules were prepared from different proteins using terephthaloylchloride. They were further reacted with alkaline hydroxylamine with the aim of destroying ester and anhydride bonds in the wall and attaching hydroxamic groups to the membrane. The resulting polyhydroxamic microcapsules exhibited interesting iron complexing properties which could be improved by esterification of the free carboxylic groups of the wall with benzyl alcohol using a carbodiimide, prior to the hydroxylamine treatment. These biocompatible chelating microcapsules have potential applications as contrast enhancing agents for diagnostic imaging or in extracorporeal detoxification in heavy metal poisoning cases.

The last part is devoted to a novel microencapsulation process avoiding acid dichlorides. The principle is based on a transacylation reaction between a polysaccharide bearing numerous esterified carboxylic groups, such as propylene glycol alginate (PGA) and proteins. The O to N transfer of acyl groups starts upon alkalinization and results in the formation of a film consisting of a protein directly bound to the acidic polysaccharide through amide bonds. This reaction was successfully applied to emulsion systems giving stable and degradable microcapsules.

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Comparative study on microwave and conventional methods for protein hydrolysis

The rate-limiting and most crucial step of an amino-acid analysis, after the recent advances in chromatographic methods, lies in the preparation of protein hydrolysates. The traditional protocol (Moore et al., 1958) utilizes 6M hydrochloric acid at 100°C for 24 and 72 hours and has been the standard method for the past thirty years. Protein (lysozyme) hydrolysis for amino acid analysis by means of a commercial microwave oven was first reported in 1987 (Chen et al., 1987). Afterwards Gilman and Woodward (1989) suggested a usefull microwave vapor phase hydrolysis for the analysis of amino-acids when sample amount is limited (methionyl human growth hormone).

In this work a microwave irradiation method of protein hydrolysis in food is reported.

The microwave system used was a Microwave Digestion System, Model MDS 2000 (CEM Corporation). The unit can operate at 0–100 percent full power (630 ± 50 watts). The MDS 2000 is equipped with an inlet/outlet port to allow the tubing connected to the vessel and the Pressure Controller to pass through the cavity wall without allowing microwave leakage.

Microwave hydrolysis were conducted in duplicate for 5, 10, 20, 30 minutes at two different power steps. Vials containing proteins were placed inside a Teflon PFA digestion vessel (CEM Corporation) along with 8ml of 6M HCl. The vessels were then connected to a purge trap and to a manifold for evacuation, nitrogen purge, pressure monitoring and controlling and a safety release mechanism.

After hydrolysis the samples were removed from the vessel and placed in the vacuum evaporator to remove liquid and then redissolved in 50ml of pH 2.2 starting chromatographic buffer.

All amino-acid analysis were made using a Beckman amino-analyzer 118BL system.

The food proteins chosen for hydrolysis were: standard bovine serum albumin (BSA), milk, durum wheat semola and pasta proteins. The amino-acid composition data from different heating procedures were compared with the literature values, based on its amino-acid sequence, for BSA and with the classic method results for the other food proteins.

Although microwave heating is frequently used to prepare samples for atomic absorption or emission analysis it is not yet widely used for the hydrolysis of proteins. This research confirms that microwave hydrolysis is an important and useful tool in protein research.

H. Suzuki

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Sarcosine oxidase: structure, function, and the application to creatinine determination

Determination of creatinine is important in the clinical laboratory. Jaffé reaction has long been used to determine creatinine, but the method suffers from various interferences. To overcome this problem, the enzymatic methods were invented and have been used widely. Sarcosine oxidase has a critical role in the enzymatic method. Of sarcosine oxidases, *Corynebacterium* enzyme has been studied extensively in kinetic and structural aspects. The enzyme contains noncovalently bound and covalently bound FADs, and consists of 4 nonidentical subunits (A, B, C, D). The covalently bound FAD is bound to the subunit B. The rate of oxidation of sarcosine was explained by the rates of the oxidation and reduction of the bound FADs. From the chemical modification of the enzyme with iodeacetamide, the amino acid sequence around the noncovalently bound FAD is suggested and the modification changed the enzyme so that the only noncovalently bound FAD functions in the oxidation of sarcosine.

Synthesis and Biosynthesis

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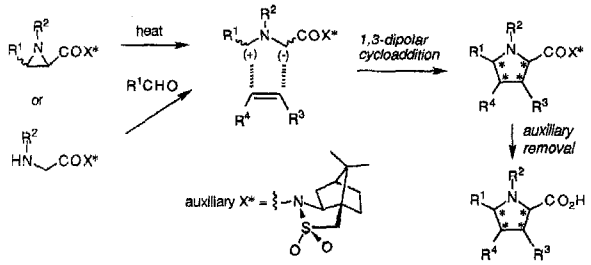
The development of new chiral "cycloadditive" synthons for the asymmetric synthesis of amino acids

It is generally recognized that azomethine ylide cycloadditions represent a very powerful method for the assembly of substituted pyrrolidine ring systems. With an eye on asymmetric synthesis of pyrrolidine-containing targets, much effort has been expended to develop a suitable chiral auxiliary for the azomethine ylide dipole. There are 5 issues which need to be addressed when evaluating the resulting chiral azomethine ylides for use in asymmetric synthesis. These are: (1) availability of the auxiliary, (2) control of ylide geometry, (3) diastereofacial selectivity, (4) endo/exo selectivity, and (5) auxiliary removal/recovery. In this talk, we will report on the use of Oppolzer's camphor-derived sultam as a recoverable chiral auxiliary. The chiral azomethine ylides will be shown to serve as useful c^3 ("cycloadditive, 3-atom") reagents for the asymmetric synthesis of highly functionalized prolines and related derivatives which are useful intermediates for the asymmetric synthesis of pyrrolidine-containing natural products and conformationally restricted peptide analogues. A convenient synthesis of the starting homochiral aziridine-2-carboxylates, which are themselves useful a^3 reagents for amino acid synthesis, will also be present.



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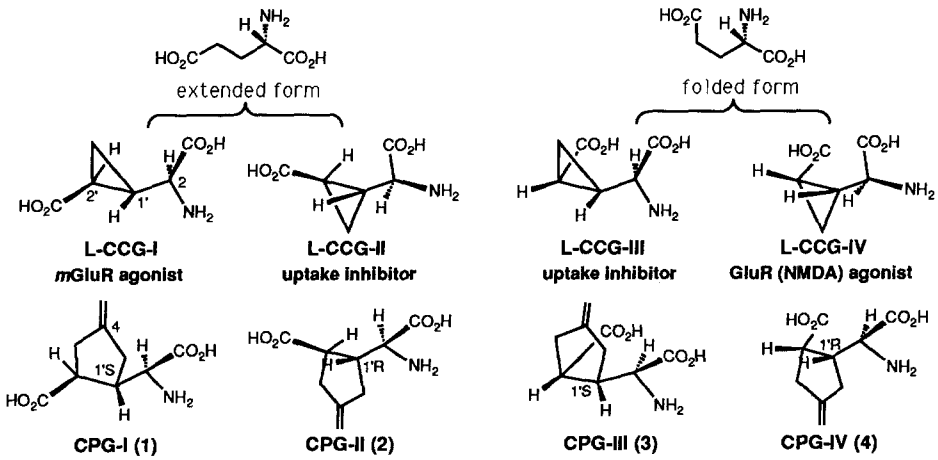
Stereoselective synthesis of conformationally restricted amino acids. New glutamate agonists

L-Glutamic acid has currently received much attention because it is an excitatory neurotransmitter in the mammalian central nervous system and a potent excitotoxin that causes various acute and chronic brain diseases. In addition, its action is thought to be closely related to the construction of memory and early learning. Glutamate receptors are classified into (1) ionotropic glutamate receptors that are further divided into the NMDA, kainate and AMPA subtypes, and (2) metabotropic glutamate receptors. It is reasonable to assume that different conformations (e.g., extended or folded form) of L-glutamate activate the different types of glutamate receptors. We have synthesized four diastereomers of L-2-(carboxycyclopropyl)-glycines (CCGs) as conformational variants of L-glutamate. Electrophysiological as well as biochemical studies using these CCG isomers provided evidence that each subtype receptor recognizes a specific conformation of L-glutamate. These results suggest that the conformation of L-glutamate that activates ionotropic glutamate receptors, especially NMDA receptor, is the folded form and that the conformation of L-glutamate that activates the metabotropic glutamate receptors are the extended form (conformational requirements of glutamate receptors).

In conjunction with the above studies, we planned to develop new glutamate agonists, L-2-(2-carboxy-4-methylenecyclopentyl)glycines (CPG-I ~ IV, **1–4**), which were expected to add further informations as to the conformational requirements of glutamate receptors. In the CPGs, the ring strain and the angle strain that characterize the CCG's structure are minimized, and the exomethylene group allows for further structural modifications. We wish to describe here the stereoselective syntheses of (2*S*, 1′*S*, 2′*S*)-**1** (CPG-I), (2*S*, 1′*R*, 2′*R*)-**2** (CPG-II), and (2*S*, 1′*R*, 2′*S*)-**4** (CPG-IV). Among them, CPG-IV (**4**) was found to be a potent agonist of kainate receptors.



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Synthesis and biological action of peptidoamines: Carcinine and analogs

In 1975, carcinine (β-alanalylhistamine) was discovered by Arnould in cardiac tissue of the crustacean *Carcinus maenas* and has since been identified in several tissues of the rat, guinea-pig, mouse and human in level as high as than those reported for the related imiodazole compounds: camosine (β-alanylhistamine), histidine and histamine.

A radioisotopic study showed that carcinine is metabolically linked to both histamine and camosine. Histamine containing peptidoamines were also found in bee venom and their simple dipeptide derivatives (glycylhistamine) was shown to be a radio protective agent. We have synthesized several peptidoamines by a new simple and economic "one pot" method.

The carcinine (β-alanylhistamine), glycylhistamine, sarcosylhistamine and glycylglycylhistamine were synthetisized from Boc-β-alanine (N-tert-butoxycarbonyl-β-alanine), Boc-glycine, Boc-sarcosine, and Boc-glycylglycine respectively and histamine hydrochloride in presence of BOP (benzotriazol-l-yl-oxyltris (dimethylamino-phosphonium hexafluorophosphate), which was used as coupling agent. The final products were directely then obtained by release of the protecting tert-butoxycarbonyl group with hydrochloric acid in ether.

The structure and purity of the compounds were checked by ^1H and ^{13}C NMR spectroscopy elemental analysis (C, H, N, Cl) and acid-base titration. The yield is 80%, 92%, 85% and 68% for carcinine, glycylhistamine, sarcosylhistamine, glycylcglycylhistamine respectively.

In biological action, the immunoreactivity related to carcinine appears very intense in the herat of *Carcinus maenas*. More surprising is its distribution at the cellular level: it is localised essentially in plasmatic membranes (Epicarde's Cells), in the myocard (gliale's cell of perineurium). This special distribution of carcinine suggests that it may play a role protection of the myocardical elements.

P. Meffre, E. Branquet, P. Durand, C. Ariente-Fliche, J. Braun, L. Vo-Quang, and F. Le Goffic

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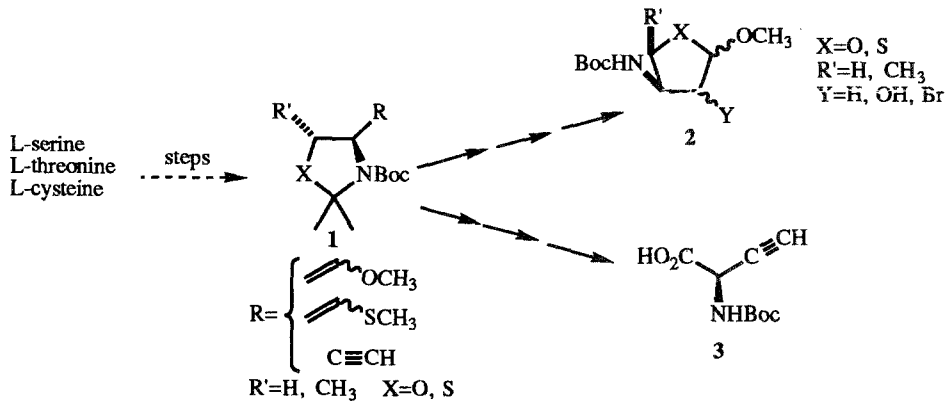
Different aspects of synthetic approaches including enzymatic enantioselective synthesis of α-amino acids

Our group has been involved for many years in the synthesis of unusual α-aminoacids (β,γ-unsaturated α-aminoacids and derivatives and aminocyclopropane carboxylic acid derivatives) as potential enzyme inhibitors.

We would like now to present new developments in this synthetic area.

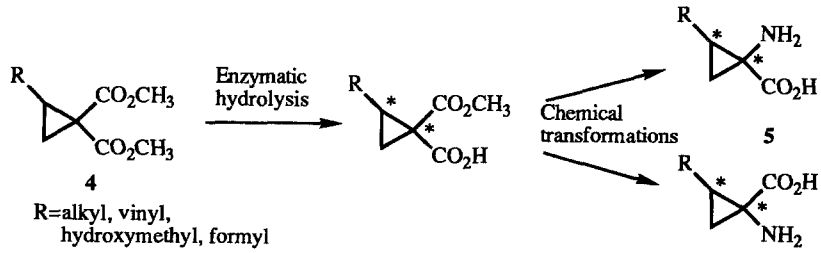
In the first part, the synthesis of unusual optically active oxa or thiazolidine derivatives **1** starting from natural aminoacids L-serine, L-threonine, L-cysteine and their use in the synthesis of unusual amino sugars **2** will be discussed.



The type of methyl 2,3-dideoxy 3-amino furanoside derivatives **2** could be used as intermediate in the synthesis of aminosugar components of new nucleosides, anthracyclines or macrolides analogues with antibiotic, antitumoral or antiviral properties.

Moreover, results in the synthesis of chiral non racemic ethynylglycine derivative **3** will be shown. Ethynylglycine is a natural unusual α-aminoacid with antimicrobial activity against gram-positive bacteria and is an irreversible inhibitor of alanine racemase. Its inherent lability and the uncertainty concerning the absolute configuration of the chiral center in the natural compound make a synthesis of optically active ethynylglycine a challenge.

In a second part, new results concerning the synthesis of enantiomerically pure aminocyclopropane carboxylic acids **5** by enantio and diastereoselective enzymatic hydrolysis of substituted racemic cyclopropane dicarboxylic acid diesters **4** using different hydrolytic enzymes and subsequent chemical transformations will be presented.



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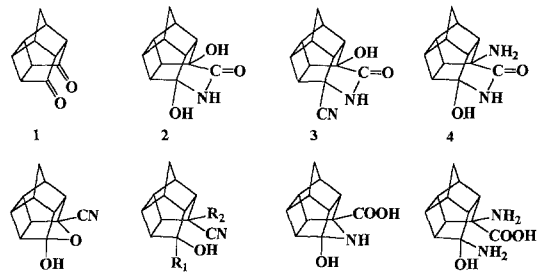
Synthesis of novel amino acids with cage structures

It has long been recognised that hydrocarbon moieties promote the transport of drugs containing them across cell membranes and increase their affinity for lipophilic regions in receptor molecules. The incorporation of a rigid cagelike hydrocarbon system into drugs for this purpose has the added advantage that metabolic degradation is retarded by the inherent stability and steric bulk of the cage skeleton, thus prolonging the activity and reducing the frequency of drug administration to the patient. For example, areas in which the introduction of an adamantane substituent has been shown to result in longevity of drug action, increased drug potency, speed of action and receptor site specificity include antibacterial activity, anabolic action and analgesic activity.

Amino acids are important building blocks for the synthesis of certain drugs. Very few amino acids with cage frameworks are known. We have sought to synthesise α-amino acids by utilising the dione **1** as substrate in Strecker reactions. **1** is easily obtained from the Diels-Alder adduct of cyclopentadiene and p-benzoquinone by intramolecular photocyclisation.

Treatment of **1** with one equivalent sodium cyanide in water produced the lactam **2**. With two equivalents or more sodium cyanide **3** was obtained. Treatment of **1** with aqueous sodium cyanide, ammonium chloride and ammonium hydroxide, produced the amino lactam **4**. The conversion of **1** to the corresponding lactams proceeds via the cyclic ether **5** which suffers cleavage to form **6**. The endo orientated nitrile group in **6** is surprisingly smoothly hydrolised to the corresponding amide which is converted to the lactam. The amino acids **7** and **8** are obtained from **2** and **4** respectively by alkaline cleavage of the C–N bond.



J. J. Cappon, K. D. Witters, P. J. E. Verdegem, A. C. Hoek, J. Raap, and J. Lugtenburg

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Synthesis of specifically stable-isotope labelled L-histidine for protein studies

L-Histidine is an important amino acid in protein chemistry because of the unique properties of the imidazole ring in the side chain. Under physiological conditions the imidazole ring can function as proton acceptor and as proton donor. Histidine residues therefore show internal buffer activity and are often found in active sites and cofactor binding sites in proteins. In the photosynthetic reaction centre of *Rhodobacter sphaeroides* histidine residues are found in crucial positions. They coordinate the Fe^{2+}-ion and the Mg^{2+}-ions in the bacteriochlorophylls, and form hydrogen bonds with both quinone cofacters.

To study the role of these histidine residues with spectroscopic techniques such as solid-state MAS NMR, specifically ^{13}C- or ^{15}N-labelled histidines have to be incorporated into the photosynthetic reaction centre. For this reason, a new synthesis is developed for the labelling of L-histidine. This scheme starts from simple, commercially available enriched synthons and allows the specific ^{13}C- and ^{15}N-labelling of every position or any combination of positions. The histidines are obtained on a gram scale, without any scrambling or dilution of the labelled material. First, a 1,5-disubstituted imidazole is constructed using tosylmethylisocyanide (TosMIC) as central intermediate. The key step is the coupling of 1-benzyl-5 chloromethylimidazole to a bislactimether, which introduces the right chirality. Hydrolysis and deprotection of this compound affords L-histidine in good yield and high optical purity. Several ^{13}C- and ^{15}N-labelled L-histidines have been prepared following this method.

(1′-^{15}N)-L-Histidine is incorporated into the photosynthetic reaction centre by growing the bacteria on a synthetic medium containing the labelled histidine. The degree of incorporation is determined by GC-MS analysis of the protein hydrolysate.

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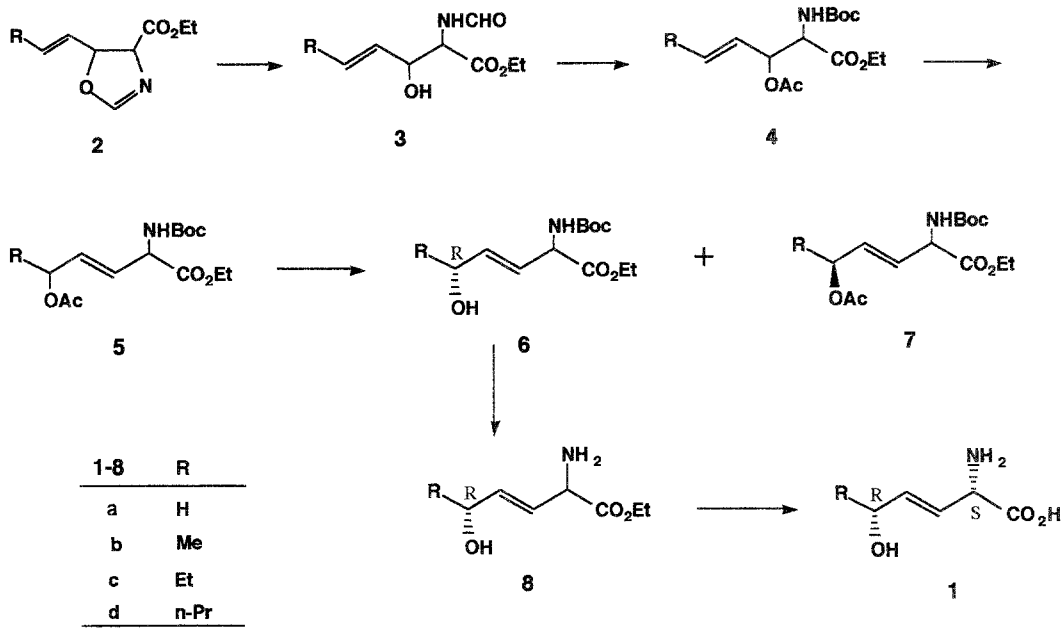
Synthesis of optically active β-dehydro-δ-hydroxy-α-amino acids by hybrid process

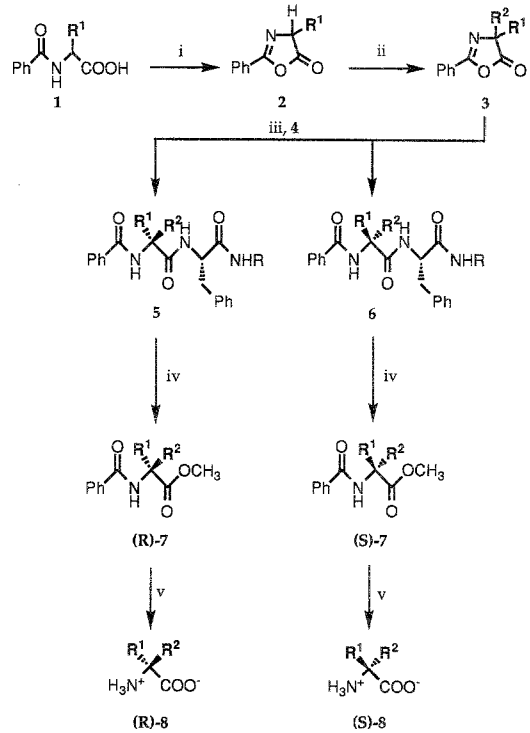
In view of the increasing number of biologically active vinylglycine derivatives which possess hydroxyl group in their side chain, recently much attention has been focused on the efficient synthesis of these compounds. We present herein a convergent synthesis of optically active β-dehydro-δ-hydroxy-α-amino acids

1. The procedure involves a chemoenzymatic hydrolysis of allylic acetate **5** as a key step.

The starting compound, oxazoline **2**, prepared from ethyl isocyanoacetate and α,β-unsaturated aldehyde, was transformed into the β-acetoxyallylglycine derivative **4**. The Pd(II)-catalyzed allylic rearrangement of **4** afforded (*E*)-allylic acetate **5**, which then treated with lipase (Amano P) to give optically active **6** {(*5R*)-**6b** ~ **6d**} in good yield. Finally, the amino ester **8** was enzymatically (chymotripsin) hydrolyzed to yield (*2S, 5R*)-**1** in moderate yield.



1-8	R
a	H
b	Me
c	Et
d	n-Pr



Scheme. **i:** DCC,CH_2Cl_2; **ii:** NaH, DMF, R^2-X; **iii:** L-PheNHR (4), NMP, 50–80°;
iv: CF_3SO_3H, MeOH, 50–80°; **v:** aq, HCl/dioxane, 100°.

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Synthesis, properties and applications of functionalized, optically pure α,α-disubstituted amino acids

A novel versatile synthesis of α,α-disubstituted amino acids starting from recemic N-benzoylated amino acids **1** (*Scheme*) has been developed.

The key α-alkylation of 4-monosubstituted-2-phenyl-1,3-oxazol-5(4H)-ones **2** to the 4,4-disubstituted-2-phenyl-1,3-oxazol-5(4H)-ones **3** has been reinvestigated and more convenient and reliable conditions (*Scheme*, ii) have been found. The (rac)-4,4-disubstituted azlactones **3** were then treated with a optically pure amine **4**, which was easily obtained from Boc-L-Phe, to yield the separable diastereomeric peptides **5** and **6**. Selective amide cleavage using triflic acid in methanol gave in high yields the optically pure α,α-disubstituted amino acids (R)-and-(S)-**8**.

Among the various α,α-disubstituted amino acids synthesized so far by this method, the synthesis and conformational properties of (R)-and-(S)-α-methyl aspartic acid (R^1 = CH_2COOH, R^2 = CH_3) and (R)-and-(S)-α-methyl glutamic acid (R^1 = CH_2CH_2COOH, R^2 = CH_3) and their potential use as N-terminal helix stabilizing templates will be discussed.

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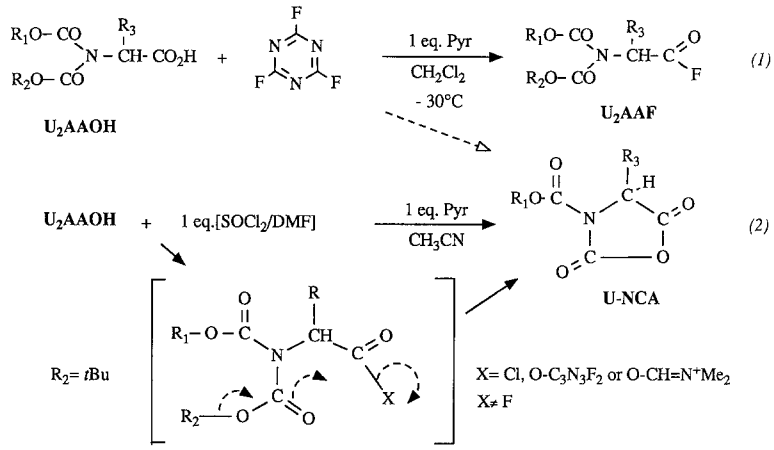
CNRS-CERCOA, Thiais, France

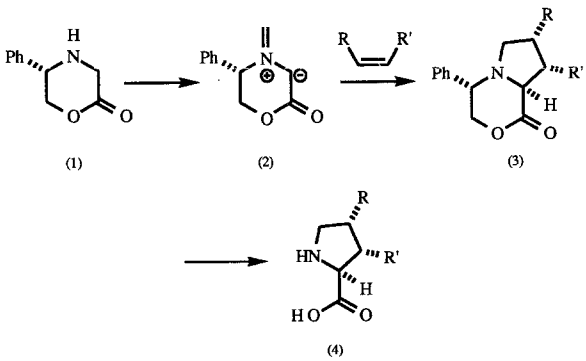
Synthesis and use of some functionalized aminoacids

In order to obtain peptide analogues containing a central pyrrolid bond, as potential mechanism-based inhibitors of the HIV-1 proteinase, activated derivatives of amino acids possessing easily removable N-protecting groups and devoid of an exchangeable NH hydrogen were required. Treatment of a N,N-*bis*(Boc) amino acid pyridinium salt with cyanuric fluoride at low temperature furnished the corresponding *bis*(Boc) amino acid fluoride (Boc_2AAF; *eq. 1*). Use of the Vilsmeier reagent, instead of the cyanuric fluoride, led to a N-Boc amino acid N-carboxyanhydride (Boc-NCA; *eq. 2*). From a mixed N-Z,N-Boc amino acid salt a N-Z,N-Boc amino acid fluoride and a Z-NCA were respectively obtained. The very sensitive Young test showed that during the coupling of the N-benzoyl-L-Leucine N-carboxyanhydride or the N-benzoyl N-Boc-L-Leucyl fluoride with ethyl glycinate no significant racemization was observed. Owing to the high electronegativity and the small size of the fluorine atom, the *bis*(urethane) amino acid fluorides (U_2AAFs) are efficient acylating agents for amines and pyrrole anions. A series of Boc and Z-NCAs has been prepared with a high degree of chemical and optical purity without the use of phosgene and the unstable *tert*-butyl chlorocarbonate.



Synthetic approaches to functionalised pyrrolidines

This research concerns the application of a [3 + 2] cycloaddition reaction to the synthesis of chiral functionalised pyrrolidines, and attempts to elaborate these to a class of organic compounds known as the kainic acid family. Kainic acid and closely related congeners – domoic acid and acromelic acid – have attracted considerable interest due to their potent insecticidal, anthelmintic and neuroexcitatory properties. The morpholinone (**1**) reacted with paraformaldehyde to give the chiral, stabilized azomethine ylid (**2**) which was trapped by a dipolarophile to give cycloadducts of type (**3**) (see scheme below).



The morpholinone (**1**) was reacted in this manner with a range of dipolarophiles. NOE difference experiments have permitted the assignment of the *endo*- and *exo*-stereochemistries of the adducts obtained. In addition, three structures have been confirmed by x-ray crystallographic analysis.

Electron-poor alkenes (eg where R is a carbonyl functionality) gave higher yields than electron rich alkenes (eg where R is a phenyl group). In all the reactions discussed there was complete facial selectivity with respect to the azomethine ylid; approach of the dipolarophile was always from the face opposite to the bulky phenyl group of the ylid. The observed regioselectivities were, in general, what was expected from application of FMO theory. The dominant stereochemistry of addition was *exo*.

A number of these cycloadducts were then deprotected by hydrogenolysis to give the free amino acids (**4**). The elaboration of these products towards kainic acid, and related structures, is currently under review.

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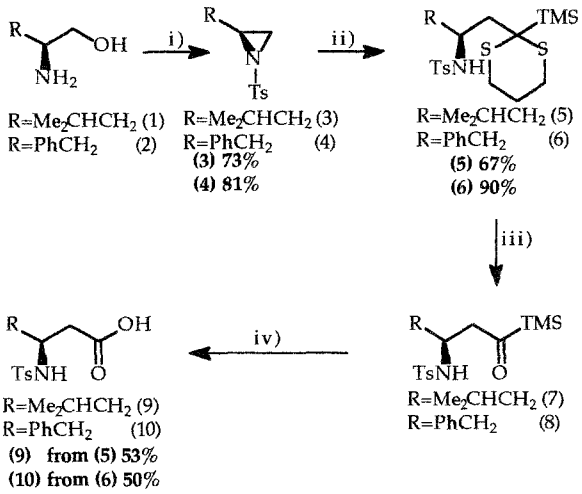
The stereoselective synthesis of β-amino acids

Introduction. The majority of synthetic methods that have been reported for the synthesis of β-amino acids involve the use of an external chiral agent to confer enantioselectivity in the reaction. Few methods are known which make use of the innate

chirality of α-amino acids. Our work has been involved with the synthesis of β-amino acids from β-amino alcohols, which are themselves readily available from α-amino acids.

Results. Our route of β-amino acids is shown below.



i) 2TsCl, $5Et_3N$, 2DMAP, CH_2Cl_2, room temp; ii) 2-TMS-1, 3-dithiane anion, −78°C to 0°C; iii) $HgCl_2$, $CaCO_3$, HgO, acetone, reflux; iv) H_2O_2, NaOH, 40°C.

The ring opening reaction of the aziridine (3) or (4) at −78°C occurs stereoselectively at the least hindered secondary position of the aziridine to yield dithiane (5) or (6) in good yields. Removal of the dithiane moiety, followed by treatment of the acyl silane (7) or (8) thus produced with alkaline H_2O_2 produces the N-protected β-amino acids (9) and (10) in good overall yields.

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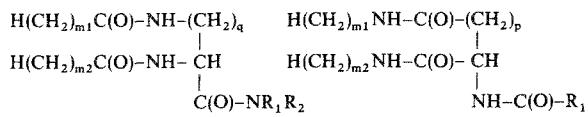
Synthesis and evaluation of non ionic amphiphilic compounds from amino-acids: Molecular mimics of lecithins

Amphiphile molecules are compounds of great scientific and practical interest due to their multifunctional properties as surfactants, emulsifiers, membrane-forming compounds .... They can form a wide variety of supramolecular aggregates closely correlated with their chemical structures and ionic character.

Monodisperse non-ionic surfactants based on Lysin, Aspartic or Glutamic acid were synthesized. These compounds containing two hydrophobic chains and one or two polyoxyethylene glycol chains with a methoxy group capping the terminal hydroxyl function could be regarded as "Non Ionic Molecular Mimics of Lecithins" due to their structural resemblance to lecithins.

The chemical structure of these amphiphiles is as follows:



-Lysine(K) q = 4; R_1 = H or $(C_2H_4O)_2-CH_3$; and $R_2 = (C_2H_4O)_{2n}CH_3$; Code: m1m2**K**02 or m1m2**K**22

-Aspartic Acid(**D**) p = 1; $R_1 = CH_2O(C_2H_4O)_{n1}-CH_3$; Code: m1m2**D**n1

-Glutamic Acid(**E**) p = 2; $R_1 = CH_2O(C_2H_4O)_{n1}-CH_3$; Code: m1m2**E**n1

The preparation is based on trifunctional α-amino acids such as L-lysine, L-aspartic acid and L-glutamic acid which are modified with fatty acids or amines from 6 to 12 carbon atoms. The non-ionic group is a monodispersed polyoxyethylene linear chain prepared in our lab. The synthesis of these molecules has been carried out in two or three steps with an overall yield of (50–60)%.

The physico-chemical and toxicology study reveals that they are interesting amphiphile structures with biological applications as biocompartible surfactants.

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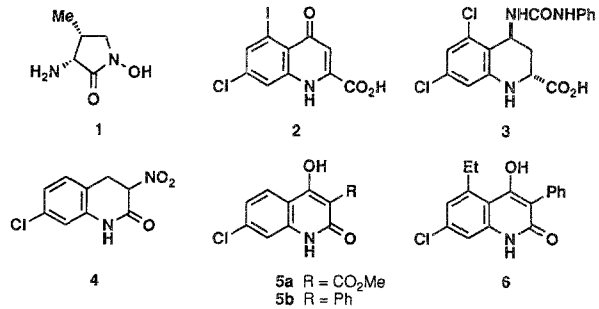
Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex, United Kingdom

Design of brain-penetrating glycine-site N-methyl-*D*-aspartate receptor antagonists

Antagonists acting at the glycine modulatory site of the N-methyl-*D*-aspartate (NMDA) subtype of excitatory amino acid receptor offer potential for the treatment of cerebral ischaemia, epilepsy, head injury, neurodegenerative diseases and schizophrenia. Studies with the prototypical glycine-site partial agonist, L-687,414 (**1**), have shown anticonvulsant and neuroprotective properties, but existing antagonists, such as 5-iodo-7-chlorokynurenic acid (L-683,334, **2**) and L-689,560 (**3**), are severely compromised by poor *in vivo* activity following systemic administration. Thus **2** and **3** have high *in vitro* affinity for the glycine site (IC_{50} values for inhibition of [^{3}H]-L-689,560 binding to rat brain membranes are 0.014 and 0.004 μM respectively), and are active in blocking audiogenic seizures in DBA/2 mice after intracerebroventricular (I.c.v.) but not intraperitoneal (i.p.) administration (ED_{50} values for **3** are 6 μg/mouse i.c.v. and >100 mg/kg i.p.). We have sought to improve *in vivo* properties by synthesising analogues of **2** and **3** in which the highly polar anionic carboxylic acid group, which is likely to limit brain penetration, is replaced by alternative acidic bioisosteres.



Using compounds **2** and **3** and related antagonists we have developed a pharmacophore for the glycine-site and used this to design benzolactams **4–6** as potential acidic, non-carboxylic ligands. These compounds were found to retain *in vitro* affinity and show significantly improved *in vivo* activity in comparison with **2** and **3**. Thus the α-nitro lactam **4** (L-698,544) has the expected acidity (pKa 5.8) and is an effective carboxyl mimic with an IC_{50} of 0.41 μM and ED_{50} of 13.5 mg/kg i.p. Although the 4-hydroxy-2-quinolone ester **5a** (L-695,902) has the required acidic properties (pKa 4.4) it displays modest affinity (6.5 μM) but has remarkably good potency *in vivo* (ED_{50} 12.5 mg/kg i.p.). The 3-phenyl derivatives **5b** (L-698,532, pKa 5.4) and **6** (L-701,315) have considerably improved affinities for the glycine-site (IC_{50} values of 0.17 and 0.007 μM respectively) showing that the α-phenyl lactam moiety can act as a novel glycine bioisostere. The enolic β-dicarbonyl moiety in **5a** can be considered as a vinylogous carboxylic acid possessing much improved ability to cross the blood-brain barrier.

J. Hiebl

Hafslund Nycomed Pharma AG, Nycomed Drug Research, Linz, Austria

Synthesis of diaminosuberic acid derivatives

Replacement of the cystine disulphide linkage in cysteine with CH_2CH_2 gives with nonproteogenic amino acid, 2,7-diaminooctanedioic acid (2,7-diaminosuberic acid). This amino acid is stable to reduction in biological media. Synthesis of various protected derivatives of diaminosuberic acid useful in peptide synthesis is presented.

Synthesis of protected diaminosuberic acid by dimerisation of photochemically generated radicals was investigated. In a one pot procedure BOC–Glu–OBzl was transformed to the thiohydroxamic acid derivative (Barton ester) which was then exposed to a 500W lamp. The yield of this reaction is very low (< 10%).

Synthesis of different protected diaminosuberic acid derivatives was successful using the Kolbe-electrolysis of glutamic acid derivatives. Some side products of the Kolbe reaction were isolated and characterised. The extent of racemisation during the electrochemical dimerisation was studied.

Finally, polyfunctional protected diaminosuberic acid derivatives were selectively deprotected yielding very useful starting materials for the synthesis of peptides of pharmaceutical interest. In addition, some results of the synthesis of 2,5-diaminohexanedioic acid derivatives are presented.

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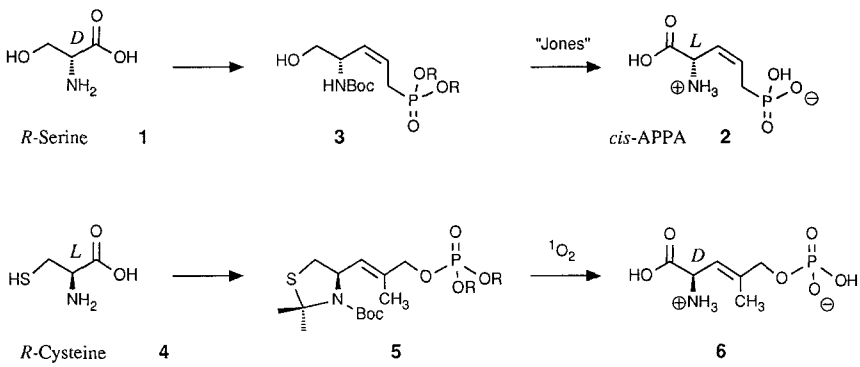
Central Research Laboratories, Ciba-Geigy AG Basel, Basel, Switzerland

Synthesis of unusual amino acids from serine and cysteine

Despite numerous elegant methods for the preparation of amino acids in enantiomerically pure form, there is still a need for improvements, especially when complicated and sensitive structures with β,γ-unsaturated or aromatic sidechains are concerned. By converting *R*-serine **1** to *S*-amino acids (*e.g. cis*-APPA **2**) manipulations in the presence of the carboxylate are avoided. This allows the construction of delicate and sophisticated residues. The crucial oxidation of intermediate **3** at the end of the sequence is, however, often thwarted by low yields and partial recemization. The analogous transformation of *R*-cysteine **4**, on the other hand, leads to thiazolidines **5**, which can be oxidized under much milder conditions by singulet oxygen of *R*-amino acids (*e.g.* **6**).



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Fungal metabolites. Synthesis of a voltage dependent channel forming peptide, hypelcin A-III, from *Hypocrea peltata*

Hypelcin A is a mixture of peptide antibiotics isolated from the fruit bodies of Hypocrea peltata. Isolation and structure elucidation studies performed on this mixture revealed the nine components to be peptaibols, carrying several residues of unusual amino acids, α-aminoisobutyric acid (Aib) and isovaline (Iva), and two proline residues; protected by an acetyl group at the N-terminal and containing the amino alcohol leucinol (Lol) or isoleucinol at the C-terminal. It has been shown that hypelcin A, has uncoupling activity on liver mitochondria, induce permeability change of phosphatidylcholine bilayers, and cause fusion of egg yolk L-α-phosphatidylcoline small unilamellar vesicles. Thus it can be concluded that hypelcin A has membrane modifying properties and voltage dependent channel forming activity

like alamethicin, and its components may serve as models for studying biomembrane functions.

Due to the low abundance of these compounds in the natural source we planned to make the total synthesis of the components of hypelcin A, in order to confirm the structures by comparison with the synthetic one, and for further investigation of the above mentioned bioactivities on the basis of structure-activity relationship.

This report describes the total synthesis of hypelcin A-III, which has the following primary structure and is one of major components of hypelcin A:

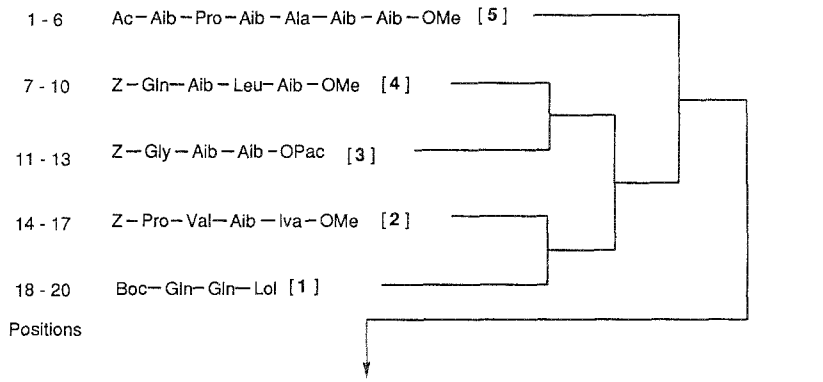
Ac-Aib^{1}-Pro^{2}-Aib^{3}-Ala^{4}-Aib^{5}-Aib^{6}-Gln^{7}-Aib^{8}-Leu^{9}-Aib^{10}-Gly^{11}-Aib^{12}-Aib^{13}-Pro^{14}-Val^{15}-Alb^{16}-Iva^{17}-Gln^{18}-Gln^{19}-Lol^{20}.

In this syntesis Iva at position 17 was used as DL-mixture, and solution phase method was used adopting the synthetic strategy initated by our previous work. The five peptides, comprising the amino acids of position 1–6, 7–10, 11–13, 14–17 and 18–20, prepared by step-wise condensation, was coupled by DCC additive method (Scheme-1), to yield the final product.

For protection of the N-terminals, benzyloxycarbonyl (Z) and tert-butyloxycarbonyl (Boc) group were used. On the other hand C-terminals were protected by methylation or phenacylation.

The structures of the peptide fragments were elucidated by mass spectromety, elementary analysis and ^{1}H-NMR techniques after purification either by recrystallization or chromatographic techniques.

The final product [D,L-Iva^{17}] hypelcin A-III, was purified by crystallization, followed by recyle HPLC with a reversed-phase ODS column which gave [D-Iva^{17}] hypelcin A-III, identical with the natural one, and [L-Iva^{17}]. Hypelcin A-III was identified by ion spray mass spectrometry (ISP-MS) and the fragment patterns showed the same amino acid sepuence with the natural one. Also the amide region of ^{1}H an ^{13}C NMR spectra of the pure [D-Iva^{17}] hypelcin A-III and natural product were in good agreement, as well as the physical constants.



Scheme 1

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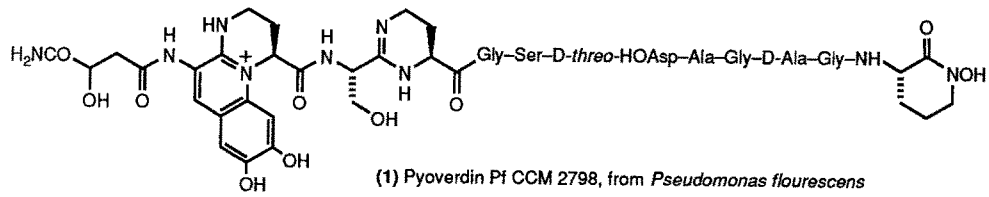
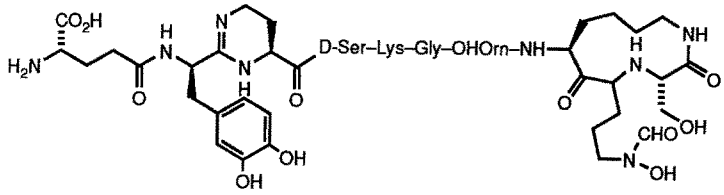
Synthesis of unusual tetrahydropyrimidine amino acids and peptide fragments from bacterial siderophores

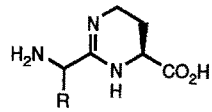
Siderophores are small molecules produced by microorganisms under iron-deficiency whose role is to transport iron into the cells of their parent organism. Fluorescent *Pseudomonas* bacteria are characterized by the excretion of yellow-green water-soluble fluorescent siderophores. These 'pyoverdins', e.g. **1**, possess a fluorescent chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline, bound to a peptidic portion. Unusual 3,4,5,6-tetrahydropyrimidine amino acids **3** can be isolated from some pyoverdins after hydrolysis; they correspond to cyclodehydrated dipeptides of 2,4-diaminobutyric acid. Also excreted are desferriferribactins such as **2**, which do not have a chromophore but do chelate iron(III). It is suggested that the desferriferribactins are biogenetic precursors for the pyoverdins, with the 3,4,5,6-tetrahydropyrimidine built up from tyrosine & 2,4-diaminobutyric acid, *cf.* **3c**, yielding the chromophore.



(1) Pyoverdin Pf CCM 2798, from *Pseudomonas flourescens*

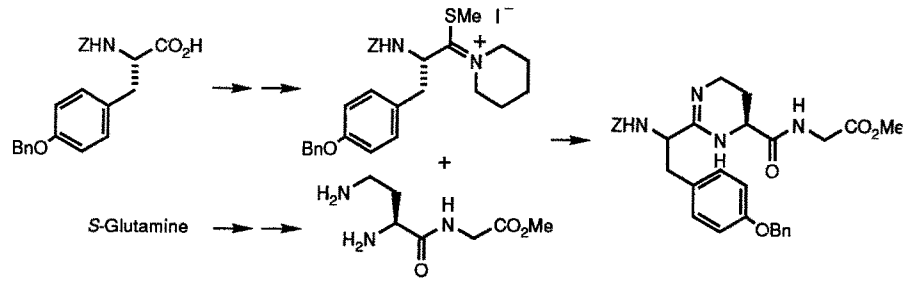


(2) Desferriferribactin ATCC 13525, from *Pseudomonas flourescens*



(3) **a**; R = CH_2OH;
b; R = $CH_2CH_2CONH_2$;
c; R = CH_2-*p*-C_6H_4-OH

We report preparation of the unusual amino acids **3** by condensation of an amino acid (thio)imidate with a dipeptide of 2,4-diaminobutyric acid, e.g. Scheme 1. Use of 2,4-diaminobutyric acid (prepared from S-glutamine in homochiral form) as an amide is necessary to avoid cyclization to produce 3-amino-2-pyrrolidone. This 'protection' is with an amino acid needed for the siderophore peptide, or with Gly–OMe; acid hydrolysis of the tetrahydropyrimidine peptides is known to cleave this C-terminal residue without opening the cyclic amidine, i.e. glycine is a true protecting group!

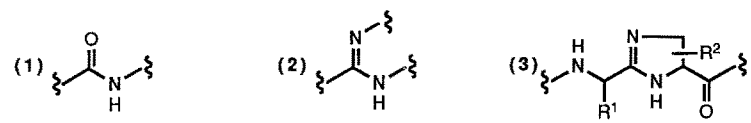


Scheme 1

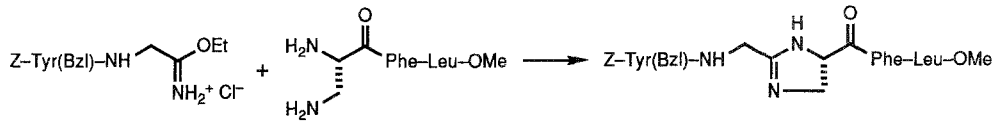
R. C. F. Jones, A. Crockett, and D. C. Rees
Department of Chemistry, University of Nottingham, University Park, Nottingham, United Kingdom

Amidines as peptide bond isosteres: Synthesis of dihydroimidazole amino acids and pseudopeptides

Replacement of crucial amide bonds in bioactive peptides is a recognised strategy of programmes aimed at either the inhibition of proteolytic enzymes or the development of agonists & antagonists at peptide receptors. These alterations can provide 'pseudopeptides' stable to proteolysis, and can impose conformational restraints that help to identify critical conformations necessary for biological activity. The relationship between amide **1** and amidine **2** functional groups led us to propose the 4,5-dihydroimidazole ring as an amide bond replacement to provide pseudopeptides containing the segment **3**.

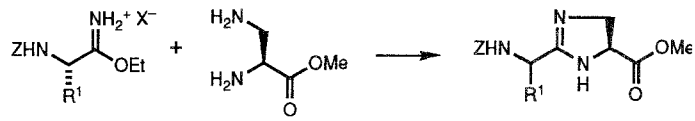


We will report the preparation of pseudopeptides **3** by both convergent and stepwise assembly routes. In the first approach a peptide imidate is condensed with a diaminopeptide, e.g. Scheme 1 to prepare an enkephalin analogue.

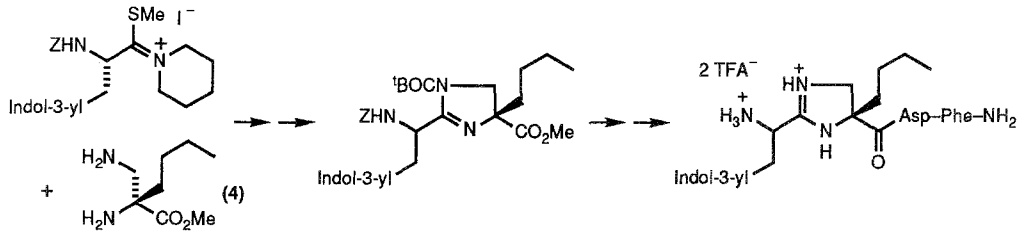


Scheme 1

In the second, and more flexible approach, a dihydroimidazole pseudodipeptide is prepared from an amino-acid imidate and an α,ω-diamino acid, Scheme 2. This unit is orthogonally protected, allowing successive manipulations at C- and N-termini to be accomplished, as shown in Scheme 3 to prepare a CCK-4 analogue. The amidine functional group in the heterocycle must be protected during these modifications. This example also illustrates the use of an optically active α-substituted α,β-diamino acid **4** in these pseudopeptides, and we will report the stereospecific preparation of this diamino acid.



Scheme 2



Scheme 3

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The solid-state catalytic synthesis of tritium labelled amino acids, peptides and proteins

It was analyzed the mechanism, selectivity and kinetics of high-temperature solid-state isotope exchange (HSCIE) – reaction of solid organic compounds with gaseous hydrogen isotopes. We have shown the kinetic dependencies of isotope atoms incorporation and their distribution at different temperatures. The described process is based on the activation of hydrogen isotopes on platinum metals, activated hydrogen spillover in the organic compound layer and hydrogenolysis resulting in the formation of isotope-substituted compounds. It was found that spillover tritium can migrate for considerable distances under conditions of spatial separation of the organic compound and the catalyst in the HSCIE reaction. Intensive isotopic exchange between hydrogen atoms of solid organic compounds and activated hydrogen isotopes takes place in the layer of solid organic compound under spatial separation. HSCIE leads to the isotopic counterbalancing of hydrogen atoms in solid and gaseous phases at temperature higher than 450 K. On reaching isotopic balancing in HSCIE, the isotopic label is evenly distributed over the amino acid molecule regardless of the chemical reactivity of the hydrogen atoms. Experimental data are well described by the isotopomer distribution and by the kinetics of isotopic balancing, calculated from the mathematical model. We have shown that the isotopic exchange between tritium activated on catalyst and the hydrogen of solid organic compound proceeds with a high degree of configuration retention of carbon atoms. HSCIE allows us to introduce the isotope label into organic compounds of different classes. HSCIE gave us the possibility to obtain for the first time uniformly tritium labelled biologically active compounds. At temperatures below 450 K the isotopic exchange proceeds selectively definite positions of organic molecules. It was found out that the ability of hydrogen atoms to exchange under HSCIE conditions can considerably differ from the similar one in liquid state isotopic exchange. It was done quantum calculation of HSCIE of amino acids, peptides and biogenic amines with activated hydrogen. It was discovered electrofilic nature of spillover hydrogen in the reaction of HSCIE. Chemical

modification of the initial compound allows to achieve different patterns of tritium distribution in the labelled product. By tritium NMR we analyzed isotope chemical shifts and spin coupling constants of uniformly tritium labelled compounds. Tritium NMR analysis of tritiated compound isotopomers demonstrated that within this temperature interval only part of the molecules is in the tritium spillover reaction zone, other one is inaccessible for activated tritium. Spillover tritium reacts with amino acids molecules situated near to the catalyst surface at minor temperature. This property of HSCIE makes it possible to use it for structural investigations of proteins and their complexes. I was shown that HSCIE allows to determine proteins situated on the external side of proteins complex. The existing production process makes it possible to synthesize tritium labelled proteins with specific activity 20–30 mCi/mg and saved activity.

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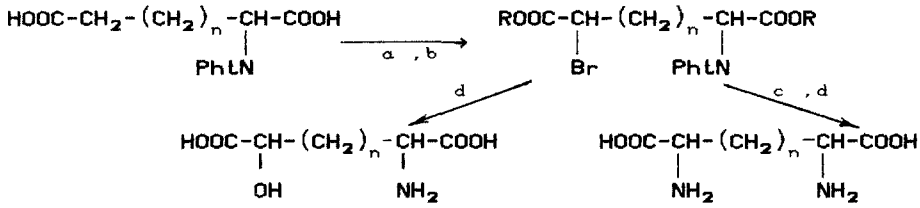
Synthesis of α′-derivatives of aminodicarboxylic acids

Well-known synthetic route to 4-substituted glutamic acids consists in carbanion generation followed by its interaction with electrophile. We have worked out an alternative preparation method for 4-substituted glutamic and 5-substituted homoglutamic (HGlu) acids. N-Phthalylglutamic or N-phthalyhomoglutamic acids were brominated in the presence of PBr_5 followed by treatment with water or alcohols. The synthesized bromoderivatives were subjected to the nucleophilic substitution reaction.



Pht = phthalyl n = 1, 2 R = H, CH_3, C_2H_5

[a] PBr_5, Bt_2, 70°; [b] ROH; [c] PhtN–K; [d] H^+, H_2O

The reaction may result in formation of (2S)- or (2R)-bromoderivatives depending on stereoconfiguration of the parent compound. All possible enantiomers of dimethyl N-phthalyl-4-bromoglutamate were prepared. The proposed approach makes it possible to obtain convenient optically active synthons from aminodicarboxylic acids. The synthesized compound structures were confirmed by the microanalysis data, NMR-spectroscopy and in some cases by X-ray analysis. Hydrolysis of glutamic acid derivatives with (2S)- or (2R)-configuration and subsequent resolution of diastereomeric mixtures led to enantiomers of 4-hydroxyglutamic acid. As result of treatment of (2RS)-halogenderivatives of glutamic or homoglutamic acids with potassium phthalimide followed by chromatografic resolution of diastereomers and their hydrolysis threo and meso diastereomers of diaminoglutaric and diaminoadipic acids have been obtained.

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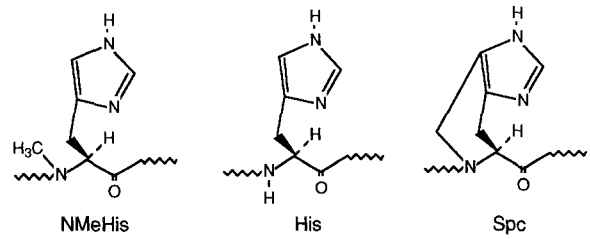
Synthesis and binding activity of conformationally constrained histidine analogs of the δ opioid peptide deltorphin-A

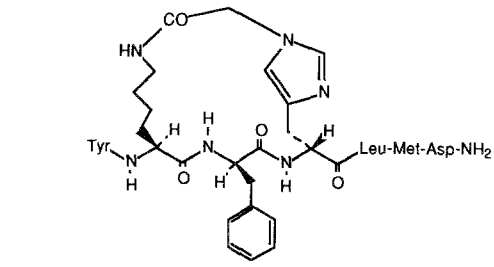
The existence of multiple opioid receptors μ, δ and κ stimulated interest on the receptor binding selectivity of opioid ligands. Recently, highly δ selective peptides were detected in frog skin by cDNA methodology and subsequently isolated and sequenced: deltorphin-A (H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2), deltorphin-B (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) and deltorphin-C (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2). In this investigation, we focused on modifications to deltorphin-A, in particular on the importance of the His^4 residue.

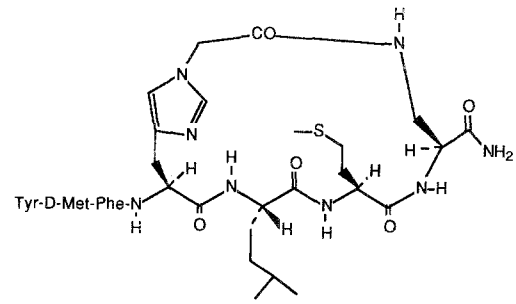
We previously demonstrated its substitution with various amino acids (e.g., Phe, Tyr, Gly, Asp or Lys) drastically modified its inherent high δ affinity ($K_i\delta = 0.41 \pm 0.07$ nM; $K_i\mu = 343.8 \pm 12.8$ nM) and δ selectivity ($K_i\mu/K_i\delta = 839$). Affinity and selectivity are related to the conformation of the peptide which is characterized by a C-terminal folded structure that generates a highly hydrophobic surface above the plane of the N-terminal β-turn.

In order to obtain insight and confirmation of the potential bioactive conformers of deltorphin-A, we prepared a series of conformationally constrained analogs around the His^4 imidazole side chain and evaluated them by use of radioreceptor binding assays for δ and μ sites. First, we adopted two kinds of constraints: (a) local conformational restriction of the peptide backbone by replacement of His with NMeHis and Spc,

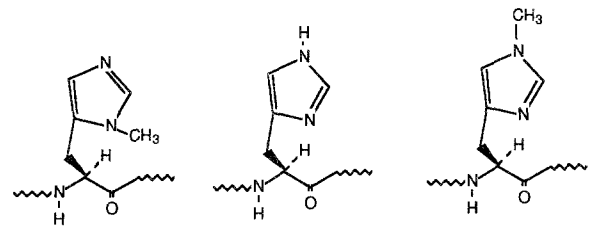


and (b) extensive conformational restriction through peptide cyclization between side chain of His^4 and D-Lys^2 or A_2pr^7, the latter residues being substitutions for D-Met^2 and Asp^7. The cyclic peptides are as follows:





Finally, we tested the effect of modifying the nitrogens in the imidazole ring of L- and D-enantiomers of His[4] by side chain alkilation to yield the corresponding His (1-Me)[4] and His (3-Me)[4] analogs of deltorphin-A:



J. Pospíšek, T. Barth, J. Velek, I. Svoboda, V. Kašička, K. Ubik, I. Bláha, and V. Černá

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N-Methyl-D-phenylalanine: synthesis and use in peptide analogues

In the course of our long-term project concerned with the synthesis of biologically active peptide analogues containing phenylalanine, we decided to incorporate a modified phenylalanine residue into the peptide. The amino acid was modified by N-methylation. The introduction of an amino acid with such a modification should alter the conformation of the peptide in the region of the peptide bond formed by N-methylated phenylalanine and could result in higher metabolic stability of the peptide. N-Methyl-L-phenylalanine and its protected forms have already been described, whereas the preparation of D-stereoisomer has not been reported. We synthesized N-methyl-D-phenylalanine protected by a Boc- or Cbz group: the free amino acid was liberated from the latter. The protected (Boc.Cbz) D-phenylalanine was treated by methyl iodide in the presence of natrium hydride. The dicyclohexylamine salt of Boc-N-methyl-D-phenylalanine was prepared in the yield of 63%. For the formula $C_{27}H_4N_2O_4$ the composition was calculated; 70.4% C, 9.63% H, 6.08% N; found 70.58% C, 9.63% H, 5.35% N. The amino acid was further purified by RP-HPLC. Cbz-N-Methyl-D-phenylalanine, prepared by a similar procedure, was treated by HBr and the product was purified by ionex chromatography on Dowex 50. N-Methyl-D-phenylalanine was obtained in a yield of 93%. For $C_{10}H_{13}NO_2$ (m.w. 179.2) the composition was calculated; 67.01% C, 7.31% H, 7.81% N; found: 66.88% C, 7.26% H, 7.74% N. Four tripeptides with L or D phenylalanine in position 2 and N-methyl-D-phenylalanine or its stereoisomer in position 3 were synthesized by a condensation method using DCCI. The synthesized tripeptides designed to be used in the semisynthesis of the larger peptides were, so far, characterized by elemental analysis and by their mobility on RP-HPLC.

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Synthesis and biological evaluation of pro-drugs of L-tryptophane and L-5-hydroxytryptophane

Deficiency in the serotonine (S-HT) neurotransmission is one of the important features of affective disorders. As L-tryptophane and L-5-hydroxytryptophane are precursors in the biosynthesis of serotonine, these amino acids are frequently used in the treatment of depression.

A major inconvenience, however, in the use of these compounds is their limited brain bioavailability: the oral absorption is low, their extracerebral metabolisation is high and only 1% of the available amino acid is transported through the blood-brain barrier.

We prepared a series of prodrugs of L-tryptophane and L-5-hydroxytryptophane to bypass the above problems. Following derivatives are prepared:
- Pseudotriglyceride esters
- N-alkyl derivatives
- Dihydropyridine derivatives

These compounds have been designed to increase the oral absorption, to decrease the metabolic degradation and to increase the transport through the blood-brain barrier. Their synthesis and some aspects of their biological evaluation will be reported.

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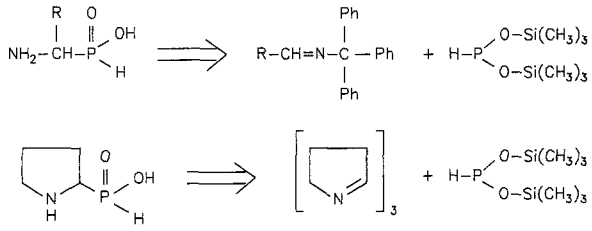
New methods for the preparation of aminophosphinic acids

1-Aminoalkylphosphinic acids are isosteres of amino acids and useful building-blocks in the design of peptidomimetics. They could be considered as transition state mimicking moieties and, hence, be used in the development of enzyme inhibitors.

Unlike 1-aminoalkylphosphonic acid, the synthetic accessi-

bility to these acids is rather limited and only a few methods are described in the literature.

We developed an efficient high yield method for the synthesis of functionalized 1-aminoalkylphosphinic acids. The starting materials were the tritylimine of the corresponding aldehyde and bis(trimethylsilyl)phosphonite. For the synthesis of proline, a pyrrolidine trimer was used as starting material.

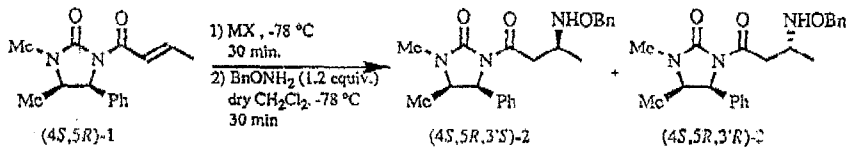


The hydrolysis of the heterocyclic compounds affords the β-amino acid.

Moreover optically active β-amino acids, have been obtained through diastereoselective 1,4 addition of benzylhydroxylamine to α,β-unsaturated imides in the presence of Lewis acids.

Benzylhydroxylamine was added in the presence of various Lewis acids at −78°C to the (4S,5R)-3-butenoylimidazolidin-2-one, obtained from the magnesium salt of (4S,5R)-1,5-dimethyl-4-phenylimidazolidin-2-one and butenoyl chloride.

A turnover of diastereoselectivity was observed when Ti or Al derivatives have been used as Lewis acids.



The (-)-(R)-3-amino-butanoic acid was obtained from major product **3** when $TiCl_4$ was utilized as Lewis acid by rduction of the N–O bond with zinc/copper in acetic acids and subsequent hydrolysis of the imidazolidin-4-one.

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Dipartimento di chimica "G. Ciamician" Università e C.S.F.M.-C.N.R., Bologna, Italy

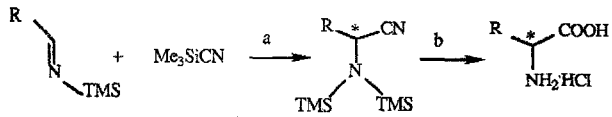
Enantioselective synthesis of α-amino acids starting from N-trimethylsilylimines

The utilization of natural and unnatural amino acids continues to grow. Because of their widespread use, new and versatile methods for the asymmetric synthesis of α-amino acids, especially those which involve catalytic enantiocontrol, are of special practical interest.

During our studies on *N*-metallo imines we tested the possibility to perform a Streker synthesis of α-amino acids through an asymmetric hydrocyanation of *N*-trimethylsilylimines.

To this aim *N*-trimethylsilylimine of benzaldehyde, chosen as representative example, was reacted in different conditions with trimethylsilylcyanide in the presence of binaphthylic C2-symmetry derivatives of zinc and aluminum.

The results obtained on this and other imines will be the care of our contribution.



R = aryl, alkyl
TMS = trimethylsilyl-

a: (R)-1,1'-binaphthalene-2,2'-diol, $ZnMe_2$ or $AlMe_3$
b: HCl conc. reflux

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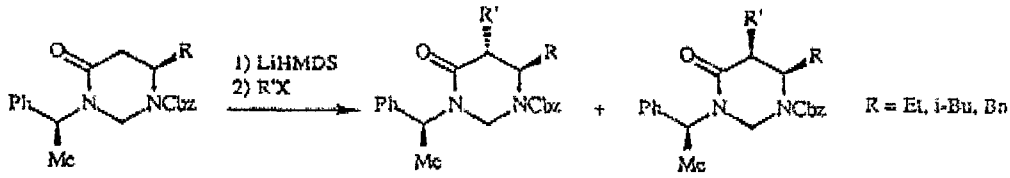
Synthesis of chiral α-substituted β-amino acids and β amino acids

β-Amino acids are receiving increasing interest due to the importance of these compounds as components of natural products and as starting materials in the synthesis of antibiotics. Among the methods for the synthesis of enantiomerically pure β-amino acids, the use of perihydropyrimidin-4-ones as precursors is particularly attractive, in fact the alkylation of these compounds with introduction of a new stereogenic center allows to obtain α-substituted β-amino acids.

The alkylation of lithium enolates of 6-alkyiperihydropyrimidin-4-ones affords 5-substituted derivatives with high trans stereoselection. The addition of alkylating agents was made under a variety of conditions and the trans/cis ratio ranges from 87/13 to 99/1 depending on the bulkiness of R-



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[1] Lomonosov Institute of Fine Chemical Technology and
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Biosynthetic production of stable isotope labeled amino acids using methylotroph *Methylobacillus flagellatum*

To obtain various ^{2}H- and ^{13}C-labeled amino acids the overproducing fermentation of obligate methylotroph *Methylobacillus flagellatum* was stidied using labeled methanol as the only source of carbon and energy.

To detect the level of biosynthetical isotopic enrichment the excreted amino acids isolated from culture media and amino acid mixtures of total protein subjected to acid (6 N HCl and 6 N ^{2}HCl) or alkaline hydrolysis were converted into N-dansyl- (trifluoroacetyl- and benzylhydroxycarbonyl-) methyl esters for mass spectrometric analysis.

According to the data of electron impact mass apectrometry, verified by nuclear magnetic resonance, methylotroph microorganisms are feasible for the preparation of ^{2}H- and ^{13}C- analogs of amino acids due to labeled methanol bioconversion.

Comparative data testify to high efficiency of dansyl derivatization for mass spectrometric analysis of complex amino acid mixtures both of culture media and cell protein hydrolyzates. The developed method can be used for the express analysis of multicomponent amino acid mixtures.

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In vitro biosynthesis of cyclosporin A- and SDZ 214-103 analogues

Cyclosporin A is a cyclic undecapeptide which exerts immunosuppressive, antiinflammatory, antifungal, and antiparasitic activities. It is commonly used in transplantation surgery and in the treatment of autoimmune diseases. To date, the nephrotoxicity of the drug (and partially its immunosuppressivity, too) forbids its wide use in a lot of indications described for cyclosporin A like type I diabetes, psoriasis, malaria and multi drug resistance in cancer chemotherapy. Therefore, search for new cyclosporin A analogues either immunosuppressive and non-nephrotoxic or non-immunosuppressive but still exerting one or more of the other properties of cyclosporin A remains an important attempt.

The structure of cyclosporin A strongly suggests a non-ribosomal biosynthesis mechanism. We have been able to prepare an enzyme fraction from extracts of a high producer strain of *Beauveria nivea* which catalyzes the synthesis of CyA. To our surprise purification yields one single high molecular weight protein chain, which activates all constituent amino acids of CyA in their unmethylated form as adenylates (measured by amino acid dependent ATP/pyrophosphate exchange) and binds these covalently as thioesters. At this stage, *N*-methylation of the amino acids 1, 3, 4, 6, 9, 10, and 11 takes place, followed by peptide bond formation and cyclization. Thus, at least 40 reaction steps are carried out by one polypeptide chain.

In 1988, the structure of a new cyclic peptolide, named SDZ 214-103, and produced by the fungus *Cylindrotrichum* Bonorden was published, which exerts similar biological activities to cyclosporin A ([L-threonine, L-leucine, D-2-hydroxyisovaleric acid]CyA). We were able to prepare an enzyme fraction from crude extracts of the mycelium of *Cylindrotrichum* Bonorden, which is capable of synthesizing peptolide SDZ 214-103 *in vitro*. Cyclosporin A is no reaction product of the peptolide synthetase and cyclosporin synthetase does not synthesize peptolide SDZ 214-103 *in vitro*. Peptolide 214-103 synthetase is clearly distinct from cyclosporin synthetase; however both enzymes have in common the large size and as far as studied the principal biosynthetic mechanism.

The molecular masses of cyclosporin synthetase and peptolide SDZ 214-103 synthetase have been determined by 3% SDS-PAGEs and cesium chloride density gradient centrifugation in an analytical ultracentrifuge to be about 1.4 MDa and 1.38 MDa, respectively.

We were able to establish an *in vitro* synthesis system for cyclosporins at suboptimal temperature. Since for new cyclosporins no references exist, it was necessary to produce sufficient quantities for structural proof. Preliminary structural proof was done by fast atom bombardment mass spectrometry. A second criterion to confirm a cyclosporin structure is the immunosuppressive activity of this class of cyclopeptides. This was done in an *in vitro* system. All new cyclosporins so far synthesized *in vitro* exert immunosuppressive activity in *in vitro* assays.

The substrate specificity of both related enzymes will be discussed and it will be shown by several examples that both enzymes strongly differ in their substrate specificities not only for position 8 (amino versus hydroxy acid), but also for most of the other sites. Even position 1, which recognizes in both enzymes the unusual amino acid Bmt, has not the same specificity in both enzymes.

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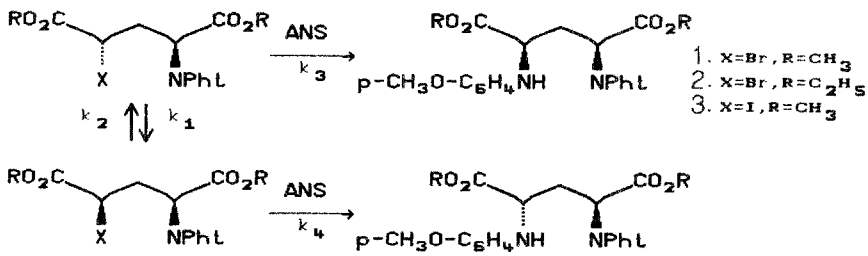
Diastereoselective substitution of halogen in 4-halogeno-N-phthalyl-L-glutamates

Reactions of dimethyl (2S,4RS)-4-bromo-N-phthalylglutamate with arilamines were found out before to be diastereoselective in relation to threo epimer of the product. Kinetic studies of nucleophilic halogen substitution in substrates (1–3) by anisidine (ANS) in ethanol and acetonitrile were provided to determine stereochemical factors of substitution.



Concentrations of reactants were defined by HPLC. Ratio of stereomeric products was shown to depend on the rates of halogenderivatives epimerisation (k_1, k_2) and relative rates of substitution (k_3, k_4). Substitution proceeded through the close transition state and was accompanied by the Walden inversion.

The reaction rate increased from substrate 1 to 3 and the stereoselectivity (k_4/k_3, Tab.) for 1–3 decreased when ELOH was replaced by CH_3CN. It may be accounted for by the solvation effects of different functions in molecule which changed its conformation. k_4/k_3 ratio was lower for diethyl ester (2) than that for dimethyl ester (1). Similarly k_3/k_3 ratio was lower for iododerivative (3) than that for bromoderivative (1). Our results make it possible to choose suitable conditions providing high yield of desired stereomer.

Substitution rate constants ($+10^3$ s^{-1}), 341 K.

Comp.	Ethanol			Acetonitrile		
	k_3	k_4	k_4/k_3	k_3	k_4	k_4/k_3
1	2,1	10,2	4,9	1,7	7,0	4,1
2	1,9	7,8	4,1	1,5	5,9	3,9
3	2,9	12,7	4,3	2,9	10,1	3,4

K. G. Bhansali[1] and A. M. Kook[2]

[1] College of Pharmacy and Health Sciences, Texas Southern University, Houston, and [2] Department of Chemistry, Rice University, Houston, Texas

Synthesis and identification of 1,2-benzo-8-alanyl-3-phenoxazone and 1,2-benzo-7-alanyl-3-phenozxazoneisomers

By reacting tyrosine with 1-nitroso-2-naphthol in presence of nitric acid, two isomers 1,2-benzo-8-alanyl-3-pheno-xazone (I) and 1,2-benzo-7-alanyl-3-phenoxazone (II) are produced.

Amino acid isomers (I) and (II) have very similar ^{13}C and ^1H NMR spectra. However, this is not uncommon in heterocyclic systems. The analysis of ABX spin system of the C-ring shows that isomer (II) is highly second-order, whereas, isomer (I) is clearly more first order. These effects can be calculated and compared with established substituent chemical shifts (SCS) values. A comparison of the published ^1H NMR spectra of these isomers with similar quinoline and naphthalene derivatives showed similar ABX chemical shift and J coupling effects.

Both isomers were screened for their antiviral/antitumor activities by drug development laboratories at the National Cancer Institute of the National Institutes of Health. Isomer (I) showed antileukemic activity *in vitro* while isomer (II) is inactive.

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[1] University Halle, Institute of Biochemistry, Department of Biochemistry and Biotechnology, Halle, Federal Republic of Germany

[2] Bulgarian Academy of Sciences, Institute of Molecular Biology, Sofia, Bulgaria

Some new canavanine derivatives: synthesis and biological activity

L-Canavanine (2-amino-4-guanidinooxy)butanoic acid) is a non-protein amino acid of plant origin. It is a guanidinooxy analog of L-arginine and is a potent inhibitor of growth of many types of microorganisms, viruses, plants, insects and animal cells. It supresses the formation of the enzymes of arginine cycle. Canavanine acts also as an allelochemical and can be exuded by roots. Here we report the synthesis and the biological effects of some new derivatives of this natural antimetabolite.

During the preparation of canavanine derivatives our major efforts were directed towards obtaining of the C-protected canavanines. The methyl ester of canavanine was prepared by acid-catalyzed esterification. As Nα and N_G-aminoprotection we have used a t-Butyloxycarbonyl group (Boc) which was readily removed by HCl-saturated organic solvent. Using Boc-Cav(Boc)-OMe as a starting substance we have obtained protected amide (Boc-Cav(Boc)-NH_2) and hydrazide (Boc-Cav(Boc)-$NHNH_2$) by ammonolysis or hydrazinolysis, respectively.

Inhibition studies were carried out with plant seedlings (cress and amaranth), tomato suspensions culture, the microorganisms *E. coli pR 55*, *Proteus vulgaris*, *Bacillus subtilis*, *Bac. cereus*, *Sarcina lutea* and *Candida albicans* as well as the enzyme arginase. The most active compound in all test systems was canavanine (C). Canavanine amide and canavanine methyl ester inhibited significantly the growth of tomato cells, and canavanine hydrazids (CH) was inhibitory on plant radicle growth. C and CH were active against *Bac. cereus*, *Bac. subtilis* and *Pr. vulgaris*. The canavanine derivatives were no substrates of rat liver arginase and showed no inhibitoty activity against this enzyme. The lack of substrate properties of the canavanine derivatives for arginase may reflect the lowered antimetabolic potential of these arginine analogs in comparison to canavanine.

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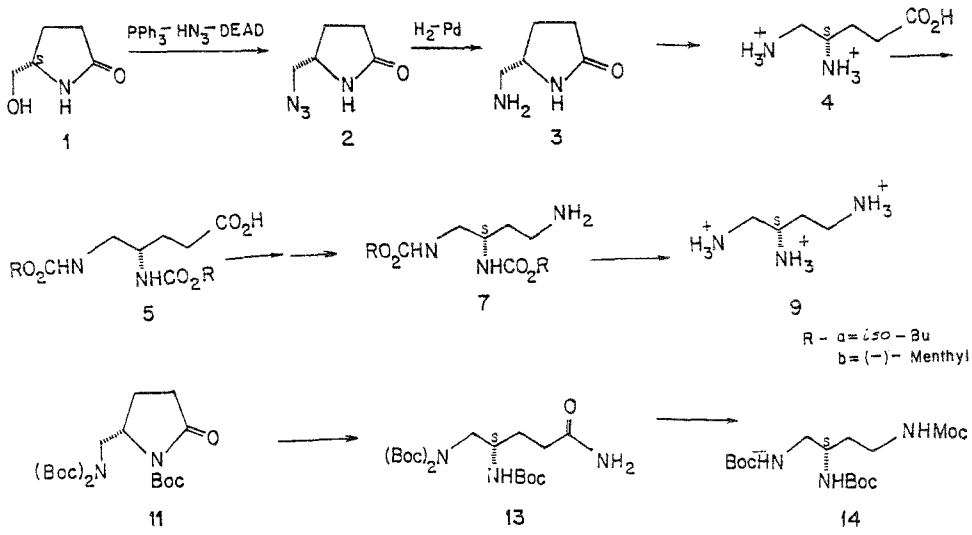
Department of Chemistry, Technion – Israel Institute of Technology, Haifa, Israel

Synthesis of chiral 1,2,4-triaminobutanes

Vicinal diamines and triamines are important as cis-platinum analogues and as intermediates in the synthesis of reagents for radiolabelling and imaging. Previously, we have elaborated a method for racemic 1,2,4-triaminobutanes, bearing preferancially removable protecting groups, by Bamberger ring-cleavage acylation of substituted imidazoles. Now, the chiral 1,2,4-triaminobutanes are reported starting from (*S*)-pyrroglutamic acid.

(S)-5-(Hydroxymethyl)-pyrrolidone (**1**) was converted by the Mitzunobu reaction with hydrazoic acid, to (*S*)-5-(azidomethyl)-2-pyrrolidone (**2**), which was catalytically reduced to amine (**3**). The latter was hydrolysed with 6M HCl to (**S**)-4,5-diaminovaleric acid (**4**) and acylated with *i*-butyl chloroformate or (-)-menthyl chloroformate. The diacylated product (**5**) was converted to acyl azide and submitted to the Curtius rearrangement yielding (*S*)-N^1,N^2-di-*iso*-butoxycarbonyl-1,2,4-triaminobutane (**7a**). **7** was further acylated to 1,2,4-triacyl derivatives or hydrolysed to (*S*)-1,2,4-triaminobutane (**9**). Alternatively, (**3**) was exhaustively *t*-butoxycarbonilated with Boc_2O to (*S*)-5-(di-Bocaminomethyl)-N^1-Boc-2-pyrrolidone (**11**), which underwent selective ring-opening to N^4,N^5,N^5-tri-Boc-4,5-diaminovaleramide (**13**), following Hofmann reaction with NaOBr in MeOH, to N^1,N^2-di-Boc-N^4-Moc-1,2,4-triaminobutane (**14**).



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Solid-phase synthesis of phosphopeptides related to small heat shock protein, HSP27

Establishment of a practical procedure for synthesis of phosphopeptides is strongly desired at present to elucidate the biochemical significance of protein phosphorylation. The most important subject in the synthesis of phosphopeptides was to find suitable phosphate-protecting groups which can be utilized for the Boc strategy. Although the phenyl group was widely used for this purpose, we recently proposed the 4-nitrobenzyl (Bzl(4NO_2)) or cyclohexyl (cHex) groups, which were found to be quite stable to TFA, but removable with trifluoromethanesulfonic acid (TFMSA) and thioanisole in TFA without the cleavage of *O*-phosphono moiety. In the present study, the usefulness of these groups for acid stable phosphate-protection was demonstrated by the solid-phase synthesis of phosphoserine (PSer)-containing peptide related to small heat shock protein HSP27.

Boc-Ser[PO(OcHex)$_2$]-OH employed as a starting material was obtained as fine prisms, while Boc-Ser[PO(OBzl(4NO_2))$_2$]-OH was crystallized only as DCHA salt. From the standpoint of their application to the solid-phase peptide synthesis using automatic synthesizer, the former is more useful than the latter. The synthesis of HSP27-(79–89)-CysNH2 was carried out by use of the standard protocol of the benzotriazole active ester method in the system of software Ver. 1.40 NMP/HOBt *t*-Boc on *p*-methylbenzhydrylamine resin. After completion of the chain assembly, the resin was treated with lM TFMSA-TFA in the presence of thioanisole or pentamethylbenzene as additive to isolate the desired PSer-containing peptide, though it was obtained as the disulfide form.

H-Arg-Ala-Leu-Asn-Arg-Gln-Leu-PSer-Ser-Gly-Val-Cys-NH_2

H-Arg-Ala-Leu-Asn-Arg-Gln-Leu-PSer-Ser-Gly-Val-Cys-NH_2

(HSP27-(79–89)-CysNH$_2$)$_2$

We are currently studying the application of more acid sensitive group such as benzyl than the cHex group to the synthesis of HSP27-(85–94)-CysNH$_2$ and the result will be also discussed.

H-Leu-Ser-Ser-Gly-Val-PSer-Glu-Ile-Arg-Gln-Cys-NH_2

HSP27-(85–94)-CysNH$_2$

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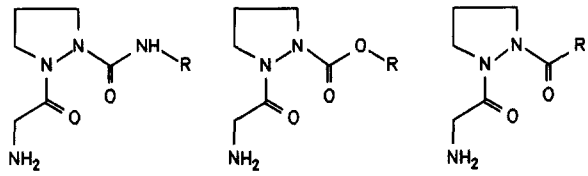
Synthesis and biological evaluation of azapeptides as potential inhibitors of dipeptidyl peptidase IV

Dipeptidyl peptidase IV (DPP IV, E.C. 3.4.14.5) is a serine protease with a specificity for hydrolyzing peptides and proteins with a penultimate proline residue. This peptidase is located on the surface of the plasma membrane of a variety of cells. Among the leukocytes, DPP IV is almost exclusively found on T cells and it's expression is strongly enhanced after T cell stimulation. Although the physiological importance of this enzyme is not completely elucidated, evidence is available that DPP IV plays a significant role in the regulation of the immune response. Hence, inhibitors of DPP IV could be important drugs in diseases where the immune system is involved. Azapeptides are known as mechanism based inhibitors of serine proteases. A series of azaproline analogues have been synthesized and enzymatically evaluated. Gly-Pro being a good leaving group, we prepared Gly-azaPro residues with urea-, carbamate- or amide moieties:



Gly-Pro-4-Me-2-NA was used as substrate to determine the inhibitory activity of these compounds against DPP IV. The synthetic methods and biological results will be reported.

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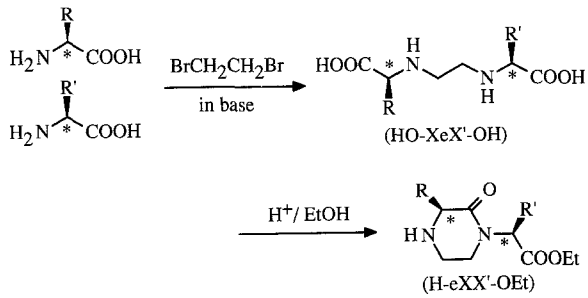
The synthesis of chiral *N,N'*-ethylene bridged dipeptides as the unit of pseudopeptide

For the purpose of studying the structure-activity correlation and the resistance to protease *etc.*, several pseudepeptides have been synthesized. Authors have studied macrocyclic pseudopeptides including the *N,N'*-ethylene bridged dipeptides(eXX) with the chirality. Recently, the covenient synthetic method of eXX (two steps as following scheme) was reported. Furthermore, the eXX' which is constructed from different amino acids could be synthesized and used as the unit of enkephalin analogues. Applying of these methods, authors synthesized several eXX' using (L)-lysine(Z){=K(Z)}, (L)-tyrosine{=Y}, (L)-phenylalanine {=F} and glycine{=G}. As the result, it is found that eK(Z)G-, eYG- and eFG-OEt are obtained in preference to their corresponding retro forms, eGK(Z)-, eGY- and eGF-OEt.



R. Polt, F. Porecca, L. Szabò, J. Ramza, and V. J. Hruby

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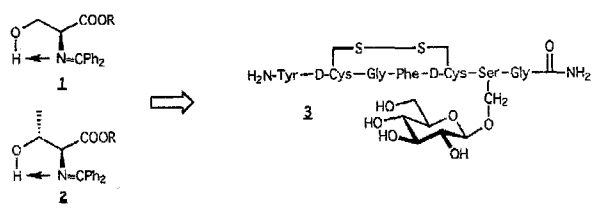
Synthesis of glycosyl-enkephalin analogues which rapidly cross the blood-brain barrier to produce analgesia in mice. An entirely new class of "designer drugs"



The use of D-serine and D-threonine Schiff base esters **1** and **2** permits the selective construction of either α- or β-linked glycosides, as desired. Use of standard Fmoc-based solid-phase peptide synthesis provided a series of O-linked glycopeptide enkephalin analogues **3**. Interperitoneal (i.p.) injection of these compounds into mice produces and long-lasting analgesia comparable to morphine as demonstrated by both the tail-flick and hot-plate assays. Control experiments show that the drugs are acting at opiod receptors in the brain, not peripherally or at the spinal column. It is believed that the glucose transporter enzyme is responsible for the transport process.

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Enzymatic peptide synthesis, using *Pseudomonas aeruginosa* elastase (PsE)

Introduction. Enzyme-catalyzed peptide synthesis, either in aqueous or non-aqueous solvents, has by now been widely experienced. Various proteases have proved to be helpful for such syntheses, mainly serine proteases (trypsin, chymotrypsin, pancreatic elastase ...). One of the limitations of the use of enzymes is that no protease in able to synthesize all the possible peptide bonds. So, every enzyme which allows new possibilities of synthesis enlarges the usefulness of enzymatic peptide synthesis. *Pseudomonas aeruginosa*, while similar to thermolysin, a metalloproteinase from *Bacillu thermoproteolyticus*, possesses a broader specificity. We present here the synthesis of N-protected dipeptides catalyzed by this enzyme.

Results and discussion. Pseudomonas aeruginosa elastase (Nagase, Japan), when added at a 1 μM concentration to a mixture of 1 M Z-Ala and of 1.5 M Phe-NH_2 induced a precipitate which appeared within 10 min. Similar results were obtained with amides of Leu, Tyr, Val, Ile, Ala and Trp, but at a slower rate. No precipitation occured with amides of Gly, Pro, Ser, Arg and Glu, even at a higher nucleophile excess. The N-protected dipeptides were recrystallized and checked for purity. Rates of synthesis were determined by HPLC from initial linear slopes of product versus time curves. The linearity of the rates of synthesis with enzyme concentration was established for the synthesis of Z-Ala-Phe-NH_2 in the range 0–2 μM. For the synthesis of Z-Ala-*X*-NH_2, the rates were in the decreasing order: Phe > Leu > Tyr > Ile > Val > Ala. Their hydrolysis rates were in the same order and the results are consistent with the specificity of the enzyme determined from the hydrolysis of the corresponding tetrapeptides. The main advantage of using PsE is that it allows the coupling of Tyr in position P'_1. Furthermore, in the case of Z-Ala-Phe-NH_2, the rate of synthesis is thirty times higher with *Pseudomonas elastase* than with thermolysin. We could then synthesized N-protected dipeptides with the structure Z-*X*-Phe-NH_2 with X = Ala, Met, Trp, Asp, Ser, Tyr, His. After crystallisation their purity was over 95%, but the yield were quite low. For development of substrates for a conductimetric assay of PsE, we have enzymatically synthesized (either with thermolysin or PsE) Z-Ala-Ala-*X*-Ala-NH_2 with X = Phe, Tyr, Leu. With Z-Ala-Ala-Phe-Ala-NH_2, we could propose an assay of the enzyme in the range 0.2–1 nM. In all cases, standard deviations were lower than 5%.

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Synthesis of phosphono peptides – State of art

Replacement of carboxylic moiety in amino acids by phosphonic group leads to an interesting class of substances – aminophosphonic acids. The term phosphono peptides was coined by Martell in 1975 to designate peptide analogues containing aminophosphonic acids in place of aminocarboxylic acids at any position in the peptide chain.

Introduction of aminophosphonate into the peptide molecule offers several structural possibilities: it may appear as N-terminal or C-terminal amino acid; may be inserted into the peptide chain or phosphonic moiety may be located in a side chain of a peptide.

The synthesis of phosphono peptides of any type presents special problems resulting from unique properties of aminophosphonic acids. First, the phosphonic group posses both electrophilic and nucleophilic properties and thus may interfere with standard coupling reactions yielding unexpected products. Second, esterification of phosphonic moiety, as well as cleavage of phosphonate esters is much more difficult than the same reactions of carboxylic group. This causes that special, selective methods of blocking and deblocking of phosphonate moiety are required.

In this communication the methods for the preparation of all structural types of phosphono peptides will be presented and their scopes and limitations will be discussed.

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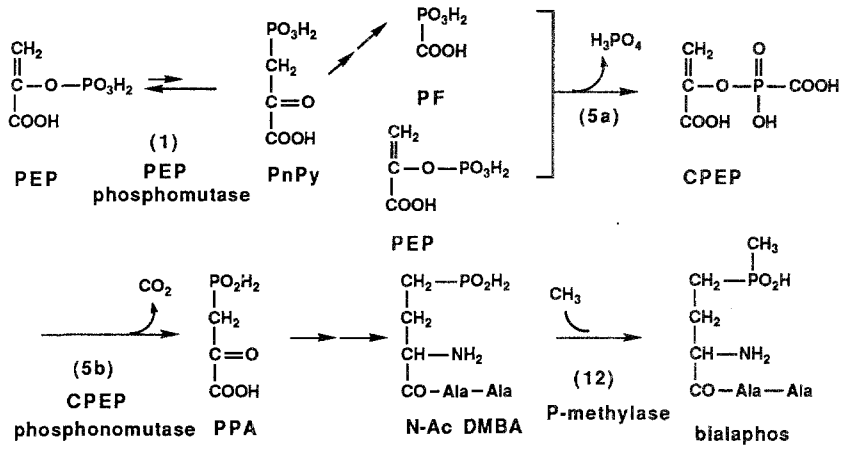
Studies on the biosynthesis of bialaphos, a unique tripeptide containing a C–P–C bond

BA (bialaphos) is a herbicide produced by *Streptomyces hygroscopicus* and is characterized by the presence of a unique C–P–C bond. The biosynthetic pathway of BA has been proved to consist of at least 13 steps. The results will be explained with special emphasis on the three unprecedented C–P bond formation reactions included in the biosynthesis of BA.

One is an intramolecular rearrangement of phosphoenolpyruvate (PEP) to form the C–P bond of phosphonopyruvate (PnPy) catalyzed by PEP phosphomutase (Fig. (1)), another is a carboxyphosphonoenolpyruvate (CPEP) phosphonomutase reaction (Fig. 1 (**5b**)) analogous to that catalyzed by PEP phosphomutase, and the last is P-methylation of phosphinic acid derivatives (Fig. (**12**)).

These two enzymes catalyze very similar reactions, namely the rearrangement of phosphate esters to form a C–P bond. Their enzymatic properties are, however, different from each other, the most striking difference between them being the reaction equilibrium. PEP phosphomutase is characterized as possessing a very strong ability to catalyze the reverse react-ion, *i.e.*, the equilibrium between PEP and PnPy is greatly in favor of the formation of the phosphate ester (PEP).

On the other hand, CPEP phosphonomutase catalyzes irreversibly the rearrangement of CPEP with concomitant decarboxylation resulting in the formation of phosphinopyruvic acid



(PPA). The amino acid sequences of these two enzymes showed a very high similarity each other.

The remaining enzyme catalyzes P-methylation of phosphinic acid derivatives (Fig. 1, (**12**)). This reaction was studies by overexpression of the corresponding gene in *S. lividans*. By the use of the cell free system of this transformant, the methylgroup donor and acceptor for P-methylation were determined to be methylcobalamine and N-acetyldemethylbialaphos (N-Ac DMBA), respectively. So far, P-methylation enzyme has never been reported.

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Synthesis of phosphonic analogues of γ-glutamyl peptides and depsipeptides

Replacement of one or more amino acid residues in a peptide chain with the aminoalkylphosphonic acid moiety to give phosphonopeptide offers numerous structural possibilities. These compounds are interesting not only from structural and chemical

point of view but also because of their biological activity. They exhibit antibacterial inhibitory and neuroactive properties.

Continuing our study on the synthesis of phosphonopeptides and phosphonodepsipeptides we have synthesized several analogues of biologically active natural peptides namely ophthalmic and norophthalmic acids containing phosphonic analogues of glycine and/or glycolic acid. Ophthalmic (γ-L-glutamyl-α-L-aminobutyrylglycine) and norophthalmic (α-L-glutamyl-α-L-alanylglycine) acids isolated from calf lens are competitive inhibitors in the reaction catalysed by glyoxalase.

The synthesis of phosphonopeptides and depsipeptides were accomplished by the 1 + 2 method. The key intermediates were P-terminal di-p-nitrobenzyl phosphonodipeptides and didepsipeptides obtained from N-t-butoxycarbonyl amino acids by coupling with di-p-nitrobenzyl amino and/or hydroxymethylphosphonates followed by removal of the N-t-butoxycarbonyl group. These compounds were coupled with α-benzyl ester of N-benzyloxycarbonyl-L-glutamic acid and next simultaneous deprotection of phosphonic carboxylic and amino groups was achieved by catalytic hydrogenation.

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Enzymatic peptide synthesis in nearly anhydrous medium

If stepwise chemical peptide synthesis is now a well-established procedure, there is room for the development of new methods in the convergent synthesis of large peptides which is based on segment coupling. Enzymatic catalysis, avoiding both the risk of racemization and the need of lateral chain protection during the chemical activation, appears to be a good alternative strategy.

Reverse action of proteases is a popular procedure for peptide bond formation, either under kinetic or thermodynamic control, but due to the presence of water, undesired hydrolysis may take place on the new formed bond or on sensitive site(s) already contained in the fragment peptide sequence(s).

In order to avoid this effect, water content has been minimized and the enzyme (α-chymotrypsin) subjected to modification by amphiphilic reagent (Polyethylene glycol). The resulting adduct soluble in organic solvent has been active in peptide coupling in t-amylalcohol/toluene containing 0.5% water (w/w)

For recycling the biocatalyst, a copolymerisation of acrylic derivatives of both enzyme and PEG has been performed and the resulting gel shown to be an efficient re-usable catalyst of peptide synthesis mediated in t-amylalcohol (containing 1% water). A drastic reaction rate increase has been found by applying ultra-sonic irradiation through the reaction vessel according to a better substrate and product diffusion across the gel. In contrary to the native enzyme, the supported-biocatalyst activity has been preserved

In this poster, we will show our preliminary results concerning a new supported biocatalyst made of trypsin instead of α-chymotrypsin. Interesting results have been obtained specially with O-carboxamidomethyl esters as acyl donors: the enzyme primary specificity for basic residues is lost during the coupling whereas the resulting peptide bond is not subjected to the reverse hydrolysis.

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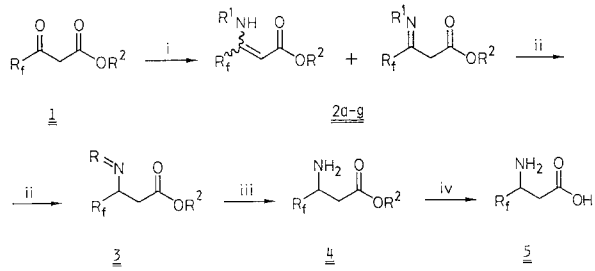
A new entry to racemic and optically active β-polyfluoroalkyl-β-amino acids through biomimetic transamination of fluorinated β-ketocarboxylic esters

β-Amino acids are important natural products and can be found in peptides, terpene, alkaloids. They are also useful building blocks in the synthesis of β-lactam antibiotics. For these reasons the development of new, expedient routes to β-amino acids constitutes an active area of investigation.

In view of the peculiar and useful properties that the introduction of fluorine can impart to biologically active molecules, it becomes clear the importance of fluorine containing β-amino acids as potentially bioactive compounds and probes in methabolic studies.



R_f = polyfluoroalkyl; R^1 = benzylamine, (*R*)- or (*S*)-α-methylbenzylamine; R^2 = alkyl

Scheme. i benzylamine (or (*R*)- or (*S*)-α-methylbenzylamine), benzene, Dowex-50 (H^+-form), reflux; **ii** base; **iii** 2N HCl, Et_2O, 25–30°C; **iv** 6n HCl, 100°C

We would like to report a conceptually new approach to β-polyfluoroalkyl-β-amino acids **5** in racemic or optically active form *via* biomimetic transamination of fluorinated β-keto carboxylic esters **1** with benzylamine (racemic version) or (*R*)- and (*S*)-α-methylbenzylamine (asymmetric version) (Scheme).

We have found that β-perfluoroalkyl-β-ketocarboxylic esters **1** give corresponding enamines **2** in moderate to good yields by treatment with benzylamine under acid catalyses. Unexpectedly, we have found that in the presence of triethylamine these enamines easily undergo two 1,3-proton shifts at room temperature and give corresponding Schiff bases **3** of β-amino esters. When the β-perfluoroalkyl chain is replaced by a β-fluoroalkyl chain (e.g. when a β-trifluoromethyl residue is replaced by a β-difluoromethyl one) either elevated reaction temperatures or the employment of stronger bases (e.g. DBU) are required in order to have a complete conversion of the enamine **2** into the Schiff base of the β-amino ester.

It is possible to conclude that there is a qualitative correla-

tion between the easiness in the prototropic shift of the enamine and the electron withdrawing effect of the alkyl chain in the β-position.

The asymmetric version of the process occurs through the same chain of events with the difference that (*R*)- or (*S*)-α-methylbenzylamines are used instead of benzylamine. The highest asymmetric induction (75% e.e.) was obtained by employing Dabco at 160°C.

Analysis

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Ultra-trace determination of amino acids and γ-glutamyl peptides in single mammalian neurons and microdialysates using optimized argon ion laser-induced fluorescence detection and microcolumn liquid chromatography

Discrete studies of neurotransmitter release, using sampling techniques remains unprecendented owing to the demands on temporal (a few milliseconds) and spatial resolution (the width and diameter of the synaptic cleft is on the order of 20 nm and 0.6 μm, respectively). However, synaptically derived molecules may be monitored in the extracellular fluid after pharmacological treatments or in certain pathological situations using microdialysis. In some situations also intracellular species needs to be monitored in order to unambigously interpret and elucidate the specific role of neuroactive compounds. To successfully analyze the content of single neurons or microdialysates containing pM concentrations of amino acids and peptides, ultra-sensitive analytical techniques are needed.

In the present report, determination of neurotoxic and putative transmitter amino acids and di- and tripeptides in single mammalian neurons and brain microdialysates is demonstrated. O-phthaldialdehyde/β-mercaptoethanol was used as a reagent to form highly fluorescent derivatives which were subsequently separated using microcolumn LC. By elaborate optimization of the high-power argon ion laser-induced fluorescence detection and derivatization conditions, picomolar concentrations could be determined in complex sample matrices without the need for any pre-concentration. Furthermore, different approaches to selectivity-enhanced determination at this low concentration level is presented. Neurons, measuring 10–35 μm in diameter were freshly dissociated from the *Bulbus olfactorius* of rat brain using an enzymatic procedure. The technique described is easy to perform and requires no sub-μl sample volume transfers.

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Department of Paediatrics, Free University Hospital Amsterdam, Amsterdam, The Netherlands

The simultaneous stable isotope ratio analysis of leucine, phenylalanine and keto-isocaproic acid in blood plasma by gas chromatography/negative ion mass spectrometry. Use of pentafluorobenzylesters

In the framework of a study to stimulate the immediate postnatal growth and development of severely intra uterine growth retarded preterm newborns by use of rhGH (recombinant human growth hormone) we can report on stable isotope ratio analyses by gaschromatography/negative ion mass spectrometry and isotope ratio mass spectrometry (IRMS).

For the estimation of protein turnover we developed one rapid procedure for the extraction and derivatization of leucine, phenylalanine and keto-isocaproic acid in human plasma. After a phase transfer derivatization, the keto- and amino acid(s) were analyzed as the pentafluorobenzyl (PFB) esters by ammonia negative chemical ionization gaschromatography/mass spectrometry. Mass spectrometric analyses were performed on a VG 30-253 quadrupole mass spectrometer.

The procedure was used for: quantification based on stable isotope dilution analysis; measurement of isotopic enrichments in human plasma after adminstration of the labelled analogues. The overall sensitivity was sufficiently high to study isotopic enrichments in the range of 0.3%–10% APE using plasma samples of 100 μl. The moment of plasma sampling, during the isotopic enrichment experiments, was established by monitoring the $^{13}CO_2$ production in the expiratory air by IRMS.

This rapid analytical method does not require extensive chromatographic plasma cleanup before derivatization; allows the simultaneous measurement of concentration and isotopic enrichment of leucine, phenylalanine and keto-isocaproic acid using a single 100 μl plasma sample.

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Direct stable carbon isotope analysis of amino acid enantiomers at nanomole levels by combined GC/Isotope Ratio Mass Spectrometry

The stable carbon isotope compositions of amino acids and their respective enantiomers can provide important information with respect to geochemical processes and biosynthetic pathways. This type of research has been hindered, however, by the difficulties encountered for isolation of sufficient quantities (microgram to milligram) of individual compounds for combustion and $\delta^{13}C$ analysis by conventional isotope ratio mass spectrometry (IRMS). The development and refinement of a combined gas chromatography/isotope ratio mass spectrometry (GC/IRMS) method for the direct $\delta^{13}C$ analysis of individual amino acid enantiomers at nanomole levels virtually eliminates all of the requisite preparative, chromatographic steps required for $\delta^{13}C$ analyses of amino acids in complex mixtures (e.g. hydrolyzates) by conventional IRMS. The GC/IRMS system consists of a Hewlett-Packard 5890 gas chromatograph interfaced to a VG PRISM isotope ratio mass spectrometer via a combustion furnace/water trap.

Unlike hydrocarbons, amino acids are not volatile and thus require derivatization (e.g. trifluoroacetyl isopropyl esters) prior to analysis by GC/IRMS. In addition to the introduction of carbon, distinct, reproducible fractionations occur for each ami-

no acid type during derivatization. Derivatization of amino acid standards of known $\delta^{13}C$ composition, however, permits computation of the original, underivatized amino acid $\delta^{13}C$ values in natural samples via an empirical correction for the carbon introduced during the derivatization process. In general, the $\delta^{13}C$ values for underivatized amino acids calculated from the derivative values determined by GC/IRMS are within 0.25 per mil of the original, underivatized $\delta^{13}C$ values determined by IRMS. In summary, the development of GC/IRMS for the direct $\delta^{13}C$ analysis of individual amino acid enantiomers at nanomole levels opens up new avenues of research in Biochemistry and Geochemistry. Several examples in which this new methodology was employed for $\delta^{13}C$ analysis of amino acid enantiomers in natural systems will be presented.

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A new fluorescent chiral derivatizing agent for amines and amino acids

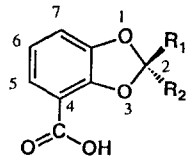
(+)-TBMB carboxylic acid 1 and (+)-TBB carboxylic acid 2 were developed as chiral discriminating agents of amines and alcohols. They were designed to be useful for the enantiomer anlaysis by ^{1}H-NMR, HPLC and circular dichroism (CD). The latter 2 has stronger solvating ability than 1 by replacing the Me group of 1 by H. The utility was evaluated with some amino acids by these three different analytical methods.

Amino acid methyl esters were derivatized in pyridine with the agents in the form of acylchloride or acylfluoride form. A pair of diastereomers thus obtained from (+)-1 and racemic amino acid was analyzed in each method to give following results:

1) For the ^{1}H-NMR analysis, the *tert*-Bu group gave strong singlet diagnostic for the enantiomeric purity of amino acids. *Tert*-Bu signal of D-amino acids was more deshielded than that of L-amino acids except for proline.

2) With normal phase HPLC (silica gel, 5 cm × 0.5 cm ϕ: n-hexane/ethyl acetate), D,L-isomers of amino acids were well separated. By us ing fluorescence detector (Ex. 310 nm, Em. 380 nm), D,L-isomers could be determined in a few pico-molar amount on the column. Particularly, for hydrophobic amino acids (alanine, phenylalanine, leucine, methionine), the separation was accomplished on the TLC plate (5 cm × 5 cm, UV o fluorescent detection). In most of the case D-isomer was eluted faster than the L-isome on silica gel, and this was rationalized by supposing the three dimensional structure of the diasteremers and their interactions with the silica gel face.

3) For the circular dichroism (CD) analysis, the benzoate chromophore of the TBMB acid induced strong Cotton effects selectively for aromatic amino acids like phenylalanine. The sign of the Cotton effects changed reflecting the D,L-configuration. Thus, this approach was found to be useful for the selective detection and D,L-analysis of aromatic amino acids on HPLC-CD analytical system.



	R_1	R_2	
(+)-1	CH_3	*tert*-Butyl	[(+)-TBMB carboxylic acid]
(+)-2	H	*tert*-Butyl	[(+)-TBB carboxylic acid]

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Perspectives on the origins and history of amino acids through compound specific isotope analysis

Investigations of amino acids in terrestrial and extraterrestrial geologic materials have been problematic. This is because it has not been possible to determine with certainty whether amino acids in these materials are entirely indigeneous or, to some extent, reflect the addition of amino acids at later times via exchange with the surrounding environment. We have recently demonstrated that amino acids retain their stable carbon and nitrogen isotopic integrity during racemization. It may, therefore, be possible to establish the origin(s) of amino acids in terrestrial and extraterrestrial materials by comparing the stable isotope compositions of the D- and L-enantiomers of individual amino acids in these samples. The recent development of a combined gas chromatography/isotope ratio mass spectrometry (GC/IRMS) method permits, for the first time, the direct $\delta^{13}C$ analysis of D- and L-enantiomers of amino acids at nanomole levels.

The GC/IRMS analysis of amino acid enantiomers in the Murchison meteorite revealed that these components are significantly enriched in ^{13}C relative to biogenic systems on Earth, thus confirming an extraterrestrial origin. A comparison of these values with the $\delta^{13}C$ values of amino acid enantiomers synthesized in laboratory simulations (e.g. spark discharge) permits a preliminary assessment of the mechanisms for prebiotic synthesis on the meteorite parent body.

The GC/IRMS analysis of amino acid enantiomers in marine and terrigenous fossils and sediments has been used to establish the indigeneity of these compounds and to assess the effects of diagenetic reactions (e.g. hydrolysis) on their respective stable isotope compositions. The results of simulation experiments on the diagenesis of proteins and peptides will be presented and discussed within the context of interpreting the stable isotope record of fossil materials.

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Applications of phenylthiocarbamyl (PTC)-amino acid analyses to the quantitation of protein hydrolysates and free amino acids present in physiological fluids

Reaction of amino acids with phenylisothiocyanate (PITC) yields phenylthiocarbamyl (PTC) derivatives which are subsequently separated by High Performance Liquid Chromatography (HPLC) analysis on reverse phase colums with UV detection at 254 nm. The aim of the present work was to evaluate the linearity, precision and accuracy of the PTC method. A mixed diet (based on soya, corn and barley meals) and chicken excreta, as proteinaceous materials, as well as poultry plasma and muscle, as representative physiological samples, were selected for quan-

titation of amino acids. Amino acid analysis was determined by a Waters HPLC system with an automatic sample injector. Nova Pack columns (150 mm × 3.9 mm I.D. and 300 mm × 3.9 I.D.) were used for hydrolysates and physiological samples, respectively. Derivatized amino acids were eluted by means of a single binary gradient. An internal standard quantitation procedure was used (AAD and NLE for hydrolysates and physiological samples, respectively).

The linearity was evaluated at ten different concentration levels, within a range of response from 0 to about 10^{-2} μmoles/ml for hydrolysates and from 0 to about 10^{-3} μmoles/ml for physiological samples. The correlation coefficients obtained varied from 0.962 (TRY) to 0.999 (ASX), from 0.972 (PRO) to 0.997 (SER), from 0.938 (SER) to 0.994 (GLU) and from 0.960 (LEU) to 0.999 (ALA) for the amino acids examined in diet, excreta, plasma and muscle samples, respectively.

The precision of the injection was calculated as the relative standard derivation of the response after 10 consecutive analysis from the same vial. The overall coefficients of variation (CV) were 1.6 and 2.2% for the hydrolysates and physiological samples, respectively.

The reproductibility of analysis was evaluated by subjecting 11 different individual samples to the entire procedure. The CV ranged from 2.6 (LYS) to 13.3% (PRO), from 5.8 (SER) to 13.2% (PRO), from 2.0 (LYS) to 13.7% (ASX), and from 1.5 (THR) to 39.0% (ILE), for the amino acids in diet, excreta, plasma and muscle samples, respectively.

To assess the accuracy of the method, each sample was spiked with graded levels of internal standard, whose recovery (as percentage) was calculated at the end of the procedure. Values ranged from 74.8 (VAL) to 121.1% (ARG), from 77.4 (LYS) to 114.0% (ILE), from 57.8 (THR) to 119.4% (VAL), and from 66.7 (GLU) to 97.8% (ILE) for the amino acids in diet, excreta, plasma and muscle samples, respectively.

It is concluded that the reproducibility and accuracy of the PTC method are poor for several of the amino acids studied. Further investigation will be required to validate this procedure for complete AA analysis with a wide range of sample matrices.

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An analytical system for estimation of total- and protein-nitrogen in biological materials including a new ultramicro decomposition and an adapted coulometric titration analysis

Introduction. The analysis of total-and protein-nitrogen is of importance and has been achieved mostly with the well-known Kjeldahl procedure. We improved this procedure with particular regard to thermal digestion and final volumetric analysis with the principle aims of optimal adjustment, a miniaturization and simplification of both parts of the analysis to a better practical handling. The absolute principle of the original procedure should be preserved.

Principle and methods. Digestion. A new apparatus for digestion carries out parallel sample decompositions with sulphuric acid using an automatic run off. In this way it simultaneosly develops and controls graduated ranges of temperature in digestion vessels, so that different temperature gradients are produced. These gradients act as valves and promote steam-off processes, or they prevent the removal of the decomposition acid. In such a way the control of thermic processes at high temperatures in concised open vessels is reproducibly possible. Thus the digestion processes are accelerated by physical means only.

Catalytic substances or decomposition salts which especially disturb the electrochemical analysis, need not be used. A loss of the initial volume or of concentrated acid (for digestion) does not occur.

In this way, a faultless analysis of nitrogen immediately after the decomposition is possible and the expensive destillation step of the original Kjeldahl procedure can be avoided.

Volumetric analysis. The nitrogen in a deflned part of the neutralized mineralizate is estimated by means of the hypobromite procedure using an automatic titrator. The volumetric analysis is done by means of a coulometric titration. The automate indicates biamperometrically the redox processes in the measuring cell, currently processes the measuring signals and controls without assistance by means of regulating algorithms the titration until the equivalence point is reached. The dead-stop-principle finished the reaction.

Results and discussion. For the digestion normal reagent vessels are sumcient. They are resistant to concentrated sulphuric acid up to a temperature of +420°C. In these the solid or fluid material (5 ... 1000 μl) of biological origin is placed. For decomposition sulphuric acid (96%, 100 μl) and for oxidation hydrogen peroxide (30%, 100 μl) has been used.

The apparatus for digestion contains 30 reagent tubes and uses various ranges of temperature up to a maximum of +420°C. The total mineralization is finished in a time of 2.5 h. The initial volume of concentrated sulphuric acid was maintained at a volume of 100 μl. The volume of water for cleaning the reaction tubes reached with 1000 μl a minimum.

The mineralizate was neutralized by sodium hydroxide and a deflned part of this has been titrated. The titration was done in a special cell, which were filled with carbonate buffer (pH 8.6) and the reactive element bromine. The value of the analysis could be converted immediately and stoichiometrically in the nitrogen-value, because the chemical reaction equations, the electrode gross-reaction and the Faraday's law have been absolute validity.

The total procedure for analysis of nitrogen represents in its combination of digestion and electrochemical analysis a physical method, which is absolutely adjustable and does not need any chemical calibration. The method comprises a broad concentration range of nitrogen of 50 g/l to 0.5 mg/l, by a lower limit of 0.25 μg nitrogen. The relative error in series is 1%. A comparison of this method with the original Kjeldahl procedure shows the complete correlation of the nitrogen values.

This analytical system can play in its totality or in its partial methods like digestion and electrochemical processes an important part for the analysis of trace elements, too.

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Determination of 3,5-dinitrobenzoylated taurine and hypotaurine by high-performance liquid chromatography

A simple method for the determination of taurine and hypotaurine by high-performance liquid chromatography was developed. After biological samples were deproteinized, taurine and hypotaurine were converted to 3,5-dinitrobenzoyl derivatives by reaction with 3,5-dinitrobenzoyl chloride in the presence of triethylamine. For analysis of these derivatives, a C18 reversed-phase column was used, and the absorbance at 254 nm was recorded. Taurine and hypotaurine were derivatized in the yield

of 91.4 ± 3.3 and 85.6 ± 2.6%, respectively. The calibration curves were linear between 4 and 500 μM, and the recoveries of taurine and hypotaurine added to biological samples were quantitative. Contents of taurine in human urine, serum, rat liver and heart were determined to be 0.92 ± 0.33 μmol/mg of creatinine, 159 ± 19 μmol/ml, 9.77 ± 0.44 and 22.0 ± 2.2 μmol/g of wet weight, respectively. Hypotaurine contents in rat liver and heart were 0.13 ± 0.02 and 0.23 ± 0.02 μmol/g of wet weight, respectively. This method may be applicable to the assay of cysteinesulfinate decarboxylase (EC 4.1.1.29).

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Simultaneous determination of amino acids and phosphoamino acids by HPLC at Picomole level

A reversed-phase high performance liquid chromatographic method is described for the separation of phosphoamino acids simultaneously with non-phosphorylated amino acids. The method involves precolumn derivatization of amino acids with dimethylaminoazobenzene sulfonyl chloride (DABS-CI) followed by separation of all the DABS-derivatives by HPLC. The separation was carried out on a Supelcosil LC-18 T column (15 cm × 4.6 mm I.D.), 3 μm particles, at room temperature using as solvent A, 25 mM potassium dihydrogen phosphate buffer at pH 7.1 and as solvent B, acetonitrile and methanol 70:30.The detection of DABS-amino acid derivatives was performed in the visible region, at 436 nm, with a sensitivity at picomole level. The procedure allows the baseline separation of about 40 DABS-amino acids and by-products in 38 minutes. Phosphoserine, phosphothreonine, and phosphotyrosine are resolved within the first 14 min. The method allows the determination of phosphorylated amino acids at picomole level even in presence of a great excess of other amino acids.The sensitivity can be further increased using a narrow-bore column. The method, in conjunction with an appropriate hydrolysis procedure which permitsto recover the amino acids in their phosphorylated state, makes it possible to follow the phosphorylation and dephosphorylation of proteins detecting the specific amino acid residues involved without the need to use radioactive or immunodetection methods. This could be of great utility in studying protein phosphorylation this process being one of the main regulatory mechanisms for controlling of intracellular function in physiological and pathological states.

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Efficiency of the RP-HPLC analytical method for determination of essential amino acids in some edible insect species

The acquaintance of the total sum of essential aminoacids which rate the quality of proteins is absolutely necessary in order to know the nourishing value of food. Nutrition is centered in the disponibility or lack of proteins in the daily diet. Now a days, a large quantity of protein supplies come from conventional foods which are very expensive, specially those derivated from animals, therefore a high percentage of population do not have access to consume them. Nevertheless there are nonconventional sources, which exist as part of the everyday diet of lots of communities all over the world. Some of this sources are certain insect species. Therefore, it is necessary to know the nutritive value of this renewable natural resource. Insects have those aminoacids which are essential to human beings. They can be qualified as "proteinic concentrates" due to their high proteinic content and to their high digestibility.

A faster and more sensitive method than conventional ion-exchange chromatography was required to evaluate the protein composition in edible insects samples in order to meet their quality as food complement according to their essential aminoacids content. Reversed-phase high-performance liquid chromatography (RP-HPLC) is recognized as a powerful method for determination of amino acids. This paper describes the method developed for this purpose. A liquid chromatographic "system gold" from Beckman was used. The O-phthaldialdehyde precolumn derivatization was performed manually and the adducts were detected by fluorescence. Applying optimized conditions (using gradient elution) the nine essential amino acids can be separated in less than 43 minutes, in the lower pMOL 3–10 range, in a 3 uM ultrasphere XL-ODS column. Quantitative analysis of amino acid microamounts in hidrolized samples of insects, by external standard method, gave highly reproducible results with a mean coefficient of variation of less than 2% and the correlation coefficient of 0.9999(r). The kindness that this technique offers, compared to ion exchange technique are: reduced analysis times, best resolution, less sample amount, less reagents amount and hence, less expenses. These advantages allow to have more analysis with less resource, this means a grater knowledge in this topic can be reached, which is very important in countries, like ours, where the living economy is prior.

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The analysis of amino acid enantiomers by ligand-exchange chromatography

Ligand-exchange chromatography (LEC) of eighteen essential α-amino acid enantiorners has been investigated by using a commercially available column. The separation of D- and L-enantiomer is based on the stability of the mixed-ligand complex formed on the chiral stationary phase. Three bivalent cations of first transition-metals Ni, Cu, and Zn have been used for the achiral aqueous mobile phase and tested for their efficiencies in separating the individual amino acid enantiomers. The effect of column temperature has been studied by valying the temperature from 25°C to 50°C for aqueous mobile phase containing Cu^{2+} ions. The resolu-tions between individual amino acid enantiomers containing aliphatic side chains or longer hydrophilic side chains are favored at high temperature. While those amino acid enantiomers with shorter hydrophilic side chains usually showed a maximum resolution around a column temperature 30°C. Eight of the eighteen amino acid enantiomers can be resolved well for practical usage at temperature 50°C by using Zn^{2+} ion as the complexation metal. Most of the eight enantiomers contain π-electrons or nonbonding electron pairs on their side chains. Only phenylalanine, tyrosine, and tryptophan can be separated by using Ni^{2+} ion as the complexation metal in the present LEC at a temperature 50°C. The elution orders of the amino acids were determined mainly by the polarities of the side chains. However, The structures of the side chains on different enantiomers affect the distortion of the te-trahedral on α-carbon and determine the most stable configuration of enantiomers. The relative thermal

stabilities for D- and L-enantiomers will not be changed drastically to reverse their elution orders by alternating the column temperature. The applications of LEC can thus be more fruitful by varying the column temperature and the kind of complexation metal.

The technique of LEC has been used to prove that only L-aspartic acid of the racemic DL-aspartic acid can be decarboxylated by *Pseudomonas dacunhae* to form L-alanine. Sufficient amount of L-glutamic acid and L-aspartic acid must be provided to execute this biotransformation. The analysis of DL-amino acid enantiomers in the medium with LEC was performed by 0.125 mM Cu^{2+} aqueous mobile phase under a column temperature of 30°C and 50°C. The retention times of amino acid enantiomers in the biotransformation medium are a little faster at 50°C. However, the resolutions between amino acid enantiomers in the reaction medium are better at the column temperature of 50°C. To have a successful application, we have to use a mobile phase containing low concentration of Cu^{2+} ions to move L-alanine to a higher retention time. We also found that L-threonine was present in the final biotransformation medium. The metabolism of DL-aspartic acid to L-threonine by *Pseudomonas dacunhae* should be studied further.

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Present procedures of amino acid analysis by GC, HPLC and CZE and presentation of a new approach to extremely rapid amino acid analysis

Methods of amino acid determination by gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) by means of precolumn, on-column and postcolumn derivatization have been reviewed and documented by presentation of slides. Strong and weak points of the corresponding procedures are figured out. Approximately ten procedures, elaborated during 80s, proved to be worth pursuing as they were worked up to sophisticated and reliable methods. Among them we find approaches using, e.g. o-phthalaldehyde, phenylisothiocyanate, dansyl and dabsyl chlorides, napthalenedialdehyde, fluorenylmethyl chloroformate, fluorodinitrodiethylaniline and others for amino acid determination by HPLC. In GC it is, e.g. formation of acylated alkyl esters, persilylated compounds and oxazolidinones. A new procedure is presented, that enables one to derivatize and analyze amino acids by GC in five minutes using chloroformates and aqueous solutions. An example of amino acid determination in human serum is shown. This procedure enables one to determine amino acids by cheapest and quickest way at present.

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A new, stable and sensitive HPLC method for the determination of enantiomeric purity of amino acids

Amino Acid analysis has been greatly enhanced by the use of a variety of precolumn derivatizing reagents. Fluorescent tags like ortho-phthaldehyde (OPA) and fluorenyl methyl chloroformate (FMOC-Cl) have demonstrated excellent sensitivity while phenylisothiocyanate (PITC) remains the most widely used tag, even though it is UV adsorbing. For the determination of chiral purity, each of these reagents, as well as others like Dansyl chloride or naphthylamide, have been used to various degrees of success on cyclodextrin based HPLC. Each has its limitations, ie, long term stability, background, or inability to separate certain amino acid racemates including secondary amino acids like proline and its metabolites. Fewer of these reagents have been successful with peptides.

Recently, a novel derivatization reagent, 6-Aminoquinolyl-N-Hydroxy succinimidyl carbamate, was introduced by Millipore Corporation under the trademark Accq-Fluor. This reagent has demonstrated rapid formation of a highly stable asymmetric urea compound with excellent fluorescent characteristics. A dramatic shift in fluorescence emission maximum compared to the reagent peak allows for the direct injection of the sample reaction with no interference from excess reagent. No racemization during the derivatization reaction could be detected.

Thirty-three amino acid racemates including proline and a variety of di-and tri-peptides with two chiral centers have been resolved on either the bonded native cyclodextrins or one of the cyclodextrin derivatives. The most useful derivative was the S or R naphthylethyl carbamate of beta cyclodextrin. A new, highly stable mobile phase defined as a polar organic mode was used extensively in the separation process. The composition was essentially acetonitrile/methanol 90-99/10-1 with small amounts of glacial HOAc and TEA (0.1 to 1.0% each). The system being essentially anhydrous leads to long term column stability. In addition, only small adjustments in composition are needed to afford selectivity enhancement. Resolution factors ran as high as 8.5.

A reversal in elution order was observed in separations based on a reversed phase solvent as opposed to a polar organic phase solvent. In addition, as little as 0.0075% of the D enantiomer of Phe could be detected in an excess of the L-enantiomer. No change in the enantiomeric ratio could be detected over a seven day period at room temperature.

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Methods for the separation and quantitative determination of the stereoisomers of 2,6-diaminopimelic acid in rumen bacteria

Bacterial cell walls are structurally diverse, yet most Gram-positive and Gram-negative bacteria contain peptidoglycan, which consists of polysaccharide strands cross-linked through short peptides. The amino acids in these peptides can vary, but 2,6-diaminopimelic acid (DAP) is commonly present.

Because DAP is thought to be unique to bacteria, it has been extensively used as a marker to measure bacterial biomass in several ecosystems, including that of the rumen. However, questions about its accuracy as a marker have been raised as a result of investigations into its metabolism by rumen microorganisms.

We have previously shown that DAP can be metabolised in both its free and bacterially-bound forms, by both rumen bacteria and protozoa, both *in vitro* and *in vivo*. The fact that DAP exists in three stereoisomeric forms, *meso*-, DD- and LL-, undoubtedly influences its metabolism by rumen microorganisms, yet nothing is known about these aspects of DAP metabolism.

Several methods for estimating DAP have already been reported, but they are generally incapable of resolving all three stereoisomers. To advance our knowledge of DAP metabolism, we have developed two chiral methods; one for DAP separation and one for DAP analysis.

The first method is capable of separating the three DAP stereoisomers by chiral HPLC without derivatisation so that the isomers can be subsequently used as substrates for microbial incubations and enzymic studies. The conditions for separation were optimised so that samples of up to 250 μg DAP could be eluted from a chiral column (MCl gel CRS 10w; 100 × 4 mm I.D.) with a mobile phase of 2mM $CuSO_4$ and 2% methanol, at a temperature of 40°C. With this system the peaks were completely resolved with retention times of 9.7, 15.7 and 38.9 min for the DD-, *meso*- and LL-isomers respectively, as confirmed by CD spectral analysis.

The second method, for the rapid assay of DAP stereoisomers, depends upon the derivatisation of DAP with N^2-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent). Separation was by reverse-phase HPLC using a column of LiChrospher 100 RP-18 (250 × 4 mm I.D.) which was developed with an eluent composed of a mixture (70:30 v/v) of 0.05 M triethylamine in phosphate buffer (pH 3.0) and acetonitrile. Complete resolution was achieved with retention times of 24.9, 33.6 and 38.8 min for the *meso*-, LL- and DD-DAP isomers respectively.

Standard mixtures of DAP stereoisomers and acid-hydrolysed rumen bacteria were used in the development of these chiral HPLC systems. No conversion of the DAP stereoisomers occurred as a result of the acid-hydrolysis procedure. Mixed rumen bacteria were isolated from goats and preliminary results showed that their DAP concentrations ranged from 0.34 to 1.10 mg DAP per g bacterial dry matter, and that the vast majority was in the form of *meso*-DAP, with only traces of the DD- and LL-isomers present.

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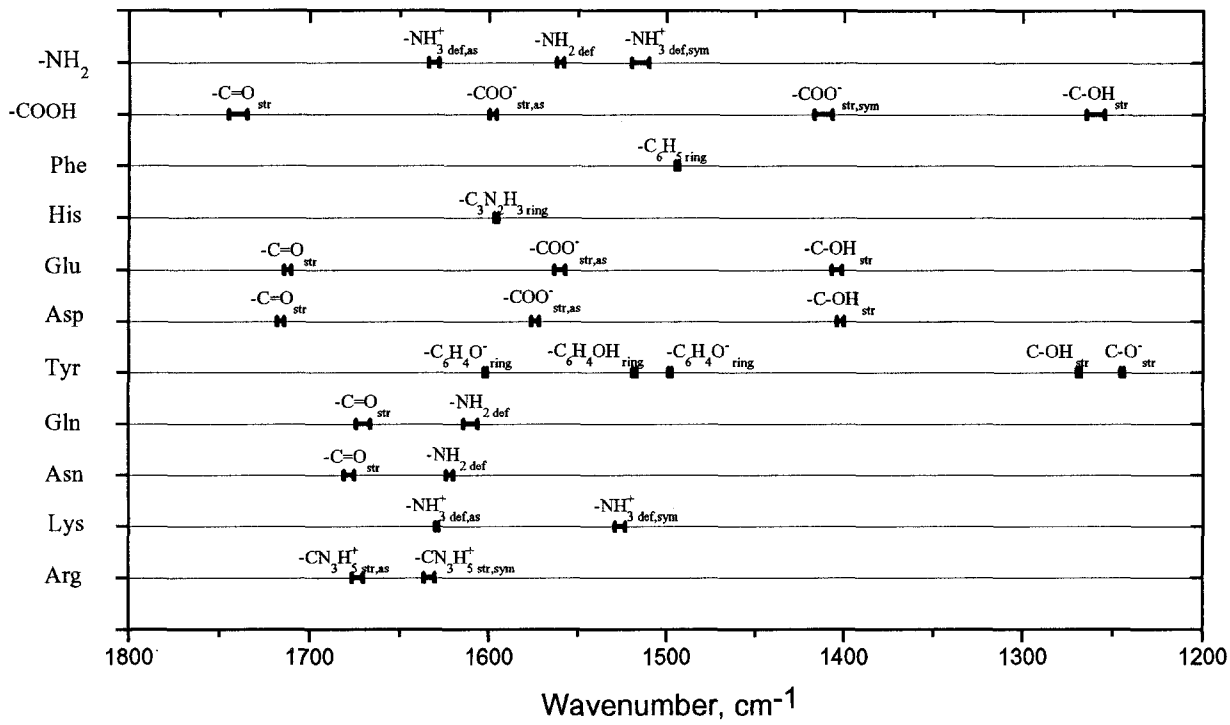
Trace analysis of amino acids in Permian/Triassic boundary sediments by electron capture gas chromatography

Here presented is a method for trace analysis of twenty-one amino acids, involving conversion of amino acids into corresponding amino acid derivatives by reaction with n-propanol and pentafluoropropionic anhydride. The amino acids with α-aminoisobutyric acid and isovaline as their N(O)-pentafluoropropionyl n-propyl esters were separated by fused-silica capillary gas chromatography, and determination was achieved by using split injection and electron-capture detection or flame-ionization detection. By using N(O)-pentafluoropropionyl amino acid n-propyl esters, it was shown that the electron-capture detection response was 300–600 times more sensitive than flame-ionization detection response. The electron-capture detection limits were in the low picogram range. This method was applied to trace determination of amino acids from Permian/Triassic boundary sediments.

In addition, also resolved on Chirasil-Val were N(O)-trifluoroacetyl, N(O)-pentafluoropropionyl, N(O)-heptabutyryl isopropyl and N(O)-pentafluoropropionyl, n-propyl esters of some DL-amino acids which are interesting in the field of biogeochemistry. Better resolution could be obtained by using N(O)-pentafluoropropionyl isopropyl and n-propyl esters. Coupled with electron-capture detection, the N(O)-pentafluoropropionyl derivatives will prove to be a applicability to amino acid dating.

Vibrations: str- stretching; def- deformation; ring- vibration of aromatic ring; sym- symmetrical; as- antisymmetrical.



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Infrared absorption band library of amino acids and analogies in water solution

Infrared spectra of the 20 naturally occurred amino acids and their analogies in water solution were obtained at the various pH values in the 1800–1000 cm^{-1} region. The absorption band parameters (position of maximum, ν_0, cm^{-1}; intensity at the maximum, E_0, 1 mol^{-1} cm^{-1}; band half width, $\Delta\nu_{1/2}$, cm^{-1}; band shape parameter, f_G) were determined for the main characteristic vibrations and the bands were assigned to the vibrations of particular groups in the molecules. It has been found that amino acid residues of arginine, asparagine, glutamine, aspartic and glutamic acids, lysine, tyrosine, histidine, and phenylalanine have the most intensive absorption in the considered spectral region.

Chart of characteristic frequencies of the main absorption bands of amino acids with ionized and deionized side chain in the water solution is represented below (see page 136).

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Column chromatographic resolution of chemically unmodified amino acids and its application for different purpose

We have been interested in the presence of D-amino acid in biological world (e.g. utilization by *Halobacterium salinarium*, incorporation into peptides by ribosome as well as non-ribosome system, biochemical and physiological functions). Not only that, racemizations of amino acids during the course of geological diagenesis have also been investigated. Those researches would encourage new field of D-amino acid study.

In order to carry out biological study, *in vitro* or *in vivo*, the optically pure form of D- and L-amino acids must be prepared, if possible, without modifying amino acids with chemicals. In this sense, the leading methods for resolutions of racemic amino acids might not be recommended. We have established the system that the native form (unmodified) racemic amino acids could be separated using a native cellulose column and a cyanocobalamin-bound ODS column.

The cellulose specially selected in terms of particle size and source was packed into a glass column (3 m long and 0.85 cm in diameter). Native DL-amino acids charged onto a bed was eluted (0.5 ml/min) by the pyridine-ethanol-water system of which composition was changed according to amino acid species analyzed and the eluant was monitored at 570 nm. Using the above method all the amino acid racemates could be separated.

The solution containing cyanocobalamin was passed through a ODS-packed stainless column (25 cm long and 0.45 cm in diameter) until red-colored solution appeared at the bottom of a column. Then DL-amino acids were eluted (0.5 ml/min) with acetate buffer containing Cu(II), after which copper was eliminated by passing the resolved through a Dowex column.

The optical purity of enantiomers thus obtained was measured using a spectroporalimeter, showing that all the enantiomers resolved were more than 99% in purity. D- or L- amino acid was then provided for bioassay. It was known that they were utilized as normal.

The above methods were useful especially for separating a small amount of radioactively labeled amino acids for biochemical study. Since the commerically available one-handed were sometimes racemized in a small quantity, those had to be purified prior to use. Also, the radioactive racemic mixtures chemically synthesized were also separated using the above method to give the pure form. Of course, the method can be utilized for usual separation of derivatized amino acids, which gave good result as well.

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Ligand-exchange chromatographic resolution of DL-amino acids using nucleic acids and coenzymes

We have studied many kinds of nucleic acids and coenzymes to determine whether they can be utilized as chiral additives to resolve DL-amino acids. When we tested four ribonucleoside 5′-monophosphate (5′-AMP, -CMP, -GMP and -UMP) [of which ribonucleic acids (RNAs) consist] as chiral additives for ligand-exchange chromatography, nine representative DL-amino acids were resolved using 5′-AMP or -GMP. 5′-GMP gave better results than those with 5′-AMP. When four deoxyribonucleoside 5′-monophosphate (5′-dAMP, -dCMP, -dGMP and -dTMP) [of which deoxyribonucleic acids (DNAs) consist] were tested, similar chromatograms to those observed by using ribonucleoside 5′-monophosphate were obtained. Adenosine and four different adenine ribonucleotides (3′-AMP, 5′-AMP, 5′-ADP and 5′-ATP) were also tested, among which 5′-ADP was known to be the best chiral additive.

On the other hand, we tested several coenzymes which contain nucleic acids, e.g. nicotinamide adenine dinucleotide (NAD) and flavine adenine dinucleotide (FAD). Fourteen of sixteen DL-amino acids tested were resolved using NAD or FAD as a chiral additive for ligand-exchange chl omatography.

An ODS silica gel column (250 mm × 4.6 mm i.d.) was used for the present study. The eluent usually contained 0.2 mM nucleic acid or coenzyme and 0.2 mM cupric acetate. It was shown under the condition that separation factor (α) and resolution rate (Rs) were dependent upon the concentration of chiral additives.

We applied this method to obtain pure one-handed amino acids from their racemic mixtures, and the optical purity of the enantiomers thus resolved was estimated to be greater than 99%. The resolved enantiomers (e.g. [^{3}H]-labeled D- or L-valine and -leucine) could be used for biochemical study.

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Fast atom bombardment mass spectrometric analysis of amino acids and peptides

It is well known that conventional MS ionisation techniques lead to molecular decomposition of underivatised amino acids

and produce uninterpretable mass spectra. Molecular ions are seldom observed We now wish to report on the utilisation of fast atom bombardment mass spectrometry (FAB MS) as a tool for underivatised amino acid analysis.

FAB MS has become a powerful structural tool since the first reports of its use in 1981. Samples are ionised in a suitable matrix by bombardment with a beam of accelerated neutral xenon atoms produced by a FAB gun. This can yield both positive and negative ions which are sputtered from the surface of the matrix.

The dipolar character of neutral amino acids poses a problem and requires suitable preionisation in the matrix. Two alternatives are possible and should be used supplementary. Using a sodium hydroxide containing matrix and recording in the negative ion mode strong $[M - H]^-$ molecular ions are observed for most amino acids. With a hydrochloric acid containing matrix strong $[M + H]^+$ molecular ions are registered when recording is done in the positive ion mode.

In the majority of cases studied glycerol proved to be a suitable matrix for both positive and negative ion FAB analysis. In cases where solubility poses a problem m-nitrobenzylalcohol can be used as a matrix.

Sequence analysis of peptides can be accomplished from FAB mass spectra as bombardment with the neutral atom beam causes fragmentation of the peptide backbone and can provide significant, if not complete, sequence information. FAB spectra provide sequence information from both the N and C termini. A FAB-MS study was carried out on synthetic, underivatised neuropeptides which are both N and C terminally blocked and thus not readily amenable to chemical sequencing. The utilisation of this technique is demonstrated by the complete sequencing of a [D-Trp^6, Pro^9-CONHEt]-GnRH peptide.

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High-performance liquid chromatography as a tool in studying the abnormalities in neurological diseases.

The separation and quantitation of biologically important substances and especailly neurotransmitters such as excitatory amino acids ,catecholamines , serotonin and their metabolites have become increasingly important and critical for the advances in neurosciences. High-performance liquid chromatography (HPLC) is one of the most important arsenals for the analysis of increasingly complex material such as plasma, CFS and brain tissues.

In order to study the functional role of various neurotransmitters in the brain, it is necessary to assess their concentrations in a compartment of the body which reflects their correlation in the brain. Cerebrospinal fluid (CSF) is considered to be the compartment where substances of central nervous system (CNS) origin are easily available to forfiet the vital information on the function of the brain. Various studies have shown abnormalities in CSF monoamine and amino acid levels in various neurological and psychiatric disorders and in some cases it has provided with a helpful information for diagnosis assessment of cure.

In this study two HPLC methods of analysis are applied to study the changes in catecholamines, amino acids, serotonin and their metabolites in patients with asecptic meningitis, cerebrovascular disorders and multiple sclerosis under remission, excerbation and chronic progressive state. The analytical assays used are sensitive, stable and reproducible . The results accumulated in this study are compared with age-matched healthy subjects and the discussed in the light of their variations.

Pharmacology and Toxicology

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.Effects of the lysine analogue, S-2-aminoethylcysteine, on the synthesis, assembly and secretion of collagen in embryonic chick tendon cells

The effects of the lysine analogue S-2-aminoethylcysteine on the acylation of various amino acids and on their activation onto embryonic chick tendon tRNA has been studied. It was found that the analogue competed efficiently with lysine for activation onto tRNA without affecting significantly the activation of other amino acids. At 10 uM. the analogue suppressed the activation of lysine by 50% and at 500 uM, the suppression was greater than 90%. In a tRNA activation assay, S-2-aminoethylcysteine was found to have a binding affinity for the synthetase at least equal to that of lysine (km for lysine: 1.6 uM: Ki for S-2-aminoethylcysteine: 1.4 uM). In the incubation of tendon cell cultures, it was shown that S-2-aminoethylcysteine had a marked inhibition on the incorporation of ^{14}C-proline but not ^{14}C-tyrosine or ^{14}C-cysteine into protein. The analogue also markedly inhibited the secretion of ^{14}C-proline labeled proteins and minimally affected the secretion of ^{14}C-tyrosine labeled proteins. Amino acid analysis of collagen extracted from tendons incubated with 500 uM S-2-aminoethylcysteine, demonstrated direct incorporation of the analogue into the protein. The ^{14}C-proline labeled collagen which had been synthesized in the presence of S-2-aminoethylcysteine was shown to be susceptible to digestion with pepsin at 25°C but not at 15°C. indicating that the incorporated S-2-aminoethylcysteine interferes with the formation of the collagen triple helix. The high binding affinity of SAC for the lysyl tRNA synthetase and the demonstration of its incorporation into tendon cell collagen. indicate that this analogue should be a powerful tool to further study the role of lysine on collagen structure and how altered structure affects its synthesis and secretion. Also because this lysine analogue has been shown to have a very low toxicity level in vivo and because it caused selective inhibition on procollagen synthesis and secretion, the analogue may prove to be very effective in the treatment of a variety of fibrotic diseases.

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Biological activity of phosphonic acid analogues of amino acids

For many years our laboratory has been engaged in synthesis of aminophosphonates and evaluation of their biological activities.

Aminophosphonates are interesting class of synthetic, as well as naturally occurring compounds, that can be thought to be analogues of amino acids in which a carboxylic function was replaced by a phosphonic or related groups:

Members of this class of phosphorus-containing compounds differ from the more prevalent organophosphates by virtue of their P—C linkage. Being the structural analogues of amino acids they exert their activity mainly as amino acid antimetabolites. As inhibitors of metabolic processes, they act as antibacterial agents, neuroactive compounds, anticancer drugs or pesticides, possible application of which range from medicine to agriculture.

Our investigations of biological activity of aminophosphonates go along following main lines: elucidation of enzyme inhibitors (glutamine synthetase, leucine aminopeptidases and tyrosinase): search for antibacterial agents, including transport problems within bacterial cell wall and search for plant growth regulators.

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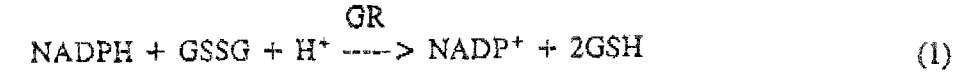
Rational design of peptide-based inhibitors of TR as potential antitrypanosomal drugs

Reduced glutathione plays a fundamental biological role in mammalian redox defence and in the course of this becomes oxidised to glutathione disulphide. Reduced glutathione is regenerated from glutathione disulphide (1) by the NADPH-dependent flavoenzyme glutathione reductase (GR) equation (1).



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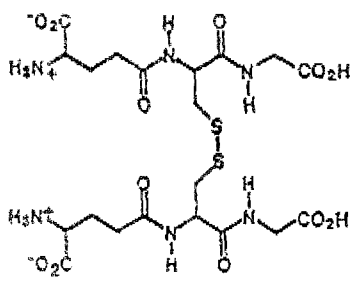

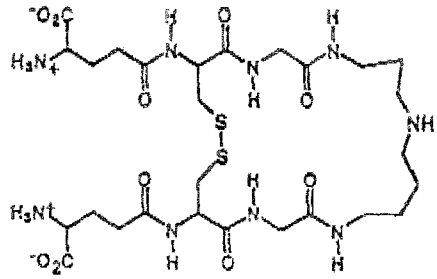

This system is absent in trypanosomes and leishmanias, the causative agents of tropical diseases such as African Sleeping Sickness, Chagas' disease and Leishmaniasis. Instead, these parasites rely on an analogous thiol, trypanothione (2), and its cognate enzyme trypanothione reductase (TR) for cellular protection against redox damage. TR does not recognise glutathione as a substrate, and likewise GR does not recognise trypanothione. This mutual substrate exclusivity makes TR an important target for rational drug design. Comparison of the crystal structures of TR and GR revealed an enlarged active site in TR, characterised by a large hydrophobic cleft which accommodates the spermidine bridge of trypanothione.

We have designed a series of novel peptide-based inhibitors. Our molecular graphics analysis suggested that, although structurally unrelated to the native substrate, these potential inhibitors would occupy the trypanothione-binding site of TR. Kinetic studies confirmed the inhibition to be competitive with respect to trypanothione for Benzoyl-Leu-Arg-Arg-β-Naphthylamide, with K_i 13μM and also selective for TR in that human GR was not inhibited. It is proposed that, whilst these inhibitors use some of the binding determinants of trypanothione, they also incorporate novel binding interactions compared to trypanothione itself. Specificity is attributed to the designed complementarity to the hydrophobic region of the TR active site.

By nature of their readily variable functionality, peptides represent useful lead ligands in inhibitor design. Peptide-based inhibitors are easily synthesized and provide a means of rapidly rationalising key interactions in the active and other ligand binding sites. We are currently linking peptides with other non-peptidyl inhibitors developed in this laboratory to increase potency. The potential of using peptides to incorporate two distinct ligand-binding sites is also under investigation.

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Antitumoral cyclic peptide analogs of chlamydocin

Chlamydocin, extracted first from Diheterospora chlamydosporia 20 years ago ,exhibits potent cytotoxic activity in vitro

(IC_{50} = 0.36 ng/ml) against P815 mastocytoma mouse cell proliferation. Unfortunately, this activity quickly fades under in vivo conditions and is destroyed within a few minutes when chlamydocin is incubated in blood serum. Chlamydocin is the cyclic tetrapeptide c[Aib-Phe-D-Pro-Aoe] containing the uncommon aminoacid Aoe = (2S,9S)2-Amino-8-oxo-9,10-epoxydecanoic acid. The keto epoxidic group of Aoe which is required for biological activity appears to be too reactive against nucleophilic attack, thus leading to epoxide opening before having reached its target.

Other cyclic tetrapeptides, all from fungus origin, share the same structural features with chlamydocin, namely the presence of Aoe, of an iminoacid such as proline or pipecolic acid and of one D-amino acid. One of them, HC toxin from Helminsthosporium carbonum, a potent host-specific agent against sensitive maize hybrids has also a considerable in vitro cytotoxic activity, although 10 times lower than chlamydocin. This result seems to imply that the cyclopeptide moiety is a part of the pharmacophore important for target recognition.

Basing on this assumption, we have prepared a series of cyclic peptides belonging either to the chlamydocin or to the HC toxin family. With the purpose of preserving the high in vivo activity by increasing the stability of the alkylating function, a lysine was introduced in the place of Aoe. This provided a free amino function on Nε for the anchoring of one of the well known alkylating β-chloroethyl-nitrosourea, amidoepoxy or N,N-di-(β-chloroethyl)-4-amino-benzoyl (nitrogen mustard) groups.

One analog belonging to the chlamydocin family and bearing a β-chloroethylnitrosourea group was found to be potent at inhibiting L1210 cell proliferation and had a higher therapeutic index than the reference compound bis-β-chloroethylnitrosourea (BCNU) on the in vivo P388-induced leukemia model.

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The relative ability of buthionine sulfoximine and other γ-glutamylcysteine synthetase inhibitors to both deplete rat hepatic glutathione and alter drug metabolizing enzyme activities

L-Buthionine sulfoximine (S-(n-butyl)homocysteine sulfoximine; BSO) is a potent and specific inhibitor of γ-glutamylcysteine synthetase, a key enzyme in controlling glutathione (GSH) synthesis. Recent investigations have revealed that chronic BSO treatment of rats also increases UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST) activities in liver subcellular fractions and alters acetaminophen disposition in whole animals. Thus chronic BSO treatment cannot be used in toxicity studies on the assumption that GSH depletion is the sole effect on hepatic xenobiotic metabolism. Analogs of BSO differ in the extent and duration for which they are able to deplete hepatic GSH concentrations but have been considered less suitable GSH depleting agents because of other effects, particularly CNS toxicities. This study was undertaken to determine whether two BSO analogs, L-prothionine sulfoximine (PSO) and L-hexathionine sulfoximine (HSO) might offer an advantage over BSO by depleting GSH without inducing drug metabolizing enzyme activities. PSO and HSO were synthesized from L-methionine by a sodium/liquid ammonia reduction and coupling to the appropriate alkylbromide, followed by conversion to the sulfoximines using sodium azide/sulfuric acid. BSO was available commercially. All three compounds depleted hepatic GSH concentrations when given orally (6 mmol/kg) as a suspension in methyl cellulose. BSO was the most effective agent, depleting GSH to below 50% of controls within 3 hours and keeping it below 50% for at least another 15 hours. Maximal depletion of over 90% occurred around 8 hours. HSO exhibited similar behavior, but it was slower to deplete GSH, caused less maximal depletion, and allowed a quicker return of GSH to normal. PSO was less effective than either BSO or HSO. It did not deplete hepatic GSH below 50% at any time. Over the 24-hour period following administration, PSO depleted GSH by 29%, HSO by 44%, and BSO by 65% compared to normal controls. When the sulfoximines were administered at the same dose for three consecutive days, and drug metabolizing enzymes examined 24 hours after the last dose, PSO and HSO treatments had not significantly increased any activities. However, BSO treatment had produced signiflcant changes ($p < 0.05$): a 42% increase in (UGT (4-nitrophenol aglycone), a 30% increase in UGT (1-naphthol aglycone), and a 28% increase in GST activities. UGT activities towards morphine, estrone, and testosterone were not significantly elevated by any treatment, nor were microsomal epoxide hydrolase activity, P450 concentration, P450-dependent 4-nitroanisole demethylation, or cytoplasmic sulfotransferase activity. HSO treatment significantly decreased P450-dependent 4-nitroanisole demethylase activity (by 22%). Thus although PSO did not alter enzyme activities, it was less useful than BSO because depletion of GSH was much less efficient. HSO also showed lower GSH depleting ability than BSO and, although it did not induce UGT or GST activities, it had the disadvantage of altering cytochrome P450 concentrations. Overall, none of the three sulfoximines had the ideal property of depleting GSH to a high extent without affecting xenobiotic metabolizing enzymes.

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Cytotoxic activity of isothiocyanate derivatives of some non-aromatic amino acids toward cancer cells

The aim of this study was to assess the cytotoxic activity of 6 novel isothiocyanate (ITC) derivatives of non-aromatic amino acids toward both Ehrlich ascites carcinoma (EAC) cells and P388 murine leukemia cells in vitro. Cytotoxic activity was assessed as a degree of incorporation inhibition of [^{14}C] adenine and [^{14}C] valine into TCA-insoluble fraction of both tumor cells. The inhibitory effect was characterized by IC_{50} values (molar concentration of compound required for 50% reduction of the incorporation rate). ITC-ethylester of L-alfa-alanine, ITC-ethylester of gama-butyric acid and diethylester of 2-isothiocyanate-butanedicarboxyl acid (ITC-aspartic acid) show little effect on the incorporation of both precursors (IC_{50} values for adenine and valine were in the range of 120 to more than 600 μmol/1). Diethylester of 2-ITC-D,L-aspartic acid, ethylesters of 2-ITC L- and D- valine significantly inhibited incorporation of both ^{14}C-precursors into appropriate macromolecules of both cancer cells, according to concentration dependence. This has been con-

firmed not only by percentage inhibition but also by IC_{50} values (35 to 150 μmol/l). The IC_{50} values for valine were lower than for adenine. This means that for the tested drugs biosynthesis protein is "more sensitive" than is the biosynthesis of nucleic acid.

On the basis of our previous results it is convenient to use the IC_{50} adenine: IC_{50} valine ratio (R) as a suitable parameter to indicate the possible primary mode of action of the substance investigated. All ratios are in the range 0.98 to 2.5. Such ratios are typical also for other biologically active compounds which interfere with energy-generating systems of cells. In a first approach to determine the mode of action of the cytotoxically active compounds, the konetics of ONA, RNA and protein synthesis inhibition were examined using isotope incorporation. On the basis of primary screening, two of the most active compounds, namely L- and D-isomers of ethylester 2-ITC valine, were chosen for further biochemical study.

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Polymeric carriers of *cis*-hydroxyl-L-proline: potential agents for inhibiting collagen accumulation

Cis-hydroxy-L-proline (cHyp) is an L-amino acid that inhibits collagen accumulation in vivo but is toxic if administered over long periods of time. To circumvent this problem, we developed two polymeric carriers of cHyp and tested their bioactivity in vitro. We prepared poly(ethylene glycol) conjugates in which cHyp was attached to a polymeric backbone, poly(PEG-Lys), by the carboxylic groups of the lysyl residues. cHyp was bound to the PEG based carrier through a hydrolyzable ester linkage or a more stable amide bond. Biological activity was investigated by the ability of the polymeric cHyp derivatives to inhibit growth of cultured flbroblasts, an antiflbrotic effect since cells grown on plastic require secretion of collagen to attach and grow. The polymeric cHyp derivatives were added to cultures at 75 ug/ml, a non-inhibiting concentration for free cHyp; grow at 6 days was inhibited by 39% (ester-linked conjugate) and 62% (amide-linked conjugate) compared to nonbioactive *trans*-Hyp in equimolar amounts. There was no inhibitory effect of poly(PEG-Lys) plus nonconjugated cHyp. Cells grown on a collagen substrate in the presence of these compounds were not inhibited, showing that growth inhibition was due to an antifibrotic effect. Cell viability was not affected. We conclude: (1) copolymers of cHyp are nontoxic to cells and have prolonged antifibrotic effects, (2) the antiflbrotic effect requires conjugation of cHyp to poly(PEG-Lys), and (3) an equimolar concentration of the amide polymer has a greater inhibitory effect on fibroblast growth than the ester polymer. The more proloned bioactivity of the amide polymer is probably due to the more stable amide bond producing gradual release of the agent from the polymeric backbone. This delivery system which releases cHyp slowly may improve the antifibrotic effect of cHyp in vivo by allowing targeting of the agent to sites of fibrosis and may reduce toxicity.

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Amino acid induced renal hemodynamic response in adolescents with IgA Nephropathy treated with enalapril

Five males (range of age 13–17 yrs), with IgA Nephropathy (IgAN), proteinuria >1 g/day and normal GFR, were treated with Enalapril (E) 0.25 mg/kg/day in order to reduce urinary loss of protein. Before (t_0), after 15 days (t_{15d}) and 6 months (t_{6m}) of treatment with E, the five patients underwent an infusion of amino acids (AA) for 2 hrs (290 mg/min/1.73 m^2 of Freamine 8.5%). The renal hemodynamics was studied before (Pre-AA) and during (Post-AA) the infusion of AA through the following paramethers: GFR (Clearance of Polyfructosan S) and RPF (Clearance of PAH), at the three different observation times with respect to E treatment: t_0, t_{15d} and t_{6m}. 24 hrs proteinuria was also detected the day preceeding the infusion tests. Results are given as means $\pm$ sd. The increase from baseline value of GFR (ΔGFR) and RPF (ΔRPF) during AA infusion is expressed according to the following formula: [(Post AA – Pre AA value)/(mean of Pre AA and Post AA value)].Differences in proteinuria were analyzed as Log of individual value. ANOVA was used for statistical analysis. *: $p < 0.05$ vs to value.

	GFR (ml/min/1.73m2)	RPF	ΔGFR	ΔRPF	Proteinuria (g/day)
t_0	105.5 ± 8.6	538 ± 73.8	0.20 ± .12	0.19 ± .12	1.8 ± 0.8
t_{15d}	106.5 ± 7.3	584.7 ± 106	0.14 ± .08	0.12 ± .02	1.3 ± 1.6
t_{6m}	109.7 ± 9.2	616.7 ± 62*	0.08 ± .06	−0.09 ± .25*	1.0 ± 0.8*

After treatment with E, together with a reduction of proteinuria, patients showed an increase in RPF reaching statistical significance only after 6 months, while GFR remained statistically unchanged. The ability of AA infusion to induce an increase in GFR was reduced along E treatment, and blunted with regard to RPF. Our data confirm that in patients with IgAN, E maintains its efficacy on proteinuria, and suggest that, over time, the drug inhibits the renal hemodynamic response to the infusion of AA.

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Glomerular hemodynamics and tubular handling of amino acids after intravenous infusion of different amino acid mixtures

Six healthy male subjects (23–25 yrs of age) underwent the infusion of the two following solutions of Amino Acids (AA):

Solution 1: Essential AA (FEs 5.4%, Baxter)
Solution 2: Mixture of essential and non essential AA (Freamine III 8.5%, Baxter) Both solutions were infused at the rate of 145 mg/min/1.73 m^2 bsa for 2 hrs. Before and during infusion the following test and determinations were performed: clearance of Polyfructosan S for GFR, urinary sodium excretion (Na_u), plasma concentration and fractional excretion (FeAA) of essential and non essential AA. Results are given as means ± sd. Student t test for paired data was used for statistical analysis *: $p < 0.05$ before vs during infusion of AA.

	Solution 1		Solution 2	
	Before	During	Before	During
Plasma AA (mcMol/l)				
Essential	913 ± 150	2822 ± 455*	911 ± 149	2076 ± 291*
Non Essential	1702 ± 221	1758 ± 176	1936 ± 326	3383 ± 668*
Total	2615 ± 362	4579 ± 564*	2847 ± 442	5459 ± 943*
GFR (ml/min/1.73 m^2)	99 ± 5	104 ± 20	102 ± 7	121 ± 6*
FeAA (%)				
Essential	1.1 ± 0.3	1.4 ± 0.6	1.0 ± 0.4	1.7 ± 0.7
Non Essential	1.8 ± 0.4	1.9 ± 0.3	1.5 ± 0.3	5.6 ± 2.0*
Total	1.5 ± 0.2	1.6 ± 0.4	1.3 ± 0.3	4.2 ± 1.5*
Na_u (mMol/100 GF)	0.20 ± .09	0.21 ± .09	0.19 ± .08	0.27 ± .09*

Results. Although the increase of total plasma AA obtained with both solutions 1 and 2 was of similar magnitude, a significant renal hyperfiltrative response was observed only when non essential AA were infused.

While FeAA during infusion of solution 1 did not significantly change, it increased during infusion of solution 2, and this was almost entirely due to the non essential component. Urinary Na excretion only raised during the infusion of solution 2.

Conclusion. Essential AA, in our experimental conditions, do not elicit a renal hyperfiltrative response. During loading essential AA are more completely reabsorbed then non essential ones. The increase in urinary Na excretion during AA infusion was coupled with the increase in FeAA which, in its turn, was observed when non essential AA were infused. Hyperfiltration, increase in Na and AA urinary excretion are therefore linked to the same experimental condition: infusion of non essential AA. Our data, however, do not allow to draw conclusions on wheter a cause-effect relationship exists between hyperfiltration, Na and AA excretion.

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Long-term treatment with calcium ketoglutarate in end stage renal disease corrects secondary hyperparathyreoidism

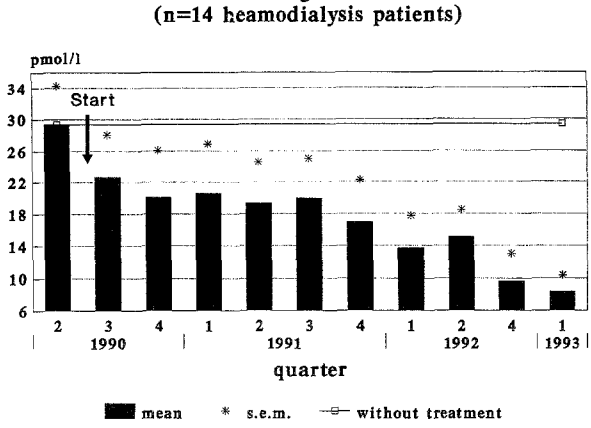
Because of serious side effects of aluminiumhydoxide as phosphate (P) binder, calcium (Ca) compounds recently are used in therapy of P accumulation in end stage renal disease. However, Ca carbonate often shows the risk of hypercalcemia, whereas Ca acetate tends to provoke gastrointestinal discomfort. In addition, Ca salts of ketoanalogues of essential amino acids (KKA), commonly used as supplements of low protein diet, are potent P lowering substances. Ca α-Ketoglutarate (Ca-ket) showed metabolic effects similar to those observed in KKA.In vitro studies have shown that Ca α-ketoglutarate (Ca-ket) has a high and rather pH independent P binding capacity.

As a consequence, Ca-ket has been investigated as a P binder in haemodialysis (HD) patients. A soluble preparation of Ca-ket was given to 16 HD patients in a mean dosage of 4.5 (0.975 g elemental Ca) g/day for a long-term period of 33 months. Serum P dropped from pre-study mean 2.6 ± 0.1 mmol/l (mean ± s.e.m.) to 1.9 ± 0.07 mmol/l ($p < 0.001$) after 6 months, stabilizing then for the observation period. Ca/P ratio in serum markedly increased and intact parathyroid hormone (iPTH) levels continously normalized in 14 patients investigated from 29,3 ± 4.9 pmol/l to 8.3 ± 2.0 ($p < 0.001$). The 2 non-responding patients showed severe hyperparathyreoidism (HPT) and were excluded from the study. Side effects, in particular hypercalcemia and gastrointestinal discomfort, did not occur.

It is concluded that in long-term treatment with Ca-ket secondary HPT is corrected or even normalized as a consequence of P binding and correction of Ca/P ratio.

iPTH serum concentration in pmol/l during 33 months, n = 14, mean (±s.e.m.)

month:	before	3	6	9	12	15	18	21	24	30	33
mean:	29.4	22.6	20.1	20.5	19.3	19.9	16.9	13.7	15.1	9.6	8.3
s.e.m.:	(4.9)	(5.4)	(6.0)	(6.3)	(5.2)	(5.1)	(5.3)	(4.1)	(3.4)	(3.4)	(2.0)



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The opioid mechanism of interferon-alpha action

We have utilized the drug discrimination technique as means to investigate the neurochemical mechanism of action of cytokines such as interferon-α (rIFN-α). Rats were trained to discriminate the opioid agonist ethylketocyclazocine (EKC, 0.3 mg/kg, i.p.) from saline. rIFN-α (2,4 and 6 million U/kg, i.m.) was shown to elicit EKC-like responses, indicating the involvement of the opioid system in the cytokine's behavioral effect. This rIFN-α induced EKC-like responses was suppressed by co-administration of naloxone (1 mg/kg, i.p.) with the cytokine. Potentiation of EKC response from a submaximal dose (0.1 mg/kg) was also observed by rIFN-α (1 and 2 million U/kg). rIFN-α exhibited analgesic properties in mice. When compared on a molar basis, rIFN-α (i.m.) produced greater analgesia, as measured by hot-plate (55°C) assay, than that produced by morphine. ED_{50} values were 3700 and 0.39 nmole/kg, respectively. Interaction between rIFN-α and morphine was further evaluated in mice made tolerant to the cytokine or morphine by administering the drug (rIFN-α, 9 μg/kg, i.m.; morphine, 7 mg/kg, i.p.) three times daily. Tolerance developed more rapidly in animals injected repeatedly with rIFN-α than those injected repeatedly with morphine. Analgesia was reduced to 3% on the fourth day of rIFN-α treatment. Cross tolerance between rIFN-α and morphine was shown. On the last day of measurement, when the two groups of tolerant animals were challenged with both rIFN-α and morphine, there was no difference in the degree of analgesia produced by either drug. The role of opioid receptor subtypes in the mediation of rIFN-α analgesia activity is discussed.

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Measurement of atrial natriuretic peptide (ANP) metabolism and identification of its *in vivo* generated endogenous metabolites using a new tracer method in humans

Either infusion or the *bolus* injection of pharmacologically active doses of ANP have generally been used to measure the main kinetic parameters of the hormone in humans, even if the high dose of ANP administered in these studies greatly increased the hormonal circulating levels, thus inducing a major perturbation of the system and possibly of the kinetic parameters of the hormone. Therefore, we set up a tracer method, which only minimally perturbs the steady state conditions of ANP system, with the aim of studying the *in vivo* metabolism of the hormone.

The circulating form of ANP (αh_{1-28}ANP, Ser^{99}-Tyr^{126}), monoradioiodinated in last amino-acid (Tyr), was injected into 7 healthy normal men on an unresctricted diet (sodium intake ranging from 80 mmol/day to 300 mmol/day). HPLC was used to purify the labeled hormone and the principal labeled metabolites present in venous plasma samples collected up to 50 min. after injection. A linear gradient from 20% to 50% acetonitrile in 0.1% trifluoroacetic acid was applied over 60 min; flow rate was 1 ml/min and 1 ml fractions were collected into polypropylene tubes by means of an automated fraction collector. The main ANP kinetic parameters were derived from the disappearance curves of the [^{125}I]ANP which were satisfactorily fitted by a biexponential function in all subjects.

A complete separation of labeled ANP from its labeled metabolites was achieved by the HPLC technique. The first chromatographic peak eluted in the 12–14 fractions (23% gradient) showed an identical elution time to mono-iodotyrosine. As early as 1.5 min after tracer injection, the mono-iodotyrosine peak represented the main labeled metabolite of injected tracer, and it remained the principal labeled peak throughout the first 5 min. At least three other peaks due to *in vivo* generated labeled metabolites were identified in the chromatograms; two peaks were eluted ahead (fractions 30–33, 30% gradient, and 35–37, 31% gradient, respectively) and one behind (fractions 46–49, 34% gradient) the elution peak of the labeled ANP.

Newly produced ANP initially distributes in a large, plasma equivalent space (12.1 ± 3.6 l/m^2 body surface); the hormone rapidly leaves this space due to both degradation and to distribution in peripheral spaces, as indicated by the single pass mean transit time through the sampling space (3.9 ± 1.2 min.). The mean residence time in the body (22.7 ± 23.1 min.) and the plasma equivalent total distribution volume (30.9 ± 12.0 l/m^2) indicate that ANP is also widely distributed outside the initial space. Metabolic clearance rate values were distributed across a wide range (from 740 ml/min./m^2 to 2581 ml/min./m^2), and were shown to strongly correlate (R = 0.973) with the daily urinary excretion of sodium.

In conclusion, our data indicate that: 1) tyrosine is the main endogenous metabolite of the hormone; 2) newly produced ANP is rapidly distributed and degraded; 3) the body pool of the hormone can be considered as a combination of two exchanging spaces; 4) circulating ANP is no more than 1/15 of the body pool); 5) MCR of ANP is closely related to sodium intake, at least in normal subjects on a free sodium intake diet.

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Pharmacolgic studies of L-tryptophan depletion device in human plasma

Systemic depletion of amino acids (e.g., L-asparagine and L-tryptophan) as a means of curbing cancer cell growth has long been of interest. L-tryptophan, unlike L-asparagine, is an essential amino acid; target cells have no means of synthesizing new stores. Systemic administration of E. coli tryptophanase has limited clinical use because of the enzyme's exogenous cofactor requirement of pyridoxal phosphate. A novel tryptophan-degrading enzyme (tryptophan side chain oxidase) from Pseudomonas XA does not require this short-lived cofactor. The application of this tryptophan oxidase in an extracorporeal treatment was studied in three patients with refractory acute lymphocytic leukemia. Three days prior to and during the therapy, patients A and C were on a low tryptophan (< 100 mg/day) diet and patient B received tryptophan-free hyperalimentation. By means of a COBE 2997 Spectra Pheresis Unit, patients' plasma was continuously passed through a tryptophan depletion column containing immobilized tryptophan oxidase. Three plasma volumes were pheresed 4 hours daily, 5 days per week for 2–3 weeks, and plasma tryptophan levels were measured by HPLC. All samples of column effluent had unmeasurable tryptophan levels. Blood tryptophan levels decreased after therapy, but rebounded the next day. These preliminary studies indicated intensive mobilization of tryptophan, and patient compliance with a tryptophan-free diet for an extended period was difficult. Development of this method will require further studies of the effects of the duration, interval, and frequency of use of this column, as well as the diet, on therapeutic efficacy. However, this study demonstrated that this tryptophan-depletion device is efficient in reducing circulating plasma tryptophan levels and is well tolerated by patients.

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Nutrient-enhanced drug absorption reversed by gut peptide antagonist in vivo

The gut peptide, cholecystokinin (CCK), is released into the blood stream from endocrine cells in the small intestine in response to food intake. Systemic CCK stimulates pancreatic endocrine and exocrine secretions, gall bladder contractions to secrete bile, slows gastric emptying and alters fasted-state intestinal motility and blood flow in response to nutrient input. A number of CCK antagonists have been synthesized which are specific for CCK receptors in the gastrointestinal (GI) tract.

Drug plasma levels following oral administration are often remarkably different when certain drugs are taken with meals as opposed to in the fasted state and this can be of significant therapeutic consequence. For example, lipophilic drugs are poorly water soluble and their dissolution rate and availability for GI absorption differs under fasted and fed-state conditions. The lipophilic antiepileptic agent, phenytoin, is better absorbed in the fed-state and drug plasma levels are elevated when the drug is co-administered with a caloric load in dogs. Non-invasive measurements indicated that fed-state delays in gastric emptying provide smoother delivery of dissolved drug for intestinal absorption than is the case in the fasted state. However, other fed-state factors were observed to affect drug plasma levels.

Drug co-administration with lipid calories promoted higher drug levels than from other equivalent caloric sources. As might be expected for a lipophilic drug, lipid/bile salt mixed micelles should promote drug dissolution in the intestine to enhance drug plasma levels. The benzodiazepine CCK-A antagonist, MK329, has been shown to block canine gallbladder biliary secretion in both the fed and fasted state. Administration of MK329 was employed as a non-invasive *in vivo* tool to evaluate the role of lipid-induced biliary secretion on drug plasma levels.

Compared to the fasted state, phenytoin plasma levels in four dogs were found to be substantially enhanced by co-administered oleate and Intralipid. (A similar observation from mixed meal co-administration in the gastric emptying study prompted the search for in vivo tools to further detail this fed-state effect.) Drug plasma levels with co-administered lipid were consistently higher than those following carbohydrate or protein administration. Oral and intravenous pretreatment with MK329 prior to phenytoin-lipid co-administration usually reduced drug plasma levels to those observed under fasted-state conditions. In those cases where MK329 failed to reduce lipid-enhanced drug blood levels, protracted drug plasma level lag times were observed. The results suggest that the CCK antagonist blocked biliary-enhanced drug dissolution in the small intestine but did not consistently block lipid-induced delays in gastric emptying.

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Influence of treatment with triiodothyronine (T3) or dexamethasone on renal amino acid transport in immature and adult rats

Little is known about postnatal development of renal handling of amino acids (AA). The effects of repeated administration of hormones on renal transport of AA were barly investigated as yet. In contrast, it is well established that renal tubular secretion of drugs and xenobiotics at the basolateral membrane of the renal tubule can be stimulated by repeated administration of hormones or xenobiotics.

It was the aim of this study a) to give basic values of AA composition and handling in the late neonatal period and in adult Wistar rats, b) to characterize effects of treatment with T3 or dexamethasone at the brush border membranes of the proximal renal tubules, and c) to investigate the effects of both hormones after administration of an AA-load (glutamine, leucine, taurine). For this purpose the renal transport of AA was measured before and after treatment with the two hormones in 10- and 55-day-old anaesthetized rats without AA load and after administration of the three AA mentioned. AA load was calculated to obtain approximately a 10-fold increase of physiological AA concentrations in plasma.

In the late neonatal period plasma concentrations were enhanced in 5 of 24 AA (β-alanine, serine, tyrosine, glycine, histidine). The concentrations of tryptophan, valine, and leucine were lower in young rats. The renal clearances of AA were also lower in immature animals. Because of the significantly lower GFR the filtered load of AA is lower in young rats. Therefore, their AA transporting carriers are able to reabsorb AA from ultrafiltrate as in adults. After AA load, the plasma concentration of the respective AA increased about 8- to 10-fold; various transaminating reactions could be proved.

Repeated administration of T3 (20 μg/100 g b.wt. for 3 days) or dexamethasone (60 μg/100 g b.wt. for 3 days) caused significant changes of AA plasma concentrations only in young rats. In the kidney, the reabsorbed fraction of AA was enhanced after treatment with both hormones in young animals, whereas in adults the two hormones were without influence on tubular reabsorption of AA. However, after AA-load the fractional excretion (FE) of AA was decreased after hormone treatment in adults, too. In 10-day-old rats, FE was reduced in 8 of 22 AA, indicating stimulatory effects of hormones on tubular AA carriers also without AA-load.

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Pharmacological effects of glycyl-L-proline and other N-derivatives of imino-acids on collagen metabolism

The amount of collagen produced by cells is modulated by different mechanisms. One of these is the basal degradation of newly synthesized collagen which concerns approximately 15% of the collagen synthesized by normal connective tissue cells. The increase in degradation may be the manifestation of a quality control system that rids the cells of non functional protein molecules. But degradation can also be increased when cells are exposed to various pharmacologic agents.

First we showed that basal degradation of newly synthesized collagen can be modulated by a dipeptide: Glycyl-L-Proline. When normal fibroblasts were cultivated in presence of Glycyl-L-Proline the rapidly degraded collagen was at less multiplied by a factor of 2 and this fact was observed at different concentrations: a dose effect was demonstrated from 0.18 μmol/l of Glycyl-L-Proline. Moreover, we demonstrated an increased amount of gelatinases. These gelatinases were showed by zymography using a polyacrylamide gel including type I collagen denatured at 100°C.

It is interesting to point out that this dipeptide is an intermediate of the collagen degradation and its concentration increased during an hereditary disease: the prolidase deficiency. Fibroblasts from prolidase deficient patient presented collagen metabolism disturbances and in particular an increase of basal degradation of newly synthesized collagen.

We showed that a pharmacological agent was able also to stimulate the basal degradation of the newly synthesized collagen: N-Acetyl-Hydroxyproline. In this case the rate of collagen rapidly degraded reached 150% of the control at the concentration of 0.2 μg/ml of N-Acetyl-Hydroxyproline in the culture medium. The quality of the synthesized collagen is normal in particular the hydroxylation rate. So the increase of the degradation seemed due to the stimulation of proteases.

These both agents, Glycyl-L-Proline and N-Acetyl-Hydroxyproline presented similar effects on collagen metabolism. The chemical structures will be discussed, according to their common N-substitution.

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Biochemistry and pharmacology of the NO/cGMP signal transducing pathway in cardiovascular and nervous systems

Nitric oxide (NO) is involved in signal transduction and cellular communication in various biological systems, especially in blood vessels and in the nervous system. It has an exceptionally high affinity to heme-containing soluble guanylyl cyclase, which is stimulated several hundred-fold by submicromolar concentrations of NO. Thus, many of its physiological effects, e.g. vascular relaxation, inhibition of platelet aggregation and coupling of neural activity to increased cerebral blood flow are probably due to accumulation of intracellular cGMP, which stimulates specific protein kinases, regulates ion channels and affects different phosphodiesterases.

NO is synthesized from the guanidino group of L-arginine in an NADPH-dependent reaction that is catalyzed by various NO synthase (NOS) isozymes. Endothelial cells and neuronal tissues contain constitutively expressed NOSs, which require Ca^{2+} for their activities and produce a rapid and transient NO signal in response to hormones and neurotransmitters. Many other cells, e.g. macrophages, hepatocytes, and smooth muscle cells express Ca^{2+}-independent NOS upon activation by cytokines and endotoxin. Expression of inducible NOS results in the release of substantial amounts of NO, which seems to be responsible for the sustained vasorelaxation seen in patients with septic shock syndrome.

We have purified and characterized constitutive neuronal NOS from porcine brain. The enzyme turned out to be a 160 kDa homodimer of unique complexity, containing heme-iron, FAD, FMN and tetrahydrobiopterin (H_4biopterin) as prosthetic groups. The flavins apparently shuttle electrons from NADPH to the catalytic heme site. These reducing equivalents are required for the activation of molecular oxygen, which is incorporated into both NO and the co-product of the NOS reaction, L-citrulline. When the enzyme is not saturated with L-arginine or H_4biopterin, oxygen activation uncouples from NO synthesis, resulting in the generation of superoxide and H_2O_2. Thus, reduced intracellular availability of L-arginine or H_4biopterin may not only decrease the rates of NO formation but may give rise to the generation of cytotoxic oxygen species and peroxynitrite by Ca^{2+}-activated NOS.

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Biosurfactants with potentially antioxydative properties of the perfluoroalkylpeptidoamine type

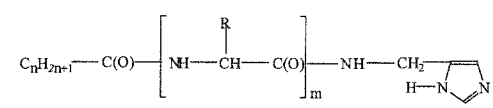
We synthetized an amphiphilic molecules using the simple and direct way. These surfactants are ade of hydrocarbon or perfluorocarbon chains as hydrophobe tail and a peptidoamine as hydrophilic head. The peptidoamines are carcinine or analogs. The following steps are made:

i) The hydrocarboxylic acid tail are commercial product. The perfluorocarboxylic acid tail is obtained by oxydation of corresponding alcohol.

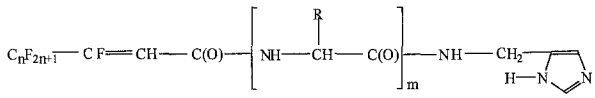
ii) The peptidoamines have been synthetised by coupling α-aminoacid or oligopeptide to histamine.

iii) The two preceeding moieties have been linked together to obtain the amphiphilic molecule.

The structure of synthetised compounds are:



and



Physicochemical properties of these surface active agents in binary systems with water are evaluated. These amphiphilic molecules present a surface tension and Critical Micellar Concentration comparable with other non-ionic surfactants.

An evaluation of agressivity of these amphiphilic molecules was carried out by measuring the hemolytic activity and cell viability of hybridoma in culture in their presence. A comparison of commercial compounds generally considered as biocompatible has been carried out.

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The effect of ovariectomy on phenylalanine and tyrosine metabolism

Although the regulatory activity of steroid hormones on amino acid metabolism has been described, no information is published on the effect of ovariectomy. We studied the influence of ovariectomy in Wistar rats determining the amino acids phenylalanine and tyrosine in liver, kidney, plasma and urine. 32 animals were used in the study, 12 animals were sham operated, 9 animals were ovariectomized and 11 rats were ovariectomized and supplemented with estradiol. No quantitative changes were detected comparing liver and kidney phenylalanine and tyrosine between the groups (sham operated rats liver phenylalanine 2.53 nM/mg $\pm$ 1.07; liver tyrosine 1.95 nM/mg $\pm$ 0.92; kidney phenylalanine 2.16 nM/mg $\pm$ 0.53; kidney tyrosine 1.80 nM/mg $\pm$ 0.39. Ovariectomized rats showed liver phenylalanine 3.07 nM/mg $\pm$ 1.14; liver tyrosine 2.63 nM/mg $\pm$ 1.01; kidney phenylalanine 2.30 nM/mg $\pm$ 0.74; kidney tyrosine 1.93 nM/mg $\pm$ 0.63. Ovariectomized and estradiol supplemented rats presented with liver phenylalanine 2.84 nM/mg $\pm$ 1.40; liver tyrosine 2.35 nM/mg $\pm$ 1.28; kidney phenylalanine 1.91 nM/mg $\pm$ 0.26, kidney tyrosine 1.67 nM/mg $\pm$ 0.23.).

When, however, the phenylalanine/tyrosine ratio in the liver was evaluated, ovariectomized rats showed a significant decrease of the quotient ($p = 0.001$). The phenylalanine/tyrosine ratio was restored by estradiol replacement.

Our findings show that phenylalanine and tyrosine metabolism is under estradiol control. The effect on the metabolic changes could be mediated by enzyme systems as phenylalanine hydroxylase, tyrosine hydroxylase and tyrosine aminotransferase.

Our results would be compatible with previous reports on the stimulatory effect of estradiol on these enzymes.

The kidney phenylalanine/tyrosine ratio was unaffected by ovariectomy and/or estradiol replacement which can be easily explained by different pools, enzyme activities, filtration/reabsorption effects, etc.

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Identification of the motilin pharmacophore through single amino acid substitutions

Motilin is a 22 amino acid polypeptide (FVPIFTYGELQR-MQEKERNKGQ) that regulates fasting gastrointestinal motility patterns through stimulation of discrete receptors on gut smooth muscle cells and enteric neurons. Exogenous administration of the hormone accelerates intestinal transit time and enhances gastric emptying in healthy human subjects. In addition, motilin infusion stimulates the emptying of solids and liquids in patients with diabetic gastroparesis.

Previous research demonstrated that the N-terminal tetradecapeptide of motilin retained most of the binding affinity and biological activity of the full molecule. Subsequently, alanine and D-amino acid scans of this bioactive sequence identified Phe[1], Val[2], Ile[4] and Tyr[7] as critical residues for motilin agonist activity. In order to more clearly define the physicochemical basis for the high affinity interaction between motilin and its receptor, we have examined several series of analogs of [Leu[13]]motilin (1–14) (IC_{50}4.4 nM) in which structural features of these key amino acid residues have been systematically modified. Compounds were assayed in a motilin receptor binding assay employing a rabbit antral homogenate. Biological activity was measured in a contractility assay using rabbit duodenal smooth muscle strips.

Acetylation of the N-terminal amino group {[N-AcPhe[1], Leu[13]]motilin (1–14)} or substitution of this functionality by hydrogen {[des-NH_2-Phe[1], Leu[13]]motilin (1–14)} afforded analogs with significantly reduced in vitro potencies (IC_{50}s 26 and 110 nM, respectively). In contrast, biological activity was relatively insensitive to modifications of the Phe[1] side chain, with the caveat that introduction of hydrophilic functional groups {[β-Pal[1], Leu[13]]motilin (1–14)} adversely affected binding affinity (IC_{50}56 nM). Replacement of Val[2] or Ile[4] by a variety of natural and unnatural amino acids produced several highly active peptides with potency primarily a function of the lipophilicity of the side chains (IC_{50}s 2.8–3.5 nM).

Minor structural alterations of Tyr[7] were not tolerated unless the modified side chain contained both an aromatic ring and a hydrogen bond donor. For example, deletion of the aromatic hydroxyl group {[Phe[7], Leu[13]]motilin (1–14)} or saturation of the aromatic ring {[Cha[7], Leu[13]]motilin (1–14)} was detrimental to in vitro bioactivity (IC_{50}s 6.3 and 174 nM, respectively), whereas incorporation of the 3-indole ring of tryptophan {[Trp[7], Leu[13]]motilin (1–14)} produced an analog that was more potent than the parent peptide (IC_{50} 3.0 nM).

Based on these structure-activity relationships, the physicochemical features responsible for the expression of motilin agonist activity include: 1) a basic N-terminal amino group; 2) hydrophobic contributions from Phe[1], Val[2], and Ile[4]; and 3) π-electron density and hydrogen bond donation from the Tyr[7] side chain.

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The posttranslational modification of C-terminal amino acids of the ras super family proteins

The ras genes are originally discovered as oncogenes of Harvey and Kirsten murine sarcoma viruses. Later, the ras gene was identified as one of the transforming genes isolated from various human tumor tissues. The gene codes for $p21^{ras}$ protein whose molecular weight is about 21,000. The ras protein is now categorized as a member of small molecular weight GTP binding proteins; also referred to as ras super family proteins. The ras family proteins have GTP binding as well as GTPase activity that a single mutation in certain position of the GTP binding domain knocked down the GTPase activity, thus caused the ras to become oncogenic. Current view on the activity of the ras proteins *in vivo* is that they might be functioning as a signal transducer between membranous receptors and downstream protein kinases.

From the structural studies of yeast RAS protein, we pointed out that the RAS protein undergoes extensive posttranslational modification that is essential for proper sub cellular localization and expression of the function. In the case of yeast RAS2 protein, the modification involves (1) removal of methionine from the amino terminus; (2) farnesylation of cysteine residue at the C-terminal region via thio-ether linkage; (3) removal of three amino acid residues from the C-terminus, thus the farnesylated cysteine becomes new C-terminus; (4) methyl esterification of the C-terminal carboxyl group; (5) palmitoylation via weak ester bond whose exact position is yet unclear.

To obtain clearer view on the modification event, we are developing *in vitro* system to reconstitute the entire process of the modification event. For that purpose, we are using *Schizosaccharomyces pombe* system for several reasons, (*i*) molecular size of the pombe ras protein is closer to mammalian ras proteins than RAS proteins of *S. cerevisiae*; (*ii*) we have extensively characterized biochemical features of the *S. pombe* ras protein; (*iii*) the pombe ras is believed to be functioning as a signal transducer for mating and sporulation processes. In addition, the genetics system is as powerful as that of *S. cerevisiae*.

The attempt to purify Cys-isoprenyl transferases from *S. pombe* revealed the existence of several molecular species that showed different substrate specificity to various GTP binding proteins. The cloning effort of the transferase gene also suggests the existence of several genes that might encode isoprenyl transferases that are essential for the posttranslational modification of ras and other small molecular weight GTP binding proteins.

The role of the C-terminal modifications in the activity of ras is yet unclear; however, we hope that we will have clearer view on their functional meanings in near future.

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The purification and characterisation of cystathionine-β-lyase from plant suspension cultures of *Echinochloa colonum*

In higher plants methionine is synthesised from aspartate in a complex pathway that also includes the synthesis of lysine, threonine and isoleucine. Cystathionine-β-lyase is a second enzyme unique to the synthesis of methionine. The enzyme has now been purified from *Echinochloa colonum* suspension cultures to a specific activity of 11360 nmol min^{-1} mg^{-1} protein, some ten fold higher than previously reported; with a purification factor of 5680 and a 62% yield. The enzyme ran as a single band following native polyacrylamide gel electrophoresis which coincided with a single activity staining band. Following SDS-denaturing polyacrylamide gel electrophoresis and silver staining, a single band of molecular weight 41 kDa was determined. Polyclonal antisera raised against the highly purified native enzyme protein, detected one single band following both native and denaturing polyacrylamide gel electrophoresis of crude extracts of *E. colonum* suspension culture cells. A similar protein was shown to be present at lower concentrations in crude extracts of roots, stress and young leaves of two week old plants of *E. colonum*.

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Chemical constitution and toxic activities of aroma concentrate by heating aspartic acid and fructose

Volatile products of aspartic acid-Fructose (AF) interaction at 60, 100 and 120°C were obtained. These products were analysed by gas liquid chromatography. The components identified included different chemical classes, such as short-chain aldehydes, furans, alcohol and alkylated pyrazine derevatives. The short-chain aldehydes and mono and di-alkylated pyrazines were found predominant at 60°C whereas furan and poly alkylated pyrazine derevatives were relatively abundant at 100°C and 120°C.

The toxicological studies on aspartic acid-Fructose interaction products were due to determine the acute median letal dose (LD_{50}) of volatile aroma concentrate of AF-60, AF-100 and AF.120°C via gasteric administration. The LD_{50} of AF-120 was found to be 0.482 g/100 gm body wt, which is the least LD_{50} in proportion to the others.

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Structure and function of gramicidin S synthetase

Antibiotic gramicidin S, a cyclic decapeptide $(_D\text{-Phe-}_L\text{-Pro-}_L\text{-Val-}_L\text{-Orn-}_L\text{-Leu})_2$ is produced by different strains of *Bacillus brevis*. The nonribosomal synthesis of gramicidin S is catalyzed by at least two multifunctional enzymes designated gramicidin S synthetase 1 and 2 (GS1 and GS2), which have molecular weights of 120,000 and 510,000, respectively. In the biosynthesis of gramicidin S, the individual amino acids are activated by ATP through the aminoacyl adenylates analogous to the activation by aminoacyl tRNA synthetase. The aminoacyl moieties are then transferred to thiol groups on the enzymes. GS1 activates, thioesterifies, and racemizes phenylalanine, and GS2 activates and thioesterifies other constituent amino acids. Peptide elongation is initiated by the transfer of $_D$-phenylalanine from GS1 to GS2. The subsequent polymerization process *via* 4′-phosphopantetheine leads to the formation of the enzyme-bound pentapeptides, and then two such pentapeptides cyclize to form gramicidin S by head-to-tail condensation. About 18 reactions are involved in the synthesis of gramicidin S. Many peptide antibiotics in

prokaryotes and lower eukaryotes are produced nonribosomally through similar thiotemplate mechanism. To elucidate the relationship of structure and function of such complicated enzymes, we cloned GS1 (*grs1*) and GS2 (*grs2*) gene from *B. brevis* Nagano. Gramicidin S synthetase genes were organized in an operon of about 18 kb containing three genes in the following order, *grsT*, *grs1*, and *grs2*. *grsT* encoded a protein of 256 amino acids with unknown function, which is homologous to fatty acid thioesterases. It is located at the beginning of the *grs* operon. *grs1* gene encoded 1098 amino acids and *grs2* gene encoded 4451 amino acids. The deduced amino acid sequence of *grs2* gene had four conserved and repeated domains of about 600 amino acids, which was highly homologous to amino-terminal half of GS1 (about 45–50% identity). The four domains were separated by non-homologous sequence of about 500 amino acids. The domains had also significant homology (20–70%) to other peptide synthetases of bacterial and fungal origin and other adenylate-forming enzymes such as tyrocidine synthetase 1, ACVS, an enzyme involved in penicilline biosynthesis, EntF, and AngR. These results suggest that the activation site of the constituent amino acids consist of individual domains. Proline domain was proven at the 5′ terminal quarter of *grs2* gene. The computed analysis revealed several conserved motif in the domains. SGTTGXPKG, the consensus sequence among the adenylate-forming enzymes, may be phosphate-binding loop related to ATP-binding. Recently, we found a new functional motif, QVKIRGXRIE from the analysis of mutant gene defective in amino acid activation. This motif may be related to the recognition of amino acid or the formation of aminoacyl adenylate.

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On the oxidation of thia- and selena-diamines by plant diamineoxidase

Animal and plant diamineoxidase are active on different thiadiamines, that is cystamine, homocystamine, lanthionamine, homolanthionamine, cystathionamine, and on the corresponding selenadiamines. The arising aminoaldehydes spontaneously cyclize into an internal Schiff base, which may then undergo further degradation for the cleavage of a S–C bond due to the presence of the azomethine linkage in the ring.

The animal enzyme and the plant ones -from pea or lentil seedlings- show kinetic parameters very similar for thia- and selena-diamines, thus indicating that, like for other enzymic systems, the substitution of a sulfur atom by a selenium one in the substrate does not appreciably affect the enzyme specificity.

Among the different thia- and selenadiamines, cystathionamine and selenacystathionamine, –NH2.CH2.CH2.X.CH2.CH2.CH2.NH2–, being asimmetrical thioethers are fit to test if the diamineoxidases can discriminate between their two aminogroups. We have therefore studied the oxidation of these two diamines by purified lentil seedlings amineoxidase with the aim to test if only one or both the possible aminoaldehydes –NH2.CH2.CH2.X.CH2.CH2.CHO– or –CHO.CH2.X.CH2.CH2.CH2.NH2– were formed.

Cystathionamine was incubated with diamineoxidase in the presence of aldehyde dehydrogenase (Sigma) and NAD, following NAD reduction at 340 nm. When NAD reduction stopped the reaction mixture was loaded on the aminoacid analyzer. It was demonstrated that carboxymethyl-thia (or selena)-homocysteamine –COOH.CH2.X.(CH2)3.NH2– and carboxyethyl-thia-(or selena)-cysteamine –COOH.(CH2)2.X.(CH2)2.NH2 were formed, in a molar ratio varying in repeated tests between 1,5–2, and in a total amount corresponding to the amount of NAD reduced.

These results indicate that either aminogroup of the diamines considered can be oxidized by diamineoxidase. The different amounts of the carboxyderivatives recovered after the action of aldehyde oxidase may be due to a different affinity of this enzyme for the two aminoaldehydes, and not to a discrimination of the amineoxidase between the two aminogroups of the diamines. Both hypothesis deserve further investigation.

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Transamination of some selena-aminoacids by the so-called glutamine transaminase

The enzyme called glutamine transaminase -E.C. 2.6.1.15- exhibits maximal activity towards phenylpyruvate and 2-oxo-4-methylthiobutyrate as amino acceptors, and phenylalanine and methionine as amino donors.

It has been shown that the enzyme is also active on selenalysine -a lysine analog having the 4 methylene group substituted by a selenium atom- and on the higher homolog selenahomolysine (Se-3-aminopropylselenocysteine).

It seemed interesting to study the activity of the enzyme on selenomethionine and 2-oxo-4-methylselenobutyrate, in comparison with the two correspoding sulfur analogs, to have informations about the possible effects of the substitution of the sulfur by a selenium atom in these substrates.

Glutamine transaminase from bovine brain has been used. Reaction conditions were as described in L-Selenomethionine was a commercial product, from Fluka; 2-oxo-4-methylselenobutyrate was obtained by action of snake venom L-aminoacid oxidase on selenomethionine. Transamination was followed by determining the new formed aminoacid on the aminoacid analyzer.

Incubating selenomethionine with 2-oxo-4-methylthiobutyrate and methionine with 2-oxo-4-methylselenobutyrate it has been possible to calculate the Vmax, Km and Kcat for all the four substrates, following the procedure described by Velick. The values obtained were almost identical for all the four compounds, thus showing that the substitution of the sulfur atom of methionine or of the corresponding ketoderivate by a selenium one does not affect the substrate specificity of the transaminase. Similar general conclusion was drawn from the kinetic parameters for transamination of thialysine and selenalysine, thiahomolysine and selenahomolysine.

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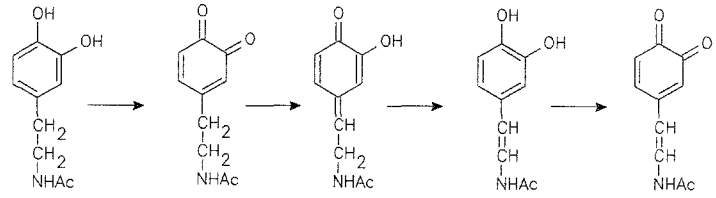
Modification of amino acid residues in the sclerotization of invertebrate structural proteins

Structural proteins may be stabilized by formation of interchain cross-links after extracellular deposition. Various types of

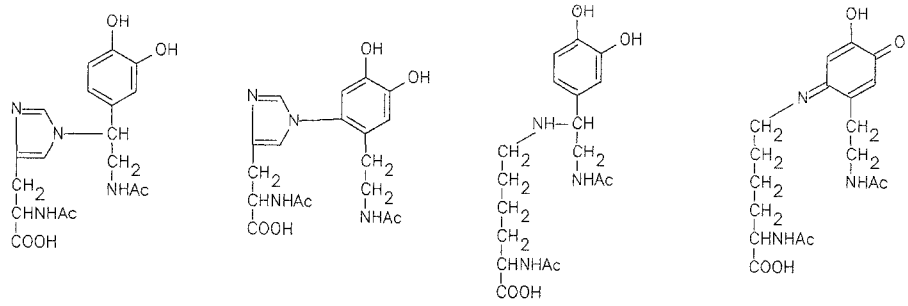
cross-linking mechanisms can be found in different organisms; one mechanism of wide-spread occurrence utilizes protein-bound lysine residues, which after oxidation to aldehydes can react with other amino acid residues in neighbouring chains. Another commonly used mechanism utilizes oxidation products of low-molecular weight phenols to establish cross-links between protein chains. Phenolic sclerotization occurs in many groups of invertebrates, and the insect cuticle is the system which has been investigated in most detail.

Insect cuticle is an extracellular layer covering the whole animal, and consists of chitin filaments imbedded in a protein matrix. Cuticles vary in properties from very soft and pliable structures to extremely hard materials. The differences depend both upon the degree of sclerotization and upon variuos modifications in the enzymatically catalyzed reactions. The main features of the reactions are: 1) an ortho-diphenol (either *N*-acetyldopamine (**I**), or *N*-beta-alanyldopamine) is oxidized to the corresponding ortho-quinone (**II**), which either can react with the cuticular proteins or be enzymatically converted to the corresponding para-quinone methide (**III**). This product can also react with proteins, or in some cuticles it may be isomerized to a 1,2-dehydrodopamine derivative (**IV**), which after oxidation to an unsaturated ortho-quinone (**V**) tends to react with ortho-diphenols.

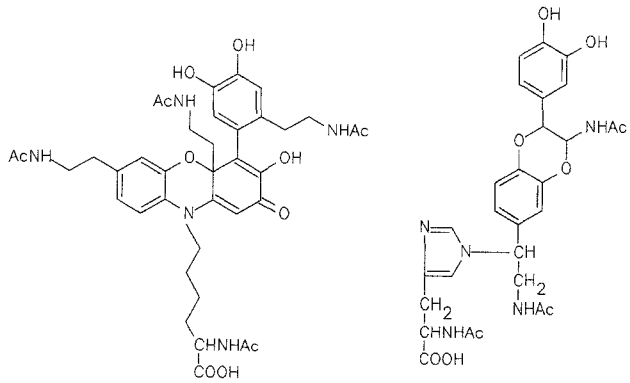
Several amino acid residues have been suggested to be involved in reactions with the quinones, but all attempts to isolate substituted amino acids from sclerotized cuticle have so far been in vain. We have now investigated the problem in another way. After incubation of pieces of cuticle together with *N*-acetyldopamine and *N*-acetylated amino acids the reaction products were separated by reversed phase HPLC, and adducts between *N*-acetyldopamine and amino acid appearing in significant



I **II** **III** **IV** **V**



VI **VII** **VIII** **IX**



X **XI**

amounts were collected for characterization and identification. Their stuctures were established by means of mass spectrometry combined with ^{1}H- and ^{13}C-NMR.

Two adducts were obtained from *N*-acetyldopamine and *N*-acetylhistidine (**VI** and **VII**), and three adducts were identified from *N*-acetyldopamine and alpha-*N*-acetyllysine (**VIII**, **IX** and **X**), as well as from *N*-acetyldopamine and beta-alanine. Some types of insect cuticle can add more *N*-acetyldopamine residues to the primary adducts to give more complicated polymerization products (**XI**). The relationship between the various products and sclerotization will be discussed.

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Prediction of protein folding types from amino acid composition by correlation angles

A protein is usually classified into one of the following four folding types: all-α, all-β, $\alpha + \beta$, and α/β. On the other hand, a protein can also be expressed as a vector of a 20-D space, in which its 20 components are defined by the composition of its 20 amino acids, respectively. Thus, the similarity between any two proteins in their amino acid composition can be quantitatively described in terms of their mutual projection in the 20-D space. The larger the projection value between the two vectors is, the higher their similar extent would be. Based on such a physical picture, a new method, the maximum projection method, has been proposed for predicting the folding type of a protein according to its amino acid composition. In comparison with the existing methods, the new method has the merits of yielding a higher rate of correct prediction, displaying a more intuitive physical picture, and being convenient in application.

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Specific modification of tryptophan residues in peptides and proteins by L-Tryptophan 2',3'- oxidase from *Chromobacterium violaceum*

We isolated from *Chromobacterium violaceum* (ATCC 12472) a new enzyme designated L-tryptophan 2',3'-oxidase (or L-tryptophan: Oxygen 2',3'-oxidoreductase) which catalyzes the formation of a double bond at the C_α–C_β position of tryptophan residues.

When the enzyme was incubated in the presence of N-acetyltryptophanamide (NATA) we observed a total conversion into a α,β-dehydro-NATA under several experimental conditions between pH 3 and pH 8. Using a variety of tryptophan derivatives, we demonstrated that the enzyme is highly specific for unsubstituted indole containing compounds. The catalysis requires the presence of the α-carbonyl group which specifies the stereoselectivity towards formation of L-enantiomers. These results will be discussed in terms of a possible catalytic mechanism.

L-tryptophan 2',3'-oxidase not only acts on isolated tryptophan side-chain but is also capable of dehydrogenating tryptophan residues in peptides and proteins. Its stability in both reducing and denaturating conditions (urea 3–5 M, SDS 0,1–2%, DTT 10–200 mM) and toward high temperature allowed us to use this enzyme in conditions that are denaturing for most proteins and which, therefore, yield their hidden tryptophan acessible to modification.

One major feature of L-tryptophan 2',3'-oxidase resides in the fact that it does not lead to side-product formation. This particularity allows the direct routine synthesis of pure α,β-dehydrotryptophanyl peptides and proteins, a situation which is of obvious practical interest for either structure-activity relationships studies or design of molecules having new pharmacological and/or pharmacokinetic properties. Finally, the presence of a double bond in a peptide or a protein will be of great use to introduce stable or radioactive isotopes (^{2}D, ^{3}H, ^{125}I).

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Regulation effect of the side chain on the N-phospho-amino acids—mechanism for the phosphoprotein

The amino acids could be N-phosphorylated in aqueous basic media with dialkyl phosphite by one pot reaction. Due to the presence of phsophoryl group, the N-phosphoamino acids were promoted to the reactive compounds, which would be derived into four types of products: namely the ester exchanges on the phosphoryl, migration of the phsophorylgroup, esterification and the self elongation to peptides. It was found that the free carboxylic group was essential for this activation, and the side chains had profound effect on their reactivity. For example, the α-amino acid was promoted but not the β-amino acid, the serine was activated but not the cysteine, the aspartic acid was promoted but not the glutamic acid. Through the model compounds investigation, it showed that the side chains of the amino acids had the regulation and diffrentiation effect on the reactivity of N-phosphoamino acids selectively and specifically. These results indicats that the intrinsic relationship between the phsophoryl group and the amino acids side chains might be the clue to the mechanism for the phosphoprotein.

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Enantiomeric conversion of racemic amino acid mixtures via an oxidase-aminotransferase coupled system

Porcine D-amino acid oxidase, E. coli branched-chain amino acid aminotransferase and excess L-glutamate are used to convert gram amounts of racemic amino acid mixtures to the L-form enantiomer. Aliphatic and aromatic proteinogenic amino acids were obtained with 99% enantiomeric purity in 90% isolated chemical yields. Nonbiological amino acids such as 2-aminobutyrate and 2-amino-4-pentenoate (allylglycine) are also substrates for this enantiomeric conversion system. Further research is being conducted to extend the practical substrate range of this reaction system. In addition we are studying procedures which more efficiently shift the equilibrium distribution of the aminotransferase reaction toward the desired amino acid.

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Overhydration with 1.5% glycine solution in conscious sheep

With the aim of studying the pathophysiological background of the "TUR syndrome", we gave 6 conscious ewes an intrave-

nous infusion of 57 ml/kg of 1.5% glycine solution over 40 min, which was equal to a total glycine load between 50–67 grams. Isotonic saline infusions served as controls. Central haemodynamics were monitored and the distribution of the administered water between different body fluid compartments was estimated. Plasma atrial natriuretic peptide (ANP), arginine vasopressin (AVP) and plasma renin activity (PRA) was followed. In addition plasma concentrations of protein, K, Na, creatinine, urea and plasma osmolality were measured repeatedly for up to 4 h. The urinary excretions of Na, K, creatinine, urea and solutes were also followed.

Both infusions caused an considerable initial cardiovascular strain which was normalised within 30 min after the infusion.

Infusion of glycine resulted in a decrease in the plasma Na concentration from 144 ± 3 to 114 ± 4 mmol/l. The plasma osmolality decreased from 290 ± 2 to 280 ± 1 mosmol/l, and remained low. There was a median 6-fold increase in plasma vasopressin concentration, while saline did not elicit vasopressin release. Despite the absence of electrolytes in glycine solution, the urinary excretion of sodium amounted to 106 ± 40 mmol.

Glycine infusions resulted in a 4-fold increase and saline in a doubling of the plasma ANP concentration, despite a more pronounced volume expansion from saline. The ANP level remained significantly elevated for 2 h after glycine infusion.

The PRA decreased by about 50 per cent in response to both infusions. However, PRA returned to the baseline level at the end of the glycine infusion, whereas it remained depressed during the entire follow-up period after saline infusion. This may be explained from the calculations of fluid distribution between different compartments suggesting that, in contrast to saline, glycine tended to be accumulated intracellulary.

In conclusion, i.v. infusion of 1.5% glycine solution in sheep causes transient circulatory strain and induces an osmotic diuresis with a substantial loss of electrolytes and solutes. In addition there is a specific ANP- and AVP stimulating effect which may on one hand contribute to hypovolaemia, hypotension and natriuresis and on the other hand cause maximal water retention and persisting hypoosmolality. Urea and creatinine clearances increased only in response to isotonic saline. Glycine infusion was even followed by reduction of the creatinine clearance, indicating an impairment of glomerular filtration.

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Glycine toxicity in transurethral surgery

Irrigating fluid is used during endoscopic procedures to give the surgeon clear vision by removing blood and pieces of resected tissue from operating field. Glycine 1.5% in sterile water is the most widely used solution for this purpose. However, irrigating fluid in contact with severed human tissues can be absorbed into the body. This is known to occur frequently during transurethral resection of the prostate (TURP) which is, next to cataract, the most common surgical operation in the Western society. In a recent case series of 700 TURP operations in our hospital, glycine absorption was found in 46% of the operations, and the absorption volume exceeded 1,000 ml (uptake of >15 g of glycine) in 8% of them.

Due to the toxic properties of glycine and a huge volume load, excessive absorption may give rise to symptoms which are usually summarized as the "TUR syndrome". This comprises symptoms affecting the circulatory and nervous systems. Symptoms of glycine toxicity may occur when more than 20 g of glycine is absorbed. In these cases, there is a marked increase in the serum concentration of glycine and also of most other non-essential amino acids. The patients may have an elevated blood ammonia level, although a rise in the serum glutamate concentration seems to be a more consist feature that distinguishes patients who experience glycine toxicity symptoms from those who do not.

We have performed studies of the pharmacokinetics of an excess amount of glycine. At the end of an intravenous infusion of 1,000 ml of 1.5% glycine solution over 20 min in 10 male volunteers, the distribution volume for glycine at 20 min was 26.8 ± 4.71 and the half-life was 41 ± 7 min. Between 5 and 10% of the glycine load was excreted unchanged in the urine. However, other data show a markedly longer apparent half-life and a higher percentage of excreted glycine in the urine for larger amounts of glycine.

Recent methods to detect fluid absorption before the patient develops symptoms of the TUR syndrome include ethanol monitoring, which means that a tracer amount of ethanol (1%) is added to the irrigating fluid and that the transfer of fluid to the patient is indicated by measuring the ethanol content in the exhaled breath.

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Measurement of serum and urinary nitrate as indices for nitric oxide (NO) production; inhibition of no synthesis and blood pressure regulation in rats

It has become evident that inhibition of synthesis of nitric oxide (NO) from L-arginine causes blood pressure rises. However there is no direct evidence to show the underlying mechanism. We measured the hemodynamic characteristics and both serum concentrations and amount of urinary excretions of nitrate ions (NO_3^-) after inhibition of NO synthase (NOS) by N^G nitro-L-arginine (NNA).

When NNA was administered for 5 days by giving it with the drinking water (1 mg/ml), blood pressure was significantly elevated throughout the study period. At the 2nd day, pulse rate and urinary excretions of norepinephrine significantly decreased, but urine volume and amount of sodium and potassium excretion were not influenced. At the 4th day, urinary excretions of dopamine significantly decreased. Mean femoral arterial pressure was significantly elevated in the group treated with NNA when measured directly at the 5th day. Thereby, since cardiac output decreased, total peripheral resistance was significantly increased when measured by using radioactive microspheres with a reference sample method. Among the organs and tissues examined, regional blood flow was significantly decreased in the liver and spleen. The vascular resistance was significantly increased in all organs and tissues except the brain, heart, ileum and colon. When NNA, 10 mg/Kg, was injected bolusly into the femoral vein using urethane-anesthetized rats, blood pressure markedly increased and cardiac index decreased. Blood flow was significantly decreased at the lungs, liver, kidneys, intestine and adipose tissues.

Hemodynamic studies revealed that the main cause of the hypertension with NNA was elevation of peripheral resistance, particularly at the liver, kidneys and intestine. Therefore, at the 2nd day, baroreceptor reflexes inhibited sympathetic outflow, but

was reset at the 4th day. Since urinary excretions of NO_3 markedly decreased with NNA, and NO_3 is known to be derived from NO, NNA was found to inhibit synthesis of NO *in vivo*. Furthermore because amount of urinary NO_3 significantly and negatively correlated with blood pressure, the cause of hypertension could be due to reduced production of NO.

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Antiviral and immunological activity of glycoproteins from marine brown Alga Colpomenia sinuosa

Algae are an important source of therapeutically useful substances. Based on ethnopharmacognosy; immunodulatory activities of some marine brown algae have been studied. The brown alga *Colpomenia sinuosa* yielded mannogalactan-containing glycoproteins. As test material, retentates were obtained by ultrafiltration and fractionations from a subsequently performed Sephadex G-50 chromatography. The second and fourth fractions contained high amounts of glycoproteins with relative molecular masses 60.000 and 80.000 D respectively. Administration of these glycoprotein-containing fractions strongly inhibited the incorporation of (^{3}H)-thymidine into mitogen-stimulated human lymphocytes as observed in the lymphocyte transformation test. These suppressive effects were confirmed by corresponding results from chemiluminescence test of isolated and stimulated human granulocytes as well as, from a photometrical method using isolated and stimulated peritoneal macrophages from guinea pigs. Furthermore, extracts injected intraperitoneally in mice caused a suppression of antibody-forming cells as shown in the hemolysis plaque assay. The bioassays were performed on CV-1 cells infected with HSV-1 (herpes simplex virus) and with BHK cells infected with VSV (vesicular stomatitis virus). The glycoprotein-containing fractions II and IV of Sepnadex G-50 chromatography exerted an antiviral activity and reduced the number of plaques by up to 80–100% respectively for HSV-1 and 40–70% respectively for VSV.

Sulfur containing Amino Acids

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Effects of taurine on the phosphorylation of a specific protein in a mitochondrial subcellular fraction of the rat retina: partial characterization of the ~20 K molecular weight phosphoprotein

While the physiological actions of taurine (2-aminoethanesulfonic acid) in the rat retina and other excitable tissues such as brain and heart are numerous (Brain Res. Reviews 16: 151, 1991; Physiol. Rev. 72: 101, 1992) the exact mechanism(s) of action of this ubiquitous sulfur compound in mammalian tissues is (are) not known. However, one possible mechanism of action for taurine at the molecular level is its inhibitory effects on the phosphorylation of specific proteins found in the rat retina (Neurochem. Res. 17: 831, 1992), brain (Brain Res. 553: 89, 1991) and heart (Adv. Exp. Med. Biol. 315: 309, 1992). A second postulated mechanism of action for taurine is its role in Ca^{2+} modulation. Taurine has a biphasic effect on Ca^{2+} uptake, i.e., increases Ca^{2+} uptake at low Ca^{2+} concentrations in the rat retina but prevents Ca^{2+} accumulation in situations of high concentrations in the rat retina and Ca^{2+} paradox model in the rat heart. It has also been suggested that the stimulatory effects of taurine on Ca^{2+} uptake and the inhibitory effects of taurine on protein phosphorylation may be causally related in the rat retina. In these studies a physiologic taurine concentration of 34.2 ± 2.1 mM was determined to inhibit by 50% the phosphorylation of a ~20 K molecular weight protein. (The concentration of taurine in the retina is 50–80 mM.) Phase separation experiments utilizing Triton X-114 indicate that the ~20 K phosphoprotein is a soluble protein found in the aqueous fraction. No evidence was observed to indicate that the ~20 K phosphoprotein partitions into the detergent phase thus suggesting that it is not an integral membrane protein. Subjecting the ~20 K molecular weight protein (after phosphorylation with [γ-^{32}P]ATP) to different treatments such as acetone, chloroform/methanol, NaOH (both 0° and 100°C), trichloroacetic acid (100°C), and pronase and ribonuclease A indicate that the phosphate moiety is incorporated into protein via a phospho-ester bond. Digestion of the isolated ~20 K phosphoprotein with trypsin and 6 M HCl and analysis on 2-dimensional high voltage electrophoresis using cellulose mylar plates indicate that serine and threonine residues are phosphorylated. Phosphorylation of the serine residue(s) predominates other phosphorylation of the threonine residue(s). Kinase activators and modulators such as cAMP, cGMP, phorbol ester, calmodulin, and Ca^{2+} have no effect on the phosphorylation of the ~20 K molecular weight protein. Kinase inhibitors were also tested for their effects on phosphorylation. Staurosporin and W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] were found to be inhibitors while chelerythrine was observed to be an activator of the phosphorylation of the ~20 K molecular weight protein.

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Sulfur containing cyclic ketimines: functional trends

The cysteine derivative lanthionine ketimine (LK), detected in bovine brain (about 0.5 nmol/g) and in other mammalian tissues, binds specifically and with high affinity to isolated brain membranes. This binding has been studied in detail with [^{35}S]LK synthesized from [^{35}S]L-cysteine. It is reversible, pH dependent and the labeled ligand is released unchanged upon addition of excess unlabeled LK. Binding is highly specific and the ligand is displaced only by other ketimines and by catecholamines (adrenaline, noradrenaline, dopamine, isoproterenol), and it is not displaced by adrenergic antagonists. Displacing curves of

[^{35}S]LK by catecholamines, of [^{3}H]adrenaline by ketimines and double-label experiments with [^{3}H]dihydroalprenolol show that LK binds to sites which do not bind adrenergic antagonists, which are not identical with α- or β-type adrenergic sites and which interact in some way with catecholamine-binding sites. LK binding has been found present also in other bovine tissues membranes as heart and kidney. With turkey erythrocytes membranes, LK shows an effect on [^{3}H]dihydroalprenolol displacement by adrenaline, shifting the curve to lower affinity; with bovine brain homogenate a significant improvement of about 30% of basal adenylate cyclase activity by LK and other ketimines is observed.

From these results a physiological role in central nervous system may be inferred for LK, indicating that ketimines have a yet undiscovered role in brain. Identification of involved brain area by autoradiography on tissues slices and behavioural and electroencephalographic studies on rats and mice after LK injection are in progress in order to recognize the role of this class of natural sulfur-containing, amino acids-related compounds.

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Regulation of cysteinesulfinate-dependent metabolism of cysteine by dietary protein, dietary sulfur amino acids and acid-base balance

Cysteine (CYS) may be metabolized by both cysteinesulfinate-dependent and cysteinesulfinate-independent pathways. The cysteinesulfinate-dependent routes of CYS catabolism appear to be highly regulated in liver in response to dietary changes. CYS is oxidized to cysteinesulfinate (CSA) by cysteine dioxygenase (CYS-DO). The CSA may then either be decarboxylated by cysteinesulfinate decarboxylase (CSA-DC) to form hypotaurine (which is further oxidized to taurine) or transaminated to the putative intermediate, 3-sulfinylpyruvate, which decomposes to yield pyruvate and sulfite (which is further oxidized to sulfate). To determine the relationship between changes in enzyme activities and changes in CYS catabolism, rats were fed diets that contained 10%, 30% or 60% casein or 10% casein with supplemental L-methionine (0.3% or 1.0%) or L-cystine (0.24% or 0.8%) for 3 weeks. Hepatocytes were then isolated. Enzyme activities were measured, and metabolism of [^{35}S]CYS and [^{35}S]CSA by hepatocytes was determined. CYS-DO activity in hepatocytes isolated from rats fed 30% and 60% casein was 4- and 5-times that in cells from rats fed 10% casein. In contrast, CSA-DC activity was decreased in rats fed 30 or 60% casein to 14% or 7% of the level observed in rats fed 10% casein. Total catabolism of [^{35}S]CYS by hepatocytes was greater in hepatocytes isolated from rats fed higher levels of casein, whereas taurine production from [^{35}S]CSA was less in hepatocytes isolated from rats fed higher levels of casein. Taurine production from CYS, as a percentage of total catabolism, was also lower in hepatocytes from rats fed higher levels of casein; this was presumably a consequence of the decreased CSA-DC activity. CYS-DO activity in hepatocytes isolated from rats fed 10% casein supplemented with 1.0% L-methionine or an equisulfur amount of cystine was 12.6-times or 7.5-times that in hepatocytes from unsupplemented control rats. CSA-DC activity was not significantly affected by methionine or cystine supplementation. Total catabolism of CYS was greater in hepatocytes from rats fed supplemented diets than in those fed the 10% casein basal diet, whereas total catabolism of CSA was the same for all dietary groups. Taurine production, as a percentage of total CYS catabolism, increased from 22% in hepatocytes from rats fed the basal diet to 50% in cells from rats fed the diet supplemented with 0.8% cystine and to 62% in cells from rats fed the diet supplemented with 1.0% methionine. This appeared to be due to increased flux of CYS to CSA, which would generate a higher intracellular CSA concentration. Production of acidosis by feeding rats excess chloride did not affect the activity of either CYS-DO or CSA-DC. The excretion of excess sulfur and nitrogen as taurine may minimize sulfate-induced acidosis when excess sulfur amino acids are being catabolized, whereas taurine production may be restricted when protein or a mixture of amino acids is being catabolized to conserve the carbon chain of CYS for gluconeogenesis or oxidation.

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Physiological activities of S-(1,2-dicarboxyethyl)glutathione as an intrinsic tripeptide present in liver, heart and lens

In 1963 S-(1,2-dicarboxyethyl)glutathione (DCE-GS) was isolated from calf lens. However, the physiological significance and biochemistry of this peptide were unknown. First of all we established the analytical method of DCE-GS. Using this method the peptide was found to be present in concentrations of 119, 71.6 and 27.4 nmol/g tissue in rat lens, liver and heart, respectively. The concentration of DCE-GS in lens decreased rapidly during the development of cataract and parallel to that of glutathione. In rat liver, it was primarily located in the cytosolic fraction. The DCE-GS-synthesizing enzyme was found to be highest in rat liver and partially purified from liver cytosolic fraction. The enzyme had M.W. of 53 kDa and was monomeric. The substrates for the enzymic synthesis were GSH and L-malate and the Km values for GSH and L-malate were 2.3 and 4.0 mM, respectively. Changes in the level of DCE-GS with time were determined during regeneration of rat liver after partial hepatectomy. The level increased in regenerating rat liver, reaching a maximum level (4.7-fold) on day 2 and reverted to the normal level in 1 week. The peptide showed strong inhibitory effects on blood coagulation and platelet aggregation. The inhibitory effect on blood coagulation was almost the same as those of EDTA or citrate. But DCE-GS did not show chelating activity. As for ADP- or thrombin-induced platelet aggregations, it exerted a potent effect on the secondary aggregation, while it was less active in the primary aggregaton. The mechanism of the inhibition by DCE-GS on platelet aggregation will be mentioned in detail. DCE-GS showed an anti-inflammatory effect which was evaluated by testing the inhibition of the experimental conjunctival edema of rats. This peptide also inhibited the histamine release from rat mast cells induced by the compound 48/80. It was added to the mast cells before addition of the compound 48/80 and an inhibition of the histamine release up to 96% at a 1 mmol/l concentration occurred. Furthermore, it displayed an antianaphylactic effect in rats using antibody against chiken egg albumin. Analogues or derivatives of DCE-GS were synthesized and tested for those inhibitory activities. However, there was no other peptide having stronger effects than DCE-GS.

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In vivo and in vitro effect of (aminooxy)acetate on the cysteine metabolism in rats

Sulfate is a main final metabolite of L-cysteine metabolism in mammals. The main route of this metabolism is cysteinesulfinate pathway (CSA pathway). The first metabolite is CSA, which is then transaminated to sulfinopyruvate by aspartate aminotransferase (AAT) and finally metabolized to sulfate. In addition to this pathway, we have reported the presence of a new sulfate-forming pathway in rat liver mitochondria and proposed a scheme, in which L-cysteine is metabolized to sulfate through 3-mercaptopyruvate pathway (MP pathway or transamination pathway). The first reaction of this pathway is the transamination of L-cysteine, which is shown to be catalyzed by AAT. In both pathways, the transamination reaction catalyzed by AAT is involved. In the present study, we examined the effect of (aminooxy)acetate, a potent inhibitor of AAT, on the cysteine metabolism in rats.

(1) In vitro effect: Incubation of rat liver mitochondria with 10 mM L-cysteine, 10 mM 2-oxoglutarate and 10 mM GSH resulted in the formation of 4.6 and 1.5 μmol/mitochondria from 1 g of liver per h of sulfate and thiosulfate, respectively. Substitution of CSA for Cys resulted in the formation of 25.0 of sulfate and no thiosulfate. These reaction was strongly inhibited by AOA at more than 2 mM. (2) In vivo effect: Subcutaneous injection, twice a day, of 25 mg/kg of body weight of AOA inhibited sulfate excretion after Cys administration by 50%. The excretion of taurine, another main Cys metabolite of CSA pathway, increased, and the increase corresponded to the decrease in the sulfate excretion. Sulfate formation by liver mitochondria prepared from rats injected with AOA was found to be also inhibited. These findings show that the transamination reaction is important in the cysteine metabolism both through CSA and MP pathways.

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Identification of cystathionine mono-oxo acids, S-(3-oxo-3-carboxy-n-propyl)cysteine and S-(2-oxo-2-carboxyethyl)homocysteine in the urine of a patient with cystathioninuria

Cystathioninuria is an autosomal recessive hereditary disorder, and phenotypical homozygotes lead to persistent excretion of large amounts of cystathionine in the urine due to cystathionine γ-lyase deficiency. We have previously reported that S-(3-hydroxy-3-carboxy-n-propyl)cysteine, S-(2-hydroxy-2-carboxyethyl)homocysteine, S-(2-carboxyethyl)cysteine, N-monoacetylcystathionine, perhydro-1,4-thiazepine-3,5-dicarboxylic acid, N-acetyl-S-(3-hydroxy-3-carboxy-n-proryl)cysteine, N-acetyl-S-(2-carboxyethyl)cysteine were identified as cystathionine metabolites in the urine of patients with cystathioninuria.

Cystathionine ketimine has been reported in the urine samples from normal subjects by D. Cavallini et al.

We suggested the existence of precursors of cystathionine ketimine, S-(3-oxo-3-carboxy-n-propyl)cysteine and S-(2-oxo-2-carboxyethyl)homocysteine in the urine of patients with cystathioninuria, but these cystathionine mono-oxo acids have been identified yet.

Novel cystathionine mono-oxo acids have been detected in the urine of a patient with cystathioninuria using liquid chromatography-mass spectrometry with an atmospheric pressure ionization interface system, and amino acid analyzer.

The determination of these cystathionine mono-oxo acids and cystathionine ketimine took advantage of the selective absorbance at 380 nm of the phenyl-isothiocyanate-ketimine interaction product. The total concentration of these compounds found in the urine samples of a cystathioninuric patient and six healthy subjects were respectively 3611.3 μg and 148.4 μg per g of creatinine. The cystathioninuric patient excrets 20 times more cystathionine mono-oxo acids in the urine than those in healthy subjects.

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S-Adenosylmethionine synthesis and its regulation

Rat liver S-adenosylmethionine synthetase (AdoMet synthetase) appears as high-Mr (tetramer) and low-Mr (dimer) forms. Both are inhibited in the presence of GSSG at pH 8. The K_i values are 2.14 and 4.03 mM for the high- and low-Mr forms, respectively. GSH modulates the inhibitory effect of GSSG, but has no effect when added alone. At a total glutathione concentration of 10 mM a K_{OX} of 1.74 was calculated for the high-Mr form, whereas this constant was 2.85 for the low-Mr AdoMet synthetase. No incorporation of ^{35}S-GSSG was observed in either of the enzyme forms, and inhibition of the activity was correlated with dissociation of both AdoMet synthetases to a monomer. The data obtained by GSSG incubation seem to suggest that oxidation leads to the formation of an intrasubunit disulfide.

The AdoMet synthetase sequence presents several consensus motifs for the action of protein kinases. We have studied the possible regulation by protein kinase C of rat liver AdoMet synthetase. Both enzyme forms, tetramer and dimer, are phosphorylated in vitro by this kinase in the same residue, Thr 342 of the sequence. Phosphorylation of the dimer leads to its dissociation, being this the first time that a fully active monomer has been obtained. The kinetics of the monomer have been studied, and a Km_{Met} of 931.9 μM, a Km_{ATP} of 708 μM and a V_{max} of 66.8 nmol/min/mg have been calculated. Alkaline phosphatase treatment of both enzyme forms produces a reduction in their activity with no effect in the oligomeric state. On the other hand, Protein kinase C phosphorylation of the alkaline phosphatase-treated AdoMet synthetase forms leads to the dissociation of the dimer to originate a monomer. Rephosphorylation occurs again in the same residue, Thr 342 of the sequence. This amino acid is the most exposed residue of the whole sequence as deduced from an hydrophaticity profile.

In summary, all the data available in vitro seem to suggest the implication of the ratios GSH/GSSG and protein phosphorylation in the regulation of rat liver AdoMet synthetase.

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Structural and functional properties of bacterial S-adenosylhomocysteine hydrolase

S-adenosylhomocysteine hydrolase (SAHase) catalyzes the reversible hydrolysis of S-adenosylhomocysteine (SAH) to L-homocysteine (Hcy) and adenosine (Ado), thus accomplishing the double function of clearing a potentially harmful compound such as SAH and of regenerating free homocysteine, which is essential for the reactivation of methyl cycle through methionine formation. SAHase has been purified and extensively studied from a variety of animal and plant sources. In order to assess other structural and kinetic features of bacterial SAHase which can be expected to be different from the more deeply investigated eukaryotic enzyme, we studied, a prokaryote strain in which this enzyme was quite abundant. SAHase was purified to homogeneity from the Gram negative bacterial strain *Acinetobacter calcoaceticus* 501. The molecular weight of the native enzyme, estimated by gel permeation, was about 288 KDa, while sodium dodecyl sulfate polyacrylamide gel electrophoresis yielded a relative molecular mass of 48 KDa. It could be thus tentatively concluded that SAHase from bacterial cells has the structure of a hexamer. The determination of the coenzyme content gave 4 mol of NAD^+ and 2 mol of NADH per mol of enzyme. The isoelectric point of native SAHase was at pH 5.1. When assayed in the hydrolytic direction, the Km for SAH and the Vmax of the enzyme for this substrate were 84 μM and 357 μmol/min/mg, respectively; in the synthetic direction, instead, the Km for adenosine and the corresponding Vmax value were 1.6 μM and 37 μmol/min/mg, respectively. Substrate analogs were tested for their ability to act both as inhibitors and as inactivators of the enzyme. Among these compounds, arabinofuranosyl adenine (Ara A) appeared as the most powerful competitive inhibitor (K_i = 18 μM) as well as the strongest time-dependent inactivator. The common feature of all the assayed analogs was the presence of the adenine ring in their molecular structure. It can thus be concluded that, in agreement with eukaryotic SAHase, the presence of the adenine moiety is an essential element in substrate and/or inhibitor interaction with this bacterial enzyme.

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Spontaneous and enzymatic changes of aminoethylcysteine ketimine

A minoethylcysteine ketimine (AECK) is the cyclization product of the keto acid coming from the α-deamination of aminoethylcysteine, called also thialysine. AECK is known to undergo autoxidation under slight alkaline conditions and dimerization in the neutral and acidic range. An unknown red product is also formed by staying under the latter conditions. Autoxidation is accelerated by some enzymatic and non enzymatic catalysts. AECK, under O_2 bubbling, 38°C, pH 8.5, revealed the formation of various products envisaged by TLC and HPLC. Among these, the sulfoxide of the ketimine (AECK-SO) has been identified as one of the main products. Addition of 2,4-dinitrophenylhydrazine at the end of autoxidation yelded a precipitate identified as the osazone of the mesoxalic semialdehyde, suggesting the production of 2-hydroxy-AECK (i.e., the product of addition of the aldeyhde-keto acid with cysteamine). Extraction with chloroform of the final oxidation solution of AECK followed by preparative TLC gave other compounds among which a thiazane bicyclic product, 2-hydroxy-thiomorpholeine and thiomorpholine-3-one have been identified by NMR and Mass analysis. AECK has been detected in mammalian (bovine) brain and the dimer in human urine, underlining the possible physiological significance of these compounds and derivatives in mammals. AECK-SO can be reduced to chondrine reopening the problem of the meaning of the presence of chondrine in the urine of japanese people, attributed so far only to the habit of this population to eat marine algae.

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Selective modulation of GSH levels and sulfurtransferases activity in Ehrlich ascites tumor bearing mice

The aim of the experiments was to evaluate the effect of i.p. administration of some sulfur compounds (S-comp) upon the GSH level and the activity values of 3-mercaptopyruvate sulphurtransferase (MPST) (EC 2.8.1.2) and rhodanese (EC 2.8.1.1) in homogenates of tumor bearing (TB) mouse liver and Ehrlich ascites tumor cells (EATC).

GSH plays an important role as a cellular protector. Thiazolidine derivatives (TD), products of condensation of L-cysteine (cys) with formaldehyde (thiazolidine-4-carboxylic acid – CF), acetaldehyde (2-methyl-thiazolidine-4-carboxylic acid – CA) and pyruvate (2-methyl-thiazolidine-2,4-dicarboxylic acid – CP), as well as thiocystine T-cys) and cys, but not methionine (met) and thiosulfate ($S_2O_3^{2-}$) successfully elevated glutathione (GSH) levels in TB mice livers.

At the same time, CA, results in a significant drop of GSH concentration value in EATC, whereas the others do not result in an elevating of GSH level. Thus, the studied S-comp through their selective influence upon the level of GSH in the liver and in cancer cells, seem to be the interesting compounds for further studies on anticancer therapy.

The activities of MPST and rhodanese are much lower in EATC than in TB mice liver. All studied S-comp, especially CP and CA increase the activity value of MPST in TB mice liver. CP, CF, CA and t-cys result also in elevation rhodanese activity but in contrast to non TB mouse liver met and $S_2O_3^{2-}$ do not cause any increase in this enzyme activity and cys even seems to decrease it. The above findings indicate that in TB mouse liver the pathway of metabolic changes through 3-mercaptopyruvate and GSH synthesis is induced.

In EATC, all studied S-comp had no effect in the rhodanese activity value while MPST was activated only by CP and CA. Our investigation "in vitro" also proved that in EATC homogenate in contrast to mice liver homogenate no synthesis of sulfane sulfur containing compouds from TD and cys was observed. It means that all S-comp in cancer cells had no effect both on sulfane sulfur metabolism mainly associated with rhodanese as well as on GSH synthesis.

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Biosynthesis of 3-mercaptolactate-cysteine mixed disulfide

3-Mercaptolactate-cysteine mixed disulfide [S-(2-hydroxy-2-carboxyethylthio) cysteine, HCETC] is a constituent of normal human urine and excreted in large amounts in the urine of patients with β-mercaptolactate-cysteine disulfiduria. Studies using rat tissues have shown that 3-mercaptolactate portion of HCETC is formed by the reduction of 3-mercaptopyruvate, the intermediate of 3-mercaptopyruvate pathway of cysteine metabolism. However, the biosynthetic pathway of HCETC is not established. We studied the formation of 3-mercaptolactate-glutathione mixed disulfide [S-(2-hydroxy-2-carboxyethylthio) glutathione, HCETG] and degradation of HCETG to HCETC using homogenates of rat tissues.

When the homogenate of 100 mg of liver, heart, kidney, brain or blood was incubated with 10 μmol of 3-mercaptopyruvate, 10 μmol of GSH and 5 μmol of NADH at 37°C for 30 min, 0.92, 1.22, 0.28, 0.58, or 0.21 μmol, respectively, of HCETG was formed. In the same reactions, 0.01, 0.09, and 0.45 μmol of HCETC was formed with the reaction with liver, heart and kidney homogenate, respectively. HCETG (0.42 μmol) was also formed when the liver homogenate was incubated with 10 μmol of D,L-3-mercaptolactate and 10 μmol of GSH. When a homogenate of 170 mg of kidney was incubated with 5 μmol of HCETG at 37°C, HCETG was completely degradated within 30 min and HCETC corresponding to over 80% of HCETG was formed. These results seem to indicate that HCETG is one of the precurcers of HCETC biosynthesis.

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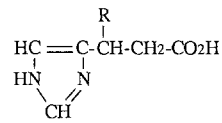
Participation of S-containing amino acids in the metabolism of histidine: formation of S-[2-carboxy-1-(1H-imidazol-4-yl)ethyl]cysteine by enzymatic degradation of S-[2-carboxy-1-(1H-imidazol-4-yl)ethyl]glutathione with rat kidney homogenate

S-[2-Carboxy-1-(1H-imidazol-4-yl)ethyl]glutathione (I), N-{S-[2-carboxy-1-(1H-imidazol-4-yl)ethyl]cysteinyl} glycine (II) and S-[2-carboxy-1-(1H-imidazol-4-yl)ethyl]-N-γ-glutamylcysteine (III) have been tested for possibility as physiological precursors of two urinary compounds, S-[2-carboxy-1-(1H-imidazol-4-yl)ethyl]cysteine (IV) [Kinuta M et al (1992) Biochem J 283: 39–40] and S-[2-carboxy-1-(1H-imidazol-4-yl)ethyl-2-thioacetic acid (V). [Kinuta M et al (1991) Biochem J 275: 617–621]. Characterization and identification of compounds here were performed by FAB-MS, high-voltage paper electrophoreses and elemental analyses as well as chemical syntheses. Compound I was synthesized in 72% yield by incubating the reaction mixture of urocanic acid and 3-fold excess glutathione (GSH) at 65°C for one week, which was accompanied by formation of II in 15% yield. Compound III was produced by partial hydrolysis of I in HCl. By incubation of I with rat kidney homogenate in a Tris-HCl buffer of pH 8 at 37°C for 2 h, II and IV were formed in the yield of 6% and 80%, respectively; on the other hand, III was not found in the reaction mixture. In degradation of II as the substrate with the kidney homogenate, IV was generated in 87% yield. An addition of glycylglycine to the reaction mixture of I and the kidney homogenate resulted in increase in the catabolism of I to form IV possibly via II. These results suggest that I is degraded to IV by actions of enzymes, γ-glutamyltransferase and dipeptidase, just like the metabolism of GSH. However, little degradation of I occured in the use of rat liver, brain, heart or spleen homogenate as the enzyme source, indicating that formation of IV occurs mostly in only kidney under physiological conditions.

In incubation of IV with rat kidney homogenate in a phosphate buffer of pH 7.4, IV was further metabolized to V. In this reaction, S-[2-carboxy-1-(1H-imidazol-4-yl)ethyl]-3-thiolactic acid (VI) was also formed; additionally, VI has been isolated from normal human urine by using ion-exchage column chromatography. These results suggest the formation of S-[2-carboxy-1-(1H-imidazol-4-yl)ethyl]-3-thiopyruvic acid (VII) as a metabolic intermediary to form V and VI. Namely, V may be yielded by two successive reactions, the first decarboxylation of VII to form the corresponding thioacetoaldehyde-adduct and the following oxygenation; VI is generated by reduction of VII possibly with lactate dehydrogenase. From these results, we propose a new alternative pathway of histidine metabolism through adduction of GSH and/or cysteme to urocanic acid as follows: histidine → urocanic acid → I → II → IV → (VII) → V and VI.



I : R= HO_2C-CH(NH_2)-(CH_2)$_2$-CONH-CH(-S-CH_2)-CONH-CH_2-CO_2H

II : R= -S-CH_2-CH(NH_2)-CONH-CH_2-CO_2H

III : R= HO_2C-CH(NH_2)-(CH_2)$_2$-CONH-CH(CO_2H)-CH_2-S-

IV : R= -S-CH_2-CH(NH_2)-CO_2H

V : R= -S-CH_2-CO_2H

VI : R= -S-CH_2-CH(OH)-CO_2H

VII : R= -S-CH_2-CO-CO_2H

Neurochemistry-Neurobiology

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1S,3R-ACPD: Behavioural effects and c-fos expression in the basal ganglia

1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), a selective metabotropic excitatory amino acid receptor agonist, was injected (i.p. 15 mg/kg) to rats of 150 to 180 g body weight in order to examine behavioural changes and induction of c-fos.

Open field with hole board: Locomotion and the number of head dips (looking into holes) was increased by 1S,3R-ACPD. Pretreatment with haloperidol (0.5 mg/kg i.p.) reduced all activities. 1S,3R-ACPD was able to enhance locomotion, the number of head dips and rearing in haloperidol pretreated animals.

Sniffing was strongly enhanced by 1S,3R-ACPD
The onset of 1S,3R-ACPD-induced effects occurred 300 min after administration of the drug and persisted for 30–60 min.

Catalepsy was induced by 0.5 mg/kg i.p. haloperidol. 1S,3R-ACPD was not able to antagonize haloperidol-induced catalepsy.

C-fos induction was examined in brains of rats that have received the same pharmacological treatment (15 mg/kg, 1S,3R-ACPD, 300 min before perfusion). 1S,3R-ACPD induced strong c-fos expression in the nucleus accumbens, but not in the striatum (caudate, putamen). Coadministration of haloperidol strongly suppressed c-fos expression in the nucleus accumbens.

Conclusion: 1S,3R-ACPD did not antagonize catalepsy which is thought to be mediated by the dorsal striatum. In this structure 1S,3R-ACPD did not induce c-fos expression. 1S,3R-ACPD has stimulant actions on behaviours (such as locomotion) that are thought to be mediated by the nucleus accumbens. These behavioural effects may be related to c-fos expression in this structure.

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Amino acid replacements and pseudopeptide/nonpeptide mimics for selected insect neuropeptide families

The first member of the insect kinin, sulfakinin, pyrokinin, insectatachykinin, and myosuppressin neuropeptide families were isolated from the cockroach or locust on the basis of their ability to either stimulate or inhibit contractions of the isolated cockroach hindgut. Subsequently, these families have been found in a variety of other insect species and associated with such physiological functions as pheromone production, diuresis, diapause induction, oviduct contraction, and melanization. A discussion of pseudopeptide analogs utilizing acyl group, reduced-bond, unnatural amino acid, and peptoid strategies is followed by biological evaluation data on several non-peptide analogs that mimic the activity of insect neuropeptides. Carboxy-acyl, carboxymethylphenylalanine and tetrazolylphenylalanine replacements for the acidic Tyr(SO_3H) complex in the sulfakinins led to analogs that retain biological activity. Current knowledge of the active conformation adopted by the pyrokinin, insect kinin, and sulfakinin neuropeptide families at the receptor site is presented, based on spectroscopic and computer molecular dynamic calculation studies on active restricted-conformation analogs. The utility of this knowledge to the development of potent pseudopeptide and nonpeptide mimetic agonist/antagonist analogs of insect neuropeptides is discussed.

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SDZ EAB 515, a competitive NMDA antagonist, exerts anxiolytic and stress attenuating properties in mice

SDZ EAB 515, a biphenyl analogue of AP7, was found to be a potent and selective competitive NMDA-antagonist, as determined by measuring the antagonism of NMDA-induced depolarisations in the rat neocortical wedge preparation (pA2 6.94). Insertion of a biphenyl moiety in the chain of the AP7 molecule increases the NMDA antagonistic activity relative to that of AP-7 by a factor of about 40 and inversed enantioselectivity from R to S.

Evidence for putative 'anxiolytic' properties was obtained from ethological studies of mice, placed on an elevated Plus-Maze, 1 hr after receiving vehicle or orally administered SDZ EAB 515 (0.01, 0.1, 1, 10 mg/kg), SDZ EAA 494 (0.32, 1, 3.2, 10 mg/kg, 30 min pretreatment), Diazepam (0.1, 0.32, 1, 3.2 mg/kg) and MK-801 (0.01, 0.032, 0.1, 0.32 mg/kg). SDZ EAB 515, MK-801, like diazepam caused the mice to peer over the edges of the open arms (Head-Over-Edge) significantly more than controls. In contrast, SDZ EAA 494 exerted no such effect. Although SDZ EAB 515 (0.3–3 mg/kg, po) tended to accentuate the corticosterone (CS)-response to a non-social stressor (trolley ride), it selectively attenuated the CS-rise in response to a social stressor (aggressive male mouse). In contrast MK-801 (0.1–10 mg/kg po) potentiated the CS-response to social stress whereas Diazepam (0.3–10 mg/kg, po) reduced CS-responses to both social and non-social forms of stress. SDZ EAA 494 (0.3–10 mg/kg, po) was without clear effects on either form of stress.

SDZ EAB 515 may, therefore, represent a new type of NMDA-antagonist, which, unlike SDZ EAA 494 and the NMDA channel blocker MK-801, could be useful for treating stress-related disorders and, like diazepam, possess anxiolytic properties.

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Release of brain dopamine, its metabolites and the effect of Cholecystokinin on the ingestion of intraorally administered sucrose in male rats

It has been recently shown in rates that implantation of osmotic minipumps filled with cholecystokinin octapeptide (CCK-8) inhibits food intake. Hence it is likely that the inhibitory effect of CCK-8 on ingestive behaviour is a physiologically rele-

vant phenomenon- an issue which is extensively debatted ever since CCK-8 was first considered as a satiety peptide.

Various brain areas such as parabrachial nucleus, hypothalmic paraventricular and ventromedial – all contain CCK-binding sides, however variety of ways of intracerebral injection of CCK-8 failed to effect food intake. One of the suggestions being that CCK-8 interact with another transmitter in the brain to control food intake. One of such candidates may be dopamine (DA) as it coexists with CCK in some neurons in which it released during ingestion of food.

In order to support CCK-DA interaction in control of food intake, deprivation of food depleted DA from CSF and various parts of rats brain and feeding or injection of CCK-8 in presence and absence of D1- and D2- antagonist, this study has been conducted to study the effects of above experimental conditions on the variation in the levels of DA and its metabolites. The results obtained are discussed.

Attempt has also been made to study the function of excitory amino acids under these conditions.

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Effect of neuropeptide Y(NPY) on the synthetic enzyme of norepinephrine

Neuropeptide Y(NPY), a 36-amino acids peptide, has been identified as the co-transmitter in adrenergic neurotransmission. Heteroregulation of this peptide with norepinephrine (NE) has also been mentioned recently; NPY potentiates the vasoconstrictive action of NE in postsynaptic sites and NPY decreases the release of NE from nerve terminals via the presynaptic receptors. However, regulation of this peptide with catecholamine in the nerve terminals remains obscure.

In the *in vitro* assay of dopamine-β-hydroxylase (DBH) activity, NPY inhibits the formation of NE from dopamine in the presence of purified DBH. Effect of NPY was produced in a dose-dependent manner and this inhibition was obtained in a non-competitive fashion. Also, NPY has the ability to reduce the activity of tyrosine hydroxylase in the *in vitro* assay using the electrochemical detection (HPLC-ECD). The concentration required to inhibit tyrosine hydroxylase seems higher than that sufficient to block the activity of DBH in NPY treated samples. However, NPY lacks the ability to influence the activity of dopa decarboxylase even at a higher dose.

The obtained results suggest that NPY can regulate the synthesis of NE in additional to the heteroregulation with catecholamine in adrenergic neurotransmission.

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Neurotransmission and receptor study of the novel amino acid containing dihydropyridines

Series of novel type of compounds comprising amino acids (glutamate, aspartate, γ-aminobutyric acid, taurine, etc.) bound with 1,4-dihydropyridine molecule were investigated in behavioural and neurochemical tests.

Preliminary data showed that some effects typical for endogenous ligands were observed in their action: neuroregulatory activity, low effective doses (micrograms), lack of strong dose-effect dependence (plateau effects), etc.

In present conditioned avoidance response (CAR) studies in rats a long-lasting intensification of neurotransmission processes was obtained: elevation of brain monoamine level was maintained 2–3 weeks after interruption of drug-treatment and was even more expressed than during treatment. Particularly pronounced inhibition of CAR extinction was observed in taurine-containing compound studies.

In receptor binding studies *in vitro* these compounds have no direct binding with brain membrane receptors. Antagonism, potentiation to NMDA-induced seizures (in mice) has been found to depend on amino acid moieties, geometry of structure and dosage.

Data obtained indicate that this intriguing novel class of compounds significantly differs from typical 1,4-dihydropiridines: they loss calcium antagonistic properties and acquire the neuropeptide-like trigger functions. Revealing of structure determinants and activity peculiarities is under study.

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Glycinoceptive structures in the cerebral cortex

Glycine is known to play an important role as an inhibitory transmitter in the spinal cord where glycine and glycine receptors have been demonstrated biochemically and were visualized immunohistochemically at the light and electron microscopic level. There is increasing evidence that glycine is involved in synaptic transmission in supraspinal structures, in particular the cerebral cortex, too. To unravel the possible existence of glycine receptors in the cerebral cortex we have investigated the cortices of adult rats and during ontogenesis using the monoclonal antibody 4a (MAb 4a) – directed to the ligand binding site of the glycine receptor – for immunohistochemical and biochemical analysis. MAb 4a-reactive sites were visualized light microscopically and ultrastructurally by means of immunoperoxidase. Quantitative analysis of glycine receptors was achieved by dot receptor immunoassay (DORA). In adult brains mAb 4a-immunoreactivity was prominent in pyramidal neurons and their apical dendrites. At the ultrastructural level the immunoprecipitate was mainly associated with postsynaptic thickenings. Using DORA significant concentrations of mAb 4a-antigen were detected in adult rat cerebral cortex that amounted approximately one tenth of that measured in in the rat spinal cord corroborating previous evidence ror a rostro-caudal gradient in glycinergic transmission. In the developing rat cerebral cortex at birth high levels of immunoreactivity were found that transiently increased during the second postnatal week. Subsequently a decline to adult values was observed. In line with these biochemical findings, immunocytochemistry revealed a change in the distribution of glycine receptor-immunoreactivity within two weeks after birth from a dense labeling of closely packed neurons in the superficial layers to a predominant labeling of pyramidal neurons comparable to adult patterns. Biochemical analysis of the mAb 4a antigen indicates the cerebrocortical glycine receptors to correspond to the neonatal isoform previously identified in newborn spinal cord. The findings provide first hints to a glycinergic system operational at the cerebrocortical level.

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Achatin-I, an endogenous *Achatina* peptide having a D-phenylalanine residue, as a neurotransmitter and a neuromodulator.

A neuroexcltatory tetrapeptide having a D-phenylalanine residue (Gly-D-Phe-L-Ala-L-Asp), termed achatin-I, has been isolated from the ganglia of an African giant snail (*Achatina fulica* Ferussac) by a collaboration with Suntory Institute for Bioorganic Research (Osaka), University of Santo Tomas (Manila, Philippines) and our group (Gifu) (Kamatani et al., Biochem. Biophys. Res. Commun., 160, 1015–1020, 1989). Among the eight possible stereoisomers of achatin-I, only achatin-I showed marked excitatory effects on the three *Achatina* giant neurone types (PON, periodically oscillating neurone; TAN, tonically autoactive neurone; and v-RCDN (ventral-right cerebral distinct neurone), indicating that the effects are stereo-specific. Achatin-II having no D-amino acid residue (Gly-L-Phe-L-Ala-L-Asp) was also *Achatina* endogenous, but showed no direct effect on these neurones. Among the more than twenty *Achatina* giant neurone types tested, the ten types were excited by achatin-I; the peptide is considered to be an excitatory neurotransmitter of these neurones. By testing the effects of achatin-I and its derivatives, the effects were evidenced to be structure-specific for achatin-I.

The fast component of the excitation of an *Achatina* neurone type, TAN, caused by 5-hydroxytryptamine (5-HT) was facilitated by achatin-I at 3×10^{-6} M, which was lower than its ED_{50} for the direct TAN excitation, whereas the late component of the excitation was not affected. It is considered that achatin-I is acting in the *Achatina* ganglia as a neuromodulator. Achatin-II, as an agonist of achatin-I, also facilitated, but ten times less, the fast component of the 5-HT excitation. Of the neuroactive peptides originally isolated from Mollusca, the effects of oxytocin and APGW-amide, which was isolated from both a prosobranchia (*Fusinus ferrugineus*) and *Achatina fulica*, on the *Achatina* giant neurones were suppressed by achatin-I. On the other hand, the effects of FMRFamide were facilitated.

We looked for the drugs antagonistic to the achatin-I excitation using an *Achatina* neurone type, PON, by testing the known blockers of the classical neurotransmitters, such as acetylcholine blockers, GABA blockers, L-glutamate blockers, 5-HT blockers, dopamine blockers, α- and β-adrenalin blockers and histamine (H_1- and H_2-) blockers. Among the about twenty histamine (H_1-) blockers, a few drugs including triprolidine and trimeprazine antagonized the achatin-I excitation of PON. It is considered that the antagonistic effects of these drugs are not due to their anti-histamine (H_1-) effects, since the majority of H_1-blockers tested were ineffective on the achatin-I excitation.

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Nitric oxide synthase and glutamate-induced neurodegeneration

A substantial body of knowledge has accumulated suggesting that nitric oxide (NO) is involved in pathogenetic mechanisms of glutamate-mediated neurodegenerative processes. The biosynthetic enzyme for NO in the brain, NO-synthase, is largely co-localized with reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), which can be easily detected histochemically at the light microscopical level. Using the tetrazolium salt BSPT (yielding an osmiophilic formazan on reduction) to adapt to the electron microscopy, we were able to demonstrate NADPH-d distinctly on membranes of the endoplasmic reticulum. A number of cells including a few non-neuronal elements (light microscopically 'negative') show membranes and single membrane patches labeled by electron-dense formazan deposits.

In the hippocampal formation of the rat heavily stained neurons are sparsely distributed. They are found to be relatively resistant to glutamate-related neurotoxins, such as to the NMDA-receptor agonist quinolinic acid, and contrary to findings in the literature, to AMPA as well. Electron microscopically positively stained neurons as well as an increased number of glial cells with stained endoplasmic membranes were seen in lesioned areas.

In cell cultures of the cerebral cortex we have found a substantial increase in the neurodegenerative potency of glutamate analogs if arginine, the substrate for NO production, was added to the culture medium.

Quinolinic acid microinjected unilaterally into the rat striatum led to a complete loss of NADPH-d-positive neurons whereas the contralateral side appeared to be unaffected. Contrastingly, parallel biochemical studies have shown that striatal tissue which displayed massive excitotoxin-induced nerve cell loss (glutamate decarboxylase was used as a marker enzyme for the success of the lesion), and a pronounced reactive astrogliosis, revealed marked increase in NO-synthase activity (to 202% of controls, $p < 0.005$ by t-test). These data indicate that astrocytes activated by quinolinic-acid mediated neurodegeneration express high activity of NO-synthase, which possibly contributes to neuronal damage.

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Factors affecting neurotransmitter glutamate release from rat cerebellar slices

Previous experiments showed that aminooxyacetic acid (AOAA) and phenylsuccinate (Phs) inhibit the Ca^{2+} dependent release of glutamate from primary cultures of cerebellar granule cells. With these experiments was also shown that the presence of glutamine increased the release of this neurotransmitter. Therefore it has been suggested that, in this preparation, aspartate aminotransferase (AAT), the mitochondrial ketodicarboxylate carrier (MKC) and phosphate activated glutaminase (PAG) are obligatory steps for the production of neurotransmitter glutamate.

These studies were extended to cerebellar slices, as preparation closer to the physiological condition. In this preparation, Ca^{2+} dependent K^+ stimulated release of the neurotransmitter was studied by using D-[^{3}H]-aspartate (D-Asp), which is not metabolized and labels the exogenously accessible glutamate pools, also including the transmitter pool.

Therefore rat cerebellar slices were preincubated for 2h in a Krebs-Bicarbonate glucose medium and incubated for 30 min in the same medium containing 1 μC D-Asp, in the presence or absence of the following effectors: AOAA (5 mM) as inhibitor of AAT, Phs (5 mM) as inhibitor of MKC, glutamine (1 mM) as substrate of PAG, NH_4^+ (4 mM) as inhibitor of PAG and ketone bodies (3 mM) as inhibitors of glycolysis. Consequently the slices were superfused with D-Asp-free incubation medium either in the presence or in the absence of Ca^{2+} plus Mg^{2+} (10 mM). After 50

min of superfusion, the [K^+] (5 mM) was increased to 56 mM (replacing Na^+} for 4 min and returned to the original [K^+] for the following 26 min. Under these conditions the following results were obtained

a) A statistically significant increase in the K^+ stimulated Ca^{2+} dependent release of radioactivity was observed by AOAA (30%), Phs (188%) and NH_4^+ (68%)
b) Gln and ketone bodies had no effect

As shown previously increased Ca^{2+} dependent, K^+ stimulated radioactivity release corresponds to decreased neurotransmitter glutamate release. Therefore the above results support the suggestion that AOAA, NH_3 or phenylsuccinate inhibit neurotransmitter glutamate release.

That ketone bodies have no effect, leads to the suggestion that glycolysis is not participating in the supply of neurotransmitter glutamate pool, in this preparation. On the contrary, the NH_4^+ result shows that PAG is essential for the production of this neurotransmitter, although glutamine, contrary to the cell preparation do not change the stimulated release of radioactivity from the slice preparation. This preparation contains glial cells with a high affinity uptake system for glutamate. Therefore it is plausible to suggest that the glutamate released from the neurons it is taken up by the glial cells. These cells provide glutamine through an exclusive and active glutamine synthetase reaction that can replenish glutamine in the neurons for neurotransmitter glutamate production.

On the other hand as with cultured cells the Phs inhibition support the hypothesis that glutamate derived through the PAG reaction, has to enter the mitochondria before supplying the neurotransmitter glutamate pool.

The result of AOAA shows that AAT participate in the supply of neurotransmitter glutamate in rat cerebellum. This is in keeping with previous observations in primary cultures.

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The effect of an antagonist at the glycine binding site of the NMDA receptor on catalepsy

The dopamine/glutamate balance controls motor behavior via the basal ganglia output nuclei. The output from the striatum to the thalamus is mediated by a direct and an indirect pathway. Dopaminergic excitatory effects on the direct path can be obtained via a dopamine D1 receptor, inhibitory effects on the indirect path via a dopamine D2 receptor. An overlap between the receptor distributlon is controversially discussed. The highest density of NMDA receptors is found within the striatum as compared to the other basal ganglia nuclei. Glycine is a positive modulator of the NMDA receptor and enhances its efficiency.

Blockade of dopaminergic receptors in rats results in akinesia and rigidity (catalepsy), symptoms which can also be seen in parkinsonian patients. Since NMDA antagonists reduce these symptoms in rats, blockade of the glycine binding site may has also anticataleptic potential in a more physiological way. A first group of male Sprague Dawley rats was treated with the dopamine D2 antagonist haloperidol (0.5 mg/kg i.p.) and either a 5 μl intraventricular injection of the glycine antagonist 7-chlorokynurenate (7-CLKYN) (10–40 nmol) or a 0.5 μl intrastriatal (anterodorsal) injection of 7-CLKYN (2.5–10 nmol). A second group was treated with the dopamine D1 antagonist SCH 23390 (0.5 mg/kg i.p.) and either a 5 μl 40 nmol 7-CLKYN intraventricular injection or a 0.5 μl 10 nmol 7-CLKYN intrastriatal injection. In a catalepsy test (bar, podium and vertical grid) descent latency was tested.

Intraventricular as well as intrastriatal injection of 7-CLKYN antagonized the haloperidol-induced catalepsy. Since an intraventricular injection of 7-CLKYN reduced the muscle tone, the anticataleptic effects of the intrastriatal injection was not as pronounced as the intraventricular injection. An anticataleptic effect of 7-CLKYN against SCH 23390-induced catalepsy was not seen.

The results show, that in the case of dopamme D2 mediated catalepsy a sufficient anticataleptic response via the glycine binding site can only be obtained if glycine binding sites at the striatal and additionally at a perhaps lower basal ganglia level (substantia nigra reticulata or globus pallidus interna) are blocked. The result, that a glycine site antagonist is ineffective to reduce dopamine D1 mediated catalepsy implies that within the striatum NMDA receptor with different affinity for glycine must exist, since NMDA antagonists reduce dopamine D2 as well as D1 mediated catalepsy.

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Central noradrenergic depletion by lesions of the locus ceruleus affects dopaminergic and glutamatergic mediated locomotion

Parkinson's disease (PD) is characterized by a degeneration of the nigrostriatal dopaminergic (DA) pathway as well as by a marked cell loss in the locus ceruleus (LC), the main origin of the noradrenergic (NA) innervation in the brain. To study behavioral effects after lesion of the LC, male Sprague-Dawley rats were bilaterally injected with 1 μl of the neurotoxin 6-hydroxydopamine (6-OHDA, 6 μg/μl, dissolved in 0.1 mg/ml ascorbic acid) under an angle of 30° in rostro-caudal direction. The coordinates were: AP = −2.6 mm (interaural); L = ±0.9 mm (bregma); V = +3.4 mm (interaural); [Paxinos and Watson, 1986]. For sham lesion, controls received 1 μl ascorbic acid (0.1 mg/ml). One week after surgery, locomotor activity was tested in an open field. Rats were tested under drug-free conditions and after application of the DA D2 antagonist haloperidol (0.2 mg/kg, i.p.), the DA D1 antagonist SCH-23390 (0.1 mg/kg, i.p.), the indirect DA agonist DL-amphetamine (2 mg/kg, i.p.) and the non-competitive NMDA receptor antagonist dizocilpine (0.04 mg/kg, i.p.) respectively. Following a drug-free period of one week, rats were decapitated and their brains were dissected for neurochemical analysis. In lesioned rats there was a significant reduction in the tissue levels of NA in the prefrontal cortex ($p \leq 0.01$), in the nucleus accumbens ($p \leq 0.05$) and in the posterior striatum ($p \leq 0.05$). However, there was no difference in the levels of DA, DOPAC, serotonin and 5-HIAA. Compared to controls, 6-OHDA-lesioned animals did not differ in spontaneous locomotor activity. Treatment with haloperidol or SCH-23390 reduced locomotion in 6-OHDA animals to a greater extent ($p \leq 0.05$) than in controls. DL-amphetamine and dizocilpine enhanced locomotion but – to a lesser extent – in 6-OHDA animals ($p \leq 0.05$). The present study demonstrates that a destruction of NA projections of the LC increases the effects of DA antagonists and decreases the actions of psychostimulants. These findings indicate that a NA cell loss in the LC system may worsen the motor-symptoms in PD.

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EP- and SNR-lesions reduce neuroleptic-induced catalepsy

In Parkinson's disease (PD) the degeneration of the nigrostriatal dopaminergic pathway results in an imbalance of the interaction between dopamine and glutamate in the striatum. Due to a dopaminergic deficit there is a functional glutamatergic overactivity. This leads to an increased activity of the striatopallidal pathway and consequently of the subthalamic nucleus (STN). The STN sends glutamatergic projections to the output nuclei of the basal ganglia – the medial part of the globus pallidus (GPm, entopeduncular nucleus (EP) in the rat) and the substantia nigra pars reticulata (SNR). The excessive activity of the STN is responsible for the abnormally increased activity of GPm/EP and SNR in PD. The present study investigated the function of EP and SNR in mediating the PD symptoms akinesia and rigidity. In rats, EP and SNR were bilaterally lesioned with the selective NMDA-agonist quinolinic acid (30 nmol/0.5 μl/side and 2 × 30 nmol/0.5 μl/side respectively). The effects of these lesions on dopamine-antagonist-induced catalepsy were tested. Catalepsy is a syndrome consisting of akinesia and rigidity and thus, is regarded as an animal model for PD. The selective D1-antagonist SCH 23390 (0.5 mg/kg) and the D2-antagonist haloperidol (0.5 mg/kg) were injected in order to induce catalepsy. The degree of catalepsy was measured by descent latencies from the vertical grid. Both EP- and SNR-lesion antagonized SCH 23390 and haloperidol-induced catalepsy. Furthermore locomotor activity was investigated in the open field after D1- and D2 receptor blockade: Neither EP- nor SNR-lesion increased locomotor activity. The histological verification of the lesions demonstrated, that a part of the substantia nigra pars compacta was simultaneously lesioned with the SNR. The effect of this lesion on dopamine and dopamine metabolites (DOPAC, HVA) in the anterior striatum, the posterior striatum and the nucleus accumbens was analysed post mortem with HPLC with electrochemical detection. The levels of dopamine and DOPAC were reduced in these structures.

In conclusion, EP- and SNR-lesion antagonized catalepsy. This antagonism consisted of an anti-rigidity effect, but not of an anti-akinetic effect. Catalepsy induced by both D1- and D2 receptor blockade is mediated by the EP and the SNR.

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Effects of an excitotoxic lesion of the entorhinal cortex on spatial learning in the rat

The entorhinal cortex (EC) supplies the hippocampus via the perforant path with excitatory input from neocortical areas. These glutamatergic neurons in layer II of the EC projecting to the hippocampus are also primarily affected in Alzheimer's disease (AD). AD is characterized by an initial impairment in working memory that is similar to learning impairment induced by lesions in the hippocampal formation. Since the hippocampal formation is especially sensitive to spatial learning, we chose a spatial task in order to test EC-lesioned animals. We bilaterally lesioned the more medial part of the rat EC by infusion of the NMDA-agonist quinolinic acid (60 nmol/0.5 μl). Animals were tested postoperatively in an 8-arm radial maze. Four of the eight arms of the maze were baited by a food pellet. The baited arms remained in constant room coordinates during all trials of one experiment. Animals had 5 trials per day. They started in every trial from a different start arm that was randomly chosen from the four non-baited arms. This paradigm assured allocentric orientation since extramaze cues had to be used to find the goal arms. Errors were defined as first entry into an unbaited arm (reference memory error) or reentry of an already visited arm (working memory error). Rats with bilateral lesions of the EC made more total errors in 5 trials until having found all goal arms, but also constantly reduced their number of errors. The learning impairment was due to a stronger increase in working than reference memory errors. After 10 days of testing, the animals were submitted to reversal learning. The former start arms were changed to goal arms and the goal to start arms. As the learning impairment in the first part of the experiment was not very pronounced, we decided to further challenge the glutamatergic system with 0.04 mg/kg i.p. of the non-competitive NMDA-antagonist dizocilpme (MK-801), i.e. a dose of dizocilpine without behavioral effects on its own. Injection of dizocilpine resulted in a pronounced impairment of the lesioned group as seen by an increase in both reference and working memory errors but had no effect on controls.

The present study shows that an excitotoxic lesion of the EC induces spatial learning impairment in rats. Further challenge of the glutamatergic system results in more pronounced learning impairment. The results might be discussed in the context of AD as (1) the EC is a region primarily affected in AD and (2) the lesion-induced learning impairment has some similarity to the impairment in the initial state of AD.

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Excitatory amino acid innervation of the neostriatum tonically regulates neuropeptide expression: A cellular in situ hybridization analysis

The effect of N-methyl-D-aspartate (NMDA) receptor blockade on the expression of proenkephalin, protachykinin and prodynorphin mRNAs in the caudate-putamen and nucleus accumbens was assessed with the non-competitive NMDA receptor antagonist MK801.

Administration of MK801 for seven consecutive days increased the abundance of all three neuropeptide mRNAs in the CPU and NAc. (1) PE mRNA abundance was increased from 26–46% in the anterior CPU and dorsal and ventral CPU but was unaffected in the NAc. (2) PT mRNA was increased in the NAc (33%) as well as anterior CPU (27%), dorsal CPU (43%) and ventral CPU (67%). In the ventral CPU, PT mRNA abundance doubled when the dose of MK801 increased two-fold (from 67% to 119% above control). (3) PD mRNA was elevated in dorsal and ventral regions of the CPU (49% and 24%, respectively) and in anterior CPU (50%). In the NAc PD mRNA was increased only at the higher dose (0.1 mg/kg) of MK801. The abundance of tyrosine hydroxylase and procholecystokinin mRNAs in mesencephalic cells of the substantia nigra and ventral tegmental area was unaffected by the same treatment. These observations demonstrate that NMDA receptor activity plays a significant role in the regulation of neuropeptide expression in the caudate-putamen and accumbens of the rat brain. NMDA receptor activity, putatively via glutamate, exerts tonic inhibition of PE, PT and PD mRNAs in the CPU and PT and PD mRNAs in the NAc.

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Structure-activity relationship of neuropeptide Y: Relationship between formation of α-helical part and binding activity to Y_2 receptor

Neuropeptide Y(NPY) is a single-chain peptide consisting of 36 amino acid residues. This peptide is abundant in brain and is thought to be involved in the regulation of food intake, memory processing, circadian rhythm, etc. Its C-terminal fragments including a long α-helical segment can bind effectively to the Y_2 receptor on hippocampal membranes. To reveal the relationship between formation of the α-helical part and binding activity, we prepared several peptides including the helical part. NPY(12–36) exhibited about one order of magnitude less potent inhibitory effect than the intact NPY on ^{125}I-NPY binding to porcine hippocampal membranes. Among the peptides, in which *N*-terminal Ala of NPY(12–36) was replaced with Asp, Asn, Glu, Gln, Lys, or Arg, E12-peptide inhibited the ^{125}I-NPY binding with slightly higher potency than NPY(12–36), whereas the other peptides exhibited almost the same potency as that of NPY(12–36). CD spectroscopy under the same conditions as those of the binding assay (25°C, pH 7.5) revealed that the trend in α-helix-stabilizing ability of *N*-terminal amino acid was found to be as follows: Ser ≫ Gln > Asn ≫ Asp > Glu > Lys > Ala > Arg. This result suggests that the presence of *N*-terminal residues which have side chains to supply hydrogen-bond partners for unpaired main-chain NH groups is important for stabilizing the helix. Our result is consistent with the Presta and Rose hypothesis (1988) and observations in proteins (Richardson and Richardson, (1988) and short peptides (Forood et al., (1993). In contrast, deamidation of the *C*-terminal amide group of NPY(13–36) resulted in large decrease in the α-helical content, suggesting that the interaction of a positive charge at the *C*-terminal region with the helix dipole is also important for the stabilization. However, there was no apparent relationship between the formation of the α-helical part and the binding activity to the receptor. Introduction of Trp to the positions 23–27 of NPY(13–36) as a fluorescent probe reduced the binding activity to the receptor to about 10% that of NPY(13–36). Further studies on the conformation of these peptides are in progress.

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Neurochemical correlates of the interaction of dopaminergic, GABA-ergic and glutamatergic systems in the motor loop of patients with Parkinson's disease

The idea of basal ganglia organization have changed markedly over the past decade, due to significant advances in our understanding of the anatomy, physiology and neuropharmacology of these structures. Current evidence suggests that the basal ganglia are organized into several structurally and functionally distinct circuits that link the cortex, the basal ganglia (substantia nigra, striatum, globus pallidus, subthalamic nucleus) and the thalamus, with each circuit focused on a different portion of the frontal lobe. The basal ganglia contain neurotransmitter-specific compartments that bring different inputs under different neurochemical control. The classic neurotransmitters GABA (γ-aminobutyric acid) and glutamate/aspartate are, respectively, the probable inhibitory and excitatory modulators responsible for a majority of neurons in the mammalian brain. The role of dopamine within the basal ganglia appears to be complex, and many issues remain unresolved. However, there is recent evidence that the nigro-striatal dopaminergic projections exert contrasting effects on the basal ganglia output nuclei (external and internal segment of the globus pallidus, substantia nigra pars reticulata).

Dysfunction of the basal ganglia leads to the so-called extrapyramidal movement disorders. These disorders comprise a spectrum of abnormalities, that range from the hypokinetic disorders (of which Parkinson's disease is the best-known example) at one extreme to the hyperkinetic disorders (exemplified by Huntington's disease and hemiballismus) at the other. Both extremes of this movement disorder spectrum can be accounted for by postulating specific disturbances within the basal ganglia-thalamocortical motor circuit. Parkinson's disease is characterized by a progressive loss of nigro-striatal dopamine neurons that modulate striatal activity. The concept of the motor loop predicts that this loss has functional consequences in the activities of GABA-ergic and glutamatergic pathways. In order to investigate this prediction, we measured the concentrations of aspartate, dopamine, GABA and glutamate in relevant regions of the "motor" loop from patients with Parkinson's disease and individuals with no apparent history of neurological and psychiatric disorders. Such assay may give an indication of presynaptic neuronal activity and of which neuronal systems have been affected by the disease. Furthermore, glutamate binding sites experiments were used to give an idea of the functional state of the target neurons. The results do not confirm the postulated mode of operation of the motor circuit in Parkinson's disease.

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Mechanism of cysteine neurotoxicity in the immature rat brain

Cysteine (CYS) is a unique excitotoxin since it does not conform to the established structure-activity relationship for excitotoxins. The oxidized CYS metabolites cysteine sulfinate (CSA) and/or cysteate (CA) have been proposed to mediate CYS toxicity, but this hypothesis has not been experimentally tested. The problem was approached in 3 different ways: a. The levels of CSA in regions vulnerable to CYS were measured after systemic administration of CYS to neonatal rats. b. The cerebral pathology developing after CYS injection was compared with that of CSA. c. The sensitivity of CYS and CSA toxicity to the NMDA receptor antagonist dizocilpine was investigated.

Results: a. Administration of CYS (1 mg/g) produced a large increase (19-fold) in the levels of CSA (HPLC analysis) in the frontal cortex. However, the absolute levels amounted only to 90 nmol/g protein. CA was below detection limit in both control and CYS-injected rats. The levels of CSA in the frontomedial cortex (a vulnerable region) after administration of a toxic dose of CSA (3 mg/g) were 83 μmol/g protein, i.e. 3 orders of magnitude higher than after CYS administration. b. There were some similarities between the distribution of CYS and CSA toxicity, e.g., pyramidal cells of the subiculum and cortical neurons around the rhinal fissure were quite vulnerable. However, the intracortical distribution of cell death differed, and CSA but not CYS killed neurons of the medial habenula and tectum. c. Dizocilpine completely prevented CYS-induced lesions. CSA toxicity

was only partially inhibited by dizocilpine although the damage in the subiculum, for instance, was much more widespread after CYS injection. These results indicate that it is unlikely that CSA (and/or CA) mediates CYS neurotoxicity. Alternatively, the sulfhydryl group of CYS reduces the redox site of the NMDA receptor-ion channel complex and thereby permits endogenous glutamate (GLU) to overcome the threshold for toxicity. Rat pups were administered GLU (0.5 mg/g, s.c.), CYS (0.5 mg/g) or GLU + CYS (0.5 mg/g + 0.5 mg/g), and neuronal injury in the arcuate nucleus was studied histologically. There was no damage in animals given GLU or CYS, but all animals injected with GLU + CYS carried arcuate lesions. Previous studies suggest that the dose of GLU used was just below the threshold for toxicity, and that the dose of CYS was less than half of the dose toxic to arcuate neurons. This indicates that CYS potentiates GLU toxicity in vivo, possibly by the mechanism described above. That the redox site is involved in CYS toxicity is further strengthened by the finding that the oxidant pyrrolloquinoline quinone (5 μg/kg) abolished the neurotoxicity of CYS (1 mg/g). We therefore suggest that the toxicity of CYS is linked to the sulfhydryl group rather than to formation of excitotoxic structural analogs.

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Strikingly elevated glycine and other amino acid levels in CSF in primary melanosis of the central nervous system

The ratio CSF/plasma arnino acid concentration is constant for each amino acid and very low for most of them, owing to processes of active transport and/or diffusion (blood-brain barrier). This ratio may be modified by some aminoacidopathies and other pathological states or iatrogenic injuries. Changes in CSF amino acid profile compared with their plasma levels may therefore be of value in the understanding of the pathological findings of the CNS.

Elevated levels of most amino acids, specially glycine (150–250 μmol/L, determined by ion exchange chromatography), were repeatedly observed in the CSF of a patient with primary melanosis of the CNS. Hyperproteinorraquia, hypoglucorraquia, and high CSF levels of lactate and pyruvate were also detected. However, amino acids and organic acids were normal in plasma and urine. Clinically, the 6-year-old patient had a long-term history of pseudo-tumor (intracraneal hypertension, and progressive involvement of intracraneal pairs and peripheric nervous system). Pathological examination at autopsy showed an atypical proliferation of melanocytes in the meninges at different sites of the CNS together with a melanine storage in the cerebral cortex, brain stem, thalamus and other various points of the CNS. The diagnosis of primary meningeal melanoma was established.

The association of these clinical, biochemical and pathological features has never been reported, to our knowledge. In spite of the high ratio CSF/plasma glycine (0.4–1, normal values < 0.02), non-ketotic hyperglycinemia was excluded owing to the moderate increase in other amino acids in CSF, together with normal glycine levels in plasma and urine. Transient non-ketotic hyperglycinemia or artefactual changes were also discarded since glycine and other amino acid levels tended to increase in 4 specimens of CSF analysed during the 2-month-period before the patient's death. Iatrogenic damage was unlikely because removal or changes in treatment did not modify the progressive course of the biochemical abnormalities.

We assumed that these increased amino acid levels in CSF may be due to a disturbance of the active carrier mechanisms related to cerebral atrophy (MRI and necropsy examination). Moreover, the metabolic interactions between host and tumor may probably have changed the amino acid composition of CSF, since tumor proliferation requires amino acid for nucleotide and protein synthesis and may also altere their oxidative processes. On the other hand, the striking elevation of CSF glycine, an inhibitory neurotransmitter which can also overstimulate the N-methyl-D-aspartate type of excitatory glutamate receptors, may also have had some implications in the clinical and pathological findings of the patient.

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Plasma and cerebrospinal fluid amino acid levels in neurodegenerative disorders

The role of aminoacids as neurotransmitters in the Central Nervous System and their significance in neurodegenerative diseases have already been reported. However information regarding their plasma and cerebrospinal fluid (CSF) concentrations is very poor.

Sixteen aminoacids were measured in the plasma and CSF of 23 patients with Amyotrophic Lateral Sclerosis (mean age 60 years), 18 with Alzheimer's disease (mean age 58 years) and 24 age-matched controls (mean age 55 years). The amino acids were taurine, theonine, serine, glutamic acid, glycine, alanine, valine methionine, isoleucine, leucine, tyrosine, phenylalanine, ethanolamine, cysteine, orninthine, lysine and histidine.

Separation and quantitation of amino acids was performed by high performance liquid chromatography (Waters instrumentation) and post column derivatisation with o-phthalaldeyhyde. The fluorencence was measured at excitation and emission wave lengths of 338 and 426 nm respectively.

A statistically significant increase was found in glycine (6.7 $\pm$ 2.0 SD μmol/l), alanine (35.2 $\pm$ 13.5 SD μmol/l), valine (20.4 $\pm$ 7.5 SD μmol/l) and lysine (23.2 $\pm$ 4.9 SD μmol/l) in the CSF of patients with Amyotrophic Lateral Sclerosis, while an increase in glycine (6.1 $\pm$ 1.6 SD μmol/l) and valine (17.1 $\pm$ 5.5 SD μmol/l) was found in the CSF of Alzheimer's disease patients as compared to the controls (5.1 $\pm$ 1.6, 27.4 $\pm$ 5.5, 14.8 + 5.0, 20.0 $\pm$ 4.5 SD μmol/l for glycine, alanine, valine and lysine respectively).

These results suggest that amino acids interfere in the pathogenesis of these neurodegenerative disorders. However, further investigation is required to clarify their exact role and their probable significance in the CSF.

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Amides of dopamine – the new dopaminergics

Dopamine and its conjugates are widely distributed among biological species and are utilized for a variety of functions. One of the most widely recognized and studied functions is neurotransmission. Dopamine represents more than 50% of the total

catecholamines content in the central nervous system. Recently is known, that is a derivative of dopamine that was designed as an orally active prodrug.

Now we present the synthesis of a new derivatives of dopamine as a potent dopaminergics or dopamine prodrugs. Basing on our experience with the synthesis of DOPA peptides it is evident that the catechol unit should be protected during the synthesis.

We have chosen the t-butyldimethylsilyl group (TBDMS) for the protection of dopamine phenolic functions.

Free dopamine (DA), in the reaction with TBDMS-Cl in acetonitrile in the presence of DBU, was selectively silylated in both phenolic positions and 3,4-bis(t-butyldimethylsilyloxy)-dopamine was thus obtained as a amorphous solid [DA(TBDMS)$_2$] (1). Next, the disilylated substrate (1) has been applied in the synthesis of N-Boc-aminoacids (or peptides) dopamine amides, using the BOP and/or Py BOP method. We thus obtained: BOC-X-DA(TBDMS)$_2$ (where X = Ala, Asn, Asp(β-OBu^t), Glu(γ-OBu^t), Glu, Phe, Pro, Try, DOPA(TBDMS)$_2$ and Leu-Phe).

For the removal of all the protecting groups as the Boc, t-Bu ester and TBDMS (for phenol protection), 95% TFA, was used as the more convenient deprotecting agent.

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Modification of GABA-mediated inhibition by beta-blockers

There is increasing evidence that the inhibitory neurotransmitter γ-aminobutyric acid (GABA) plays an essential role in mediating the cardiovascular responses to hypotensive drugs. Recently, it has been shown that $GABA_A$ receptor is an importante site of clonidine action in the central nervous system. In this paper we present our findings of β-blockers indicating significant alterations in GABAergic transmission. Binding to $GABA_A$ receptors was studied in synaptosomal membranes of selected brain areas in spontaneously hypertensive rats (SHR). Propranolol was administered intraperitoneally at a daily dose of 30 $mg \cdot kg^{-1}$ for two weeks and metoprolol in drinking water for seven weeks at a daily dose of about 70 $mg \cdot kg^{-1}$. Both drugs reduced significantly ($P < 0.01$) systolic blood pressure (SBP). The maximum diminution in SBP was reached on the 6th day of the propranolol-treatment and on the 49th day of the metoprolol-treatment. These effects were not intensified when the treatment was prolonged for one week.

Table 1. Specific [^{3}]GABA binding (fmol/mg protein) to membranes from various brain areas (* $P < 0.01$)

Brain area	Control SHR	Drug-treated SHR	
		Propranolol	Metoprolol
Hypothalamus	148 ± 13	430 ± 24	443 ± 20*
Hippocampus	194 ± 19	248 ± 22	269 ± 17
Medulla-pons	168 ± 14	359 ± 15*	388 ± 18*
Striatum	222 ± 17	341 ± 24*	322 ± 16*

The binding of [^{3}H]GABA was significantly elevated in hypothalamus, pons-medulla and striatum following propranolol and metoprolol chronic administration. The drugs had no effect on $GABA_A$ receptor binding in the hippocampus (Table 1). Specific [^{3}H]GABA binding was further characterized by Scatchard analysis. Our studies revealed that the change in GABA receptor binding in hypothalamus was due to the enhancement in the total number of binding sites while in pons-medulla and striatum in both receptor characteristics i.e. density and affinity.

We conclude that GABAergic system responds to chronic β-blockers treatment by upregulation of the $GABA_A$-receptor binding.

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Psychotic syndromes related to disturbed amino acid metabolism

The ICD-10 Classification of Mental and Behavioural Disorders (WHO, Geneva 1992) delineates within the group of acute and transient psychotic disorders the Acute Polymorphic Psychotic Disorders with or without Schizophrenia (F23.0 and F23.1). Some characteristics like sensory perceptual disturbances, transient feelings of happiness and ecstasy are reminiscent of a LSD or other hallucinogenic drug-induced state. This – in line with the former "Transmethylation Hypothesis of Schizophrenia" – made us postulate that these patients suffer from some disturbance of metabolism, resulting in the formation of psychotogenic substances.

Recent basic research endorses such a hypothesis by demonstrating that due to generation of excess formaldehyde, cyclization of monoamines yielding beta-carbolines (from indolamines) and isoquinolines (from catecholamines) can occur. Many of these substances have hallucinogenic properties. Formation of excess formaldehyde can be accomplished by an increased breakdown of serine and/or glycine (the primary source of one-carbon moieties) resulting in an excess of methylene-tetrahydrofolate which may decompose into tetrahydrofolate and formaldehyde.

The assumed disturbed serine/glycine catabolism has been studied in our patients in several ways:

1. Measuring fasting plasma levels of amino acids. Patients suffering from acute polymorphic psychoses (APP) as well as bipolar (manic) patients had significantly lower plasma serine concentrations than schizophrenics and healthy controls.
2. Challenge tests by oral loadings with serine, glycine or alanine (2 mmol/kg body weight) in a double blind fashion during symptom free episodes induces in 70% of APP patients the characteristic affective and sensory perceptual changes a few hours after the intake of serine.
3. An increased rate of disappearance of plasma serine after serine loading and an impaired glycine-serine interconversion after glycine loading could be demonstrated in APP patients.
4. In human fibroblast cultures the activity of the serine metabolizing enzymes serine-hydroxymethyltransferase and cystathione-beta-synthase is stimulated when serum from APP patients is added to the culture medium.

At this moment we succeeded in isolating the beta-carboline norharman in the blood of APP patients drawn during a psychotic episode. Low levels at the limit of detection were fourd during non-symptomatic episodes. Definite proof of the chemical structure of norharman was obtained by mass spectrometry.

The sensitivity in discriminating APP patients is increased by implementing the ratio of taurine to the product of serine and methionine (TSM-ratio). In a group of 125 chronically admitted

psychotic patients 2 patients suffering from recurrent APP could be allocated.

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Study of the serotoninergic system using α-methyl-L-tryptophan

α-Methyl-L-tryptophan [α-MTrp] is an analogue of Trp which, in the brain, is converted into α-methyl-L-serotonin [α-M5HT]. By using radioactively labelled α-MTrp we have developed methods for *in vivo* study of the brain serotoninergic system. The labelled α-MTrp was used to measure the brain serotonin synthesis rate and the influence of some pharmacological treatment (e.g., probenecid, lithium, fluoxetine) on the serotonin synthesis rate. We have also used labelled α-MTrp to measure the influence of the hypothalamic 5,7-dihydroxytryptamine (5,7-DHT) lesions on the anterograde axonal transport of serotonin. It showed that the 5,7-DHT lesions of the neuronal terminals in the dorsal hypothalamus have a profound influence on the axonal transport of 5-HT; the lesion increases the rate of 5-HT (about 80%) and the amount (by about 19 times) of 5-HT anterogradely transported from the dorsal and median raphe. Our results confirm that α-MTrp is a good tracer for the *in vivo* study of the brain serotonergic system in laboratory animals and humans. Examples to confirm this will be discussed in more detail.

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Release patterns and cardiovascular properties of endogenous inhibitory and excitatory amino acids in the hypothalamus

The hypothalamus is known to be involved in the central regulation of blood pressure. Aim of this study was to investigate the influence of peripherally induced blood pressure changes on the release of endogenous amino acids in the posterior hypothalamus (PH) of the conscious rat. To initially characterize amino acid release in this brain area, release rates of glutamate, GABA, taurine and arginine were investigated at basal conditions, as well as in response to neuroactive drugs. The PH was perfused through a push-pull cannula with artificial cerebrospinal fluid. Superfusate was collected continuously in 10 min periods for determination of amino acids by HPLC and fluorimetric detection.

Under basal conditions, fluctuations appeared particularly in the release rates of glutamate and taurine. Depolarization elicited by hypothalamic superfusion with veratridine or potassium chloride led to a concentration dependent increase in the release rates of glutamate, GABA and taurine. Blockade of sodium channels by superfusion with tetrodotoxin produced a pronounced decrease in the outflow of these amino acids. In contrast to the profound influence on glutamate, GABA and taurine release, arginine outflow was not influenced by neuroactive drugs.

A fall in blood pressure (−30 mm Hg), elicited by intravenous infusion of nitroprusside, enhanced the release of glutamate (70%) and diminished the release rates of GABA and taurine in the posterior hypothalamus to approximately 80% of control values. Conversely, a rise in blood pressure (45 mm Hg) induced by noradrenaline was accompanied by an increase in the outflow of GABA (30%) and taurine (100%), whereas glutamate release was not influenced. Arginine outflow, was not changed, either by an increase or a decrease in blood pressure.

These findings indicate that, in vivo, a considerable amount of released glutamate, GABA and taurine, but not arginine in the PH originates from neurons. Since glutamate, GABA and taurine release rates were altered in response to blood pressure changes, these amino acids seem to possess counteracting properties in the PH, thus contributing to cardiovascular homeostasis.

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Taurine release and influx and cell volume changes in brain slices

Organic osmolytes have been suggested to play an important role in regulation of cell volumes in the brain which is confined in the non-expanding skull. Taurine, long known as osmoregulator in marine animals, has recently been regarded to act also in the mammalian brain as osmolyte which is released predominantly from astrocyte upon cell swelling. We investigated this aspect of possible taurine functions with mouse cerebral cortex slices. The release of both preloaded exogenous and erdogenous taurine and the influx of radiolabeled taurine were related to the simultaneous alterations of cell volumes in the slices. The release of taurine was indeed enhanced in hypoosmotic media and inhibited in hyperosmotic media. The release was enhanced, however, also under other experimental conditions. By way of example, a partial or total omission of Na^+ or Cl^-, high K^+ concentrations and glutamate agonists stimulated the release. The release was enhanced both in media in which Cl^- was replaced by a relatively permeant organic anion acetate and in which there occurred massively intracellular swelling in the slices and in media in which an impermeant anion gluconate was used as replacer and in which there occurred no or only negligible swelling. In some cases an enhanced release of taurine was accompanied even by an intracellular shrinking. Most experimental conditions which enhanced the release simultaneously also inhibited the influx of taurine. In particular, the saturable influx of taurine into the slices was entirely abolished and the nonsaturable influx greatly diminished when 50 mM sodium ions were equimolarly replaced by potassium ions. In hypoosmotic media the saturable influx also diminished but markedly less effectively, whereas the nonsaturable influx tended to increase. Glutamate agonists also diminished the influx of taurine. There obtained a positive correlation between the enhancement of taurine release and the increase in cell volumes but only under certain experimental conditions. A negative correlation prevailed likewise in many experiments between taurine influx and intracellular volumes in the slices. We infer that the fluctuations in the levels of extracellular taurine in brain tissue cannot be solely attributed to changes in cell volumes, since other mechanisms are also likely to be involved. They include depolarization-induced exocytosis, reversal of carrier-mediated transport and inhibition of reuptake of taurine molecules liberated from intracellular compartments.

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Changes of the CCK-8 contents in some brain areas of rats exposed to hyperbaric oxygen

Thirty-two of male Sprague-Dawley rats were randomly assigned to four groups: (1) normobaric air group (Group A): (2) approximately normoxic hyperbaric nitrogen group (Group B); (3) preconvulsion of hyperoxic group (Group C) and (4) hyperoxic convulsion group (Group D). The CCK-8 contents in stritum, hippocampus and midbrain were determined by RIA. The results showed that there were no any significant difference of the CCK-8 contents in all the brain areas between Groups A and B and that the CCK-8 contents in all the brain areas between Groups A and B and that the CCK-8 contents in stritum and hippocampus in Group C were markedly lower than that in Group B (respectively $P < 0.01$ and $P < 0.05$), and that there were no obvious difference of the CCK-8 contents in all the brain areas between Groups C and D. These results indicated that the changes of the CCK-8 contents in stritum and hippocampus had no relation to hyperbaric (approximately normoxic nitrigen) exposure, and that the onset of oxygen convulsion was associated with the decrease of the CCK-8 contents *in stritum* and *hippocampus*, and that *oxygen convulsion* itself had no effect on the CCK-8 contents in all the brain areas. It is reported that CCK-8, which might be one of the endogenous anticonvulsion activated peptides, has obvious regulating function to the excitability of central nervous system. Why the CCK-8 content in some brain areas of rats exposed to hyperbaric oxygen decreased and whether CCK-8 has antagonism on oxygen convulsion should be further studied in the future.

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Plasma homocysteine levels in Parkinson's disease, Alzheimer's disease and multiple system atrophy patients

Introduction. Subtle deficiencies of serum and CSF vitamin B_{12}, often without accompanying hematological changes, have been found in about 30% of patients with Alzheimer's disease (AD). Vitamin B_{12} deficiency may be of etiological importance since it may contribute to the characteristic monoaminergic and cholinergic deficits of the disease. Vitamin B_{12} also has an important function as a co-factor of the enzyme methionine-synthase, which catalyses the methylation of homocysteine to methionine. In vitamin B_{12} deficiency states, an accumulation of homocysteine will occur, leading to an increase of plasma homocysteine. Plasma homocysteine level is therefore considered to be a sensitive indicator of vitamin B_{12} status. Accumulation of homocysteine has harmful effects as indicated by the increased risk of vascular accidents in patients with homocystinuria. Homocysteine is the precursor of homocysteic acid, an endogenous agonist of the NMDA receptor. Elevation of homocysteine could lead to increased homocysteic acid, which in turn might hyperactivate the NMDA receptor, resulting in selective destruction of cholinergic neurons as seen in AD. As far as we know, plasma homocysteine levels have not been studied in AD, nor in Parkinson's disease (PD) or Multiple System Atrophy (MSA).

Patients and methods. We measured plasma homocysteine levels in 87 PD patients without dementia; 18 PD patients with dementia (PDD); 13 MSA patients; 13 'probable' AD patients and 21 controls. Total homocysteine levels were measured by HPLC after reduction of plasma samples and are given in μmol/l.

Results

	n	age ± SD	homocysteine ± SD
PD	87	66 ± 12	18.1 ± 7.4
PDD	18	76 ± 7	18.2 ± 6.3
MSA	13	67 ± 10	27.0 ± 18.9
AD	13	65 ± 8	14.2 ± 3.9
Controls	21	66 ± 10	15.9 ± 8.2

MSA patients were found to have significantly higher ($p < .05$) homocysteine plasma levels compared with control subjects and PD and AD patients. There were no significant differences in plasma homocysteine levels between the other patient groups and the controls.

Discussion. We found a significant increase in homocysteine plasma levels in MSA patients. We did not find however, as expected, an increase in plasma homocysteine in AD. The increase in MSA patients was caused by a subgroup of patients in which by far the highest homocysteine levels were found. Three MSA patients showed a level of >40, the highest being 84.6. This may indicate that our MSA group comprised patients with an increased risk for vascular disease. In future research more attention should be paid to vascular factors in this group of patients.

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Modulation of dopamine release by a crossed cortico-striatal glutamate and cholecystokinin (CCK) pathway in the rat

There is evidence that sub-populations of nigro-striatal dopamine (DA) neurons contain the CCK octapeptide as cotransmitter, and we have recently suggested that CCK may also coexist with glutamate (Glu) in cortico-striatal pathways. The striatum may be a locus for important CCK, Glu and DA interactions, and deficits in these interactions may reflect in symptoms associated to schizophrenia and Parkinson's diseases. Thus, it is of interest to investigate in vivo the releasing mechanisms of these neurotransmitters and their reciprocal interactions.

In the present study we analyse: (1) the Ca^{2+}-dependence of the increase in CCK levels induced by K^+-depolarization; (2) the HPLC-characteristics of endogenous CCK-LI in perfusates collected from the striatum, under basal and K^+-depolarising conditions; (3) the effect of selective lesions on extracellular striatal CCK levels; and (4) the differences between the changes in extracellular CCK levels and those of extracellular levels of DA, Glu and Asp, simultaneously monitored under the same experimental conditions.

Extracellular CCK and DA, but not Glu and Asp levels, were decreased by perfusing with a Ca^{2+}-free medium, both under basal and K^+-depolarising conditions. HPLC revealed that the majority of the CCK-like immunoreactivity in the perfusates co-eluted with CCK-8. Striatal extracellular CCK levels were decreased by decortication plus callosotomy, with a parallel

decrease in Glu levels. Striatal extracellular levels of DA, DOPAC and HVA were significantly decreased in animals previously treated with a unilateral 6-OH-DA injection into the medial forebrain bundle. In these animals, however, the effect of decortication plus callosotomy on CCK and Glu levels was not further augmented.

Thus, this study supports the hypothesis of a neuronal origin of extracellular CCK and DA levels monitored with microdialysis in the striatum of the rat, and also supports the idea of a partly contralateral origin of cortico-striatal CCK and Glu inputs.

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Characterization of striatal glutamate, aspartate, GABA, acetylcholine and dopamine release monitored in rat with in vivo microdialysis

Striatal glutamate (Glu), aspartate (Asp), GABA, acetylcholine (ACh) and dopamine (DA) release was studied in rats with in vivo microdialysis. Glu (1–2 μM), Asp (0.1–0.2 μM), GABA (10–20 nM), DA (4–6 nM) were detected in the striatal extracellular space of halothane anaesthetized rats, when microdialysis probes were perfused with a modified CSF solution. ACh could only be detected when the ACh-esterase inhibitor, neostigmine was included in the perfusion medium, and this effect was dose-dependent. When perfused with 1 μM neostigmine, extracellular ACh levels were $\sim$100 nM and were increased >5 fold by K^+-depolarisation (100 mM KCl included in the perfusion medium). Under the same conditions, Glu and Asp were slightly increased ($\sim$40%), while GABA and DA were increased >20 fold. The possibility that the effect of K^+-depolarisation on Glu and Asp could be masked by potent reuptake mechanisms was studied by including dihydrokainic acid (DHKA) in the perfusion medium. The inclusion of DHKA (1 mM) two hours after the implantation of the microdialysis probes increased Glu and Asp (>5 fold), as well as ACh, GABA and DA levels (>2 fold). However, whenever DHKA was included prior to the implantation of the microdialysis probes, no significant differences were observed for Glu, Asp and DA, although GABA and ACh levels remained increased by $\sim$2 fold, as compared to control experiments. Under DHKA perfusion, K^+-depolarisation increased ACh, Glu and Asp levels by $\sim$3 fold, while GABA and DA levels were increased >10 fold. The Ca^{2+}-dependence of the increases induced by K^+-depolarisation was studied by perfusing with a Ca^{2+}-free solution including the Ca^{2+}-chelator, EGTA. It was found that GABA, ACh and DA increases produced by K^+-depolarisation were diminished (>50%) in the Ca^{2+}-free medium containing 2 mM EGTA. The effect of K^+-depolarisation on Glu levels was decreased after the inclusion of the Ca^{2+}-free medium. However, Glu levels were already increased (>2 fold) by the Ca^{2+}-free medium alone. A similar effect upon inclusion of a Ca^{2+}-free medium was observed with Asp levels, although the effect of K^+-depolarisation was largely increased under these conditions.

Thus, while the present results clearly support the idea that in the striatum of the rat GABA, ACh and DA are released by Ca^{2+}-dependent depolarising mechanisms, they do not support the idea that extracellular Glu and Asp levels monitored by microdialysis reflect Ca^{2+}-dependent release.

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Short- and long-term effects of perinatal asphyxia in rats monitored with peripheral and intracerebral microdialysis

We are developing a novel non-invasive animal model for studying hypoxic-ischemic lesions, similar to those produced under labor in clinical situations. Perinatal asphyxia is induced by maintaining pups-containing uterus horns removed by hysterectomy in a water bath (at 37°C, 30°C or 15°C) for various periods of time (0–101 min). Control and asphyctic pups are obtained from the same mother. Following various asphyctic periods, the uterus horns are rapidly opened and the pups are removed and stimulated to breathe by cleaning of the delivery fluid and by tactile stimulation of the oral region. The time of asphyxia is measured from the time when the blood circulation to the uterus is cut off until the pups start to breathe. The umbilical cord is ligated and the animals are allowed to recover on a heating pad. Several parameters are acutely or chronically recorded by direct observation or by in vivo microdialysis. Following asphyxia, pups are implanted subcutaneously with microdialysis probes in the dorsal region, before they are presented to surrogate mothers. Thus, levels of aminoacids (glutamate, Glu; aspartate, Asp), and metabolic products (lactate, Lact; pyruvate, Pyr) are monitored after delivery in both asphyctic and controls pups.

It was found that, when the pups were kept in a water bath at 37°C, neonatal asphyxia led to 100% mortality within the first 20 min period following delivery, whenever the asphyctic period was longer than 22 min. However, when the pups were kept in a water bath at 30°C, 100% of the pups recovered respiratory function following tactile stimulation and were accepted by the surrogate mothers, even when the pups were exposed to an asphyctic period as long as 41 min. All pups died when the asphyctic period was extended to 50–51 min. At 15°C, 100% survival was observed following a 101 min asphyctic period.

Subcutaneous Glu, Asp and Lact levels were increased in all asphyctic groups, while no significant increases could be seen in Pyr levels. Glu was increased >4 fold in all groups, with a maximum increase seen after a 5–6 min at 37°C (>10 fold).

We have completed series of experiments in which the pups were exposed to asphyxia and then, approximately one or six month later, the same rats were implanted with two microdialysis probes, one into the striatum and another into the substantia nigra. Monoamines and amino acids were monitored under basal and D-amphetamine-stimulated conditions. Significant changes in monoamines and GABA were observed in rats exposed to mild and severe asphyxia, while Glu and Asp levels were unchanged. Studies combining microdialysis and immunohistochemistry are now in progress.

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Glutamate antagonists potentiate striatal C-fos activation induced by dopamine D-1 agonists: role of the substantia nigra

Parenteral administration of NMDA receptor antagonists has been shown to potentiate dopamine D-1 mediated turning

behavior and striatal c-fos induction, in rats with a unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigro-striatal dopaminergic pathway (Morelli et al. 1992 J.P.E.T. 260, 402). In this study we have examined the role of specific basal ganglia areas in the synergistic effects observed. Local injections into the striatum of glutamate NMDA (MK-801 and CPP) or AMPA (NBQX) antagonists homolateral to the 6-OHDA lesion, strongly potentiates the c-fos activation induced by the parenteral administration of a low dose of the D-1 agonist SKF 38393, suggesting that this synergistic effect is due to a blockade of glutamate receptors at the level of this brain area. This positive interaction however, is observed also when glutamate antagonists are locally injected into the substantia nigra (SN) homolateral to the lesion. Thus, a low dose of an NMDA or AMPA antagonists when injected into the SN potentiates the c-fos activation, induced in the striatum, by the parenteral administration of SKF 38393. Higher doses of NMDA or AMPA antagonists when injected into the SN although inducing contralateral turning per-se do not activate c-fos in the striatum. The results suggest that besides the striatum also the SN plays an important role in the positive interaction between glutamate antagonists and D-1 receptor agonists.

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Biochemical and behavioural correlates of *NMDA* receptor antagonism

It is well known that motor activity of rats is differentially affected by distinct types of antagonists of the N-methyl-D-aspartate (NMDA) receptor subtype of glutamate receptors. Since there is evidence for an interaction between dopaminergic and glutamatergic afferents of the basal ganglia, we compared the behavioural effects of various types of NMDA antagonists with their actions on dopamine metabolism of ascending dopaminergic systems. Rats were injected with the non-competitive NMDA antagonists dizocilpine (0.33 mg/kg, i.p.) and memantine (20 mg/kg, i.p.), the competitive NMDA antagonist CGP 39551 (10 and 20 mg/kg, i.p.) or the antagonist of the strychnine-insensitive glycine receptor antagonist 7-Cl-kynurenic acid (40 nmoles, i.c.v.). Drug effects on behaviour were assessed in an open field. For the neurochemical studies rats were killed at the peak time of pharmacological drug activity, and their brains were dissected. Dopamine metabolism (DOPAC/DA ratio) was determined by HPLC with electrochemical detection. Dizocilpine and memantine increased the number of line crossings whereas neither CGP39551 nor 7-Cl-kynurenic acid produced any motor stimulation. Dizocilpine and memantine produced a marked increase of DOPAC/DA in the prefrontal cortex and nucleus accumbens whereas memantine additionally increased DOPAC/DA in the posterior striatum. Both CGP 39551 and 7-Cl-kynurenic acid did not increase subcortical dopamine metabolism and even decreased DOPAC/DA in the prefrontal cortex. These findings are compatible with electrophysiological studies where non-competitive NMDA antagonists increased and competitive NMDA antagonists decreased burst firing of midbrain A-10 dopaminergic neurons. Taken into acccount the well documented role of dopamine in the nucleus accumbens in locomotor activity, the present results support the notion that the stimulant actions of non-competitive NMDA antagonists are at least partially due to an increase of dopamine metabolism in the nucleus accumbens.

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Dopaminergic/glutamatergic balance in neuroleptic malignant syndrome

Neuroleptic malignant syndrome (NMS) is a rare complication of neuroleptic drug treatment or of sudden changes in antiparkinsonian medication. NMS is characterized by rigor, hyperthermia, elevated levels of serum creatine phosphokinase and signs of vegetative instability like diaphoresis. The pathophysiological basis of NMS is thought to be an acute breakdown of dopaminergic transmission, reminiscent of the pathobiology of Parkinson's disease (PD). Thermoregulation is also abnormal in patients with PD, rendering them susceptible both to internal heat loads e.g. derived from rigidity or infection and to external heat loads e.g. in summer. Impaired thermoregulation may be involved in the pathogenesis of the "akinetic crisis" of PD, a potentially life-threatening exacerbation of PD characterized by severe rigidity, progressive immobilization and a high risk of medical complications such as bronchopneumonia and urinary tract infection. NMS and the hyperthermic akinetic crisis may be clinically indistinguishable.

The hypodopaminergic hypothesis of NMS suffers from several shortcomings: Although there is marked central D_2-dopamine receptor occupancy even after low-dose neuroleptic treatment, only few patients exposed to neuroleptics develop NMS. That NMS may occur following long-term, well tolerated neuroleptic treatment, points to additional triggers. If all symptoms of NMS were due to acute dopamine deficiency, dopamine agonists should reverse NMS rapidly. However, the clinical experience shows that many cases of NMS take longer to remit after institution of dopaminergic medication, such as lisuride, than would be predicted from pharmacokinetic data.

It has recently been shown that amantadine is an NMDA antagonist. Clinical observations on the effects of amantadine suggest that glutamatergic transmission plays a pivotal role both in PD and NMS. First, amantadine withdrawal has been associated with NMS-like episodes in PD patients. Second, amantadine may be a useful pharmacologic agent for the management of NMS. We attribute the clinical efficacy of amantadine in NMS to its antagonistic properties at the NMDA receptor-coupled ion channel. This hypothesis is corroborated by experimental data showing NMDA antagonist-mediated reduction of body temperature and rigor and pronounced effects on vegetative parameters like respiration and blood pressure in laboratory animals. It remains to be elucidated whether non-NMDA antagonists have similar effects.

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Cerebrospinal fluid and plasma arginine, citrulline and glutamate levels of Parkinson's disease, Alzheimer's disease and multiple system atrophy patients and healthy subjects

Introduction. Nitric oxide (NO) is a recently discovered mediator of vasodilatation, neurotransmission and cytotoxicity. NO

is formed from L-arginine (Arg) by the enzyme NO-synthase (NOS), for which tetrahydrobiopterin (BH4) is a necessary co-factor. During this reaction, which can be stimulated by glutamate (Glu) via the NMDA receptor, L-citrulline (Cit) is formed. In recent years nitric oxide (NO) has been put forward as a possible causative factor in neurodegeneration. However, our earlier research showed a decreased amount of nitrate, a degradation product of NO, in CSF of Parkinson's disease (PD), Multiple System Atrophy (MSA) and Alzheimer's disease (AD) patients. In these disorders also a decreased BH4 has been established, in postmortem brain-tissue as well as in plasma as in CSF, which may lead to a reduced NO production. A reduction in neuronal NO production might also be caused by a decrease in availability of arginine. In order to shed light on this issue, we measured arginine, citrulline, and glutamate levels in CSF and plasma of the same PD, MSA ant AD patients.

Patients and methods. The study included 89 PD patients without dementia; 20 PD patients with dementia (PDD); 16 MSA patients; 12 'probable' AD patients and 23 control subjects (C) We measured CSF and plasma arginine, citrulline and glutamate levels in by a routine HPLC method.

Results

Mean ± SD CSF levels of arginine, citrulline and glutamate in μmol/l

	n	age + SD	Arg + SD	Cit + SD	Glu + SD
PD	89	66 ± 12	23.0 ± 3.6*	2.6 ± 0.8*	1.9 ± 0.5
PDD	20	76 ± 7	23.5 ± 4.5*	3.0 ± 1.3*	1.7 ± 0.6
MSA	16	67 ± 10	23.6 ± 3.9*	2.9 ± 0.9*	2.0 ± 0.9
AD	12	65 ± 8	22.2 ± 2.5	2.1 ± 0.7	1.5 ± 0.4*
C	23	65 ± 13	21.3 ± 3.6	2.2 ± 0.6	1.9 ± 0.5

*$p < 0.05$ compared with controls

In plasma no significant differences were found, with the exception of a decreased glutamate in PDD patients (35.7 ± 21.9) compared with controls (53.9 ± 23.6).

Discussion. We found a slight but significant increase in CSF arginine and citrulline in PD, PDD and MSA patients. This indicates that a presumed deficit in arginine is not the cause of a decreased cerebral nitrate production. The exact cause and the significance of the observed increase in CSF arginine and citrulline remains to be elucidated. The reduction of CSF glutamate in AD patients is in concordance with the decrease in CSF nitrate in these patients.

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Lower plasma L-tryptophan availability in major depression and activation of cell-mediated immunity

Lower plasma L-tryptophan (L-TRP) to the brain is one of the most reliable biological markers of major depression. Despite much research the pathophysiology underlying this disorder has remained elusive. Recently, evidence was reported that major depression may be accompanied by activation of cell-mediated immunity. Activation of cell-mediated immunity is known to accelerate L-TRP catabolism through production of cytokines such as interferon γ and interleukin (IL)-6. Our studies revealed that plasma L-TRP concentrations in depression were significantly related to disorders in various immune/inflammatory variables, such as Il-6 secretion, plasma neopterin and haptoglobin levels (negatively), transferrin plasma levels, dipepdidyl-peptidase-IV and prolyl endopeptidase serum enzyme activity (positively). It is hypothesized that lower L-TRP availability to the brain in major depression may be related to activation of cell-mediated immunity in that illness.

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Fasting and post prandial homocysteinemia in depressed patients

Homocysteine (HCY) increase in tissues and blood is associated with premature occlusive diseases (R. Clarke et al., N. Engl. J. Med., 1991, 324, 1149). HCY, a sulphur amino acid, is an intermediate step in cystine synthesis from methionine (MET). Hyperhomocysteinemia depends on several conditions: a genetic deficit in homozygote (homocystinuria) or heterozygote for inborn errors of HCY metabolism transulfuration or remethylation pathways – or acquired deficiency of coenzymes implicated in the HCY metabolism (B6, B12, folic acid).

In endogenous depressed patients, a drop in red cell folate has been observed (M. W. Carney et al., J. Affect. Disord., 1990, 19, 207). Moreover in elderly depressed people, a link has been shown between increased homocysteinemia and cognitive disturbances (BELL et al Acta Psychiatr. Scand. 1992, 86, 386).

We performed the measure of fasting and post prandial HCY and its precursor (MET) in 8 female depressed patients (DSM III R criteria), aged from 62 to 74 years and in 2 psychotic patients aged 58 and 74 years. The results were compared with those obtained in 10 female controls aged from 50 to 94 years without vascular, endocrine or digestive diseases.

HCY samples were prepared according to L. E. Brattstrom (Scand. J. Clin. Lab. Invest., 1988, 48, 215) and the total plasma HCY (free + protein bound + disulphides) in one part and MET in other part were measured by ion-exchange liquid chromatography with LKB alpha amino acid analyses.

In our depressed patients we have observed and increased homocyteinemia, in fasting condition in two subjects (15 and 20 micromol/l) compared with controls (9.1 ± 2) micromol/l and with psychotic patients (9.5 and 10 micromol/l) and an increased homocysteinemia in post prandial conditions in 3 subjects (18, 19 and 24 micromol/l) compared with psychotic patients (11 and 13 micromol/l) and with controls (9.2 ± 2.5 micromol/l).

MET fluctuated but without correlation HCY.

These results are in favour of a link between increased homocysteinemia and some depressed patients. Abnormalities were observed in fasting conditions and the more over in post prandial conditions.

Boron Neution Capture Therapy

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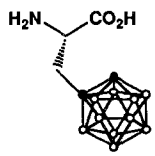
Enantioselective synthesis of unnatural amino acids for boron neutron capture therapy

Boron Neutron Capture Therapy (BNCT) holds great promise as a radiochemotherapeutic treatment for various cancers, including malignant melanoma and glioblastoma multiforme. The binary nature of this therapy shows potential for great selectivity in cell killing; the two requirements, a non-toxic boron-10-containing tumor localizing compound and a neutron source, are independently manipulable. However, there is a great need for development of new selective tumor localizing compounds.

An enantioselective synthesis of an unnatural 1,2 dicarbacarborane-bearing amino acid, L-carboranylalanine (**1**) is described. We believe that this compound is a good bioisostere for phenylalanine (**2**). It imports ten boron-10 atoms per molecule, which may significantly increase the intracellular concentration of the boron-10 species required for successful therapy. Peptide analogs incorporating carboranylalanine may bind to cell surface receptors on neoplastic cells. We wish to capitalize upon molecular processes that are upregulated in cancers, relative to the adjacent normal cells. This should permit selective targeting of peptides as selective BNCT drugs, and permit introduction of drug to the cytoplasm, thereby conferring greater therapeutic benefit. This approach may also permit us to address cancers that currently have no suitable tumor localizing drug for BNCT, such as prostate or breast cancer. We are also interested in the design and synthesis of carboranylalanine peptide analogs as probes in general structure/function studies.

Our synthetic route provides access via highly diastereoselective bromination of a carboranyl substituted imide intermediate, with subsequent azide displacement, to one of the most hydrophobic amino acids yet described, and in higher yield than previously reported. The synthetic transformations required to obtain the critical imide reveal the interesting directive effects of the carborane cage in alkylation, hydroboration, and oxidation reactions. A diastereoselective bromination introduces the latent α-amino center in a 98 : 2 ratio. Azide displacement, removal of the oxazolidone chiral template, and hydrogenolysis proceeds without erosion of stereochemical integrity, resulting in a 98 : 2 ratio of isomers in the product amino acid. This sequence is suitable for synthesis of either natural abundance boron-11 or enriched boron-10 materials, and for either the L- or D- isomer. Our route is amenable to large scale production of this amino acid and other homologs.



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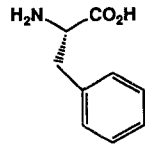

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Synthesis and optical resolution of o- and m-boronophenylalanine

p-Boronophenylalanine (p-BPA) has been effectively used for Boron Neutron Capture Therapy (BNCT) because of its selective property accumulating ^{10}B in melanoma. In this paper m-BPA and o-BPA, the isomers of p-BPA, are synthesized to understand the relationship between their structures and the selective accumulation of ^{10}B in melanoma.

The boronic acid group was introduced into the benzene rings by a Grignard reaction of m- or o-bromotoluene with trimethyl borate. The methyl group of m- or o- toluneboronic acid was brominated, and they were alkylated with acetaminomalonic acid diethyl ester anion. After hydrolysis by boiling them in HCl_{aq}-MeOH and distilling MeOH from the solution, m- or o-BPA was obtained respectively.

The optical resolution of m-BPA was effected by digestion of the racemic ethylester with α-chymotrypsin at pH 5.0 (pH stat). After the solution was evaporated to dryness, the residue was suspended in hot ethanol, and the mixture then cooled to collect L-isomer. D-isomer was obtained by saponification of the remaining D-ester after extraction into ethyl acetate. Analysis of the isomers of m-BPA by HPLC indicated that optical purities are nearly 100%. In the case of o-BPA, the selective hydrolysis of L-isomer of the ester was not observed. The HPLC analysis of the digested solution indicated the existence of four species, L- and D-isomers of o-BPA and L- and D-ethyl esters.

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Complex formation of p-boronophenylalanine with melanin-related compounds

p-Boronophenylalanine (p-BPA) has been used for Boron Neutron Capture Therapy (BNCT) for melanoma, because it selectively accumulates ^{10}B in melanoma cells. The selective accumulation of ^{10}B in melanoma might be caused by the capture of p-BPA by the melanin-related compounds that exist in melanoma cells with higher concentration than in blood. We have investigated the interaction of p-BPA with L-dopa which is one of the main precursors of melanin.

By ^{11}B-NMR experiments at the blood pH aqueous solution, two facts were observed. First, the complex formation occurred between the boronic acid group of p-BPA and two hydroxyl groups of L-dopa. Second, the complex was autooxidized to boric acid, phenylalanine and dopaquinone. These facts clearly explain the trap and release mechanism of p-BPA by melanoma cells through their L-dopaaccumulated part.

Arginine

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A comparative study on the arginine degradation cascade for sperm maturation of *Bombyx mori* and *Drosophila melanogaster*

The spermatophore of the silkmoth, *Bombyx mori*, is a reactor for a specific energy-yielding system for sperm maturation, the arginine degradation cascade. On mating, the highly viscous secretions from various glands in the male reproductive tract, which contain many enzymes and their substrates separately, are transferred to the female bursa copulatrix to form the spermatophore. In the spermatophore, they are mixed by the vigorous movement of anucleated, apyrene spermatozoa, resulting in the post-testicular maturation of nucleated, eupyrene spermatozoa. In the spermatophore, arginine-rich proteins transferred from vesicula (v.) seminalis are digested by initiatorin, an Arg-C endopeptidase of serine-protease type, from the glandula (g.) prostastica which cleaves proteins on the C-side of arginine residues and a carboxypeptidase from the g. prostatica and the v. seminalis which is activated by initiatorin. The produced free arginine is then hydrolyzed to urea and ornithine by arginase derived from the v. seminalis. Ornithine is metabolized to glutamate, followed by forming alanine and 2-oxoglutarate. This member of TCA-cycle is a preferred respiratory substrate for spermatozoa and accelerates the post-testicular sperm maturation.

Unlike *Bombyx mori*, *Drosophila melanogaster* that produces only eupyrene spermatozoa like other animals, does not form the spermatophore, and spermatozoa acquire motility in the v. seminalis of males. As found in *Drosophila*, a high glutamate-pyruvate aminotransferase activity was found in the spermatophore as well as the v. seminalis of the silkmoth. This reaches 58.3% of the whole male reproductive tract that participates in transfer of the seminal fluid.

In the male reproductive system of *Drosophila*, the concentration of arginine is low, whereas those of urea and ammonia are high. The secretion of accessory gland contains much phosphoserine. Theses substances are transferred to female uterus with spermtozoa during mating. Most amino acids increase distinctly at 30 min after the termination of mating (ATM) and decline, suggesting active degradation of transferred proteins in the uterus. As found in *Bombyx*, urea increses at the post-mating period, while ornithine shows a rather low concentration. Ornithine must be metabolized in part to glutamate. In this connection, it is notable that alanine rises markedly at 30 min following mating. As in the silkmoth, the energy metabolism of the fruit fly spermatozoa involves also arginine, ornithine, urea, and proline. These findings suggest that the occurrence of the arginine degradation cascade or related metabolic pathway in this insect.

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Subcellular fractionation and characterization of nitric oxide synthase in endothelial and neuronal cells

Endothelial and neuronal cells contain two different nitric oxide (NO) synthase (cNOS) isoenzymes which catalyze the conversion of L-arginine to NO and L-citrulline. The Ca^{2+}/calmodulin-dependent formation of NO by these enzymes plays an important role in the control of vascular tone and neurotransmission, respectively. Due to a postranslational myristoylation, the isoenzyme in endothelial cells (EC) is considered to be predominantly membrane-bound, whereas the non-myristoylated neuronal isoenzyme is believed to be mainly localized to the cytosol. However, preliminary findings suggest that a substantial portion of the neuronal cNOS activity may in fact be membrane-bound We have, therefore, investigated in detail the subcellular distribution of these isoenzymes in cultured and freshly isolated EC and in the cerebellum from various species Total cNOS activity was largely associated with the particulate fraction (~75%) of all EC types tested (bovine, human and porcine origin). Both specific cNOS activity and immunoreactivity (i.e. content) were substantially higher (~5-fold) in the microsomal fraction of EC as compared to the cytosol. These two enzyme fractions also differed in terms of their Ca^{2+}-dependency, pH optimum and inhibitor sensitivity. Subfractionation of the microsomal fraction from EC revealed that total cNOS activity was predominantly associated with the endoplasmic reticulum (~90%), but that specific enzyme activity was ~3-fold higher in the plasma membrane than in the microsomal or endoplasmic reticulum fraction. Up to 60% of total cNOS activity present in the cerebellum (rat and rabbit origin) was membrane-bound and, according to density gradient ultracentrifugation, associated mainly with the endoplasmic reticulum (~80%). This fraction also showed the highest specific activity (~2.5-fold higher as compared to the microsomal or plasma membrane fraction). In contrast to the corresponding fractions from EC, there was no major difference between the soluble and membrane-bound neuronal cNOS in terms of specific activity, pH-dependency, inhibitor sensitivity or immunoreactivity. Thus, neuronal cells seem to contain a single cNOS isoenzyme, whereas in EC either two different enzymes are present or a single enzyme, the conformation of which differs between the soluble and membrane-bound state. Moreover, these findings indicate that posttranslational myristoylation is not the only factor determining the intracellular localization of these cNOS isoenzymes. Due to the specific morphology of EC, there must be a close spatial association between plasma membrane and endoplasmic reticulum. Since these membranes may not easily dissociate during subcellular fractionation, the predominantly membrane-bound cNOS in EC, unlike the isoenzyme in the cerebellum (endoplasmic reticulum), may be either situated within the plasma membrane or localized to those regions of the endoplasmic reticulum closely associated with it. This may render the enzyme more susceptible to activation by physical stimuli, such as a shear stress-induced change in the fluidity of the plasma membrane. Continuous exposure to fluid shear stress may also upregulate cNOS expression in EC, since specific enzyme activity, immunoreactivity and basal NO release were significantly higher in freshly isolated EC as compared to cultured EC Moreover, exposure of EC in culture to elevated shear stress for several hours led to an increase in the cNOS mRNA level, content and activity.

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N^G-Substituted analogues of L-arginine: cellular transport and binding to purified nitric oxide synthase

Nitric oxide (NO) serves as an inter- and intracellular messenger in various biological systems. In neuronal tissues, NO is synthesized from L-arginine by a Ca^{2+}-dependent, constitutive NO synthase (NOS), which is inhibited by the substrate analogues N^G-methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine (L-NNA). To evaluate the mechanisms involved in inhibition of NO biosynthesis by these drugs, we investigated i) uptake mechanisms of L-NMA and L-NNA in neuronal cells, ii) binding of these inhibitors to purified brain NOS, iii) inhibitor-induced enzyme inactivation, and iv) NOS-catalyzed metabolism of L-NMA and L-NNA.

Uptake of L-[^{14}C]NMA into neuroblastoma x glioma hybrid cells (108CC15) exhibited biphasic kinetics (K_m of 3 μM and 104 μM) and was effectively antagonized by L-arginine, L-lysine, and L-ornithine, but remained almost unaffected by L-NNA. Virtually identical uptake characteristics for L-[^{3}H]arginine indicated that L-NMA and L-arginine are substrates of the same transporter. Studies with [^{3}H]-labeled L-NNA (K_m = 344 μM) showed that uptake of the nitro compound was not blocked by L-arginine and L-NMA, but most effectively inhibited by L-leucine. Kinetics and substrate specificity of L-NNA transport closely resembled that of L-[^{3}H]leucine, suggesting that L-NNA utilizes the L-leucine transporter.

Binding studies with H^3-labeled L-NNA and purified brain NOS revealed a single and saturable binding site for L-NNA (K_D = 0.2 μM). Binding did not depend on Ca^{2+}/calmodulin and was competitively antagonized by L-arginine (K_I = 2.9 μM). Association and dissociation o L-NNA were remarkably slow ($k_{on} = 4.0 \times 10^5$ $M^{-1} \times min^{-1}$, $k_{off} = 7.4 \times 10^{-2}$ min^{-1}), and the half-time of dissociation (9.4 min) closely correlated with the time necessary for surmounting NOS inhibition by L-NNA. Although apparently less potent in functional studies, L-NMA was found to exhibit the same affinity to brain NOS as the nitro derivative. We provide evidence that a rapid, turnover-independent inactivation ($t_{1/2} \sim 2$ min) of the enzyme by L-NNA accounts for this apparent discrepancy. In contrast to the nitro analogue, L-NMA induced a slow enzyme inactivation, which was associated with metabolism of the inhibitor into stoichiometrical amounts of NO and L-citrulline. Our results indicate that different mechanisms of cellular uptake and enzyme inactivation account for the inhibition of neuronal NOS by L-NMA and L-NNA.

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Modification of plasma protein by methylglyoxal under physiological conditions. Prevention by aminoguanidine and L-arginine

Methylglyoxal (2-oxopropanal) is a physiological metabolite formed mainly by the elimination of phosphate from glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. It is also formed in the oxidation of acetone and catabolism of threonine. It is metabolised by the glyoxalase system to D-lactate. The formation of methylglyoxal is increased during hyperglycaemia associated with diabetes mellitus; this has been linked to the development of diabetic complications. The median and range of concentrations of methylglyoxal in human blood (pmol/g) were: normal controls 80, 25-893 (n = 21); IDDM patients 471, 86-044 (n = 42); NIDDM patients 287, 55, 2366 (n = 105). Glyoxalase activities were risk factors for the development of diabetic complications (retinopathy, neuropathy and nephropathy).

Blood plasma from normal human donors was incubated with [^{14}C]methylglyoxal (1 μM) at 37°C under aseptic conditions for 3 weeks and the irreversible binding of methylglyoxal to plasma protein determined after ultrafiltration. The effect of scavengers, aminoguanidine (1 μM–1 nM) and L-arginine (1–100 mM), was investigated. In control experiments, 74% of methylglyoxal was bound to plasma protein. This was decreased in the presence of aminoguanidine and L-arginine where the median inhibitory concentrations were 245 μM and 14 mM, respectively.

Methylglyoxal reacts with arginine to form N_6-(3,4-dihydro-4-methylimidazol-5-on-2-yl)-2,5-diaminopentanoic acid and reacts similarly with arginine residues in proteins. Methylglyoxal reacts with aminoguanidine under physiological conditions to form two major products, isomeric triazines 3-amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine, and a minor product from the 1 : 2 stoichiometric reaction, methylglyoxal bisguanylhydrazone (a clinical anti-tumour agent). Prevention of methylglyoxal binding to plasma proteins by L-arginine and aminoguanidine in this experiment was consistent with kinetic competition between arginyl sites in plasma proteins and the scavengers.

L-Arginine and aminoguanidine have been proposed as agents for the preventive therapy of diabetic complications. Scavenging of methylglyoxal may be important in their mechanism of action. Further studies of the effect of methylglyoxal scavenging agents on the development of diabetic complications are now required.

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Regulation of the Maillard reaction by organic germanium compounds and their mode of action in the reaction between arginine and ribose

Organic germanium compounds (2-Carboxyethylgermanium sesquioxide: Ge-132, and 2-Carboxy-2-aminoethylgermanium sesquioxide: Ge-385) were found effective in suppressing the formation of Advanced Glycation Endproducts (AGE) from serum proteins, collagen and lens crystallin. We examined the Maillard reaction between amino acids and ribose and the effects of Ge-compounds on the reaction. It was elucidated that Ge-compounds could not prevent the formation of Amadori-rearranged substances, but inhibited further progress toward AGE. Specifically, Ge-385 caused the decrease of AGE after incubating the mixtures for more than 10 hours. The mode of action of Ge-compounds in the Maillard reaction was investigated by NMR to clarify the interaction between glucose and Ge-compounds. These compounds could conjugate with glucose directly through oxygens of glucose at the sites 1 & 2. This Ge-glucose complexes result in the disconnection of amino-carbonyl products to glucosone and amino residues. Ge compounds, specifically Ge-385 which has two amino residues

in its molecule, apper to be much more effective than Ge-132 in the prevention of AGE formation by making carbonyl-(NH_2-Ge-385) conjugates as the substitute of reactive amino residues on functional poly-peptides. The effects of Ge-compounds were examined in DM-rats induced by streptozotocin (STZ). Both drugs were perorally administered to STZ-DM-rats, and clinicopathological examinations revealed that glycated serum-albumin, cataract (turbidity of lens), retinopathy, neuronal conductivity, peripheral circulation were remarkably improved. Ge-compounds are the first candidates that prevent and reversibly solubilize the Maillard products which induce severe clinical complications in the patients with diabetes mellitus.

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The role of extracellular L-arginine in nitric oxide production in vascular cells

In blood vessels, the free radical nitric oxide (NO) is produced from L-arginine by a constitutive NO-synthase (NOS) which is expressed in endothelial cells and in some nerve endings, but not in smooth muscle cells. However an inducible NOS can be induced in the latter cells by bacterial lipopolysaccharide (LPS) and cytolines, resulting in cyclic GMP accumulation and pronounced smooth muscle relaxation. This induction has been involved in endotoxemia and in various vascular pathologies.

The influence of extracellular L-arginine on NO production and vascular reactivity to noradrenaline (NA) has been studied in vivo, in rats infused with LPS (10 $mg \cdot kg^{-1}$–h^{-1}) for 1 h, ex vivo in the aorta and in small resistance arteries (femoral and mesenteric) and in vitro in aortic rings and aortic smooth muscle cells exposed to LPS.

The circulating L-arginine concentration (80 μM) was not modified during LPS infusion. L-arginine injection (100 $mg \cdot kg^{-1}$ i.v.) produced no effect on blood pressure and reactivity to NA in control rats or after 1 h LPS infusion. However, when injected 5 h after the onset of LPS infusion, it enhanced the impairment of hyporeactivity to NA produced by LPS.

Ex vivo and in vitro experiments on the aorta, resistance arteries and aortic smooth muscle cells in primary cultures all showed evidence that LPS induced NO production, resulting in cyclic GMP accumulation, impairment of the vasoconstrictor effect of NA and production NO^{2-}, a degradation product of NO, in culture medium. All these effects of LPS appeared after a delay of 4–6 h, their onset was prevented by cyclohiximide, and they were blunted by N^G nitro-L-arginine methylester, and inhibitor of NOS, consistent with the induction of NOS.

Addition of L-arginine (up to 1 mM) in the organ bath had no effect on NA-induced contractions in control arteries, neither did it modify endothelium-dependent relaxations elicited by acetylcholine. However it markedly enhanced cyclic GMP accumulation and produced relaxation in LPS-exposed vessels. In these conditions, the EC_{50} of L-arginine was 8 μM in aortic rings, its maximal effect was reached at about 100 μM, and its relaxing effects was competitively antagonized by the cationic aminoacids L-lysine and L-ornithine.

These results indicate the extracellular L-arginine supply becomes a limiting factor in NO-mediated smooth muscle relaxation once NOS has been induced by LPS, both in vivo and in vitro. They support the hypothesis that an increase in L-arginine consumption following NO induction requires L-arginine entry into smooth muscle cells to provide the enzyme substrate. This mechanism may control vascular hyporeactivity in endotoxic shock.

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Role of arginine in the control of GH secretion in man

It is well known since many years that amino acids stimulate GH secretion, having arginine (ARG) the most potent GH-releasing effect in human. In fact, only ARG has been reported to potentiate the GH response to the maximal GHRH dose in man. The GH-releasing effect of ARG is dose-dependent, being 0.1 g/kg the minimal effective dose that is active even after oral administration. Concerning the mechanism of action, there is evidence that ARG has no direct effect on the somatrope cells, while likely it negatively influences the activity of hypothalamic somatostatinergic neurons. In fact, there is evidence in animal that ARG reduces plasma somatostatin levels in pituitary portal veins. In man ARG counteracts the GH negative autofeed-back mechanism, mediated by an increase of somatostatin tone. Moreover, it has been shown that the interaction between ARG and pyridostigmine, a cholinesterase inhibitor which inhibits somatostatinergic tone, has no additive effect on GH secretion suggesting a common mechanism of action of the two drugs, i.e., somatostatin inhibition. On the other hand, ARG and galanin, a neuropeptide, have a true potentiating effect on GH secretion pointing to, at least partly, different mechanisms of action. Concerning the interaction between ARG and other metabolic fuels, the stimulatory effect of the amino acid on both basal and GHRH-stimulated GH rise has been shown to override the inhibitory one induced by glucose likely mediated by an increase in hypothalamic somatostatin release. On the other hand, free fatty acids, which likely act directly on somatotrope cells, markedly inhibit the potentiating effect of ARG on both basal and stimulated GH secretion. Finally, ARG totally restores the low somatotrope responsiveness to GHRH in aging in which there is evidence for a somatostatinergic hyperactivity and a preserved pituitary GH secretory pool. Little is known about the mechanism of action of ARG at cellular level. To date, no specific receptor has been found on target cells. Recent data indicate that ARG is the physiological precursor of nitric oxide, an important vasodilator and neurotransmitter in CNS. Then, it is possible to speculate that nitric oxide could mediate also the secretory effect of ARG either on pancreatic or hypothalamic-pituitary hormones.

In conclusion, our results show that ARG plays a fundamental role in the regulation of GH secretion in man. In this contest, it is still unclear whether ARG has to be considered as a metabolic fuel or as a neurotransmitter, but it may be that these definitions are not alternative.

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L-Arginine in experimental atherosclerosis

Nitric oxide (NO) is synthesized by the vascular endothelium from the amino acid L-arginine and accounts for the biological

activities of endothelium-derived relaxing factor (EDRF). It has been demonstrated that in aorta from Watanabe heritable hyperlipidemic (WHHL) rabbit endothelium-dependent relaxation is impaired in comparison to normocholesterolemic New Zealand rabbit. A reduced synthesis and/or inactivation of EDRF have been suggested to be involved in the impaired endothelium-dependent relaxation in WHHL rabbit aorta. We recently demonstrated that the vascular content of L-arginine is significantly lower in WHHL rabbit, if compared to New Zealand rabbits. L-arginine is able to normalize endothelial function in vessels from diet-induced hypercholesterolemic rabbits. The purpose of the present study was to examine the possibility that in vitro exposure of WHHL rabbit aorta to the amino acid L-arginine might affect endothelium-dependent relaxation. The specificity of arginine was also investigated by using arginine D-stereoisomer. Experiments were carried out on WHHL rabbits of both sexes (12 months of age, 2.5–3 kg body weight). Age-matched New Zealand rabbits were used as controls. The thoracic aorta was removed, cleaned of adjacent tissue and cut into transverse rings approximately 3 mm thick. The preparations were suspended in a 30-ml tissue bath containing modified Krebsbicarbonate solution equilibrated with a 95% O_2–5% CO_2 gas mixture, pH 7.4, at 37°C. Acetylcholine or L-arginine were cumulatively added to aorta rings precontracted with EC_{50} noradrenaline to steady-state tension. In other aorta preparations, L-arginine or D-arginine were preincubated for 45 minutes before EC_{50} noradrenaline addition and subsequent cumulative acetylcholine response curve. L-Arginine (1 mM) did not modify the effect of acetylcholine on aortic isolated preparations. D-Arginine stereoisomer of L-arginine was also uneffective on vascular response to acetylcholine both in New Zealand and WHHL rabbit aorta. The lack of any effect by L-arginine indicates that the amino acid deficiency is not main cause of the impairment of endothelial function in WHHL rabbit isolated aorta. The muscarinic receptor functionality affected by atherosclerotic process and/or the increased synthesis of EDCFs could account for the reduced endothelium-dependent relaxation.

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L-arginine reduces heart collagen accumulation in the diabetic db/db mouse

Myocardial collagen accumulation is a consistent finding in diabetic cardiomyopathy. The underlying mechanism is not fully elucidated yet: increased synthesis and glycoxidation are the current concepts. As L-arginine reduced kidney collagen accumulation in spontaneously diabetic mice, we treated db/db mice for a period of 4 months with oral arginine, free base, 50 mg/kg body weight/day. Treated animals showed significantly lower heart collagen content (means = 0.24 ± 0.05 μM hydroxyproline/100 mg heart tissue) than their nontreated mates (means = 0.49 ± 0.10 μM hydroxyproline/100 mg heart tissue; $p = 0.0001$). Treated mice also showed higher amounts of soluble heart collagen as expressed by the amount of eluted collagen per total heart collagen (treated mice showed means of 0.96 ± 0.56, untreated mice showed means of 0.46 ± 0.13; $p = 0.02$). Testing the glycoxidation hypothesis of free radical cross linking as a cause of collagen accumulation we found no association with the parameters N-epsilon-carboxymethyllysine, o-tyrosine or glutathione.

Correlations of collagen with glycemic control, however, point to an association of glucose with collagen metabolism, a phenomenon documented in cell cultures at the transcriptional level. Mechanisms proposed for the arginine mediated reduction of heart collagen in the treated mice are the blocking of cross linking carbonyl species (mainly aldehydes), hypothesis compatible with the accompanying increased solubility of heart collagen; arginine mediated increased clearance of advanced stage nonenzymatic glycosylation products or increased collagenolytic activity by arginine mediated interleukin 1 alpha activation. It remains to show that the reduction of myocardial collagen accumulation is accompanied by functional benefits.

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N^G-Methylarginine derivatives in myelin basic protein from developing and mutant mice brains

Myelin basic protein (MBP), one of the major myelin protein, contains methylated arginines at Res-107 (bovine MBP). The amounts of the arginine derivatives in MBP purified from dysmyelinating mutant and different stages of normal myelinating mice brains have been studied utilyzing HPLC with a highly sensitive post-column o-phthaldialdehyde derivatization method. All three naturally occurring derivatives [MeArg, Me_2 (symmetric) and Me_2(asymmetric)Arg] were found in MBP, however, their relative concentrations varied significantly with the age of the animal. The amounts of MeArg and Me_2(sym)Arg in MBP increased as a function of the age of the brain whereas that of Me_2(asym)Arg decreased. MBP from early-myelinating mouse brain was shown to contain a high proportion of Me_2(asym)Arg which was hardly detectable in older brain MBP. MBPs isolated from dysmyelinating mutant mice brains, such as jimpy(jp/y) and quaking (qk/qk), contained much higher level of Me_2(asym)Arg relative to the other two methyl-derivatives and also in comparison with those levels in the mother brain MBP. SDS/PAGE analysis of MBPs extracted from the mutants as well as young normal mice brains indicated the presence of a high molecular weight isoform of MBP, indicate that structural integrity of myelin membrane in which MBP is embedded appears to play a pivotal role in determining the extent and the kind of Me_2Arg formation in MBP at the posttranslational level.

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N^G-Methylarginines: origin, function and metabolism

In 1968, we first described the presence in calf thymus of an enzyme which transferred methyl groups from S-adenosyl-L-methionine (AdoMet) to the guanidino (N^G-) groups of arginine residues in preformed polypeptide chain [here, calf thymus histone; J. Biol. Chem., *243*, 2108 (1968)], but not to free arginine. Subsequent studies abundantly demonstrated that the enzymatic methylation of protein-bound arginine residues constitutes one of the ubiquitously occurring posttranslational modification reactions of protein. The chemical structure of the products formed was identified to be N^G-monomethyl-L-arginine, N^G, N^G-dimethyl-L-arginine (asymmetric) and N^G, N'^G-dimethyl-L-

arginine (symmetric). These methylated arginine derivatives have been shown to occur widely in nature, in many highly specialized proteins as well as in free form.

The enzyme responsible was originally thought to be a single enzyme which is specific towards arginine residues regardless of the protein species. Thus, it was designated as protein methylase I [AdoMet: protein-arginine N-methyltransferase; EC 2.1.1.23]. However, recent observations clearly demonstrated that there is a class of enzymes which are not only specific towards the arginine side chains, but also have a second level of specificity towards protein species.

The proteins that are methylated posttranslationally will sooner or later be degraded and free methylated arginine derivatives will be released *in vivo*. Since methylated arginines can not be incorporated into protein, some of these N^G-methylarginines will be further metabolized. On the other hand, in higher organisms some of them will find their way into the kidney for eventual disposal by way of the circulatory system. The very important recent observation was that N^G-monomethyl-L-arginine acts as an antimetabolite for nitric oxide (NO) biosynthesis. Therefore, we will review the enzymology involved in the biosynthesis and the metabolism of N^G-methylarginines.

Nutrition

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Practical application of (bio-)synthetic amino acids in poultry diets

In several West European countries nitrogen (N) pollution of the environment by animal production is becoming a major problem. In poultry on an average only 40% of the N consumed is used for the production of meat or eggs. The remaining 60% is excreted via urine and faeces. Application of free amino acids (FAA) in poultry diets can improve efficiency of utilization of dietary protein, and as a result decrease N excretion. Several trials with laying hens and broiler chicks were conducted in which the requirement of amino acids which will become first limiting in a practical diet was examined. These studies indicated that the most critical amino acids in poultry diets are methionine and lysine followed by threonine, tryptophan, valine and isoleucine, and in broiler diets also arginine. From these amino acids, methionine, lysine, threonine and tryptophan are available economically. It was found that the dietary protein level in a maize-soya based layer diet can be reduced from 16.5% (normal practical level) to 14%, provided the 14% protein diet is adequately supplied witn methionine and lysine. As a resull N excretion is reduced with approximately 25%. Indications, however, were achieved that in layers utilization of FAA is lower than of protein-bound amino acids. This finding was based on the observation that substitution of 4% protein-bound amino acids for FAA led to significant poorer layer performance. In chicks, however, the substitution of 4% protein-bound amino acids for FAA did not affect performance. In conformity with practical conditions, light was provided for 16 hours a day in the layer trials and for 24 hours a day in the broiler trials. Additional studies pointed out that the difference in reaction to dietary supplementation of FAA between laying hens and chicks could be explained by the difference in the lighting system. As a result of the lighting systems used, feed was available 16 hours/day for layers and 24 hours/day for chicks. Thus, frequency of feeding seems to influence the utilization of FAA. This is supported by other who have found that in pigs free lysine was utilized much better when the feed was offered six times per day than when it was offered once a day. The difference in absorption velocity between FAA and protein-bound amino acids may be the explanation for the effect of frequency of feeding on the utilization of FAA.

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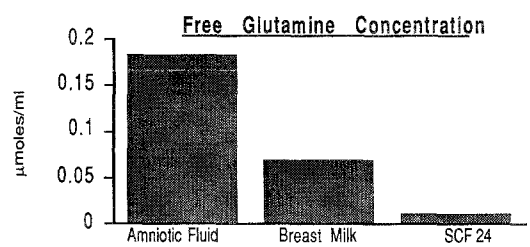
Glutamine levels in amniotic fluid, breast milk and premature infant formula: Non-essential amino acid or essential nutrient

Glutamine has many functions, it is a regulator of protein synthesis, it enhances nitrogen retention and it is a major fuel for the small intestinal mucosa. Glutamine affects the structure and metabolic function of the intestinal tract, it enhances mucosal development, promotes vilus growth and absorptive surface area, protects from gastric ulceration, promotes jejunal weight gain and decreases bacterial translocation. It has been suggested that glutamine enhances gut immune function secondary to its effects on IgA metabolism. Glutamine is present in infant formulas in extremely small quantities. This is concerning considering that in catabolic stressed states the need for glutamine is increased. Recent literature demonstrates significant advantages of glutamine enriched nutrition for adult patients in stressed states. Critically-ill premature newborns are stressed and may benefit from "custom-made" formulas, designed to meet their complex nutritional needs. Analysis was conducted by gas chromatography and thirty samples from each category were studied. The results show significant differences in the free glutamine levels of amniotic fluid and special care infant formula ($p < .001$) and between human milk and the infant formula ($p < .01$). After analyzing the glutamine content of amniotic fluid, which bathes the intestines of the fetus prior to delivery, and the glutamine content of breast milk, nature's own protective formula, and comparing these with the glutamine content of preterm infant formulas, we feel that critically-ill newborns may be better served by the addition of glutamine to neonatal nutritional therapy.



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Methionine taste; creating a palatable synthetic diet

Synthetic diets containing free amino acids as well as other nutrients, are often employed therapeutically to mitigate certain metabolic diseases. For example, the treatment of Phenylketonuria is based upon the restraint of phenylalanine intake by providing a diet replete with all necessary vitamins, calories and essential amino acids and limiting the content of phenylalanine to maintain growth and development while preventing phenylalaninemia. Such diets are difficult to consume since they have remarkably unpleasant taste. We have explored the taste of all of the dietary amino acids individually and in combinations and have observed that methionine is characterized by an unusually unpleasant taste. In an approach to developing a palatable therapeutic diet, we have synthesized hydrophobic derivatives of methionine in an attempt to evade the taste receptors and thus eliminate the bad taste of this essential amino acid. In this report we describe derivatives of methionine, namely palmityl methionine and palmityl methionine methyl ester, which are tasteless but which can be metabolized in to methionine and maintain dietary requirements for this amino acids.

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Effect of protein sources with different degradability in the rumen on blood plasma free amino acid profile of cows in early lactation

Experiments were conducted with 2 × 10 high-producing (8,000 kg FCM/year) Holstein cows over a 8-week period immediately after calving. The ruminal degradability of protein fed to the two groups of cows in the concentrate was 72% (high degradability, HD) and 55% (low degradability, LD), respectively. The daily ration of each cow consisted of 14 kg corn silage, 6 kg alfalfa hay, 0.5 kg wheat bran, 0.5 kg corn grits and 10.2 kg concentrate containing either HD or LD protein. The HD concentrate mix contained 70.2% barley, 17.5% peas, 8.8% soybean meal (fullfat) and 3.5% mineral plus vitamin mix (CP 17.5% DM; RDP 10.5% DM; UDP 4% DM). The LD concentrate mix included 73.5% corn, 9.8% soybean meal (fullfat), 4.9% corn gluten meal, 4.9% blood meal, 4.9% fish meal and 2.0% mineral plus vitamin mix (CP 23.4% DM; RDP 9.3% DM; UDP 7.6% DM). At the end of week 2, 4 and 8 of the experiment, blood samples were collected from the jugular vein of cows three hours after the morning feeding and the concentrations of blood plasma free amino acids were determined.

All the detected plasma amino acids but CYS showed significant fluctuations at different times after parturition. Total free amino acid (TFAA) concentration was significantly ($P < 0.001$) higher at postpartum week 2 than at parturition. Subsequently, it declined in the 4th week ($P < 0.01$) only to undergo another significant ($P < 0.001$) rise in the 8th week.

Of the individual amino acids, the concentrations of ALA, SER, ARG, HIS, LYS, PHE, THR and TYR determined at different times post partum showed an alternating change comparable to that observed for TFAA. On the other hand, the concentrations of ILE, LEU and MET were significantly lower 2 and 4 weeks post partum ($P < 0.01$; $P < 0.001$, respectively) than at the time of calving.

HD and LD feeds caused the biggest differences in plasma free amino acid concentration four weeks after parturition. The plasma TFAA concentration of cows of group LD exceeded that of group HD cows by about 0.2 mmol/l. This increase was primarily due to the significantly higher plasma LEU, TYR and PHE concentrations of group LD cows as compared to group HD animals. Unlike these amino acids, the plasma concentration of ILE was significantly lower in group LD than in group HD cows. No significant difference was found ($P > 0.05$) between group HD and group LD in the concentration of the other amino acids.

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Intra- and extracellular free amino acids in young beef bulls of different breed and conformation

Adequate supply of amino acids (AA) as substrates in muscle protein (MP) synthesis reactions is essential to optimize protein deposition, i.e. meat production in farm animals. In order to correctly match supply and utilisation a valid estimate of the nutritional status is imperative. Plasma concentration of free amino acids (FAA) has been proposed in this respect. FAA concentrations at the "site of action", i.e. the muscle cell, should presumably give an even better estimate. In this experiment plasma FAA (=extracellular) and FAA concentrations in muscle biopsy samples (=intracellular) were studied in young bulls of two breeds differing extremely in meat production ability: the Belgian Blue breed (BB), a highly productive breed, and the Friesian Holstein (H) which is essentially a milk breed with poor beef performances. The BB group was subdivided into two subgroups differing in animal conformation: the "normal" (BBN) and the "double muscled" type (BBDM). Each group contained 4 young bulls with an average initial weight of 340 kg. Animals were given a conventional fattening diet and were weighed fortnightly in order to estimate growth rate. Twelve and four weeks before slaughter (Periods 1 and 2) muscle biopsy samples of longissimus dorsi and venous blood samples were taken. FAA were measured after sulfosalicylic extraction by fused silica capillary gas chromatography.

Intracellular FAA concentrations ([AA]i) were for all AA substantially higher than plasmatic concentrations ([AA]e). In both periods BBDM featured higher [AA]i than BBN and H which were not different: 27.6 and 33.3 mmol/L intercellular water (H2Oi) vs 19.9 and 20.8 and 26.7 and 21.6. Total [AA]e were about ten times lower and showed no clear pattern. Consequently Intra/extracellular gradients were on average around 10 but very large differences were observed between individual amino acids. Most [AA]i were below 1 mmol/l. Some FAA occurred at much higher levels. Highest [AA]i were observed for GLU (+GLN, measured together in GC2): 7.9, 6.7, 5.4 and 9.5, 8.7, 6.0; β-ALA: 6.0, 5.9, 5.3 and 9.8, 4.8, 4.6; GLY: 4.2, 1.4, 2.6 and 3.7, 1.5, 1.7 and ALA: 1.6, 1.1, 2.9 and 1.9, 1.4, 1.6 respectively in periods 1 and 2 for BBDM, BBN and H. Where high GLN and ALA levels can be explained by the role of this AA in N transport and gluconeogenesis the high β-ALA concentrations are less clear. Ass the intracellular FAA pool is clearly the direct precursor pool for the synthesis of MP it might be interesting to verify to what extent its composition matches this of MP. The ratio

of free to protein bound (F/B) was calculated for the measured essential AA. This F/B ratio was for most AA lower than 1%. Between BBN and H no clear differences were observed. Double muscled BB showed clearly higher F/B ratios especially during the first period: .49 vs .21 and .19 and .49 vs .34 and .38. Apparently double muscling is related to a different cellular status than normal conformation, even in the same breed.

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Free amino acid regulation in the kidney of aged rats submitted to long term fasting

The design of specific nutritional regimens for the aged must account for the changes in body composition and metabolism with age.

Aim. To measure the amino acid (AA) inflow and the intraorgan free amino acid levels, in the kidney of aged rats submitted to long term starvation.

Methods. Two-yrs-old male Wistar rats (n = 20) were randomly allocated into two groups (n = 10) to be submitted to 1, or to 15 days of fasting. An arterial sample was obtained from the carotid arteria, and following rat decapitation, the left kidney was immediately taken out and frozen until analyzed. After thawing, tissue samples were treated as described by Adibi SA. Amino acid concentrations were measured by HPLC after precolumn derivatization with PITC using UV-detection. Protein content was determined according to Lowry. Blood inflow measurement was done by injection of ^{57}Co microspheres as described by Heymann MA.

Results. a. Kidney Weight (Grams. Mean ± SD): Day 1: 1.89 ± 0.28, Day 15: 1.49 ± 0.25 ($p < 0.01$). *b.* Protein Content (mg/G fresh tissue. Mean ± SD): Day 1: 123.63 ± 11.09, Day 15: 119.66 ± 5.41 ($p < 0.01$). *c.* Blood Inflow (ml/min · G tissue. Mean ± SD): Day 1: 5.17 ± 1.34, Day 15: 3.35 ± 0.62. *d.* Amino Acid Inflow (μmol · ml/min · G tissue. Mean ± SD): Total AAs (TAAs): 20.97 ± 7.86, Day 15: 12.26 ± 1.69 ($p < 0.05$); Essential AAs (EAAs): Day 1: 6.70 ± 2.69, Day 15: 3.07 ± 0.47 ($p < 0.05$); Non Essential AAs (NEAAs): Day 1: 13.99 ± 5.18, Day 15: 9.02 ± 1.21 ($p < 0.05$). *e* Intraorgan free amino acid levels (μmol/G fresh tissue): TAAs: Day 1: 22.86 ± 4.71, Day 15: 19.45 ± 3.57; EAAs: Day 1: 2.17 ± 0.48, Day 15: 1.25 ± 0.15 ($p < 0.01$); NEAAs: Day 1: 19.98 ± 4.35, Day 15: 17.49 ± 3.49.

Conclusions. 1. Kidney weight and total protein content significantly decreased after fasting. *2.* Blood inflow showed a tendency to decrease as a consequence of starvation, while organ amino acid inflow (TAAs; EAAs; NEAAs) significantly decreased. *3.* Intraorgan free amino acid levels tended to decrease but only EAAs were significantly lower. *4.* Flux studies provide important information about how organ handling of specific nutrients changes during different metabolic states. Information on kidney protein turnover in the aged is still poor and malnutrition is a common condition in the elderly.

The results presented suggest that the decreased amino acid inflows detected during long-term starvation in old rats could be partially compensated by an increased intraorgan protein breakdown. However the apparent inability to mantain the EAAs levels warns about the necessity to design specific nutritional formulae for the aged with no renal failure.

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Influence of fasting on the tissue and plasma profiles of glutamine concentrations in old rats

Starvation is usually associated with significant adaptations in glutamine (Gln) metabolism by several organs that occur despite no significant changes in the arterial glutamine concentration. However, little is known about the metabolic effects of long-term starvation in aged animals.

Aim. To study the glutamine profile in arterial blood and in several tissues (liver, heart, kidney and small intestine) during a long-term fast in old rats.

Methods. Two-yrs-old male Wistar rats (n = 48) were randomly allocated into eight groups (n = 6) to be submitted to 1, 3, 5, 7, 9, 11, 13 or 15 days of starvation. Tissue samples were treated as described by Adibi SA. Plasma and tissue Gln concentrations were measured by HPLC after precolumn derivatization with PITC using UV-detection. Results are expressed as μmol/l when refered to arterial blood, and as μmol/g fresh tissue when refered to the different organs. Simple linear regression was applied to infer the relationship between days of starvation (1–15 days) and Gln levels.

Results. a. Glutamine concentrations: Mean/SEM

Fast (Days)	Blood	Liver	Heart	Kidney	Small intestine
1	699/18	5.18/0.19	5.38/0.32	1.10/0.09	0.25/0.04
3	616/15	4.32/0.54	5.86/0.40	0.94/0.10	0.60/0.05
5	699/26	1.86/0.64	4.00/0.30	1.14/0.11	0.46/0.12
7	786/27	2.01/0.53	3.95/0.24	1.40/0.14	0.49/0.10
9	677/95	0.25/0.09	1.88/0.72	1.15/0.03	0.60/0.12
11	728/44	2.31/0.29	4.02/0.16	1.11/0.09	0.40/0.09
13	585/87	0.34/0.14	2.04/0.30	0.78/0.11	0.63/0.17
15	632/85	1.55/0.17	3.03/0.45	0.89/0.15	0.76/0.09

b. Correlation coefficient and statistical significance: Arterial Blood: −0.076, $p = 0.691$; Liver: −0.676, $p < 0.001$; Heart: −0.330, $p < 0.05$; Kidney: −0.201, $p = 0.239$; Small Intestine: 0.463, $p = 0.017$.

Conclusions. 1. This study demonstrated statistically significant decreases in liver and heart Gln concentrations, and increased Gln levels in small intestine after the starvation period. Arterial blood and kidney Gln levels remained practically unchanged. *2.* Prolonged starvation in the old rat is associated to adaptative changes in nitrogenous metabolism which essentially do not differ from those already found by other authors in younger animals. *3.* These results could suggest that during starvation Gln uptake by the intestinal tract increases, while other organs shift to release increased amounts of Gln favouring Gln balance across the splachnic viscera (e.g.: supporting renal ammoniogenesis).

In aged rats, Gln supplementation to nutrition formulae would probably favour an sparing effect on the amino acid pool in other organs (e.g.: liver), rather than improve Gln gut utilization.

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The influence of dietary amino acid levels on plasma thyroid hormones in chicks

Diets containing varying protein and carbohydrate sources such as isolated soy protein and glucose, or casein and glucose, or pure amino acids and starch, or all natural ingredients such as corn and soybean meal, cause elevations in serum 3,5,3'-L-triiodothyronine (T_3) of growing chicks when the protein level is reduced from 24% to a deficient level of 10%. This is frequently accompanied by decreases in plasma thyroxine (T_4) levels. These changes signal major effects of dietary protein on thyroid function. It is not known if this effect is a consequence of total protein or selected dietary amino acids. Therefore, in three experiments, seven different essential amino acids were added separately into an otherwise nutritionally complete diet at levels of 50–75% of the requirement established by the National Research Council. The primary ingredients in the control diets were isolated soy protein, crystalline amino acids, starch, and vitamin and mineral mixes. The experimental diets were fed to duplicate or triplicate groups of 8–10 broiler chicks each from 10 to 24 days of age. Other groups of chicks fed the control diet were pair-fed with the deficient chicks on a daily basis to account for the effect of decreases in feed intake in the deficient state on the parameters measured.

Deficiencies of all amino acids studied caused marked reductions in body weight gain and feed intake. Compared to a control diet containing adequate amounts of all amino acids, the effects of single essential amino acid deficiencies on T_3 could be divided into three groups: those having no effect on plasma T_3 (threonine and leucine); those producing an elevation in plasma T_3 compared only to pair-fed controls (arginine, lysine and methionine); and those causing an elevation in plasma T_3 compared to both pair-fed and ad libitum-fed controls (isoleucine and tryptophan). Plasma T_4 levels were unaffected by the amino acid deficiencies. It is concluded that essential amino acids have widely varying effects on thyroid hormone metabolism in chickens. Also, the effect of a dietary protein deficiency on plasma T_3 and T_4 levels is probably not a consequence of a deficiency of amino acids in general but may be related to the low intake of specific amino acids.

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Amino acid composition and nutritional properties of lupin protein isolates

Because their high content of protein among legumes, lupin seeds are considered as a protein source. Their protein quality is determinated by a reasonably good balance of essential amino acids with a high degree of digestibility. In agree of their solubility the principal protein fractions in lupin seeds are: albumins, globulins, glutelins and prolamins.

One form to improve the nutritional quality of foods is for the addition of isolated proteins as ingredients. Two process has been developed to get isolates: isoelectric precipitation and micellization.

The purpose of this work was to determine any nutritional characteristics of lupin protein isolates than has been get for isoelectric precipitation and micellization. The antinutritional factors evaluation shown that the phenolics compounds contents was lower in isolates get for micellization than for isoelectric precipitation. Both isolates have not shown quinolizidinic alkaloids toxic levels, while hemagglutinating activity was the same in the two isolates. In lupin flour and both isolates, the albumin protein fraction was the major component and in minor proportion globulin, glutelin and prolamin. However, the proportion of each one and the composition of essentials amino acids was different in the isolates. All of this results indicate interesting possibilities for the use of lupin protein isolates as food ingredients.

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Supplementation of pig diets with essential amino acids

Dense populations of animals may create major environmental problems due to the excess of mineral elements present in the excreta. The pullution with manure nitrogen is generally considered as most severe. For this reason nitrogen excretion by animals held in non-groundbound livestock units must be reduced. This may be accomplished by optimizing the utilization of dietary protein, resulting in lower protein amounts required by the animals. In monogastrics, these objectives can be achieved by simultaneously reducing the protein content in the diet and supplementing the limiting amino acids in order to maintain optimal production. Adequate amino acid fortification of low-protein diets demands accurate information on the amino acid requirements. For growing pigs the literature-reported amino acid needs are variable as they are affected by various factors such as body weight range, feed intake, feeding method, dietary energy and protein content, variation in digestibility and availability of amino acids among feedstuffs, genotype. The objective of this study was to determine the requirements for lysine, methionine, threonine and tryptophan of growing pigs in the weight range of 20 to 50 kg when fed a diet based on 59.2% barley, 28% wheat, 7% soybean meal, 1% corn oil and 3% mineral-vitamin premix. Particularly these amino acids were studied as they may become first to fourth limiting upon lowering the dietary protein content, depending on the feedstuffs in the diet. Crossbred (Belgian Landrace × Dutch Landrace) barrows were used in all experiments. In order to determine the lysine requirement, crystalline lysine. HCl was added at seven incremental amounts (from 0.27% to 0.65%) to the basal diet resulting in dietary total lysine contents between 0.81% and 1.11%. All diets were supplemented with the same amount of methionine, threonine and tryptophan, respectively, so that the dietary contents of these amino acids were 110% of the requirements reported by Cole in Recent Developments in Pig Nutrition (Butterworths, London, 1985). Also an appropriate amount of glycine was added in order to adjust the crude protein content to 12.9% in all diets. Each diet was fed ad libitum to at least five animals, individually housed in metabolism cages. Body weight increase and feed conversion were measured. Each animal was used in three five-day nitrogen-balance trials. Moreover, urea contents in blood and urine were measured as additional indicators for the efficiency of the utilization of absorbed amino acids. Depending upon the parameter measured, the optimal total lysine content in the experimental diets varied between 0.96% and 1.04% with an average of 1.03%. The procedure followed for the determination of the other amino acid requirements was similar to the procedure for lysine. Thus for

measuring the requirement for the S-containing amino acids, different amounts (0%–0.39%) of dl-methionine were added to the basal diet, yielding dietary concentrations of methionine + cystine between 0.48% and 0.87%. Optimal results were obtained with dietary methionine + cystine contents between 0.57% and 0.62%, with an average of 0.59%. For measuring the threonine requirement, the basal diet was fortfied with 0% to 0.55% of l-threonine, corresponding with 0.46% to 1.01% total threonine. Optimal threonine requirements varied between 0.66% and 0.73% with an average of 0.71%. In the experiments on the tryptophan requirement, the basal diet was supplemented with 0% to 0.20% l-tryptophan, providing dietary concentrations of total tryptophan that ranged between 0.15% and 0.35%. Optimal results were found with diets containing 0.17% to 0.22% tryptophan, with 0.20% as an average. In order to express the requirements for lysine, methionine + crystine, threonine and tryptophan in terms of ileal digestible contents in the diet, the ileal digestibility of these amino acids in the basal diet was measured using barrows which were fitted with a T-cannula. Using these values as well as the measured dietary contents of total lysine, methionine + cystine, threonine and tryptophan for optimal performance, and assuming that synthetic amino acids are totally absorbed, the following digestible amino acid requirements were found: 0.84% lysine, 0.51% methionine + cystine, 0.59% threonine and 0.17% tryptophan, yielding the ratios 100:61:70:20 in grower diets.

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Effect of the level of dietary protein on plasma and muscle free amino acid profiles in growing chickens

Many previous studies have attempted to establish a direct relationship between the amino acid (AA) content of the protein ingested and the free AA levels in tissues, with contradictory results. The aim of the present work was to obtain further information on this subject. Free amino acid levels in plasma and muscle were measured in growing chickens given continuously, above maintenance, four isocaloric (12.9 kJ ME/g DM) semi-synthetic diets containing graded levels of protein (6, 12, 18 or 24% CP). All diets were based on soya bean as the sole source of protein and were supplemented with 0.2% methionine. Following a Latin Square layout, four groups of four animals each were given the corresponding diets for periods of four days and then one chicken from each treatment was killed and samples of blood and muscle were taken for AA analysis, whereas the remaining animals were allocated to the following treatment. The same schedule was followed until the end of the experiment so that 4 replicates per treatment were obtained. Muscle samples were freeze-dried and then homogeinized in 0.1 M HCl. Plasma and muscle homogenates were centrifuged at 1500xg for 15 minutes and supernatants were mixed with norleucine as internal standard. Plasma and muscle samples were then deproteinized by ultrafiltration. Amino acid analysis was determined by HPLC, according to the Waters Pico-Tag Method with pre-column derivatization with phenylisothiocyanate.

In general, the levels of essential amino acids in plasma and muscle increased with increases in dietary protein level. Significant regressions were found between the levels of histidine ($r = 0.820$), arginine (0.765), threonine (0.755), valine (0.782), isoleucine (0.788), leucine (0.780), phenylalanine (0.563), tryptophan (0.617), lysine (0.676) and glycine (0.565) in plasma and of histidine (0.672), arginine (0.565), threonine (0.904), valine (0.797), phenylalanine (0.856), tryptophan (0.618), lysine (0.878) and glycine (-0.847) in muscle.

In is concluded that for most of the amino acids studied a quantitative agreement between the AA composition of dietary protein and the free AA levels in plasma and muscle was observed.

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Correlation of free amino acid profiles in elementary wines with grape variety by pattern recognition methods

Amino acids present in the mature grapes accumulate in the berry during maturation. After vinification, in most instances, the free amino acid profiles are dominated by proline. However, the proline content depends on exogenous factors such as fertilisation procedures and seems to be insensitive to factors related to fermentation techniques. The global free amino acid profiles in wines is dependent on grape variety, type of soil, and climatic conditions. Even for elementary wines, the vintage to vintage concentrations of individual free amino acids do not follow a normal distribution. Therefore, the normal procedures of parametric statistics do not allow to draw conclusions that show correlation between grapes and wines.

The amino acid compositions of 42 elementary wines, produced from eight selected grape varieties under standard conditions, were studied along a 7-year period. The study includes the evolution of enantiomeric composition of D-Amino acids.

The great amount of data collected is not informative about any characteristic trend within each type of wine. The amino acid concentrations exhibit great variations in a year-to-year basis. The information concealed in the data, is gathered by the use of pattern recognition techniques. The treatment of data by supervised and non-supervised techniques shows that a correlation exists between the free amino acid profiles in each type of wine and the original grape variety independent of the vintage.

Different information may be obtained from the multivariate study of the evolution of the enantiomeric composition of some D-amino acids. The pattern recognition techniques applied to treatment of data show that D-amino acids can be correlated to the fermentation techniques used.

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Study on the residual rate of the heated tryptophan solution in the case of coexistence of the food components and the food additives

We have been studying on the nutritional, physiological and hygienic significance of the reactions and the reaction products between the food components and the coexisted materials. In this study we investigated the effect of the sugars, the organic acids and the food additives on the tryptophan (one of the essential amino acid) from the nutritional standpoint.

Methods and materials: The mixture solution 5 ml of 5 mM Trp and 5 mM food components or food additives was heated at

110°C in the oil bath for 8 h (experiment 1). The mixture solution 5 ml of 10 mM Trp and 0.5 mM NaCl was heated at 100°C in the oil bath for 8 h (experiment 2). Organic acids were used 5 kinds as follows: citric acid, tartaric acid, malic acid, succinic acid and oxalic acid. Sugars were used 5 kinds as follows: xylose, fructose, glucose, sucrose and maltose. Food additives were used 3 kinds as follows: NaCl, sodium nitrite and ascorbic acid. The determination of Trp was used colorimetric method using OPA reagent (experiment 1) and HPLC method (experiment 2).

Results: A. Experiment 1. 1. The residual rates of after 8 h heat were as follows: In the case of organic acids: tartaric acid 84.3%, citric acid 86.1%, oxalic acid 88.7%, malic acid 89.3% and succinic acid 92.1%. In the case of sugars: glucose 83.9%, xylose 88.0%, fructose 92.8%, suxrose 100% and maltose 94.7%. In the case of food additives: sodium nitrite 70.2%, ascorbic acid 76.9% and NaCl 90.9%. 2. In the case of organic acids the effect of pH on the stabilities of amino acids was investigated and it was found that organic acids affect the stabilities of amino acids not only by pH and also by acid radicals. 3. The relationships between the residual rates of Trp and the coloring of the reaction mixtures were found in the same group substances but not found in the different group substances. 4. In the case of food additives, the reaction between Trp and nitric acid or ascorbic acid was reasonable but the reaction between Trp and NaCl was not reasonable, therefore the investigation of the phenomenon, a little decrease of residual rate of Trp and slight coloring, is desirable in the future. B. Experiment 2. 1. The effect of heat on the stability of Trp: The effect was not found. 2. The effect of NaCl on the stability of heated Trp: became fairly unstable (residual rate 95.9%). 3. The effect of pH on the stability of heated Trp coexisted NaCl: The effect was not found. 4. The effect of metal ions on the stability of heated Trp coexisted NaCl: The stability became remarkably bad by Cu^{2+} (0.1%–0.5%). 5. The effect of metal ions on the stability of heated Trp coexisted NaCl and ascorbic acid: The effect of Cu^{2+} was strengthed by ascorbic acid (residual rate 61.6% in the case of Cu^{2+} 0.1% and ascorbic acid 20 mg%).

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Amino acid sequence of rice reserve proteins by peptide sequencing

In Japan, rice is a staple food, supplying 35% of the caloric intake and 17% of the protein intake. Rice grains typically contain 7–8% protein. As a major reserve protein, glutelin accounts for 80% of the protein in milled rice. The next most abundant protein is globulin, which is concentrated in the bran and the embryo. Prolamin is also found in small amounts in rice but is more abundant in maize, wheat, and barley.

We purified different kinds of prolamin and globulin from rice grains. Rice prolamins can be grouped into two families, a sullfur-free family and a sulfur-containing one. We previously reported the complete primary structure of the major sulfur-free prolamin by direct peptide sequencing. The sulfur-free family has common features, such as high levels of glutamine and hydrophobic amino acids. The sulfur-containing family, which contains cysteine and methionine residues, has a lower hydrophobicity than typical prolamin. Some members of this family are also glutelin-like because of their alkaline solubility.

[Experiment] Milled rice (Oryza sativa L. Japonica cv. Nipponbare) was powdered. Prolamin fraction was extracted with 50% propanol and purified by ion exchange chromatography on a CM-Sephadex C-50 column in 8M urea, pH 4.6–5.0. The resultant main peaks were purified further by RP-HPLC on a Cosmosil C_{18} column, with a linear acetonitrile gradient (20–60%) in 0.1% TFA. Embryo globulins were extracted with saline phosphate buffer and purified by gel filtration, ion exchange chromatography and RP-HPLC. Molecular weight and protein purity were confirmed by SDS-PAGE. Digestions with trypsin, pepsin, chymotrypsin and lysyl endopeptidase were performed for determination of the primary structure. A masked N-terminal peptide was unblocked with pyroglutamate amino peptidase. Sequence analyses were performed with an Applied Biosystems model 477A sequencer equipped with a 120A PTH analyser.

[Results] The sulfur-free prolamins were eluted during 49–56% of acetonitrile. P14, the major prolamin which had been sequenced, was eluted at 53%. The next highest peak appeared at 51% of acetonitrile. The newly purified P12 prolamin was then analyzed by SDS-PAGE and electric focusing, and its molecular weight was determined to be 15.2kD. It showed a higher pI value of 7.6 than the pI 7.1 of P14. The complete amino acid sequence of P12 was determined by peptide analyses after cleavage with trypsin, pepsin and lysyl endopeptidase. The protein consisted of one polypeptide with 133 residues. The N-terminal amino acid of the protein was blocked and identified to be pyroglutamic acid. The protein was rich in glutamine (22%), asparagine (9%), leucine (16%) and tyrosine (11%). It had high hydrophobicity, hydrophobic amino acids amounting to 50% of its content. The sulfur-containing prolamins, P3, P5, P8, P9, were eluted during 35%–43% of acetonitrile, and their molecular weights were from 14kD to 24kD. They were rich in basic amino acids, especially arginine, and also had high levels of glutamic acid and aspartic acid. The purified γ_3 globulin, eluted at 46% of acetonitrile, was also analyzed and determined to have a molecular weight of 36kD. It was sequenced partially, and showed to have high levels of glutamic acid and arginine.

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The use of ^{3}H-amino acids for the measurement of fecal endogenous nitrogen excreted by rats fed bean diets

This research was done in order to evaluate the fecal endogenous nitrogen excretion in 60 Wistar rats, weighing 70 ± 9 g, by labelling their proteins with ^{3}H-amino acids. To attain this objective, a single dose of 45 μCi of L-[5-^{3}H]-arginine-monohydrochloride, [2-^{3}H]-glycine or DL-[4,5-^{3}H]-leucine, were injected **i.p.** in the rats and the radioactivity of the feces measured, after the rats been fed balanced diets (5 per treatment) containing cooked beans (*Phaseolus vulgaris*) or casein providing 10% protein or a protein-free diet, during a 4-d period. The correlation coefficients of the positive and moderate linear regressions, obtained by the least squares method, between the radioactivity of feces (μCi) and total fecal nitrogen (mg) was: $r = 0.8694$, $p < 0.0001$; $r = 0.7708$, $p < 0.001$; $r = 0.7212$, $p < 0.003$, respectively for ^{3}H-arginine, ^{3}H-leucine and ^{3}H-glycine. This fact, associated with the larger **B** angular parameter of the adjusted model ($y = Bo + Bx$) founded for the ^{3}H-Arg (0.006174) than for the ^{3}H-Leu (0.002580) and the ^{3}H-Gly (0.000824), demonstrated that the levels of radioactivity combined with nitrogen

in the feces of the rats was higher in the case of Arg, when compared to Leu or Gly, thus indicating Arg as a more appropiate protein labeller. Endogenous nitrogen excreted in the feces of the rats fed beans or casein diets, was estimated by the ratio of total endogenous nitrogen to marker nitrogen, based on the protein-free diet. The results obtained for the fecal endogenous nitrogen excretion of the rats, after the 4-d period, in the case of Arg was: casein = 36 ± 19 mg; beans cv. Carioca 80 = 66 ± 18 mg; beans cv. Aeté 3 = 59 ± 22 mg; protein-free = 25 ± 3 mg. These results showed that cooked bean diets increased fecal endogenous nitrogen of Wistar rats when compared with casein or protein-free diets.

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Digestible essential amino acid requirements of growing-finishing pigs expressed as apparent ileal digestibility

The digestible essential amino acid contents of maize and soya-bean meal diets (75.4% of maize, 19.1% of soya-bean meal and 5.5% of mineral-vitamin premix for growing pigs; and 79.5% of maize, 15% of soya-bean meal and 5.5% of mineral-vitamin premix for finishing pigs), which were introduced by NRC (1988), were determined with the Yorkshire X Landrace pigs fitted with the ileo-caecal re-entrant cannulae. The digestible threonine, valine, methionine, isoleucine, leucine, histidine, lysine and arginine contents were 4.37, 5.91, 1.86, 4.71, 10.69, 4.27, 6.52 and 6.6 g/kg for the growing pig's diet; and 3.01, 3.72, 1.73, 3.18, 6.78, 3.90, 5.01 and 4.10 g/kg for finishing pig's diet. The values of valine, isoleucine, leucine, histidine and arginine were over the requirements of their total amino acid contents introduced by NRC (1988), respectively. At the same time, the digestible essential amino acid contents in 30 batches of 40 different feedstuffs and 10 typical complex diets were determined over a period of 5 years. Similar to the maize and soya-bean meal diets, the digestible values of valine, isoleucine, leucine, histidine and arginine were over the requirements for the growing-finishing pigs in all of the tested complex diets. In practice, only the digestible lysine, methionine and threonine need considering for formulating diets of the growing-finishing pigs with a body weight from 20 to 100 kg.

Two feeding experiments with 400 pigs, initially about 20 kg and ending with 100 kg were performed, in order to correct the requirements of digestible essential amino acids. The control diet contained the same digestible lysine, mentionine and threonine as the above maize and soya-bean diets. The negative control diets contained 5.8 g/kg of digestible lysine, 1.50 g/kg of digestible methionine and 3.5 g/kg of threonine for the 20–50 kg pigs; and 4.0 g/kg of digestible lysine, 1.39 g/kg of digestible methionine and 2.6 g/kg of digestible threonine for the 50–100 kg pigs. The positive control diets contained 7.0 g/kg of digestible lysine, 2.1 g/kg of digestible methionine and 5.0 g/kg of digestible threonine for the 20–50 kg pigs; and 6.0 g/kg of digestible lysine, 1.90 g/kg of digestible methionine and 3.5 g/kg of digestible threonine for the 50–100 kg pigs. There were no significant ($p > 0.05$) differences of feeding performances between the control and positive control groups, except a significant economic benefit for control group. However, the daily gain and feed to gain ratio of the negative control group were 580 g/d and 3.17:1 for the total fattening phase comparied with 658 g/d and 3.04:1 of the control group.

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Plasma lysine content of pigs fed different fertilized corns

The present study was conducted to examine the effect of relative high N fertilizer on blood Plasma lysine content of piglets.

Pioneer 3732 corn hybrid was used in the fertilization experiment. Nitrogen fertilizer was used in treatment 2 with a level of 269 kg/ha while treatment 1 had no nitrogen fertilizer. Moisture and the content of nutrients were determined according to the standards of A.O.A.C. (1975). Amino acid composition was determined after an acidic hydrolysis, using the amino acid analyser type BIOTRONIC 5001. Thirty crossbred pigs, averaging 5.56 kg at 28 days of age, were used in an experiment utilizing a randomized block design. The experimental diets were formulated to be isonitrogenous by adding corn starch to treatment 2. Therefore, proportions of nitrogen supplied by corn, soybean meal, dried whey and corn gluten meal were identical in diet 1 and 2. By calculated analysis, the diets should have only marginally supplied the pig's requirement for lysine. On the day 21, blood samples were taken via orbital sinus puncture. The heparinized blood samples were centrifuged, and the plasma was frozen at −20 C for later analysis. Blood plasma urea was determined by the method of Marsh et al. (1965). Plasma lysine was analysed by HPLC (Shimadzu liquid chromatograph LC-4A system) with the procedure of Granberg (1984).

The crude protein content was 14% higher ($p < .001$) in corn fertilized with N (treatment 2). The lysine content decreased by 18% ($p < .01$) in corn fertilized with N (treatment 2) as compared to treatment 1. Analysis of blood urea showed an 1% increase in treatment 2. Feeding the N treated corn reduced plasma lysine by 10%. This response indicated that protein quality was effected by the N fertilizer, but the interaction was not significant.

The fertilizer can raise the crude protein content of corn by 14% and the lysine content can decrease by 18%. Such a big changes did not cause measurable changes in the animal. The blood lysine and urea did not changed significantly. These results show that it is possible to raise the protein content of corn significantly by the use of fertilizer with no adverse effects on its nutritional value for pigs. After fertilization the changes in amino acid levels in corn do not appear to have any effect on the need for amino acid supplemntation of the diets.

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Free radicals and protein metabolism: the influence of mercaptoethanol

Thiol compounds are well known for their free radical scavenging property improving tissue antioxidant status. Macromolecules as proteins or DNA are targets and modified by the action of free radicals and protein turnover may be important in the defensive systems at the cellular level degrading modified proteins and synthesizing new molecules.

The aim of the present study was therefore to investigate the possible relationship between long-term treatment of 2-mercaptoethanol (2ME, 13 μg/100 g body weight/d) and total body protein turnover in vivo using the [^{15}N]glycine-method in

rats of different ages. The parameters of nitrogen metabolism were calculated on the basis of cumulative ^{15}N-excretion curve using the standardized graphic method based on the 3-pool-model (without tracer recycling) according to Sprinson and Rittenberg resulting in the rate constants for protein synthesis as well as nitrogen excretion rate constants. Additionally, the measurement of 3-methylhistidine in urine was done.

There seems to be no overall influence of 2ME on total body protein metabolism and the results are not without contradiction. Rate constants for whole body protein synthesis were found in our experiment to be significantly lower at 15 month of age (start of 2ME supplementation at 7 month of age) in relation to untreated controls. The results were not significantly different at 11, 24 or 28 month of age.

On the other hand, the age dependency of the values of rate constants for protein synthesis were clearly demonstrated showing a decrease with age. But at 2 years of age the values were found to be relatively high. The same trend of age dependency was found for the values of rate constants for nitrogen excretion and 3-methylhistidine excretion in urine, a parameter characterizing mainly myofibrillar protein degradation.

The results were discussed taking into consideration the hypothesis of a continuous antioxidant protection by e.g. 2ME leading to reduced rates for repair of cell and tissue components by e.g. protein turnover.

Age-dependent alterations of the parameters of protein metabolism (increase at 24 month of age) were mainly attributed to hypercompensation mechanisms in which several different adaptive changes are accentuated.

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Amino acids of plant components of nutrition

The requirements of animal for amino acids are primarily determined by the amount of synthetized proteins "de novo" and their amino acid composition. Amino acids must be present at proteosynthesis in cell in sufficient amount and right ratio given by genetic information.

It is well-known that cereal proteins have got the inconvinient amino acid composition in consequence of high abundance of plolamine fraction. Prolamines have got low lysine content, less than 1 percent. The other insufficient amino acids in cereals are: threonine, methionine and tryptophan.

Amino acid content is interesting in proteins of buckwheat which is spread from point of view of rational nutrition. Buckwheat has got a yond percentual abundance of essential amino acids above all lysine (6.22–6.83%). It is in consequence of higher abundance of albumin and globulin with regard to cereals.

Legumes (pea, soya-bean, bean) have got higher content of proteins as well as the essential amino acids.

- The analyzed pea varieties have got protein content 23.7–27.8% and lysine abundance 7.3–7.6%.
- Protein content is 30.8–36.2% and lysine abundance 6.4–7.2% in the studied soybean varieties.
- Bean varieties have got protein content 25.0–27.5% and lysine abundance 6.1–7.2%.

The essential amino acid index calculated according to Oser (1951) in cereals is in the interval 60.52 (barley), 71.77 (wheat). EAAI in legumes is higher, and it 74.82 (pea), 74.90 (soya-bean) and 80.62 (bean). The highest EAAI is in legumes which have got the highest protein content. The low content of methionine and cystein but partly also valine and isoleucine is a disadvantage of legumes.

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The feeding value of cultivated amaranthus and chenopodium species and hybrids in foraging

In order to reduce the cost of foraging we should take advantage of the fact that the swine is an omnivorous animal. By feeding swine with Amaranthus and Chenopodium species, its diet can be completed with valuable protein (Szabó, 1992).

In order to estimate the yield and evaluate the composition of 5 Amaranthus species, one hybrid as well as one Chenopodium species, a model experiment liquid manure was conducted in Debrecen. We collected data by examining both whole plants and the leaves, the samples were taken at the same time. Raw protein content was lowest in Amaranthus caudatus (29.30%) and highest in the hybrid Amaranthus hypochondriacus japanicus (38.08%). These values significantly exceeded the results obtained from species collected in dry areas (Koch et al, 1967) and from those of Wetter-Szőcs (1991).

Considering the leaves only we found that A. retroflexus and A. Hypochondriacus contained similar amounts of raw protein as A. caudatus (32.50–33.22%), the highest amount was once again found in the hybrid (39.06%). 32.08% raw protein was measured in the plant Chenopodium album whereas its leaves contained 34.51%.

Among the amino acids examined, lysine is the most important for the growth of swine. Considering the entire plant we can see that there is not a great difference across species in their lysine content (6.10%).

All plants have a very low amount of methionine (0.25%) which is the other essential amino acid for swine. The leaves on the other hand contained four times the above amount (an average of 1.07%), with moderate variation across species. The amounts of cystine, which is an amino acid containing sulphur, was also measured: an average 0.38% was found in the whole plants and 0.45% in the leaves. The difference between plant and leaf content was the same for all species.

Another essential compound of swine fodder is threonine. There was no significant difference in the average amounts found in the whole plants (3.80%) and the leaves (4.00%), these values were also correlated with protein.

The average lysine content of the Chenopodium album plant (6.58%) and its leaves (7.58%) is 18% higher than those of Amaranthus plant/leaves. Chenopodium album is about there times richer in amino acids containing sulphur, but its threonine content, especially if we consider the whole plant is 20–30% lower.

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Effect of methionine deprived total parenteral nutrition on methionine and leucine metabolism in tumor bearing rats

This study was carried out to elucidate effect of methionine deprived TPN (MTPN) on leucine and methionine metabolism in tumor bearing rats. On day 1, AH109A ascites hepatoma cells ($1*10^6$) were inoculated into male Donryu rats ($n = 24$, BW: 180–220 g) subcutaneously. The tumor bearing (TB) rats were fed ad lib for 10 days. On day 10, the catheter for TPN was inserted

into the jugular vein. Non tumor bearing (NTB, n = 12) rats entered this study at this point and were also catheterized. All animals were randomized into 2 groups; standard TPN (STPN) and MTPN. MTPN was isocaloric and isonitrogenous with STPN (233 Kcal/kg/day, 1.2 gN/kg/day). On day 15, either 1-^{14}C-leucine (2.0 μCi/hr) or ^{3}H-methionine (2.0 μCi/hr) dissolved in each diet was given by a 5 hrs constant infusion. The rats were sacrificed at the end of isotope infusion. Fractional synthesis rate (FSR, %/day) of tumor, muscle, and liver was determined by Garlick's method. Endogenous leucine production (≒ whole body protein breakdown, WPBR, μmole LEU/kg/hr) and methionine production were calculated by Steele equation. Tumor weight (TW, g) at sacrifice and body weight before and after 5 days TPN (BW change, g) were measured. Data are mean (SEM), stat. by ANOVA and paired t-test, (Different superscripts indicate significant difference, and *: $P < 0.05$):

	NTB-STPN	NTB-MTPN	TB-STPN	TB-MTPN
BW change	−0.3 (4.4)^{a}	−11.0 (3.5)^{b}	−9.7 (1.6)^{b}	−17.0 (1.9)^{c}
Mus. FSR	17.6 (2.5)^{a}	9.8 (3.9)^{ab}	6.2 (1.2)^{b}	6.9 (1.8)^{b}
Liv. FSR	76.7 (12.2)	81.7 (13.6)	62.1 (11.5)	72.1 (18.5)
WPBR	74.8 (8.2)^{a}	124.6 (12.5)^{b}	116.2 (12.2)^{b}	193.4 (15.2)^{c}
TW	—	—	4.4 (0.9)	2.8 (0.7)*
Tum. FSR	—	—	55.9 (8.8)	59.0 (10.5)
Met. PRO	—	—	87.3 (13.5)	218.6 (29.5)*

We concluded that 1) leucine incorporation into protein of tumor, muscle, and liver was maintained with MTPN in TB rats, probably because methionine production was augmented, 2) body weight loss was aggravated with MTPN in TB rats, because WPBR was increased.

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Glycine and redox state dependent metabolism in isolated rat liver cells

Glycine catabolism was stimulated in vivo by feeding high glycine diets (Petzke et al., 1986). Therefore, it was of interest to study the metabolic consequences of an induced glycine catabolism to understand the biochemical mechanisms of a reduced efficiency of energy utilization caused by glycine feeding (Petzke et al., 1987).

A linear increase in the $^{14}CO_2$-formation from ^{14}C-glycine was obtained in isolated liver cells up to unphysiological high concentrations of glycine (10 mmole/l) in the medium. Simultaneously, as gluconeogenesis from lactate as synthesis of urea from NH_4Cl were inhibited by glycine additions in vitro. Glycine also inhibited the formation of $^{14}CO_2$ from [6-^{14}C]glucose just as ethanol. This effects may be explained on the base of redox state changes as a consequence of NADH production by glycine breakdown in isolated hepatocytes.

In experiments with inhibitors of the respiratory chain it was found that the malate-aspartate shuttle could be responsible for the transport of reducing equivalents into mitochondria. Rotenone and antimycin A gave nearly the same inhibitory effect on glycine degradation.

Nonetheless, glycine breakdown was verified by experimental changes of the redox state by NADH-delivering substrates like ethanol or sorbitol and by the artificial electron acceptor phenazinemethosulphate. The inhibition of the $^{14}CO_2$-formation from [1-^{14}C]glycine by ethanol or sorbitol was reversed by phenazinemethosulphate. Uncoupling of the respiratory chain also enhanced rates of glycine breakdown.

The results confirmed the significance of the metabolic pathway of glycine degradation via serine and pyruvate (Petzke and Albrecht, 1987) and the strong dependence of glycine catabolism on the redox state (Hampson et al., 1983) or the reoxydation rate of reducing equivalents, e.g. by the respiratory chain.

It was proposed that futile cycle like mechanisms as outlined by Berry (1980) with the participation of the malate- aspartate shuttle at least in part may be responsible for the dietary effects of reducing the efficiency of energy utilization or increasing thermogenesis by high glycine diets supporting the possible relationship between amino acid catabolism and calorigenic effects.

Molecular Biology and Microbiology

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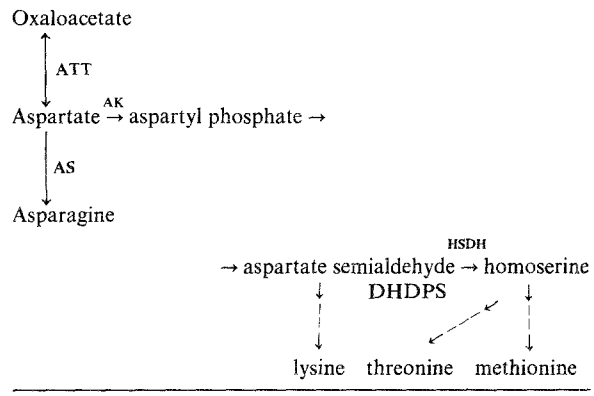
Cloning and expression of genes encoding enzymes involved in the synthesis of the aspartate family in soybean

The aspartate family of amino acids includes asparagine and the essential amino acids, lysine, threonine, methionine and isoleucine. Manipulation of genes and enzymes regulating the synthesis of these amino acids may lead to improving the nutritional value of agronomically important plants. We have cloned several important genes and studied the gene products involved in the synthesis of these amino acids.



Five isoforms of aspartate amino transferase (AAT) have been identified in extracts of soybean tissues using gel electrophoresis. Three genes encoding AAT have been cloned; one encoding the chloroplastic form; one encoding the mitochondrial form and one encoding a cytoplasmic form. The genes are differentially expressed in soybean tissues during development. All three clones have been functionally expressed in *E. coli*.

Two soybean clones encoding asparagine synthetase (AS) have been identified. The nucleotide sequences indicate that both genes are highly homologous at the 5′ end. One clone, encoding AS2, has been functionally expressed in an *E. coli* auxotroph lacking AS. The amino acid sequence at the N-terminal region of the AS2 protein reveals the proposed glutamine binding site that is highly conserved in several AS proteins, suggesting AS2 to be the glutamine-dependent form of AS.

Two clones encoding the bifunctional enzyme aspartokinase-homoserine dehydrogenase (AK-HSDH) have been distinguished. The DNA sequences indicate that these genes are very closely related and both encode proteins possessing chloroplast transit polypeptides. Southern blot hybridizations suggest the presence of a third aspartokinase gene in the soybean genome.

A single gene encoding the branch-point enzyme dihydrodipicolinic acid synthase (DHDPS) has been cloned, sequenced and functionally expressed in *E. coli*. The protein product of this gene, when expressed in soybean or expressed in *E. coli*, is sensitive to inhibition by the pathway end product, lysine.

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Mechanisms for the expression of a mutant with enhanced lysine in rice protein

Eventhough there are thousands of germplasm accessions in rice there is little known germplasm for exploitable variation in grain lysine, i.e. for nutritional quality based on protein lysine. Experiments were designed to test whether biochemical variation could be recovered In Vitro using feedback inhibitors or amino acid analogs of lysine. Exogenously applied lysine + threonine inhibit growth of rice callus. Similarly the lysine analog S(2-aminoethyl)cysteine inhibits cells in liquid cell suspensions. Hence, these inhibitors were used to recover mutants from anther-derived cells. Recovered mutants were characterized for endosperm protein lysine and crossed progeny analyzed to define the inheritance of enhanced lysine and to establish the stability of the phenotype. Enhanced lysine, up to 15% over the wild type, is inherited as a recessive character and is associated with soft opague seed similar to the opaque2 phenotype of maize. Lines have been prepared for germplasm registration.

Recent work has focused on the biochemistry and molecular genetics of the mutants using whole plants as well as liquid cell suspension cultures. One mutant cell line is constitutive for the processing of proteins for export from the cells. Data will demonstrate that this mutant exports a higher portion of its cellular proteins than the wild type grown in liquid suspension cultures. The ratio for proteins of mutant/wildtype in the extracellular media is 2.2 and for intracellular proteins the ratio is 0.93. The work shows that a greater % of protein is exported in the mutant. Some of the exported proteins are enzymatically active. This report will demonstrate that the total β1–3 glucanase activity is higher in the mutant than the control. However, not all proteins are exported or preserved equally. For example, total chitinase activity is not increased in the mutant over the control and the predominant chitinase activity is extracellular in both the mutant and wild type cells cultured In Vitro. Western hybridizations with antibodies for chitinases separated 2-dimensionally provide similar profiles for the mutant and the controls. But, there are substantially higher levels of glycoproteins in the controls as measured by Concanavalin A sensitivity and therefore glycosylation may be inhibited in the mutant. The exported proteins in the mutant are more water soluble than proteins from the controls which are solubilized from subcellular components only after denaturation with SDS. The altered glycosylation of proteins in the mutant lines may contribute to altered protein transport and solubility characteristics of the proteins. Recovered mutants are valuable for the study of protein processing in higher plants and hypotheses will be presented for future testing.

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Expression of a sodium-dependent leucine transporter from rat jejunum in *Xenopus oocytes*

We have investigated the L-leucine transport characteristics of *Xenopus* oocytes microinjected with mRNA isolated from rat jejunum. The initial rate of leucine uptake (0.2 mM, 20°C) by mRNA-injected oocytes was 3-fold higher than control oocytes injected with water. Net expressed transport activity was 80% sodium-dependent (typically 10 pmol/oocyte in 10 min). The sodium-dependent component of the expressed flux was saturable (apparent K_m 0.20 mM) and inhibited by lysine, but not by alanine or phenylalanine. The concentration of lysine required to inhibit leucine transport by 50% was 0.2 mM. Antisense hybrid-depletion experiments showed that the 4F2hc and D2 were not responsible for the expressed leucine transport activity. Maximum expression of leucine influx was found with an mRNA size-fraction of 1.5–2.25 kb (peak 2.0 kb) and substantial transport activity was also observed with mRNA in the size range 2.0–2.8 kb (peak 2.6 kb). In preparation for expression cloning of the protein(s) responsible for jejunal Na^+-dependent leucine transport, cDNA libraries have been constructed from these mRNAs using expression vectors pHAS KS^- (2.0 kb mRNA fraction) and pGEM-3Z (2.6 kb fraction). Initial screening of the pGEM-3Z library (7000 primary recombinants) identified a pool of 300 independent cDNA clones which encodes leucine transport activity and which is negative for 4F2hc and D2 as judged by diagnostic PCR. The expressed transport activity was 92% Na^+-dependent and the flux was inhibited by both leucine and lysine but not by alanine or phenylalanine. Efforts are presently underway to isolate a single positive clone from the pool. JDY is a Heritage Medical Scientist; JFE is a Heritage Medical Scholar and an MRC Scholar. SYMY is supported by a Croucher Foundation, (Hong Kong), Post-graduate Scholarship. We thank the Mary Louise Imrie Graduate Award, Faculty of Graduate and Research, University of Alberta for providing travel assistance to SYMY.

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Characterization of the promoter and enhancer regions of the ornithine transcarbamylase gene

The ornithine-urea cycle is the major pathway for detoxication of ammonia formed in amino acid metabolism and is respon-

sible for nitrogen balance of the body. The cycle is also involved in biosynthesis of arginine and nitric oxide. OTC catalyzes the second among the five enzymatic steps of the cycle. The enzyme is present in the liver and to a lesser extent in the small intestine. In the fetal liver, the level of the enzyme increases late in gestation, coordinately with other urea cycle enzymes. OTC deficiency is known in humans and mice, as an inborn error of metabolism that results in protein intolerance and hyperammonemia. The rat and human OTC genes span a region of 70–75 kb on the X chromosome. Transient expression analysis showed that the promoter of this gene is much more active in a hepatoma cell line (HepG2) than in a nonhepatic cell line (CHO). There are at least one negative and two positive regulatory elements within the 220 bp immediate 5′-flanking region. Protein factors binding to these elements were found in nuclear extracts of rat liver. In transgenic mice, the promoter of the rat OTC gene directed liver- and small intestine-specific expression, but the expression of the transgene in the liver was very low. We found a hepatoma-specific enhancer region of 230 bp located 11 kb upstream of the transcription start site. There are four protein binding sites in this region.

We found that two sites of the promoter region and two sites of the enhancer region are recognized by both HNF-4 (hepatocyte nuclear factor-4) and COUP-TF (chicken ovalbumin upstream promoter-transcription factor). Thus, these two factors were shown to have closely related binding specificities. HNF-4 and COUP-TF are orphan members of the steroid/thyroid receptor superfamily and exhibit liver-enriched and ubiquitous tissue distribution, respectively. In cotransfection analysis using HepG2 cells, HNF-4 activated expression from the OTC promoter several-fold. Therefore, this factor appears to participate in liver-selective activation of the OTC gene. On the other hand, COUP-TF was found to repress the expression from the OTC promoter. Therefore, COUP-TF may act as a repressor of the OTC gene in tissues where the gene is not expressed. Repression of a tissue-specific promoter by a ubiquitous factor and activation/de-repression by a related tissue-enriched transactivator is potentially an important mechanism for tissue-specific activation of a gene.

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A study of the vitamin B_{12}-dependent enzyme catalysing the final step of methionine biosynthesis in *E. coli*

Methionine, a sulphur-containing amino acid, is essential to humans, but is synthesised *de novo* by most bacteria. The final two steps in the methionine biosynthetic pathway involve the methylation of homocysteine. Two branches converge at this point, one deriving from aspartate and resulting in homocysteine, and the other allowing the formation and transfer of the methyl group in the form of methyltetrahydrofolate. The utilisation of methyltetrahydrofolate in homocysteine transmethylation occurs by two reactions in nature, one catalysed by a vitamin B_{12}-dependent homocysteine methyltransferase, the other by a vitamin B_{12}-independent homocysteine methyltransferase. Despite the fact that these two enzymes catalyse essentially the same reaction, they do so by different mechanisms and with different substrate requirements and efficiencies.

Our studies in Nottingham are concerned with the vitamin B_{12}-dependent homocysteine methyltransferase. This protein is of particular interest because, apart from its catalytic role, it has been implicated in the regulation of the methionine biosynthetic pathway. The overall aim of the work is to identify the site(s) involved in vitamin B_{12} binding and those regions responsible for the catalytic and regulatory functions of the holoenzyme, and in so doing construct a structure-function map of the vitamin B_{12}-dependent enzyme.

The *metE* and *metH* genes of *E. coli* have been cloned and their products identified and purified. Antibodies have been raised against the two proteins, *metE* and *metH* have been sequenced and the direction of transcription determined. Two main approaches are being used to study the vitamin B_{12}-dependent homocysteine methyltransferase. The first involves the use of a series of lacZ fusions containing overlapping segments of the *E. coli metH* gene and the examination of the fusion proteins produced for their ability to bind vitamin B_{12}. This methodology has enabled the preliminary location of the vitamin B_{12} binding site of MetH. Our second approach is to employ random mutagenesis of the *metH* gene, isolating MetH mutants deficient in various properties on the basis of methionine auxotrophy in a *metE*$^-$ background, the location of the mutations being determined by marker rescue followed by subcloning and sequencing.

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Preparation of amino acids by fermentation

Biotechnological methods of preparation have exceeded areas of amino acid application. Amino acids are used in medicine, in the food and the fodder industry and in the chemical industry, too. Amino acids can be prepared by a precursory method or by an enzymic one, but the predominant way of preparation is a fermentation.

Tryptophan has been obtained using indol as a precursor and a mutant Corynebacterium glutamicum resistant to indol. Aspartic acid has been produced in a reaction in which fumaric acid was a substrate and aspartase from a strain of Alcaligenes metalcaligenes was applied. Lysine has been accumulated in a relatively high amount by mutants of Corynebacterium glutamicum and Brevibacterium flavum. A continual increasing of lysine production was achieved by an increasing number of suitable genetic markers which the mutants had received. The lysine production increased from 20 g/L (an auxotrophic mutant) to 90 g/L (an auxotrophic-regulatory mutant) by application of mutation and selection.

Producing strains of amino acids can been improved also by methods of gene manipulation. These methods represent a perspective way applied in a future for an isolation of producing organisms used in the amino acid fermentations.

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Relationship between microbial production of gaseous unsaturated-hydrocarbons and metabolism of amino acids

We have investigated the microbial production of gaseous (C_2–C_4) unsaturated-hydrocarbons such as ethylene, propylene, 1-butene and iso-butene. We present here the relationship between microbial production of gaseous unsaturated-hydrocarbons and metabolism of amino acids.

(1) Production of ethylene by micro-organisms. There are two

pathways for the production of ethylene in micro-organisms, namely, 2-keto-4-methylthiobutyric acid (KMBA) and 2-oxoglutarate (2-OG) pathways. These substrates are transaminated derivatives of L-methionine (Met) and L-glutamate (Glu), respectively. Examples of microbes which exploit these two pathways are *Cryptococcus albidus* and *Escherichia coli* for the first pathway, and *Penicillium digitatum* and *Pseudomonas syringae* for the 2-OG pathway. Using the purified ethylene-forming enzymes (EFEs) of these pathways, we surveyed two ethylene-forming reactions. The KMBA pathway presents in most ethylenogenic micro-organisms and is catalyzed by oxygen radical-forming reactions. On the other hand, the purified EFEs of *Ps. syringae* and *P. digitatum* (2-OG pathway) simultaneously catalyzed two reactions, namely, formation of ethylene and of succinate from 2-OG with a molar ratio of 2:1. In the main reaction, 2-OG is di-oxygenated to produce one molecule of ethylene and three molecules of carbon dioxide. In the sub-reaction, both 2-OG and L-arginine (Arg) are mono-oxygenated to yield succinate together with carbon dioxide and L-hydroxyarginine, respectively, the latter being further transformed to guanidine and L-Δ^1-pyrroline-5-carboxylate. A dual-circuit mechanism has been proposed for the entire reaction, in which binding of Arg and 2-OG in a Schiff-base structure generates a common intermediate for the two reactions.

(II) Production of iso-butene by micro-organisms. The formation of isobutene by microsomes of *Rhodotorula minuta* proceeds in the presence of isovalerate (IVA), which is derived from L-leucine (Leu), NADPH and molecular oxygen. From the characteristics of the isobutene-forming enzyme, we proposed that the enzyme itself is a cytochrome P450 in microsomes. We purified a cytochrome P450 to an electrophoretically homogeneous state from microsomes of *R. minuta*, which was tentatively named P450rm. It has also been that P450rm is induced by L-phenylalanine (Phe) and several analogues of Phe. We attempted to establish a reconstituted system with high activity. Using this reconstituted system, we investigated the reaction with IVA of the isobutene-forming enzyme, P450rm, as well as the possibility that Phe and/or its metabolites are involved in reactions catalyzed by P450rm.

(III) Production of propylene and 1-butene micro-organisms. *Penicillium cyclopium* produces propylene, 1-butene and isobutene catalyzed by one enzyme which is in microsomes. These unsaturated-hydrocarbons are derived from L-valine (Val), L-isoleucine (Ile) and L-leucine (Leu), respectively.

Based on above results, it was found that many amino acids relate to the microbial production of unsaturated-hydrocarbons. Therefore, we would like to discuss on the relationship between microbial production of gaseous unsaturated-hydrocarbons and metabolism of amino acids.

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The last blind spot in histidine biosynthesis: what is the function of HisF and HisH?

The mutant UBM221 was obtained by chemical mutagenesis (NG) of *Klebsiella pneumoniae* M5a1, and can only grow with high but not low ammonia concentrations as sole nitrogen source (Laa^-) (Laa = *l*ow level *a*mmonia *a*ssimilation). The complementing gene was isolated from a gene bank of *K. pneumoniae* M5a1, subcloned and shown to be *hisF*, which codes for an enzyme called "cyclase" (MW 28 000) of the histidine biosynthesis pathway. Generally *his* mutants are histidine auxotrophs, in contrast to UBM221. P1 cotransduction of the mutation of *Kp*UBM211 together with other *his* genes, showed that the cloned *hisF* gene in fact did not suppress but complement the defect since 80% of the His^+ recombinants showed the Laa^- phenotype afterward. Therefore *Kp*UBM211 is a prototrophic *hisF* mutant.

Based on our results and previous findings it is possible to propose a hypothesis that clarifies the hitherto controversial functions of the enzymes HisF and HisH in the histidine biosynthesis. According to this hypothesis, both enzymes together belong to the *trpG*-type of the glutamine amidotransferase, wherein HisH carries the glutamine amido transfer domain and HisF the aminator domain: HisH binds glutamine and provides the activated amido group for the ammonolytical cleavage of the substrate PRF-AIC-RP (N′-5′-phosphoribulosyl-formimino-5-aminoimidazol-4-carboxamide ribonucleotide) which has been directly transferred from HisA to HisF. One of the cleaved molecules is an intermediate of the purine biosynthesis and the other one yields imidazoleglycerolphosphate after ringclosure catalysed by HisF.

It is assumed that in *Kp*UBM211 a conformational change of the HisF caused by mutation failed to effect the binding of the glutamine-HisH complex at its correct positon thus blocking the glutamine-dependent reaction of HisH. However, this defect can be compensated by NH_3-dependent activity of the glutamine amidotransferase and that is the reason why this mutant is not a histidine auxotroph, but shows the Laa^- phenotype growing with high ammonia concentrations as sole nitrogen source.

The following results support above hypothesis:
a) HisF and HisA show homology because both are binding PRF-AIC-RP. – b) HisH posseses a glutamine binding site, which is homologous to the ones of *trpG*-type of the glutamine amidotransferase, and functions as a donor of the activated amido group. – c) The "Schiffsche" base is one of the most reactive bonds of PRF-AIC-RP. It is the place of the ammonolytical cleavage. – d) A high NH_4^+ concentration is able to replace glutamine *in vitro* and *in vivo*. Then HisH is not necessary. – e) A specific *hisH* mutant was constructed using a Km cassette and a temperature sensitive vector system. This mutant shows the Laa^- phenotype, if the downstream located *his* genes are provided in *trans*.

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Aspartyl phosphates in the regulatory control of bacterial response

Bacteria respond to environmental stress by activating the transcription of genes which code for products that allow the bacteria to adapt to the new environment. The bacterium, *Bacillus subtilis*, responds to nutrient deprivation by forming spores and commitment to grow or begin sporulation depends on the action of a four-component phosphorelay that involves as the first two steps a kinase (KinA) and a response regulator (SpoOF). A unique protein phosphotransferase (SpoOB) then catalyses the transfer of phosphate from the SpoOF protein to the transcriptional activator SpoOA. The close N-terminal sequence homology between SpoOF, SpoOA and CheY (a chemotaxis regulatory factor whose structure has been recently reported Stock *et al.*, Nature, 344: 395–400 (1990)) suggests that the sporulation factors contain aspartyl pockets that are the recipients of phosphate transfer via the generation of transient aspartyl phosphate mixed anhydride species. The smaller of the two aspartate con-

taining proteins from *B. subtilis*, SpoOF (124 residues), is clearly a key component in the phosphorelay system whose size allows structure determination to be accomplished by multidimensional heteronuclear NMR spectroscopy. Therefore, experiments are in progress to obtain the complete three dimensional structure of SpoOF using this technique. Severe resonance overlap in conventional homonuclear 2D NMR spectra of the protein has led to the incorporation of ^{13}C and ^{15}N isotopic labels to give better chemical shift dispersion and so reduce resonance overlap. Initial resonance assignments derived from 3D ^{15}N-TOCSY-HSQC, ^{15}N-NOESY-HSQC, ^{13C}-NOSEY-HSQC and ^{13}C-HCCH-TOCSY experiments suggest differences exist between SpoOF and CheY. The effect of these structural changes will be used to attempt to explain the altered stabilities of their respective phosphorylated products SpoOF-P and CheY-P.

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The isoleucyl-tRNA synthetase gene alleles and expression of isoleucine/valine biosynthetic genes

Two independent mutations leading to the elevated expression of the ilvGMEDA operon in Escherichia coli were isolated. One of them provided strong isoleucine auxotrophy due to the low affinity of isoleucyl-tRNA synthetase to isoleucine. This mutation was shown to be located on 0.5 min of E. coli genetic map within the ileS gene – a structural gene for isoleucyl-tRNA synthetase. The elevated level of ilvGMEDA genes expression was found in this ileS mutant. Since ilvGMEDA operon is known to be regulated via attenuation, ileS mutation seemed to provide deattenuation of the operon.

However there was different effect of ileS mutation on expression of proximal and distal genes of ilvGMEDA operon: the ilvG gene expression was increased much less than that of the ilvA gene. Moreover ilvA gene expression on the plasmid carrying ilvGMEDA operon with deleted promotor-attenuator region was found to be also effected by ileS mutation. Thus it seemed possible that beside attenuator there is one more target within the ilvGMEDA operon effected by ileS mutation.

Another mutation designated ilvX was shown to be located on 0.5 min of E. coli genetic map closely linked to ileS mutation. In contrast to ileS mutation it didn't lead to any nutritional requrement. Expression of both ilvG and ilvA genes in ilvX mutant was 10-fold higher than in isogenic wild type strain, but there was no any effect of ilvX mutation on expression ilvGMEDA operon with deleted attenuator region.

Thus two mutations ileS and ilvX located within ileS gene locus were found to increase the expression of ilvGMEDA operon in different ways. The molecular nature of this mutations and the mechanisms of their influence on ilv operon are under studying.

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Efficient production of antimicrobial peptide by secretory expression of the gene fused with a protease inhibitor gene

SSI (*Streptomycs* subtilisin inhibitor), produced by *Streptomyces albogriseolus* S-3253 into the culture medium in a large amount, potently inhibits serine proteases such as subtilisin. We have investigated this unique secretory expression mechanism genetically, and constructed a *Streptomyces* host-vector system for efficient production of heterologous proteins or peptides of interest using the regulatory regions of the SSI gene, including its promoter, signal sequence, etc. Here we report a trial of fermentative production of an interesting antimicrobial peptide of insect origin by using the host-vector system.

Apidaecin 1b is one of unique peptides found by Casteels et al. in immune honeybee lymph, which are composed of 18 amino acid residues containing six proline and three arginine residues, and reported to inhibit specifically Gram-negative bacteria such as *E. coli* in a bacteriostatic manner. The antibacterial action, however, has not been well investigated yet. We selected this peptide as a good model for applying our system to peptide production, because *Streptomyces* is Gram-positive, and so it must be resistant to apidaecin. Moreover, if we can fermentatively produce apidaecin as a fusion protein with SSI by this system, the short peptide will be well protected from inactivation caused by various factors, since SSI is a very stable protein against heat, acidity, proteases, etc., and so acts as a protector. It is also advantageous that the product, fusion protein, can be easily monitored through the isolation procedure by the assay methods for SSI, instead of that for the peptide itself.

We first designed and chemically synthesized the gene for apidaecin 1b by using the codons suitable for *Streptomyces*, and finally constructed a secretion vector holding the whole region of SSI gene joined with the synthetic apidaecin gene *via* some spacer oligonucleotides. After the recombinant vector was introduced into a *Streptomyces* host, the resultant transformant was cultured and examined for production and accumulation of the SSI-apidaecin fusion protein in the medium. Before, during and after the purification procedure, precipitation with ammonium sulfate, followed by DEAE-cellulose column chromatography and reversed phase HPLC (RP-HPLC), the fusion protein was detected on SDS-polyacrylamide gel and subjected to other analyses. As the result, the amino acid composition was found to be identical with the theoretical value and the productivity was estimated to be greater than 200 mg/l of the culture supernatant.

The isolated fusion protein was finally cleaved with CNBr to release apidaecin from the mother SSI molecule. After purified by RP-HPLC, the recombinant apidaecin was isolated and the antibacterial activity was tested against several *Bacillus* strains (Gram-positive) as well as *E. coli* (Gram-negative). Interestingly, it was found that not only *E. coli* but also a *Bacillus* strain with weaker protease productivity were sensitive to apidaecin.

Evolutionary experiments are now in progress in our laboratory using specially devised *E. coli* host-vector system, which provides us a good screening system for a variety of apidaecin evolvants with altered antibacterial properties. This system might become a general tool for investigating the mode of action of various antimicrobial peptides, and also for their genetic improvement for practical use.

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Processing of SMR1 protein in rat submandibular glands: adrenergic- induced release of Gln-His-Asn-Pro-Arg related peptides in saliva and blood of males

A major rat-specific mRNA, which accumulates in the submandibular gland (SMG) in response to androgens, has been

characterized (Rosinski-Chupin *et al.*, 1988, Proc. Natl. Acad. Sci. USA, 85, 8553–8557). The corresponding cDNA has been cloned and the gene product named SMR1.

We had isolated from male rat SMG salivary secretions three structurally related peptides, generated from SMR1: a undecapeptide (VRGPRRQHNPR), a hexapeptide (RQHNPR) and a pentapeptide (QHNPR). They are generated from SMR1 after specific endoproteolytic processing at paired and/or multiple sites of arginine residues (Rougeot *et al.* in preparation). These three peptides are not identically distributed: the undecapeptide and hexapeptide and found in high levels in the SM gland and also released into the saliva. The pentapepride is only found in the saliva.

The salivary secretions of rats are known to be regulated by the autonomic nervous system through muscarinic receptors as well as α and β adrenoreceptors. Thus, we studied the excretory pattern of SMR1-derived peptides release, following infusion of various autonomic receptor agonists. The hexa- and undecapeptides are acutely released from the SMG into saliva of male rats, mainly under β adrenergic-stimulated conditions. This result is in accordance with SMR1 mRNA distribution in the acinar cells of SMG (Rosinski-Chupin *et al.* submitted article). Indeed, protein secretions from SMG acini have been described to be essentially under β adrenergic control. In contrast, the pentapeptide is found in the saliva mostly under α and β adrenergic-stimulated conditions. Following dual stimulation, the amounts of salivary undecapeptide are negatively correlated with those of salivary pentapeptide ($p = 0.025$, $Df = 7$, $R = 8$, by ANOVA). There is considerable evidence to suggest that the pentapeptide may be generated from the excreted undecapeptide. This step of limited proteolysis could be exerted through one of the numerous proteases secreted from ductal cells following α adrenergic stimulation (for instance, Kallikrein-related proteases synthetized and secreted from GCT cells).

Beta or $\alpha\beta$ adrenergic-induced exocrine secretion of these peptides results in the secretion of the hexapeptide from the SMG into the bloodstream. This systemic hexapeptide secretory response is not observed under the same condition in rats in which the SMG ducts and blood vessels are ligated, demonstrating that the hexapeptide originates from the SMG. In addition, under basal, α adrenergic- or chollnergic-stimulated conditions the systemic release of hexapeptide is not observed, demonstrating a β adrenergic requirement for the systemic secretion of the hexapeptide.

The physiological relevance of the tissue-specifity of these peptides, as well as their sexual difference added to their exocrine and endocrine secretion support the hypothesis of their biological involvement in male-specific behaviours.

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Multiple pathways of glutamate catabolism in anaerobic bacteria

The existence of three distinct pathways for the catabolism of glutamate to acetate and butyrate in *Fusobacterium* species was suggested by the detection, in cell-free extracts, of enzymes associated with each pathway, and by the low and high levels of radioactivity found in the carbon dioxide produced by cultures fed [1-^{14}C] and [5-^{14}C]glutamate, respectively (S. E. Gharbia and H. N. Shah, J. Gen. Microbiol. 1991, **137**, 1201). In the present study, the metabolic end products (acetate and butyrate), obtained by metabolism of ^{13}C-labelled precursors, were isolated as *p*-bromophenacyl esters by silica gel chromatography and analyzed for isotopic incorporation by NMR and mass spectrometry. Catabolism of L-[1-^{13}C]glutamate by *F. nucleatum*, a key periodontal pathogen, resulted in butyrate which was labelled with ^{13}C at carbons 1 and 3. The high level of ^{13}C in carbon-1 of butyrate is consistent only with the 2-oxoglutarate pathway, whereas the smaller ^{13}C-enrichment observed at carbon-3 can be attributed to the synthesis of butyrate from acetate. Also, acetate enriched with ^{13}C in the carboxyl group was produced in the culture, but either the mesaconate or the 2-oxoglutarate pathway can account for the formation of this labelled acetate. Preliminary experiments indicate that [$^{13}C_2$]acetate is converted to butyrate by *F. nucleatum*. Additional experiments are in progress to distinguish between the alternative sources of acetate and to verify that *Fusobacterium varium*, a gastrointestinal species, utilizes all three pathways for glutamate degradation.

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The glutamine analog, DON, mimics glutamine regulation of heat shock protein expression in *Drosophila*

The heat shock response is a reversible cellular stress response characterized by rapid and profound changes in the regulation of gene expression. At the transcriptional level in *Drosophila*, synthesis of new mRNAs coding for heat shock polypeptides (hsps) is induced within seconds, and at the translational level, the heat shock mRNAs are selected for translation while translation of normal mRNAs is suppressed. In previous studies, we have identified glutamine as a potent and specific amino acid effector of hsp expression in *Drosophila* Kc cells (J. Cell. Physiol. *146*: 180–190, 1991). Hsp expression is maximal in a balanced salt solution (BSS) containing glutamine, however expression is 100-fold or more lower in BSS containing glutamate or other amino acids. While glutamine has some effects on levels of mRNAs coding for hsps, the preponderance of the glutamine effect is exerted at a post-transcriptional level. Studies of glutamine compared with glutamate metabolism in normal and heat shocked Kc cells identified glutamine itself, not a metabolite of glutamine, as the active agent in supporting hsp expression (J. Cell. Physiol. *150*: 620–631, 1992). A glutamine analog, 6-diazo-5-oxo-L-norleucine (DON), mimiced the effect of glutamine on hsp expression while other glutamine analogs tested either had no effect or simply suppressed protein synthesis. We have studied the effects of DON on glutamine utilization in Kc cells in an attempt to identify the mechanism of this regulation.

While DON is capable of supporting hsp expression in heat shocked cells for 60–90 min in BSS containing no additional amino acids, it is an inhibitor of Kc cell growth over a period of days in normal medium. The growth inhibition was only partially rescued by high levels of glutamine. In the same time frame as the effects on hsp expression, DON reduced both the consumption of glutamine and the production of CO_2 and alanine from glutamine. Assays of glutaminase showed this enzyme activity was not affected by heat shock conditions, and that concentrations of DON effective for supporting hsp expression in heat shock inhibited glutaminase more than 80%. Thus glutaminase

inhibition correlated with decreased glutamine metabolism to CO_2 and alanine.

In contrast with these findings, NH_3 production increased in heat shock conditions in the presence of glutamine but not with glutamate (J. Cell. Physiol. *150*: 620–631, 1992), and the increase was maintained in the presence of DON. While NH_3 itself does not support hsp expression (J. Cell. Physiol. *146*, 180–190, 1991), increased production of free NH_3 consistently correlated with high levels of hsp expression in all conditions tested. Deamination of adenosine and glutaminase have been ruled out as sources of the free NH_3. Preliminary investigations of transglutaminase function in heat shocked cells show increased amine incorporation into protein in the presence of cycloheximide. Consistent with the suggested increased net transglutamination in heat shock, hsp expression is more sensitive to inhibition by false substrates for transglutaminase than is normal protein synthesis. The role of glutamine itself, and DON, in affecting activity of transglutaminases in heat shock conditions is under investigation.

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Production of amino acids and vitamins by diazotrophic bacteria: effect of the carbon and nitrogen sources

It has been reported that bacteria isolated from rhizosphere synthesizes large amounts of amino acids and vitamins, such as lysine, B_{12}, riboflavin and niacin. However, the production of those substances could be influences by the growth conditions and duration of incubation times. *Azotobacter* spp. and *Azospirillum* spp. are frequent inhabitant of the soils and rhizosphere of a wide variety of plants in diverse regions of the world.

We report the liberation of methionine, lysine, arginine, tryptophane, glutamic acid, pantothenic acid, thiamine, niacin and riboflavin by *Azospirillum brasilense* and *Azotobacter vinelandii* during batch growth in chemically-defined media amended with different carbon sources under diazotrophic and adiazotrophic conditions.

The presence or limitation of nitrogen in the culture medium, modified amino acid and vitamin synthesis by *A. vinelandii*. Therefore, our data show that carbohydrate concentration signifincantly affect the production of vitamins and aminoacids by *Azotobacter*. Lysine and pantothenic acid were the amino acid and vitamin that *A. vinelandii* produced in large amounts in chemically-defined media.

Our results suggest that culture supernatants of *A. brasilense* contain at least four substances possesing amino acids activity and four substances possesing vitamins activity. Our data show that the production of those substances are significantly influences by the growth conditions. Thus, methionine, lysine and arginine were produced in large amounts in culture medium containing fructose for 24 h at 30°C. However, the liberation of these amino acids were decreased strongly after 48 h.

When 0.5% malate or gluconate was present in the culture medium, the liberations of methionine, lysine and arginine by *A. brasilense* was drastically modified, compared with culture media amended with fructose. In this context, could be suggested that the production of those amino acid could be influenced by the carbon source added into the culture medium.

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Biology of L-lysine production with *Corynebacterium glutamicum*

With *C. glutamicum* 180000 tons L-lysine are produced annually. This bacterium has simple flux control when compared to *Escherichia coli* regarding absence of isoenzymes. There is also no apparent expression control of lysine biosynthetic enzymes.

However, studies with cloned genes revealed that unexpectedly the dihydrodipicolinate synthase within the lysine biosynthetic sequence carries significant flux control. Here the total amount of enzyme protein is critical. Another surprising outcome of a biochemical and genetical analysis was that *C. glutamicum* can synthesise lysine via a split pathway; a principle usually reserved for catabolic reactions. In a recent NMR-study we quantified the flux of the arising lysine molecule to be 30% via the dehydrogenase branch, and the remainder via the succinylase branch. This split ratio is dependent of the nitrogen availability. At high ammonium concentration 70% of flux is carried via the dehydrogenase branch. Instead, at low ammonium concentration all flux is carried nearly exclusively via the succinylase branch. Therefore, the split ratio is directly determined by the kinetic characteristics of the diaminopimelate dehydrogenase, which has a low affinity towards ammonium. Another important characteristic discovered in our laboratories is that *C. glutamicum* possesses an active export system for lysine. One hyperproducer analysed has an about threefold increased secretion activity. This probably serves to prevent piling up of unphysiological high cytosolic lysine concentrations when cellular metabolites are optimally directed towards lysine.

Thus, the apparent simple control of lysine synthesis in *C. glutamicum* masks exciting features of this organism, whose relevance to govern lysine flux will be discussed.

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Genes to enhance lysine biosynthesis in higher plants

Aspartate is the precursor of the essential amino acids lysine, threonine, methionine and isoleucine. In higher plants, biosynthesis within this pathway is controlled mainly by feedback regulation. Disruption of two regulatory loops led to the overproduction of free **lysine** and **threonine** thanks to a desensitization of two enzymes to the normal control exerted by lysine, respectively **dihydrodipicolinate syntehese (DHDPS)**, first enzyme of lysine branch, and **aspartate kinase (AK)**, first enzyme of the common pathway. As lysine and threonine are respectively the first- and second-limiting amino acids in cereal-based diets, the isolation of alleles allowing their overproduction in the free pool is of considerable interest with respect to improving plant nutritional quality.

Aspartate kinase: Although in most plants several AK isozymes feedback-inhibited generally by either lysine or threonine have been identified and separated, the precise gene-enzyme

relationship has never been clearly established for any one case. A gene coding for a bifunctional enzyme **aspartate kinase-homoserine dehydrogenase** (AK-HSDH) was isolated in *Arabidopsis thaliana* by screening two genomie banks with the carrot AK-HSDH eDNA Nucleotide sequence analysis clearly revealed that the AK and HSDH (third step in the pathway) activities are encoded by a single gene homologous to the *E. coli thrA* gene, which codes for a threonine sensitive AK-HSDH and thus a lysine insensitive AK form. A chloroplast-targeting sequence amino acid sequence was identified upstream of the known amino terminal AK conserved sequence. Gene structure analysis revealed the occurrence of 15 introns interrupting the apoprotein coding region. Among several remarkable features in the sequence, a putative kinase domain is also suggested. DNA hybridization following Southern revealed that this gene is present in single copy in Arabidopsis, even when relaxed hybridization conditions were used. Northern blot analysis revelaed a single transcript at 3.2 Kb in the poly(A)+ fraction from cell suspensions, indicating that the gene is functional in *Arabidopsis thaliana*. Putative promoter elements were found in a 2Kb upstream region of the *ak-hsdh* gene comprising three putative TATA-boxes, as well as two recognition sequences of known transcriptional factors: an *Opaque 2* and a GCN4 DNA binding site. A 270 bp fragment directly upstream of the putative initiation codon was shown to be functional when fused to the GUS reporter gene in transient expression experiments.

Dihydrodipicolinate synthase: Nucleotide sequence analysis carried out on the *dhdps* genes available from bacteria and higher plants revealed a conserved internal region among these. The soybean internal fragment was further used in Southern blot anlaysis of *Nicotiana sylvestris* RAEC-1. A *dhdps*-specific band of 1.8 Kb in the *Eco*RI digestion was used to construct a sub-library in λgt 10, that was then srceened using both the internal fragment from soybean and a full length cDNA from Populus. Preliminary sequencing of a positive candidate (pDNsrl) revealed that approximately 15 amino acids were missing at the amino side of the clone. As no intron was expected in the apoprotein sequence, a functional complementation of a DHDPS deficient strain of *E. coli* (AT997) was attempted and effective for pDNsrl in the sense orientation only. Western blotting experiments using *N. sylvestris* anti-DHDPS antibodies confirmed that the isolated clone effectively coded for DHDPS. A definite proof that the clone isolated was the *Nicotiona sylvestris dhdps-rI* gene was obtained after a DHDPS enzymatic test was carried out with extracts from the complemented *E. coli* AT997, in presence and in absence of lysine. DHDPS activity was totally insensitive to feedback inhibition by lysine, as the enzyme from the plant RAEC-1 extract. Identification of the putative nucleotide change(s) is now is progress.

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Proline overproduction and resistance to salt and water stress in mutants of *Nicotiana plumbaginiolia*

The accumulation of proline has been reported in many plant species when submitted to various types of stress (salinity, drought, cold). However, the role of proline accumulation in adaptation to such environmental injury is still the subject of debate. Our approach to this problem was to obtain plant mutants which overproduce proline to analyse their level of resistance and metabolism and to study the expression of the genes involved in proline biosynthesis.

We used haploid protoplasts of *Nicotiana plumbaginifolia* to select single gene mutants tolerant to salt (NaCl or KCl), to water stress (PEG) and also to proline analogues (azetidine-2-carboxylic acid: 2AZ trans-4-hydroxyproline: HYP). All tolerant lines produced 10–25 times more proline than the wild type when grown on a non-selective medium.

The resistance and prline overproduction were expressed at the level of the regenerated plants derived from the original cell colonies. The genetic analysis showed that the resistance to each stress factor was transmitted to progeny plants as a single dominant nuclear gene.

Such mutants represent a convenient experimental system to study the metabolism of proline and its regulation.

To anlayse the proline metabolic pathway the ^{13}C NMR techniques appeared quite appropriate. ^{13}C L-glutamate, as admitted precursor for proline biosynthesis, was adjoined to the culture medium on which wild type and mutant plants were growing, in the presence or not of natural L-proline (15–10 mM). *N. plumbaginifolia planlets* metabolized mainly L-glutamate to D-glucose and D-fluctopyranose while no ^{13}C-proline was detected. While the photosynthesis of the wild type was severely decreased by external addition of proline, the mutant was only slightly affected. The wild type supplemented with proline showed in its NMR spectrum many resonances of methylene groups of amino acids, which are presently characterized.

Similar studies are performed in both mutants and wild type submitted to salt and drought stress to assess the possible effect of a retro-inhibition exerted on gamma-glutamylkinase, the first enzym of the pathway, on proline production.

This increased knowledge of the metabolism of proline, opens ways to develop plants able to limit the inhibition of their photosynthetic system is stress conditions.

Polyamines

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Polyamine transport in *Escherichia coli*

Polyamine uptake is known to increase during cell proliferation and to be energy dependent. We obtained three clones of polyamine transport genes (pPT104, pPT79, and pPT71) in *E. coli*. Spermidine uptake was catalyzed by the system encoded by pPT104, and putrescine uptake was catalyzed by the systems encoded by pPT104 and pPT79 in the absence of spermidine. Putrescine uptake by the pPT79 system was not influenced by spermidine, whereas that by the pPT104 system was greatly inhibited. These two systems consisted of four kinds of proteins: a periplasmic substrate binding protein (potD and potF proteins for pPT104 and pPT79, respectively), a membrane associated protein having the nucleotide binding site (potA and potG proteins) and two other membrane proteins having 6 putative trans-

membrane spanning segments (potB and -C proteins for pPT104, and potH and -I proteins for pPT79). Functions of potA and -D proteins in the spermidine-preferential uptake system encoded by pPT104 were then studied through a combined biochemical and genetic approach. Spermidine uptake activity was lost when the gene for potA or potD protein was disrupted, and transformation of the cells with potA or potD gene recovered the uptake activity. PotD protein bound spermidine with a 3.2 μM dissociation constant. Spermidine uptake by membrane vesicles prepared from *E. coli* DR112 containing the genes for potA, -B and -C proteins was strongly dependent on the addition of potD protein, and its optimal concentration was 5 μM when 10 μM spermidine was used as substrate. The ATP dependency of spermidine uptake was examined with the *atp* mutant of *E. coli*. The uptake was completely dependent on ATP. When the membrane potential was extinguished by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the uptake activity was decreased by 60% even if ATP existed. This suggests that the membrane potential is also involved in the spermidine uptake. ATP was found to bind to potA protein. In the spermidine transport-deficient mutant *E. coli* NH1596, valine 135 of potA protein, which is located between two consensus amino acid sequences for nucleotide binding, was replaced by methionine. Although the amount of mutated potA protein expressed in *E. coli* cells was the same as that of normal potA protein and the mutated protein was membrane-associated, no significant spermidine uptake was observed. The results taken together indicate that potA and -D proteins are absolutely necessary for spermidine uptake in conjunction with the two channel forming proteins (potB and -C).

Excretion of putrescine was catalyzed by pPT71. The clone encoded Mr 46 kDa protein which consisted of 12 putative transmembrane spanning segments linked by hydrophilic segments of variable length. The protein was an antiport protein between putrescine and omithine (or lysine).

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Effect of inhibitors of the biosynthesis of polyamines on pal activity in *Ulmus pumila* cell suspension cultures infected with spores of *Ceratocystis ulmi*

Cell suspension cultures of *Ulmus pumila*, species resistant to *Ceratocystis ulmi*, were established from leaf undifferentiated callus. Treatment of cultures with spores of *C. ulmi* induced a large increase in the activity of phenylalanine ammonia-lyase (PAL) with a maximum after 24h. Fungal culture filtrates were ineffective in eliciting PAL activity.

Polyamines exert a crucial role in growth and morphogenesis in both higher plants (Slocum et al., 1984) and in fungi (Tabor, 1981). Based on this premise and, as previously demonstrated, in the fact that innibitors of polyamine biosynthesis inhibit mycelial growth and the germination of *C. ulmi* spores (Garcia y col., 1991, a and b), we were prompted to check the effect of difluoromethylornithine (DFMO) or Methylglyoxal-bis(guanyl hydrazone) (MGBG), both inhibitors of ODC and SAMDC respectively, in elm cells co-cultivated with *C. ulmi*.

The increase in PAL activity induced by fungal spores was abolished by the addition of DFMO (5 mM) or MGBG (1 mM). The preventative effect on the increase in PAL activity caused by DFMO was reversed by the addition of 0.5 mM putrescine or 0.5 mM spermidine. However, the inhibitory effect ol MGBG was not reversed by the addition of spermidine, probably due to the fact that, apart from affecting the polyamine biosynthesis pathway, MGBG also induces secondary effects since other processes are affected, such cellular respiration (Janne et al., 1985) and fatty acid oxidation (Nikula et al., 1984). Despite this, the addition of DFMO or MGBG to uninoculated cell cultures did not alter either PAL activity or cell growth.

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Molecular mechanism of polyamine-induced rapid degradation of ornithine decarboxylase

Ornithine decarboxylase (ODC), a key enzyme for polyamine synthesis in higher animals, turns over rapidly with a half life less than one hour, the shortest among known enzymes. The rate of ODC degradation is accelerated by polyamines in a protein synthesis-dependent but RNA synthesis-independent manner. These features makes it possible that ODC is induced dramatically by various growth stimuli in a pulse-like manner. We proposed in 1985 a working hypothesis that the ODC-destabilizing action of polyamines is mediated by antizyme, a regulatory protein which was known to be induced by polyamines and to bind with ODC, inactivating the enzyme. We recently proved the hypothesis by both in vivo and in vitro experiments. First, forced expression of rat antizyme (Zl) in transfected HTC cells elicited a rapid destabilization of ODC. Second, ODC was rapidly degraded in extracts of mammalian cells in an ATP-dependent, antizyme-dependent but ubiquitin-independent manner. Furthermore we identified 26S proteasome as the protease responsible for ODC degradation, unveiling a new role of the 26S proteasome which was widely viewed as specific to ubiquitinated proteins. Antizyme appears to destabilize ODC by exposing its C-terminal region which is thought to be critically involved in the enzyme degradation.

Northern blot analyses demonstrated that antizyme mRNA is amply expressed in cells constitutively with or without exogenous polyamines, confirming previous prediction based on effects of inhibitors that antizyme is induced at the translational level. Sequencing of a nearly full-length antizyme cDNA revealed that its major open reading frame (ORF2) lacked an initiator codon and another overlapping ORF with ATG codons in the minus one frame (ORF1) was too short to encode antizyme. The wild type antizyme mRNA was hardly translated in a reticulocyte lysate unless adequate amounts of polyamines were added, whereas synthetic single nucleotide or four nucleotides deletion mutants of the mRNA were amply translated without exogenous polyamines. Amino-terminal sequencing of the translation product of the wild-type antizyme mRNA indicated that the translation inititated at the first ATG codon of ORFl. These results strongly suggest that polyamines stimulate the translation of antizyme mRNA by eliciting a ribosomal frameshifting (plus one shift). This unique mechanism would be the first example of ribosomal frameshifting on eukaryotic mRNA and would also be a novel type of translational regulation.

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Spermidine/spermine N^1-acetyltransferase expression during rat liver regeneration

Adult liver maintains a remarkable capacity to undergo compensatory growth in response to loss of tissue after partial hepatectomy (PH) or hepatic death due to hepatotoxins.

Polyamines play an essential role in liver regeneration after PH (Luk, Gastroenterology 1986). Ornithine decarboxylase (ODC), rate-limiting enzyme for polyamine biosynthesis, is inducible during the early phases of the prereplicative period. This induction is partially dependent on an increase in the transcription-rate of ODC gene (Desiderio et al., Biochem. Pharmacol. 1990).

In the present experiments, we examine mRNA levels and activity of spermidine/spermine N^1-acetyltransferase (SAT), which controls the interconversion of higher polyamines (spermidine and spermine) to putrescine. PH of male rats was performed by removal of two-thirds of the liver. Animals were sacrificed 4, 8, 16 and 24 h after the operation. Total RNA was extracted from livers, analyzed by Northern blot and hybridized with p9.3 cDNA probe (Casero et al., J. Biol. Chem. 1991). SAT activity was measured as previously reported (Matsui & Pegg, Biochim. Biophys. Acta 1980).

SAT mRNA levels augmented 4 and 8 h after PH (2.8-fold), and decreased by 60% under control values at 24 h. SAT activity increased at 4 h (2-fold), peaked at 8 h (2.5-fold), returned to control values at 16 h and, then, remained almost constant until 24 h. Thus, SAT mRNA levels and enzyme activity showed comparable increases in the first stages of liver regeneration. It can be suggested that a transcriptional control and/or an increased half-life of the mRNA are responsible for the changes of SAT activity.

Our findings are consistent with the involvement of SAT in the control of cellular processes associated with liver regeneration together with ODC. The primary function of enhanced SAT expression at early stages post-PH might be to allow the excape of regenerating hepatocytes from eventual deleterious effects exerted *in vivo* by high levels of spermidine and spermine by contributing to putrescine formation.

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***In vivo* modulation of transglutaminase activity by polyamines**

Transglutaminases (TGases) are a class of enzymes catalyzing a Ca^2-dependent acyl-transfer reaction in which the γ-carboxamide group of a peptide-bound glutamine is the acyl-donor. Primary amine groups of many low molecular weight amines may act as acyl-acceptor with the formation of *mono-* and *bis*-substituted τ-carboxamides of peptide-bound glutamic acid. Among the enzymes catalyzing posttranslational modification of proteins, TGases have been extensively characterized from enzymological point of view. Nevertheless, the physiological role of these enzymes, particularly the intracellular TGase, is still poorly understood. In the absence of amines, TGases catalyze the formation of an ε-(γ-glutamyl)lysine isopeptide bond between endo-γ-glutaminyl and endo-ε-lysyl residues in polipeptides. A physiological role for this isopeptide in regulating the cellular proliferative state, has been proposed. Thus, polyamines, putrescine, spermidine and spermine, may act as a substrate competitor to the peptide-bound lysine moiety, resulting in a "non-lysyl" isopeptide bond. TGase-catalyzed reactions have been proposed to be involved in several biological phenomena. In cancer cells polyamines are present in elevated levels, suitable to inhibit the formation of lysyl isopeptide bond, this condition may trigger an uncontrolled proliferative state. To affirm a relationship between ε-(γ-glutamyl)lysine and polyamines levels, a highly metastatic melanoma cell line (B16-F10) was cultured in the presence of 4 mM α-difluoromethylornithin (DFMO), an inhibitor of polyamine biosynthesis and/or 1 mM theophylline, an activator of soluble TGase. Untreated B16-F10 cells showed that despite a negligible TGase activity, a remarkable intracellular synthesis of *mono*-γ-glutamylpolyamines occurred. ε-(γ-glutamyl)lysine isopeptide was not found in these cells. Inhibition of polyamine biosynthesis and/or activation of soluble TGase, resulted in increased levels of ε-(γ-glutamyl)lysine and in a striking reduction of the metastatic power and proliferation rate of the B16-F10 melanoma cell line. This finding appears to support the model, in that manipulation of the polyamine pools may be an useful tool to affect the intracellular activity of Tgases.

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Polyamine transport in eukaryotic cells

The amount of polyamines in a given cell depends not only on their rate of synthesis and degradation, but also on their passage from and to the environment. The mechanism of transport in and out of cells is at present the subject of active investigation, but further studies are still needed. Data obtained from the study of this mechanism in human lymphocytes, L1210 cells and hepatocytes in culture are here presented.

Human lymphocytes. The uptake of polyamines by human lymphocytes is saturable, dependent on energy, pH, Na+ and partially dependent on endogenous polyamine concentration. The transport system seems to be common to spermidine and spermine, which are both accumulated and partially metabolized. Polyamines are also excreted: the major catabolites released are spermidine, putrescine and N^1-acetylspermidine. However, cells preincubated with ouabain release spermidine only. The calcium ionophore A23187 further enhances this release Alkalinization with monensin or NH_4Cl also promotes release of polyamines. The effect of NH_4Cl is abolished by EGTA and diltiazem.

L1210 cells. 2-amino isobutyric acid (AIB) has been shown to markedly increase polyamine uptake in L1210 cells. To gain a fuller understanding of the mechanism involved, this effect was investigated further and the influence of hypotonicity was also studied. We were able to demonstrate that spermidine and AIB are transported by different aystems, and that hypotonic stress also promotes a marked increase in spermidine transport, which was only partially prevented by cycloheximide. Okadaic acid did not affect this increase, whereas it prevented the increase of ornithine decarboxylase activity. A23187 and NH_4Cl promoted a much higher increase in the efflux rate than in normal cells.

Hepatocytes. The rate of uptake of polyamines by hepatocytes in culture is not constant, but it increased during hepatocyte retrodifferentiation. When incubated with hepatocytes, AIB, and to a lower extent glutamine and asparagine, promoted

a marked increase in the uptake rate, and so did cycloheximide, without reversing the effect of AIB. Hypotonicity also promoted an increase and this was partially prevented by cycloheximide. Of the different hormones and factors tested, only insulin promoted an increase in the uptake. Okadaic acid was able to prevent the effect promoted by both insulin and hypotonicity. The rate of efflux was also affected by the time spent in culture: after 48 hours, it was markedly reduced. Both uptake and efflux, therefore, contribute to the increase in polyamine content in hepatocytes.

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Polyamine metabolism in rat hepatocytes cultured at different oxygen tension

Previous work by ourselves and by others has shown different zonal localization of the enzymes involved in ammonia metabolism in rat liver parenchyma. The mechanism responsible for this heterogeneity is not clear, but parameters such as O_2 concentration, hormone gradients and direct nervous signals have been suggested as factors affecting gene expression. We describe the effect of O_2 tension on ornithine and polyamine metabolism in rat liver cells cultured for 48 hours at 21% and 5% O_2 concentration to mimic arterial and venous conditions. The activity of arginase and of S-adenosylmethionine decarboxylase was 18% lower at the venous tension, whereas ornithine decarboxylase (ODC) activity increased about 4-fold, but was not followed by a corresponding increase in polyamine content: putrescine, N^1-acetylspermidine, spermidine were decreased by 50%, spermine by 27%. The modifications in polyamine content were strictly dependent on O_2 levels.

Ornithine transaminase activity was also modified: with 5% O_2, a 55% increase was observed. Since this did not seem to depend on phosphorylation/dephosphorylation, nor thiol/disulfide ratio or modification of cAMP content, a direct effect of O_2 concentration on gene expression is probable, as reported for other enzymes. Hellkamp et al. have proposed that a specific heme protein could act as O_2 sensor and gene modulator in liver. Experiments performed with $CoCl_2$ to replace the central Fe^{++} in heme, or with succinylacetone to inhibit heme synthesis, appear to support this hypothesis.

These modifications in ODC, arginase, ornithine aminotransferase and polyamines are similar to those previously detected in periportal and perivenous cellenriched preparations, suggesting that O_2 tension predominates in the modulation of liver zonation.

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Role of polyamines on the transport *in vitro* of the precursor of ornithine transcarbamylase

Biogenic polyamines putrescine, spermidine and spermine at physiological concentrations, induce the transport *in vitro* of the rat liver precursor of ornithine transcarbamylase (pOTC) into isolated rat liver mitochondria. Dissection of the whole transport process of the pOTC shows that polyamines are involved in the functional binding of this precursor to mitochondria. The sensitivity of pOTC to proteinases decreases in the presence of polyamines.

To better understand the structural characteristics by which biogenic polyamines induce the transport of pOTC into isolated rat liver mitochondria, in vitro transport experiments have been carried out in which biogenic polyamines were substituted in the incubation mixture by related compounds differing from them in a series of parameters including number of amino groups per molecule, distance between them and basicity of these groups.

Concerning the number of amino groups per molecule required to induce the transport of pOTC, ammonia and monoamines methylamine, ethylamine, n-propylamine and n-butylamine do not induce pOTC transport. However α,ω-diamines with hydrocarbon chains of 3 to 7 methylene groups induce pOTC transport as efficiently as putrescine, spermidine and spermine, although this effect decreases dramatically as chain length increases.

Experiments carried out with polyamines and related compounds in which basicity at the level of one of the nitrogen atoms was abolished clearly show that, at least two protonated amino groups per molecule are necessary to induce transport of pOTC.

All these results have allowed us to establish that: i) polyamines play a role in the transport *in vitro* of pOTC by modulating the folding of this precursor to favour its binding to mitochondria; ii) at least, two amino groups per molecule are necessary to induce to pOTC transport; iii) the distance between the amino groups and preservation of their basicity are critical in the mechanism of action of polyamines.

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Effect of growth factors on polyamine biosynthesis in normal and neoplastic cells

The ornithine decarboxylase/polyamine system plays an essential role among early events involved in the mitogenic and metabolic response of normal and neoplastic cells to growth factors. In the present study the implication of polyamines in EGF, TGF-α, TGF-β1, and orotic acid (OA) action on cultures of rat L6 myoblasts, fetal bovine myoblasts (FBM) and K 562 human chronic myelogenous leukaemia cells was investigated.

The dynamics of stimulation of protein synthesis by TGF-α was greater than by EGF in both examined cultures of myoblasts. The maximal response of FBM to TGF-α in a concentration of 100 ng/ml reached 370%, whereas EGF, in a 10 times higher concentration, stimulated protein synthesis only to 123% of control. α-Difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase (ODC), significantly abolished the metabolic response of FBM and L6 myoblasts to EGF and TGF-α. The participation of polyamines in early events evoked by growth factors was confirmed by significant stimulation of ODC and S-adenosylmethionine decarboxylase (SAMDC) activity as well as an increased concentration of spermidine and spermine in L6 cells exposed to TGF-α and EGF.

TGF-β1 as a negative growth factor inhibited cell proliferation with a significant decrease of intracellular spermidine and spermine concentration in L6 myoblasts. Similarly, TGF-β1 significantly inhibited the growth of K 562 cells. The drop in cell number was accompanied by a significant suppression of ODC and SAMDC activity in K 562 cells. The lack of a simultaneous

drop in concentration of spermidine and spermine resulted from the uptake of extracellular polyamines by leukaemic cells.

OA – a known promoter of carcinogenesis inhibited proliferation of L6 myoblasts but stimulated proliferation of K 562 leukaemic cells, and impaired their responsiveness on NK cell activity. OA both in L6 and K 562 cells inhibited ODC activity, however leukaemic cells were able to compensate for the deficit of endogenous polyamines by their uptake from the extracellular environment. This is an important observation in explaining the mechanism of tumour promotion by OA.

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$^1H/^{13}C$ Two-dimensional NMR determination of macroscopic protonation constants of spermine

We have previously reported that aliphatic polyamines, specifically interacting with negatively charged phospholipids, greately interfere with PKC association to membranes. This ability of polyamines depends on the charge distribution of its partially protonated amino groups and on the hydrogen bonding ability or acidity of the protonated base centers.

This led us to determine the pK values of the protonation sites of spermine by means of $^1H/^{13}C$ 2D-heterocorrelated NMR titration. The choise of this 2D-NMR technique was determined by the complexity of proton spectrum of spermine which make it difficult to assign all the chemical shifts. The 2D-heteronuclear correlation experiment run in the reverse detection mode is an advantageous solution to afford the cases in which ^1H NMR spectrum cannot be resolved. Furthemlore, it allowes one to obtain in one experiment both ^1H and ^{13}C chemical shifts.

Natural-abundance spectra were acquired using a spermine concentration of 50 mM in D_2O in 5 mm sample tubes with a AMX-400 WB Bruker at 40°C. TSP was used as an internal standard. The pH of each sample was adjusted by dropwise addition of concentrated NaOD and measured at the same NMR temperature using a combined glass electrod. To compensate for the effect of D_2O, 0.4 units were added to each measured pH value.

The pK values were calculated for both ^1H and ^{13}C nuclei according to the equation:

$$\delta_{obs} = \sum_{i}^{N} P_i \delta_k^i$$

where δ_k^i is the intrinsic chemical shift for the Kth signal and Pi is the fraction of the i protonated species (BHi)

$$P_i = [BH_i] \Big/ \left(\sum_{j}^{N} [BH_j] \right) = K_i \Big/ \left(\sum_{i=0}^{N} \prod_{j=0}^{i} K_j [H]^i \right)$$

where K_i are protonation constants.

For spermine, where four dissociable protons are present, the chemical shift which is observed is given by

$$\delta_{obs} = \frac{\delta_1 K_1 K_2 K_3 K_4 + \delta_2 K_1 K_2 K_3 [H^+] + \delta_3 K_1 K_2 [H^+]^2 + \delta_4 K_1 [H^+]^3 + \delta_5 [H^+]^4}{[H^+]^4 + K_1 [H^+]^3 + K_1 K_2 [H^+]^2 + K_1 K_2 K_3 [H^+] + K_1 K_2 K_3 K_4}$$

The chemical shifts of hydrogens adjacent to each base site are much less affected by the state of protonation of more remote sites than carbon or nitrogen chemical shifts which are influenced by long range effects. Therefore pK values that we have calculated from ^1H chemical shifts are more accurate than those reported in the literature from ^{13}C and ^{15}N chemical shifts.

The results obtained under our experimental conditions, serve as starting point for future NMR studies of interactions between spermine and phospholipid bilayers whose understanding is the base for the explanation of physiological spermine influence on the PKC association to membranes.

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Polyamine efflux from human colonic cancer cells

Polyamines are essential for the optimal growth and differentiation of all cells. Intracellular concentrations are regulated in parallel with growth rates with both biosynthetic and catabolic reactions being rate limiting. Polyamine transport is also important in maintaining intracellular concentrations and is responsive to the growth status of the cell.

In this study, we investigated the response of polyamine efflux to decreases in collular growth rate. The specificity of efflux was also examined and an attempt was made to characterise the mechanism involved.

HT115 (human colonic cancer) cells were grown as monolayer cultures in DMEM supplemented with 10% (v/v) horse serum under standard conditions. Intracellular polyamines were radiolabelled by growing the cells in the presence of [^3H]-putrescine. Polyamine efflux was traced by monitoring the appearance of radioactivity in the extracellular medium. Cell growth was decreased by serum-deprivation or by treatment with the cycle-specific antimetabolite, 5-fluorouracil (5-FU). Individual polyamines were acid-extracted, separated by HPLC and quantified using either fluorescence- or radiomatic detection techniques.

Serum-deprivation and treatment with 5-FU decreased the growth of HT115 cells. This was accompanied by an increase in polyamine efflux, with maximum efflux corresponding to maximum inhibition of growth. Polyamine analysis revealed spermine to be the predominant intracellular polyamine, while N^1-acetylspermidine appeared to be the polyamine effluxed selectively. The results suggest that there is a causal link between acctylation and efflux and the efflux is regulated with cell growth rate.

It may be that the extracellular polyamine content controls the efflux from cells. Altering the distribution of acctylated and free polyamines across the cell membrane by their addition to the extracellular medium caused both an increase in polyamine efflux and a change in the pattern of efflux observed. The significance of these changes is, as yet, unclear.

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Polyamines block ion channels in pituitary tumor cells

Polyamines are naturally occuring cations present in all living cells.

The major cellular polyamines like putrescine, spermidine and spermine are polyvalent cations with two three or four positive charges at physiological pH. Neuropharmacological actions of spermine and spermidine raise the possiblity that these

substances may modulate or mediate synaptic transmission. The high affinity of the polyamines to membranes of the synaptic complex suggests a physiological function in the regulation of synaptic activity. Polyamines have also been reported to play an essential role in nerve growth, nerve regeneration and survival of nerve cells. The synthesis of polyamines is increased after electrical stimulation of nervous tissue or electroconvulsive shock induced epilepsy where the intracellular concentration of polyamines can reach millimolar amounts. The interaction of the naturally occuring polyamines with biological membranes and their possible involvement in the regulation of single channel activity has not been explored in great detail.

In this study we investigated the effects of spermine, spermidine and putrescine on large conductance calcium-activated potassium-channels from rat pituitary tumor cells (GH3) with the help of the patch clamp technique. We found that all polyamines applied to inside-out patches reduce the amplitude of calcium-activated potassium-channels in a dose dependent manner. The open probability of channels decreased whereas the closing probability increased. These effects were voltage dependent increasing at more positive potentials and were only partially reversible after removal of the polyamines from the bath solution. The order of effectivness was spermine > spermidine > putrescine. The reduction of the single channel amplitude indicates that polyamines act as fast blockers. It was further found that the reduction of the open probability was reversed by an increase in the free calcium concentration of the bath solution. These experiments indicate that polyamines act also at the calcium-sensor of the channel where they compete with calcium. The data show that polyamines act as blockers at calcium-activated potassium-channels by occluding the channels and by interfering with its gating. Polyamines are not effective applied to the outside of the membrane. The results suggest that polyamines at intracellular millimolar concentrations, which can be obtained during various physiological and pathological conditions could effect electrical activity of excitable cells.

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Changes in polyamine metabolism after orthotopic liver transplantation of the rat

Since liver graft viability in cold ischemic storage is limited, long-preserved graft will not lead to successful transplantation.

Polyamine metabolism and liver regeneration are important for recovery from many forms of hepatic injury.

Here, the effect of the liver transplantation on polyamine metabolism after being preserved in Euro-Collins solution for 6 hrs at 0°C has been studied in the rat.

Orthotopic liver transplantation were performed according to a method modified from that described by Kamada et al.

Plasma ALT, AST and LDH activities elevated significantly in all animals and peaked by 4 hr after transplantation.

Hepatic ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyltransferase (SAT) activities in the liver graft increased and peaked by 8 hr after transplantation.

Hepatic putrescine contents increased and peaked by 8 hr.

The increase in [^{3}H] thymidine incorporation into DNA was observed in the liver at 24 hr after transplantation.

These results suggest that changes in polyamine metabolism prior to DNA synthesis are important for regeneration and repair of the liver graft which is damaged after orthotopic liver transplantation.

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Increased cellular levels of spermidine or spermine are essential for hepatocyte growth factor-induced DNA synthesis in primary cultured rat hepatocytes

We investigated whether polyamine metabolism is involved in hepatocyte growth factor (HGF)-induced DNA synthesis in primary cultured rat hepatocytes. Both ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) activities began to increase with 1 ng/ml HGF and reached its maximum with 5 ng/ml HGF. ODC activity increased by 4 hr after the addition of HGF and peaked by 8 hr, thereafter it decreased and returned to the control level by 16 hr. SAMDC activity increased by 3 hr after the addition of HGF and peaked by 6 hr and remained at a high level until 12 hr, thereafter it decreased and returned to the control level by 18 hr. The peaks of putrescine, spermidine, and spermine concentrations were 8 hr, 16 hr, and 16 hr, respectively. α-Difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, which caused the decreases in putrescine and spermidine concentrations, inhibited HGF-induced DNA synthesis by only 21%. The inhibitory effect of DFMO on HGF-induced DNA synthesis was reversed by exogenously added putrescine, spermidine, or spermine. On the other hand, methylglyoxal bis(guanylhydrazone) (MGBG), a reversible inhibitor of SAMDC, which caused the accumulation of putrescine and decreases in spermidine and spermine concentrations, completely inhibited HGF-induced DNA synthesis to the control level. The inhibitory effect of MGBG on HGF-induced DNA synthesis was reversed by exogenously added spermidine or spermine. Combined addition of DFMO and MGBG completely inhibited HGF-induced DNA synthesis to the control level and the inhibitory effect was reversed when spemidine or spermine was added exogenously. Contrary to these results, the inhibitory effect of DFMO and MGBG was not reversed when putrescine was added exogenously. Exogenously added putrescine, spermidine, or spermine were accumulated but each polyamine added was not converted into the other polyamines. The extent of inhibition of DNA synthesis by various concentrations of MGBG was similar to that of the inhibition of SAMDC activity. These results suggest that increased cellular levels of spermidine or spermine rather than putrescine are essential for HGF-induced DNA syntehsis in primary cultured rat hepatocytes.

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Influence of 1,25-dihydroxycholecalciferol on proliferation and ornithine decarboxylase activity in primary cultures of bovine parathyroid cells

It is now known that the aclive vitamin D is involved in the regulation of proliferation of parathyroid cells as well as in

regulation of parathyroid hormone synthesis. We examined the effects of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] on the proliferation of parathyroid cells in relation to their effects on ODC activity. Exposure of bovine parathyroid cells to serum caused an elevation in [^{3}H] thymidine incorporation by 18 h, which was preceeded by a rise in ODC activity (3.7-, 4.1-, and 3.7- fold at 4, 8, and 12 h, respectively). The interconversion reaction enzyme, spermidine/spermine N^1-acetyltransferase activity did not change by 12 h after the cells were exposed to serum. Preincubation of the cells with 10^{-8} M of $1,25(OH)_2D_3$ for 45 h abated the serum-induced rise in ODC activity by 12 h (17.6, 25.8, and 27.4% of those without $1,25(OH)_2D_3$ at 4, 8, and 12 h, respectively). This effect of $1,25(OH)_2D_3$ was dose-dependent between 10^{-11} M and 10^{-8} M. The effect of serum and $1,25(OH)_2D_3$ on ODC activity appeared at a translational or post-translational step since stationary ODC mRNA levels examined by Northern blot analysis were not altered by 12 h. We also examined the effects of 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D_3 [F_6-$1,25(OH)_2D_3$], the hexafluorointed analogs of $1,25(OH)_2D_3$. F_6-$1,25(OH)_2D_3$ was about 100-fold more potent than $1,25(OH)_2D_3$ in suppressing the serum-induced rise in ODC activity. Since altered metabolism of F_6-$1,25(OH)_2D_3$ is intimately involved in the enhancement of its activity in various systems, metabolites of F_6-$1,25(OH)_2D_3$ in paralell with $1,25(OH)_2D_3$ were examined in parathyroid cells. After 45 h incubation, a peak of F_6-$1,25(OH)_2D_3$ were detected with a greater peak observed at F_6-$1,23(S),25(OH)_3D_3$ on HPLC. However no metabolites including $1,25(OH)_2D_3$ remained after 45 h incubation. These data are in good agreement with previous reports which ascribed the greater biological activity to slower metabolism and the generation of F_6-$1,23(S),25(OH)_3D_3$ which retained its biological activity after hydroxylation at C-23(S) position. In summary, it was suggested that $1,25(OH)_2D_3$ inhibited parathyroid cell proliferation possibly through suppression of ODC activity. F_6-$1,25(OH)_2D_3$ was about 100-fold more potent than $1,25(OH)_2D_3$ in suppressing ODC activity in parathyroid cells, which might be in part due to the different matabolism of F_6-$1,25(OH)_2D_3$ in the cells.

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Identification of γ-glutamyl-polyamines in plants

The role of polyamines in plant cells is still not completely clarified, even though their involvement in growth is well established. Polyamines can be bound to several molecules with different types of binding. They are covalently linked to proteins by transglutaminase (TGase, EC 2.3.2.13). This emyme has been recently studied in plant, but its function is, at present, unknown. In chloroplasts isolated from leaves of *Helianthus tuberosus*, protein substrates of TGase have been identified as chlorophyll *a/b* proteins and RuBisCO. During the TGase assay *in vitro*, both putrescine (PU) and spermidine (SD) gave linked labelled derivatives with different efficiency (Del Duca et al., 1993).

The amino acid analysis, after exhaustive enzyme digestion of several conjugated PU derivatives formed by the *in vitro* TGase assay, indicates that an active metabolism takes place in incubated chloroplasts. For the first time in plants conjugated SD and bis-PU were detected; previously, Signorini et al. (1991) were able to find mono glu-PU and another unidentified derivative when casein was added as a substrate to the leaf extract of *Beta vulgaris*.

In *Helianthus* [^{3}H]-PU was metabolized to SD, acetyl SD (AcSD) and hypusine. PU derivatives were also conjugated as mono or bis γ-glutamyl derivatives. The low amount of free SD suggests that this PA is rapidly conjugated and metabolized. At the best of our knowledge, AcSD and hypusine were never previously found in plants. In mammals the former is an important intermediate, in particular it is the substrate of polyamine oxidase. Hypusine was demonstrated to be the substrate of TGase in mammals (Abruzzese and Beninati, 1991) and it is a component of an initiation factor of protein synthesis. This metabolism does not involve the synthesis of spermine, which has not been found . A greater amount of conjugated PA was found when 4 mM PU was administered in the TGase assay instead of 0.2 mM PU; some SD derivatives, namely N1/N8 (γ-glu)SD and hypusine, were formed in higher amounts with respect to other PU derivatives; N1,N8-bis(γ-glu)SD occurred in lower amount. These data provide the possibility to study and interpret the function of conjugated polyamines in a totally new way.

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Role of transglutaminase in lens crystallins polymerization

The lens of vertebrate eye continues to growth throughout the life, and the core or nucleus of the adult lens, still contains the cells that were formed during embryonic and fetal stages. In the lens it is possible to find a gradient from newly synthesized proteins in the outer cortex to extremely aged proteins in the deepest nucleus. This provides a unique model system for studying posttranslational modification and ageing of proteins. The occurrence of a Ca^{2+}-dependent transglutaminase (TGase) was reported in eye lenses of several mammalian species. Significant amount of ε-(γ-glutamyl)lysine isopeptides, a typical product of TGase reaction, were found in protein polymer from human cataractous lenses. However, the physiological role of TGase in the healthy lens remains as yet unknown. Two β-crystallins are the only substrates for lens TGase among the water soluble proteins. These substrates contain high TGase-affinity glutamyl and lysyl residues. The combination of these two remarkable features provides the basis for speculation on the physiological role of TGase and polyamines in the healthy lens. Indeed, polyamines act as substrates for TGase[2] and they may compete with lysine residues for the glutamyl residue of crystallins in the formation of isopeptide bonds. Induction of opacification of rabbit lens, with an experimental cataract model system, showed the formation of elevated amount of protein cross-links by spermidine. The same experiments performed in the presence of increasing concentration of spermdine (up to 100 μM) in the incubation medium, indicated that the polyamine prevented the induction of opacification. In these lenses, mono-(γ-glutamyl)spermidine was the most abundant TGase derivative observed. Our preliminary finding suggest that, in order to maintain the transparency of the healthy lens, the physiological high activity of TGase, is down-regulated by the levels of polyamines.

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Human interferon gamma – a peptide-mapping of antigenic domains

Human interferon (IFN) gamma represents a highly potent cytokine with pleiotropic activities. The structure-function relationship in IFN-gamma molecule is not fully elucidated yet. To gain further information on antigenic properties of this cytokine, we performed its study with monoclonal antibodies (mAb). Two panels of mAbs raised either to natural leukocyte IFN-gamma or against recombinant IFN-gamma were prepared and their binding sites were localized. It has been shown that the hydrophilic segments often form immunologically active structures on a protein. Therefore we selected regions in the IFN-gamma molecule with the highest hydrophilicity and the corresponding protein fragments were synthetized. Five oligopeptides spanning the primary sequence of IFN-gamma at positions 1–10, 1–17, 33–43, 61–72 and 86–98 were used in experiments. Antibodies were characterized according to their ELISA-reactivity with solid-phase bound fragments. We found that the majority of mAbs raised either to natural (4 of 7 mAbs) or to recombinant IFN-gamma (5 of 8 mAbs) showed the highest binding with the N-terminal sequences 1–10 and 1–17. In contrast, the peptide 86–98 derived from the C-terminal portion of the protein exerted the lowest antigenicity. The localization of sites recognized by the two mAbs with the strongest binding to recombinant IFN-gamma proved not possible with the set of peptides available.

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Evidence in vitro for effect of methionine enkephalin on macrophages from liver impaired mice

In this experiment, liver of mice was impaired by feeding CCl_4 and the effect of M-Enk on migration of abdominal macrophages was determined in vitro. Migration of macrophages in liver impaired and control groups were suppressed by macrophages migration inhibitory factor (MMIF) produced from Con A-stimulated spleen lymphocytes, but the suppression might be reversed by adding 1 $\mu mol \cdot L^{-1}$ M-Enk in the reaction system ($P < 0.05$ in both groups). The effect of M-Enk was more obvious in the case of impaired group than that in control group, although the change did not significantly differ from each other. It has been known that MMIF could inhibit the migration of macrophages in vitro, our results indicated that M-Enk might block the effect of MMIF and macrophages in liver impaired mice were more sensitive in response to the blockage. In order to determine whether M-Enk affect the binding between MMIF and its receptor on macrophages or interfere with the production of MMIF from lymphocytes, another experiment was performed. Macrophages were cultured in Con A-stimulated splenocytes supernatant and the distance of macrophages migration was measured in the presence of M-Enk. The data showed that M-Enke could inhibit the effect of MMIF on macrophages and macrophages from liver impaired mice were more sensitive than those from control ($P < 0.05$). When lymphocytes were preincubated in 2.5 $\mu g \cdot ml^{-1}$ Con A and M-Enk, the supernatant could no long inhibit the migration of macrophages. Macrophages from impaired group also showed a higher sensitivity compared to the control. The results suggested that M-Enk could significantly inhibit in vitro both the combination of MMIF with macrophages and the production of MMIF from lymphocytes.

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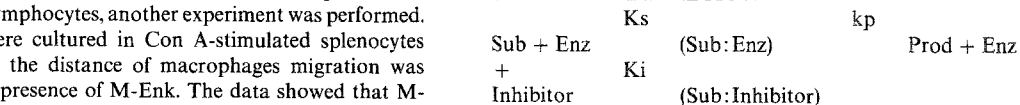
Prospect of peptides as substrate-inhibitors against the in-vitro hydrolysis of human big-endothelin to active endothelin: design and inhibition kinetics

Human Endothelin-1 (ET-1), a potent vasoconstrictor peptide released by endothelial cell, is produced from a large precursor (preproET-1) through the formation of the intermediate peptide big-Endothelin-1 (bigET-1). The physiological importance of the cleavage of bigET-1 is indicated by the reported 140-fold increase in vasoconstriction activity upon formation of ET-1. Therefore, inhibition of ET-1 production could be an important therapeutic strategy for ET-related pathologies. The recognition properties of a series of Sequence-Directed Recognition Peptides (SDRPs) with respect to bigET-1 was tested as the ability of these peptides to inhibit ET-1 formation from α-chymotrypsin degradation, in-vitro, at neutral pH. Three criteria of complementarity to the target bigET-1 peptide were followed in order to determine the SDRP sequences to be synthesized: 1) the relationship between peptide pair length and peptide affinity, 2) the site exposure of the target amino acids and 3) the opportunity of exercising an internal control for the proteolytic activity (α-chymotrypsin) by leaving unburied one of hydrolysis sites. Initial rate kinetics allowed the assessment of the inhibition mechanism via substrate-depletion (Scheme 1) and of the dissociation constants (Ki, μM) of the complexes SDRP:big-ET-1. First order rate constants (k) confirmed the biological significance of SDR-peptides, whereas any attempt of improving efficiency in complexation by emphasizing the role of hydropathic complementarity and/or three-dimensional complementarity, as suggested, in the early phase of the project by molecular modeling/energy minimization techniques, did not succeeded. Thus, as a second phase of the project, we are undertaking molecular dynamics simulation of the interacting peptides and analyzing their structural/dynamic/activity differences. We deem that modelling of conformational behaviors might provide us with a precious framework to understand and rationalize the experimental data and guide future peptide design in a more rational manner.

SUBSTRATE DEPLETION



Scheme-1

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Trypsin-catalyzed peptide synthesis by use of inverse substrates

Protease-catalyzed peptide synthesis has many advantages. The reaction is highly stereoselective, racemization-free and proceeds under mild condition with minimal side-chain protection. Thus the synthetic method has come into use in the preparation of variety of oligopeptides. However, disadvantage of this method is the loss of the product due to the hydrolysis by the protease. Trypsin is characterized by its narrow substrate specificity and this property is advantageous to avoid the unfavorable secondary hydrolysis when it is used for peptide synthesis. This concurrently suggests that trypsin is not useful as a catalyst for the preparation of wide variety of peptides.

Previously we reported that p-amidinophenyl esters derived from α-amino acids behave as specific substrates for trypsin, "inverse substrate". It was also shown that trypsin interacts with a variety of "inverse substrates" without recourse to the structure of their acyl moiety. In the present study, trypsin-catalyzed peptide coupling by use of "inverse substrates" was attempted with a view to developing a method for the preparation of wide variety of peptides.

Trypsin-catalyzed condensation was achieved by the incubation of 1 mM of acyl donor (Boc-amino acid p-amidinophenylesters), 20 mM of acyl acceptor (amino acid p-nitroanilides), and 20 μM of trypsin in DMSO-H_2O (1:1) at 25°C. The condensation products were obtained in 77–85 % yield within 10 minutes when L-amino acid derivatives were used as either acyl donor and acyl acceptor. When D-amino acids were used as the acyl donor, the condensation products were also obtained in 65–84% yield after incubation for 1 hour. On the other hand, no condensation product was obtained when D-amino acid was used as the acyl acceptor. Requirement of the structure of acyl donor and acyl acceptor on the efficiency of the coupling reaction was studied. It was shown that the method did not afford contaminants resulting from the hydrolysis of the coupling products.

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Oligopeptides with two aromatic amino acids-interaction with nucleic acids

Oligopeptides containing aromatic residues (e.g. Phe, Trp) have been proposed to serve as model systems for proteins that bind to single stranded nucleic acids (e.g. gene 5 protein of the T4 virus, gene 32 protein of the Fd virus, *E. coli* single stranded binding proteins) and for proteins that recognize damaged regions in DNA and possess endonucleolytic activity. Aromatic amino acid residues can engage in stacking interactions with nucleic acids. Such interactions may play an important role in protein-nucleic acid binding, recognition and conformation. In our previous studies on peptide-DNA interactions we found that peptides containing one or two aromatic residues caused large decreases in DNA viscosity and had large upfield shifts for the aromatic protons on complex formation. An extended chain model has been proposed that involves stereospecific interactions of the charged N-terminal lysine amino groups with the DNA backbone and of the aromatic residue of the amino acid next to it. When the aromatic group is two or three residues after the L-Lys, the interaction with DNA bases is reduced. However, the presence of two aromatic groups in a tetrapeptide enhances the interaction of both aromatic groups with DNA and significant effects on the DNA conformation are observed.

The binding of oligopeptide amides containing aromatic residues, synthesized in our laboratory, with nucleic acids was studied by NMR(399 MHz) in order to obtain detailed information about the stacking complexes formed. These amides have the following general structure: Lys-X-(Ala or Gly)$_n$-X, where X is an aromatic amino acid (phenylalanine, p-nitrophenylalanine or tryptophan). Results indicate that the degree of stacking as well as the geometry of the interaction depend on the position of the aromatic residue in the sequence, and the identity of both the aromatic amino acid and the nucleic acid. The NMR chemical shift changes obtained for the peptides with two aromatic residues suggest that the group directly following the L-Lys residue is inserted between base pairs in a partial intercalation stacking mode. The aromatic residue at the C-terminal end, however, appears to interact more indirectly, primarily through the side of the ring, and its interactions with the bases probably depend on its distance and orientation with respect to the L-Lys.

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Novel cellulose-bound combinatorial peptide libraries as a powerful tool for the rapid sceening of millions of peptides for protein and metal binding properties

A novel type of combinatorial peptide library was simultaneously synthesized on a single cellulose support. The complete combinatorial library is consisting of 400 spots, each of which contains a hexapeptide mixture XXB_1B_2XX (B_1 and B_2 are defined amino acids; X = 17 amino acids, Met, Trp and Cys were omitted for ease of handling) composed of more than 80000 single components. The 400 spots represent all 400 possible dipeptide combinations (20^2). The mixtures were synthesized by simultaneous incorporation of equimolar amino acid mixtures at the X-positions. Equimolarity of each amino acid at the random sites could be achieved using a modified synthesis protocol. The libraries were incubated with the ligands of interest, such as proteins or metal ions and subsequently analyzed quantifying the amount of ligand bound to each spot. The randomized sites of the best binding spot were further defined by synthesizing and screening 400 spots representing a combinatorial library containing additional two defined positions. The best binding spot of the second screen was then used to define the best binding hexapeptide by defining the remaining two X-positions as decribed above. The power of this method will be demonstrated for the identification of a tumor necrosis factor-alpha inhibitor and peptides that bind an anti-p24 of HIV-1 monoclonal antibody. Furthermore, novel sequence motifs that bind metals, such as nickel were obtained by this method. Nickel binding peptides are especially useful for purification of recombinant proteins using affinity tags that may interfere with protein folding and solubility, such as polyhistidine. In addition, different types of libraries containing conformationally stabalized, D- and branched peptides were prepared by this method. In summary, we present a novel and

extremely powerful method that can be used for the rapid screening of millions of peptides or derivatives that bind any given ligand.

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Freeze inhibitor properties of low molecular weight peptides from fish: Isolation and partial characterization by differential scanning calorimetry (DSC)

Supercooling is widely used as a cold survival mechanism by some poikilothermic organisms living at subzero temperatures, but supercooling can also be applied to aqueous solutions of proteins and peptides in vitro. The response of aqueous solutions to cooling depends on the structure of the solute molecules. Some molecules are good ice nucleators whereas others are effective in preventing ice crystal formation and in this way promote supercooling and vitrification. The mechanism for freeze inhibition at the molecular level is not well understood. However, it is believed that the hydration of the solutes plays an important role.

The purpose of this study is to investigate the freeze inhibitor effect of low molecular weight peptides isolated from cod (Gadus Morhua).

The freeze inhibitor fraction (FIF) was isolated by ultrafiltration followed by reverse phase chromatography on HPLC. The active fraction consist of at least two peptides with molecular weights in the range: 1000–3000 Da. Amino acid analysis of the fraction showed a high content of proline as compared to the other amino acids present. In addition, FIF also contains inosine. It is not known whether inosine is chemically bound to FIF or whether it exists in a free form. The freezing, melting and vitrification behaviour of aqueous solutions of FIF was studied by differential scanning calorimetry (DSC). Thermograms were measured during cooling from $+20°C$ to $-60°C$, and subsequent heating to $+20°C$. Scanning rate was 5°C/min. For a 20% (w/w) solution of FIF the exothermic transition was found at $-33°C$ and the corresponding melting endotherm at approximately $-10°C$. Freezing point depressions of this order cannot be explained by a colligative action, which indicate that FIF inhibit ice crystal formation via a more specific noncolligative mechanism. The amount of unfreezable water in the FIF solution as determined from the heat of fusion (ΔH_{fus}) was 0.72 g H_2O per g of FIF. The crystallization and melting enthalpies had the same numerical value. This means that approximately 72% of the water at $-33°C$ is in an unfrozen state, which indicate that FIF is highly hydrated at low temperatures. Moreover, it was found that solutions of FIF in water were capable of being vitrified. This confirms the suggestions of a high hydration number of FIF.

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Biological properties of peptides with novel inhibitors of glucosamine-6-phosphate synthase

The enzyme L-glutamine:D-glucosamine-6-phosphate amidotransferase (GlcN-6-P syntase EC 2.6.1.16.), containing an N-terminal cysteine residue, catalyses the formation of D-glucosamine-6-phosphate (eq. 1), a key molecule in the biosynthetic pathway of glucosamine containing macromolecules of the microbial cell wall

L-glutamine + D-fructose-6-Phosphate →

D-glucosamine-6-phosphate + L-glutamic acid (eq. 1)

(chitin, mannoproteins and peptidoglycan). We have shown that, GlcN-6-P synthase is selectively and covalently inactivated by a series of glutamine analogues including N^3-(4-methoxyfumaroyl)-(S)-2,3-diaminopropanoic acid (FMDP), N^3-(S)-2-bromosuccinamoyl(S)-2,3-diaminopropanoic acid, N^3-bromoacetyl- and N^3-iodoacetyl(S)-2,3-diaminopropanoic acids. Therefore, this enzyme has been proposed by us as a new target for the design of antimicrobial agents. These inhibitors are poorly active against microorganisms, however, the activity is high when incorporated into peptide carriers ("portage" transport concept). According to that concept we have synthesized a series of peptide conjugates containing aliphatic amino acids (Met, Ala, Abu, Nva, Nle, Lys) and mentioned GlcN-6-P synthase inhibitors and evaluated their activity. The obtained peptides exhibited subsatantial antibacterial (*B. pumilus, B. cereus, S. aureus and Sh. sonnei*) and especially anticandidal activity (*C. albicans, C. tropicalis, C. crusei*). Their activity has been attributed to efficient delivery of the peptide into the cell by peptide permeases and subsequent intracellular hydrolysis by peptidases to release the inhibitor inside the cell. Some of the peptides e.g. Nva-FMDP, Nva-FMDP-Nva, Lys-Nva-FMDP showed promising anticandidal in vivo activity in a generalized candidiosis model in mice.

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Homo and hetero peptides formation from N-phosphohistidine

Incubation of pure N-phosphohistidine in water, buffers and organic solvents with or without other amino acids was studied. Phosphorylated homo and hetero dipeptides, tripeptides and tetrapeptides have been discoved. Their respective dephosphorylated short peptides were also found.

These results might indicate that phosphorylated amino acids may have played an important role in prebiotic synthesis.

An intramolecular mixed anhydride mechanism with the imidazole catalysis was proposed.

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Preparation of specific antibody against neuromedin U, and its application to immunological studies on neuromedin U in human tissues

Neuromedin U is a novel neuropeptide, with a potent stimulant effect on rat uterine contraction, and has been isolated originally from pig spinal cord. Porcine neuromedin U-8 (p-NMU-8; Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2), and related peptides were synthesized by solid phase method. Anti-Neuromedin U antibody was raised in rabbits by immunizing the synthetic p-NMU-8 conjugated to BSA with carbodiimide. An anti-p-NMU-8 antiserum (ASH-15) had 40% binding activity of [^{125}I-Tyr^1]-p-NMU-8 (8,000 cpm) at the final dilution of 30,000 fold. All the neuromedin U family peptides so far determined from varous animals, cross-reacted in the same degree as p-

NMU-8 (detection limit, 10 to 20 fmol/tube) in the neuromedin U radioimmunoassay (NMU-RIA), including human neuromedin U-25 which contains p-NMU-8 sequence at its C-terminus. The effects of the chain length and substitution of C-terminal amino acid residues of p-NMU-8 on cross-reactivity were examined. The results indicated that the main antigenic determinant of neuromedin U in the RIA is a pentapeptide amide (Phe-Arg-Pro-Arg-Asn-NH_2) and the C-terminal Asn-NH_2 is an essential structure for antibody binding. Accordingly, the polypeptides of limited sequence homology with p-NMU-8, such as pancreatic polypeptide (Leu-X-Arg-Pro-Arg), neuropeptide-Y (Arg-X-Arg) and vasoactive intestinal polypeptide (Asn-NH_2) had no cross-reactivity. Using this specific antibody, the distribution of neuromedin U peptide in a human fetus in the ninth week of gestation (tubal abortion) was investigated immunohistochemically. Neuromedin U immunoreactivity was observed in epithelia of foregut and primordial bronchi of entodermal origin, and also in epidermis composed of a single layer of ectodermal cells. However, the brain, liver and trophoblast werle negative for neuromedin U in the ninth week of fetal development.

Metabolism, Transport, Enzymes

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The regulation of the interconversion of serine and glycine

The reversible interconversion of serine and glycine is catalyzed by the ubiquitous enzyme serine hydroxymethyltransferase (SHMT) (Reaction 1). This enzyme requires both tetrahydrofolate ($H_4PteGlu_n$) and pyridoxal phosphate (PLP) as coenzymes. The PLP remains bound to the enzyme during a catalytic cycle. Reaction 1 is the major source of one-carbon groups required for the biosynthesis of purines, thymidylate, methionine, and other methylated compounds. These reactions all take place in the cytosol. In eucaryotic organisms there is also a mitochondrial isoenzyme of SHMT. The physiological function of this form of SHMT is less clear, but it probably is involved in formate metabolism.

$$\text{L-Serine} + H_4PteGlu_n \rightarrow \text{Glycine} + 5,10\text{-}CH_2\text{-}H_4PteGlu_n \quad (1)$$

Recently, we have observed that SHMT catalyzes a second reaction involving folates. This is the irreversible conversion of 5,10-methenyl-$H_4PteGlu_n$ to 5-formyl-$H_4PteGlu_n$ (Reaction 2). Also, 5-formyl-$H_4PteGlu_n$ has been found to be a slow tight-binding inhibitor of SHMT. Both the mechanistic and physiological function of this second reaction of SHMT is under intense investigation.

$$5,10\text{-methenyl-}H_4PteGlu_n \rightarrow 5\text{-formyl-}H_4PteGlu_n \quad (2)$$

New methods for measuring cellular folates support earlier studies showing that 5-formyl$H_4PteGlu_n$ is a constituent of most cell folate pools. Studies prior to 1985 have not assigned a metabolic function for this compound, due in large part to the very limited knowledge about its metabolism. It has been known since the late 1940s that both formate and serine can be converted to 5-formyl$H_4PteGlu_n$ under certain physiological conditions. In the late 1950s an activity, named methenyl synthetase, was shown to exist in many cells which converted 5-formyl$H_4PteGlu_n$ to 5,10-methenyl-$H_4PteGlu_n$. In 1984 we published the purification and characterization of methenyl synthetase from rabbit liver and suggested the properties of the enzyme were consistent with a salvage pathway for converting 5-formyl$H_4PteGlu_n$ into a form which could be used in one-carbon metabolism.

The combined action of methenyl tetrahydrofolate synthetase and SHMT catalyzes a futile cycle which consumes one mole of ATP and interconverts 5,10-methenyl-$H_4PteGlu_n$ and 5-formyl-$H_4PteGlu_n$. These two enzymes control the cellular concentration of 5-formyl-$H_4PteGlu_n$. Recent studies suggest that 5-formyl$H_4PteGlu_n$ is involved as both a storage form of folate and as a regulator of one-carbon metabolism. We will review how 5-formyl$H_4PteGlu_n$ may regulate SHMT activity in response to *in vivo* levels of serine and glycine, and how it may be involved in the regulation of one-carbon metabolism.

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Maternal phenylketonuria (PKU) yesterday, today and tomorrow: the evolution of maternal PKU. Gestational carrier: a new therapeutic approach

Maternal PKU has a detrimental effect on embryogenesis. Mental retardation, intrauterine growth retardation with microcephaly, spontaneous abortion, and congenital heart disease have long been described as pathological consequences of the embryogenesis of maternal PKU. Infant pathology is not due to the genetic component of the disease, but due to the fact that the mother has elevated serum phenylalanine levels. Dietary management of PKU mothers faces considerable difficulties. Phenylalanine tolerance varies widely among PKU women and during pregnancy. There is not an established safe range of phenylalanine level that can guarantee the birth of a nommal offspring. The dietary restriction during pregnancy requires a very dedicated mother, a close-by specialty center, and many other factors not easily obtainable. The appropriate dietary restriction should be done prior to conception. Not every PKU woman conceives, and the maintenance of the very restricted diet might be in vain. Although nommal children have been delivered by affected mothers who either had benign hyperphenylalaninemia or in whom strict diet has apparently maintained maternal concentrations of phenylalanine in the low normal range from before conception, more abnormal than normal births have been reported.

The responsible physician must advise against pregnancy for the PKU woman if factors are not optimally available. Until enzymatic replacement of human phenylalanine hydroxylase by genetical engineering is available for the cure for PKU, an alternative therapy of a gestional carrier should be offered for the management of maternal PKU.

The University of Minnesota PKU Clinic and OB-Gyn Department suggest for the first time an alternate therapeutic option involving *In Vitro* Fertilization (IVF) with "gestational carrier." Gonadotropin ovarian stimulation is carried out like for patients attempting to conceive through routine IVF. Oocyte

retrieval is usually performed under sonographic guidance. Typically, the fertilized eggs are transferred two days following retrieval to a recipient whose cycle is manipulated in order to secure optimal endometrial development on the day of transfer. Following suppression of endogenous gonadotropin secretion, estrogen and progesterone are given to the recipient in order to mimic the normal menstrual cycle and are continued until the onset of placental steroid productoin. An early ultrasound is required to confirm the intrauterine nature of the pregnancy; once established, those patients have remarkable pregnancies. The route of delivery should be determined by contemporary obstetrical indications.

If successful, as anticipated, this first attempt would lead to a new avenue in the management of the metabolically abnormal mother. In addition, IVF would lend itself to pre-embryo biopsy, thus allowing for the selection of pre-embryos with at least one copy of the normal gene.

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Partial characterization of a rat cardiac mitochondrial ~44 K molecular weight protein whose phosphorylation is inhibited by taurine

Taurine (2-aminoethanesulfonic acid) is present in high concentrations in all mammalian tissues yet the physiological function of this ubiquitous sulfur compound is not known. In a recent review (Adv. Exp. Med. Biol. 315: 105, 1992) Schaffer and Azuma discuss the physiological effects of taurine on the myocardium which include antiarrhythmic activity, modulation of myocardial contraction, and cardioprotective activity. They also review the possible molecular basis underlying the actions of taurine such as its effects on osmoregulation, lipid metabolism, calcium transport, and membrane function. Another potential mechanism of action for taurine that we have pursued in recent years is its inhibitory effects on protein phosphorylation. We have demonstrated that 20 mM taurine inhibits the phosphorylation of 1) a ~20 K molecular weight protein present in rat retina (Neurochem. Res. 17: 831, 1992), 2) a ~20 K molecular weight protein (different protein) present in rat brain (Brain Res. 553: 89, 1991), and 3) a ~44 K molecular weight protein found in rat heart mitochondrial preparations (Adv. Exp. Med. Biol. 315: 309, 1992). Data from our laboratory indicate that the phosphorylation of the ~44 K molecular weight protein is inhibited by taurine in a dose dependent fashion with a 50% inhibitory concentration (IC_{50}) of 9 mM. Structure-activity-relationship experiments with a series of taurine analogues indicate that a free amino acid moiety is required for activity. Isethionic acid (the hydroxy analogue) and guanidinoethanesulfonic acid lack activity. However, the sulfonic acid moiety of taurine can be replaced with a sulfone moiety. 2-Aminoethylmethylsulfone has an IC_{50} of 3.6 mM while a structurally rigid analogue, (±)-3-aminotetrahydrothiophene-1,1-dioxide, has an IC_{50} of 15.5 mM. However, if the nitrogen is contained within the ring system (secondary amine) such as thiomorpholine-1,1-dioxide all activity is lost. Experiments utilizing the detergent Triton X-114 suggest that the ~44 K molecular weight protein is a soluble protein as it partitions in the aqueous phase. The nature of the phosphate bond was also characterized by exposure to lipid extracting solvents, acidic and basic conditions both at ice temperatures or 100°C, and proteolytic and RNA degrading enzymes. The phosphate bond was determined to be a phosphoester bond. Isolation of the ~44 K molecular weight protein and subsequent degradation with trypsin and 6 M HCl indicate that a serine residue(s) is phosphorylated. Known kinase activators such as cAMP, cGMP, and phorbol ester and the kinase inhibitor, staurosporine, had no effect on the quantity of ^{32}P-phosphate that was incorporated into the ~44 K molecular weight protein; however, Ca^{2+} and chelerythrine are potent inhibitors while W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide], a calmodulin antagonist, appears to stimulate phosphorylation of the ~44 K molecular weight protein.

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Role of the active site lysine in the mechanism of serine hydroxymethyltransferase and other pyridoxal-p enzymes

All pyridoxal phosphate (PLP) enzymes involved in amino acid metabolism have the coenzyme bound as an internal aldimine to the ε-amino group of a lysyl residue. The first mechanistic step for each of these PLP enzymes is a transimination reaction to form an external aldimine between PLP and the amino group of the substrate. In this process the lysyl amino group is displaced. Evidence with aspartate aminotransferase suggests that the displaced ε-amino group of this lysine is the base which accepts the α-proton of the substrate amino acid and transfers it to the 4′-carbon of the coenzyme to form the ketimine intermediate. Thus, the active site lysyl group appears to have two functions in aspartate aminotransferase. Similar studies have been done with several other aminotransferases. We have investigated the role of the active site lysine in serine hydroxymethyltransferase (SHMT), which catalyzes Reaction 1.

$$H_4PteGlu + Serine \rightarrow Glycine + 5,10\text{-}CH_2\text{-}H_4PteGlu \quad (1)$$

The product, 5,10-methylene tetrahydropteroylglutamate (5,10-CH_2-H_4PteGlu), is the major source of one-carbon groups in the biosynthesis of purines, thymidylate, and methylated compounds in both eucaryotic and procaryotic organisms. In addition to H_4PteGlu, SHMT requires PLP as a coenzyme. Evidence suggests that PLP functions by stabilizing a glycine anion formed after the enzyme cleaves serine to form formaldehyde at the active site. The formaldehyde then reacts with H_4PteGlu to form the product, 5,10-CH_2-H_4PteGlu. The PLP is bound as an aldimine to Lys-229 in the *E. coli* enzyme.

We have purified three mutants of *E. Coli* SHMT in which the active site lysine has been changed by site-directed mutagenesis. Of these three mutants the K229Q SHMT was the most interesting. The purified enzyme exhibits absorption maxima at 278 nm and 422 nm. The 422 nm band is characteristic of an aldimine of bound PLP. Analysis of the denatured protein showed that the aldimine was the result of the PLP being bound to the K229Q SHMT as external aldimines with serine and glycine. Removal of PLP and the amino acids could be achieved to form apoenzyme. Addition of PLP to the apoenzyme resulted in holoenzyme with an absorption maximum at 390 nm, which is characteristic of the free aldehyde form of PLP. The addition of serine resulted in a shift of the absorption maxima to 424 nm. Similar experiments were done with glycine.

The K229Q·Serine complex formed 1 equivalent of 5,10-CH_2-H_4PteGlu after the addition of H_4PteGlu. The k_{cat} for the

single turnover was about 2% of the k_{cat} value of the wild-type enzyme. Addition of 5,10-CH_2-H_4PteGlu to the K229Q · Glycine complex resulted in a single turnover of the enzyme to form 1 equivalent of H_4PteGlu. The k_{cat} for this reaction could not be determined accurately, but its value was similar to the value of k_{cat} for the wild-type enzyme. The off-rate constants of serine and glycine from their respective complexes with K229Q SHMT were also determined. In each case these values were orders of magnitude slower then the rate of release of the amino acid from the wild-type enzyme.

We conclude that in *E. coli* SHMT the active site lysine is required for expulsion of the amino acid substrate, but it is not the base which adds or removes the proton on the α-carbon of glycine. This is supported by the observation that the K229Q mutant enzyme also catalyzes the slow transamination of D-alanine at nearly the same rate as the wild-type enzyme. In this reaction removal of the α-proton is a required step.

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Regulation of myocardial calcium transport by phospholipid-N-methylation and taurine

Taurine is an ubiquitous amino acid found in very high concentration in excitable tissues. Depletion of these high intracellular stores causes several adverse effects, including the development of a dilated cardiomyopathy and retinal damage. Nevertheless, little is known regardlng the mechanism underlying the development of these pathological conditions. One theory advanced to explain the cardioprotective activity of taurine proposes that taurine functions to maintain calcium homeostasis. Yet, attempts to demonstrate direct effects of taurine on major calcium transporters have been largely unsucessful. Therefore, we explored the possibility that taurine acts indirectly to modulate calcium transport. Since there are structural similarities between taurine and ethanolamine, we felt that taurine might interfere with the metabolism of phosphatidylethanolamine, thereby altering phospholipid composition of membranes and modulating the activity of major calcium transporters. The initial studies focused on sarcolemmal phospholipid methyltransferase, which catalyzes the formation of phosphatidylcholine from phosphatidylethanolamine and serves as an important regulator of the Na^+, Ca^{2+} exchanger. Hearts perfused for 5 minutes with buffer containing 300 μM L-methionine experienced a 4-fold drop in Na^+, Ca^{2+} exchanger activity. The methionine-mediated decline in Na^+, Ca^{2+} exchange was largely blocked by inclusion of 10 mM taurine in the buffer. Taurine also decreased the incorporation of ^{3}H-methyl groups from radioactive methionine into myocardial phospholipids of isolated heart and inhibited sarcolemmal phospholipid methyltransferase activity. Studies with isolated membranes revealed that taurine was equally effective at inhibiting both sarcolemmal and sarcoplasmic reticular phospholipid methyltransferase activity. In junctional sarcoplasmic reticulum, N-methylation was found to mediate an opening of the ryanodine sensitive calcium channel and a reduction in Ca^{2+}-induced, Ca^{2+}-release. However, these effects of L-methionine were not blocked by inclusion of 10 mM taurine into the perfusion buffer, presumably because intracellular taurine levels were not appreciably altered by the 5 minute perfusion. These data suggest that exogenous taurine indirectly alters sarcolemmal Na^+, Ca^{2+} exchanger activity through the modulation of phospholipid methyltansferase activity while the intracellular taurine pool regulates Ca^{2+} release from junctional sarcoplasmic reticulum. Thus, taurine is required for normal contractile function.

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Occurence of the malate-aspartate shuttle during glutaminolysis in various hepatoma cells

In mammalian cells, cytosolic NADH lacks direct access to the mitochondrial respiration chain. Hence, an excess of reducing equivalents is afforded to the respiratory chain by way of different shuttle systems, which oxidize cytosolic NADH and transfer reducing equivalents to the mitochondrial electron transport system.

The reoxidation of cytosolic NADH was studied in chicken hepatoma cells induced by avian myelocytomatosis virus and in lines of human hepatoma cells whose mitochondria preferentially utilized glutamine as the fuel for the cellular energy metabolism.

The tunor cells showed mitochondrial reoxidation of NADH, as evidenced by the accumulation of pyruvate, when incubated aerobically with L-lactate. The involvement of the respiratory chain was demonstrated by specific inhibitors of the electron transport system. Glutamine oxidation proceeded in the tumor mitochondria exclusively via a pathway involving transamination. Malate stimulated aspartate production from glutamine. When the tumor cells were cultured in Eagle's medium with aminooxyacetate, an inhibitor of transaminase, or in the absence of glutamine, a marked reduction in the NAD/NADH ratio was observed. Involvement of various shuttles in the hepatoma cells remain to be established. However, these results indicate that the malate-aspartate shuttle was functioning in these hepatoma cells.

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The change in heart taurine and α-amino acids during cardiac insults is provoked by changes in intracellular sodium

Work on the Langendorff perfused guinea-pig hearts has shown that a reduction of the gradient for sodium ions across the cell membrane, by reduction of the bathing [Na] or exposure to Tyrode free of divalent cations to raise $[Na]_i$, provoked a time dependent loss of most intracellular free amino acids. This loss was antagonized by manoeuvres that reduce the rise in $[Na]_i$ in Ca-free media while it is increased by inhibition of the Na-pump. Complementary to this work were experiments on guinea-pig ventricular myocytes which suggested that a rise in intracellular levels of the amino acid increased the ability of the myocytes to regular intracellular sodium. This conclusion was based on the finding that myocytes isolated in the presence of taurine, showed a reduced resting intracellular sodium activity ($a_{Na}i$) and a reduced $a_{Na}i$ on exposure to cardiac glycosides or to media free of

divalent cations. Similar attenuation was also seen in myocytes isolated into media free of amino acids but when the amino acids taurine, glutamate or aspartate were introduced into the sarcoplasm through a penetrating micropipette.

When taken together the two sets of data on isolated hearts and myocytes are consistent with the presence of an amino acid-Na symport in the cardiac muscle cell membrane similar to that found in other tissues. This conclusion was further supported by measurement of the amino acids content in biopsies taken from the left ventricles of patients undergoing coronary artery bypass graft and valve replacement surgery on cardiopulmonary bypass (protected by cold cardioplegia with St Thomas' solution).

During cardiac surgery the heart undergoes a period of ischaemia and hypothermia, both of which are known to raise $a_{Na}i$ and therefore would be expected to induce a loss of tissue free amino acids. A significant fall in all the principal amino acids with the exception of alanine was seen during ischaemia. The loss of α-amino acids (unlike taurine) will depress protein synthesis and reduce energy reserves after cardiac surgery. Attempts to preserve the concentrations of α-amino acids must be balanced against the need to regulate intracellular [Na] and hence intracellular pH and calcium ions.

More recent work on isolated cardiac sarcolemmal vesicles and isolated myocytes (unpublished observations) confirm the existence of a Na/taurine symport in the cell membranes which is sensitive to membrane potential.

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Role of glutamine in regulating glutamine transport and globin gene expression in human erythroleukemia cells, K562

We have examined the mechanisms by which glutamine, a metabolically important amino acid regulates erythroid cell differentiation, resulting in an increase in globin gene expression and its own transport activity. Our studies show that glutamine uptake into K562 cells involve both Na^+-dependent and saturable Na^+-independent pathways. Both pathways exhibit heterogeneity in their glutamine uptake. The high affinity Na^+-dependent activity appears to be mediated predominantly by a system which is inhibited by asparagine, glutamine, threonine, serine, cysteine and alanine. MeAIB a model substrate for System A had no effect. This system has an apparent Km of 0.05 mM. The second Na^+-dependent system has an apparent Km of 0.14 mM, and shows a broad substrate selectivity. The Na^+-dependent uptake of 10μM glutamine did not show transeffects with most amino acids, with the exception of histidine, which was trans-inhibitory. Cation substitution showed a small but significant tolerance for Li^+. This component of uptake may resemble system N activity. There are two saturable Na^+-independent pathways with Km's of 0.16 and 0.81 mM respectively. The low Km system appears to be strongly inhibited by typical system L substrates. The second Na^+-independent component was inhibited with high concentrations of threonine, 3-methylhistidine, asparagine, and glutamine. This system appears to resemble asc activity, with respect to lack of trans-stimulation by test substrates threonine, serine, and glutamine. Glutamine deprivation upregulated the high affinity Na^+-dependent component by 10 fold. Pulse label studies demonstrate an increase in membrane proteins of molecular weights of 98, 70, and 58 KDa. In addition, RNAse protection studies suggest that the availability of extracellular glutamine appears to regulate hemoglobin expression at transcriptional level. These studies have given us the opportunity to further investigate the physiological and biochemical significance of glutamine for controlling important cellular processes during cell differentiation.

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Molecular mechanism of histamine synthesis in mouse mastocytoma cells

Histamine is an important physiological modulator of inflammation, allergic responses, gastric acid secretion, neurotransmitter and other processes. Histamine is formed by a specific enzyme L histidine decarboxylase (HDC), which is widely distributed in a variety of mammalian tissues including mast cells. Although the mechanisms of histamine actions in target cells and histamine secretion from histamine-forming cells have been widely studied, there is only a few studies on the molecular mechanism of the regulation in histamine synthesis and HDC. Mouse mastocytoma cells are the proper cell type for elucidating the mechanism underlying histamine formation in mast cells, because they synthesize HDC in response to various stimuli. We have previously shown that glucocorticoids stimulate histamine synthesis by inducing HDC, which response was synergistically potentiated by 12-O-tetradecanoylphorbol-13-acetate (TPA) in mastocytoma cells. Thus, we first purified HDC from mouse mastocytoma cells and cloned HDC cDNA and deduced its cDNA-derived amino acid sequence. The molecular mass deduced from cDNA (74 kDa) was found to be larger than that of the purified enzyme (53 kDa). The similar difference in molecular sizes was also reported in fetal rat liver HDC. These observations indicate the possible post-translational processing of nascent HDC in mastocytoma cells. Thereby we have expressed the 74 kDa HDC and its deletion mutant HDC lacking C-terminal region equivalent to 20 kDa using a baculovirus expression vector system. The expressed 74 kDa HDC was found to be essentially inactive, whereas the expressed mutant HDC to be fully active. Furthermore, the antiserum raised against a synthetic peptide corresponding to the deduced C-terminus of 74 kDa HDC, failed to recognize the purified 53 kDa HDC, indicating that 74 kDa HDC undergoes a truncation in the C-terminal region.

In addition, we cloned the gene for HDC including the 5'-flanking region from genomic library of mastocytoma cells. Transcriptional start site was determined and DNA sequence analysis showed that the promoter region have a typical glucocorticoid responsive element (GRE). Although a typical TPA responsive element (TRE) was not found, TPA markedly enhanced the promoter activity by dexamethasone in the reporter gene analysis.

These results suggest that HDC synthesis to yield fully active 53 kDa HDC peptide is transcriptionally and post-translationally regulated in mouse mastocytoma cells.

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Mechanism of gabapentin (Neurontin ®) transport across monolayers of human colon adenocarcinoma cells (CACO-2)

Gabapentin [1-(aminomethlyl)cyclohexaneacetic acid, Neurontin ®] is an analog of γ-aminobutyric acid with neuroprotective and antiepileptic properties. The structure of gabapentin closely resembles that of the bulky, hydrophobic amino acids, L-leucine and L-phenylalanine, that are substrates for the system L transporter. Noteworthy differences are the lack of a chiral carbon and the fact that the amino group is not alpha to the carboxylate functionality.

The human pharmacokinetics of gabapentin are straightforward: the drug does not bind to plasma proteins, is excreted entirely in the urine with a t_{elim} of 5 to 7 h, and is not metabolized. However, the rate of absorption is relatively slow following oral administration of drug solution, and the extent of drug absorption decreases as the dose increases. Previous work employing rat intestinal perfusion *in situ* verified the existence of a saturable transport component as the underlying cause of the dependence of fraction absorbed on dose. Everted rat intestinal rings *in vitro* were used to demonstrate commonalities between gabapentin and L-phenylalanine transport. There was a large nonsaturable component of gabapentin transport in the everted ring system which complicated interpretation of the inhibition studies.

The CACO-2 cell line is derived from human colon adenocarcinoma and demonstrates carrier-mediated amino acid transport. Studies of gabapentin and L-phenylalanine transport were undertaken in order to further elucidate the mechanism of gabapentin absorption as well as to learn more about system L expression in CACO-2 cells. Cells were grown on polycarbonate filters in Snapwell ® trays. CACO-2 cell transport experiments were conducted in the Grass-Sweeney diffusion apparatus with gas-lift mixing. The CACO-2 cell model was validated using functional, biochemical and histochemical tools, as well as with reference compounds. Transepithelial electrical resistance and [^{14}C]-PEG 4000 leakage measurements corroborated with electron microscopy that CACO-2 cells posed an effective barrier after approximately 20 d post-seeding.

In cells of passage 41 to 56, saturable transport of L-phenylalanine was 85% of total transport by day 21 in culture; this value remained relatively constant through 28 days. Permeabilities increased from 5.4×10^{-6} cm/s before day 21 to 70×10^{-6} cm/s and greater at day 21 and later. With gabapentin, saturable transport was approximately 60% of total transport by 22 days and remained relatively constant through 26 days, rising to as much as 80% of total transport; however, by day 30 in culture, the saturable component had disappeared. Permeabilities for gabapentin were much lower than for L-phenylalanine, not being greater than 10×10^{-6} cm/s at any time in culture. Inhibition studies with gabapentin were consistent with a system L-mediated component, but also indicative of a significant paracellular component.

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The peroxidase-catalyzed oxidation of oxytocin and vasopressin

Recently we have undertaken some investigations on the enzymatic action of peroxidases proving that this family of enzymes is competent of utilizing as substrates enkephalins and esorphins. Opioid peptides – characterized by the presence of a $-NH_2$ terminal tyrosine in the molecule – are easily attacked by peroxidases giving rise to dimers that are linked by a molecule of dityrosine. In the present study we have focused our attention on other two hormones, oxytocin and vasopressin, that possess on their structure a tyrosine residue internal to the backbone.

Both hormones are easily oxidized by the horseradish peroxidase/H_2O_2 system giving rise to the formation of two characteristic absorption peaks with maxima at about 290 nm and 315 nm that represent, as reported by literature, the spectral feature of dityrosine. The reaction followed on oxytocin, shows to be rapid reaching the completeness in about 10 min. The spectrum obtained utilizing vasopressin as substrate is substantially similar, the reaction proceeding more slowly. The Lineweaver-Burk plots of the oxidation of the two hormones show that the affinity of the enzyme is slightly higher for oxytocin with respect to vasopressin, being the apparent Km values 0.37×10^{-3} M and 1×10^{-3} M respectively.The two nonapeptides actually function as substrates also for lactoperoxidase, the apparent Km being in this case 0.54×10^{-3} M for oxytocin and 1×10^{-3} M for vasopressin.

The overall kinetic values confirm the finding that the oxidative coupling of tyrosine residues by peroxidases occurs at a higher K_{cat} with respect to tyrosine. Peptides seem to be preferred as hydrogen donors for peroxidases, even if tyrosine is an internal amino acid.

In order to ascertain if the action of peroxidase is able to perform the linkage between the two molecules of the hormones, a large-scale incubation of each peptide in the presence of the horseradish peroxidase/H_2O_2 system has been carried out. The hydrolysis of the incubation mixture and the subsequent analysis by the amino acid analyzer allows to detect the presence of dityrosine into the specimens. In fact, a well separate peak – with retention time of 57 min – completely overlapping with an authentic sample of dityrosine, can be observed.

The amount of dityrosine can be evaluated and the time course formation of the aminoacid can be followed.

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Role of endothelium-derived nitric oxide (EDNO) in streptozocin-induced diabetic rats

Urinary excretions of nitrate (U-NO_3), a metabolite of nitric oxide (EDNO) was measured to explore the role of EDNO on the diabetic vascular complications in rats. Diabetes was induced by injecting streptozocin, 60mg/kg, using female Wistar rats. U-NO_3 was measured by high performance liquid chromatography with an anion exchange column. Besides U-NO_3, nonfasting plasma glucose (NPG), urinary excretions of microalbumin (UMA), systolic blood pressure (SBP) and inulin

clearance (C_{in}) were measured every other week for 20 weeks. Serum, renal and aortic concentrations of NO_3 were determined at the 20th week.

In the diabetic group, NPG and UMA significantly increased after the 2nd week. SBP was not different between the groups. C_{in} increased by about 50% at the 2nd week, and then gradually returned to the baseline level. The U-NO_3 level increased by more than 280% at the 2nd week, and returned to the baseline level at the 8th week. Serum and tissue concentrations of NO_3 were not different between these two groups.

Since increases in U-NO_3 corresponded to the increase in C_{in}, it may be due to the increased clearance of NO_3. However because it also coincided with the initiation of microalbuminuria, there still remains the possibility that the increased U-NO_3 is implicated in the pathophysiological process of diabetic vascular injury.

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Roles of nitric oxide production on the hyperdynamic state in cirrhotic and partial portal vein-ligated rats

Since endothelium-derived nitric oxide (NO) is a potent vasodilator and degraded into nitric ions, we measured the serum nitrate ion (NO_3^- (S)) and the amount of urinary excretions (NO_3^- (U)) as an index of endothelium-derived NO to assess its role on hyperdynamic circulation in portal hypertension, using cirrhotic (LC) and partial portal vein-ligated (PVL) rats. Liver cirrhosi, was produced by thioacetamide administration. Systemic and splanchnic hemodynamics were determined by using tracer microspheres. The level of NO_3^- was measured using a high-performance liquid chromatography with an anion exchange column.

Systemic and splanchnic hyperdynamic circulation existed at almost the same degree in LC and PVL rats as compared to the controls (C) and the sham-operated (SHAM) rats, respectively. NO_3^- (S) and NO_3^- (U) in LC rats tended to increase as compared to the C rats (NO_3^- (S): LC 35.0 $\pm$ 1.2 v.s. C 30.8 $\pm$ 2.0 mM/L, n.s.; NO_3^- (U): LC 5.1 $\pm$ 0.4 v.s. C 3.9 $\pm$ 0.3 mmol/day, n.s.), while those in PVL rats significantly (nearly double) increased as compared to the SHAM rats (NO_3^- (S): PVL 41.1 $\pm$ 5.8 v.s. sham 22.8 $\pm$ 4.8 mM/L, $p < 0.02$; NO_3^- (U): PVL 17.7 $\pm$ 3.9 v.s. SHAM 9.4 $\pm$ 0.8 mmol/day, $p < 0.05$).

Eventually, these finding:; may indicate that the production of endogenous NO is augmented in rats with portal hypertension, particularly in PVL rats, and that the increased NO is responsible for the hyperdynamic state in these animals. It may be possible to think that the NO production is augmented in the acute phase of portal hypertension because amount of NO in both serum and urine was increased in PVL rats more than cirrhotic rats.

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Influence of tryptophan and methionine on the development of the ascites forms of Ehrlich carcinoma and sarcoma 180 in mice

After inoculation of cells from Ehrlich carcinoma or Sarcoma 180 to mice, the animals died after approximately 30 days. A single application of 50 mg endoxan did not prolong their lives. Up to 30% of the animals survived of tryptophan or methionine were additionally applied. An even higher percentage of survivals was achieved when these two substances were applied simultaneously. Even in the absence of endoxan, tryptophan as well as methionine prolonged the lives of the inoculated animals. Most effective was the simultaneous application of both substances.

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Effect of tryptophan plus methionine, 5-AZA-cytidine, and methotrexat on the adjuvant arthritis of rat

Application of complete adjuvant to rats induces an arthritis which can be reduced or prevented by a number of substances. In the frame of our studies on the genesis of the rheumatoid arthritis we are investigating the signal processes DNA methylation and adenoribosylation of proteins. We have analyzed the influence of a combination of tryptophan and methionine on the effect of 5-aza-cytidine and methotrexat on the development of arthritis. The latter two substances reduce the formation of arthritis. This effect is intensified by a combination of tryptophan plus methionine. If applied alone, these two substances show no effect on the development of adjuvant arthritis.

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The effect of ovariectomy on serum amino acids and cholesterol in the rat

As steroid hormones are known to influence amino acid metabolism we tested the hypothesis that ovariectomy should lead to significant changes in this system.

We found that after ovariectomy serum alanine was significantly decreased ($p = 0{,}0006$) in contrast to serum glycine and branched chain amino acids (BCAA).The ratio of glycine/BCAA, a parameter for anabolism or catabolism was not changed after ovariectomy. If, however, the amino acid alanine as the link to carbohydrate and lipid metabolism was introduced the alanine/BCAA atio was significantly altered ($p = 0{,}01$).

Although serum cholesterol was altered as well (increased, $p = 0.03$), no significant correlation with alanine was found. We can therefore assume that there are two independent mechanisms for lipid and amino acid changes af er ovariectomy.

The most prominent finding was that estradiol replacement after ovariectomy restored increased cholesterol levels but did not restore alanine levels. Other ovarial hormones must be incriminated for the regulation of alanine metabolism. The anabolic effects of estradiol as decreasing glycine and BCAA were noticed which rules out insufficient estradiol replacement.

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Leucine metabolism in tumor bearing rats with administration of insulin like growth factor-1 (IGF-1) versus insuline

Anabolic effect of IGF-1 was compared to that of insulin in tumor bearing rats, using 1-^{14}C-leucine constant infusion method.

Male Donryu rats (n = 24, BW: 150–170 g) were used in this experiment. On day 0, AHlO9A ascites hepatoma cells ($2*10^6$) were transplanted subcutaneously. The rats were fed a standard diet ad lib for 10 days. On day 10, the animals were catheterized for TPN under pentobarbital anesthesia and TPN was begun. Standard TPN solution was compcsed of 90% glucose and 10% fat as non-protein calorie scurces and amino acids mixture. 250 kcal/kg/day and 1.5 gN/kg/day were given as a full strength infusate. On day 14, the rats were assinged into 3 groups, 1) STPN, 2) STPN + IGF-1, 3) STPN + insulin. Either IGF-1 (4.5 mg/kg/day) or insulin (0.09mg/kg/cay) was dissolved in STPN solution and was infused continuously for 24 hrs. On day 15, 1-^{14}C-leucine was dissolved in each diet and was given by a constant infusion (2 μCi /hr). At the end of isotope infusion, the animals were sacrificed and muscle, liver, plasma, and tumor were collected. Intracellular leucine specific activity (SA) and protein bound leucine SA were measured to determine fractional synthesis rate (FSR, %/day), using Garlick's equation. Endogenous leucine production was calculcted by Steele's formula to evaluate whole body protein breakdowr rate (WPBR, μmole LEU/kg/hr). Data are mean (SEM), statistical calculation by ANOVA (Different superscripts indicate significant difference):

	STPN	IGF-1	INS.
Muscle FSR	9.0 (0.8)^a	13.4 (1.4)^b	11.3 (0.3)^{ab}
Liver FSR	111.1 (4.6)	107.5 (5.3)	111.9 (9.4)
Plasma protein FSR	31.2 (2.7)	30.8 (4.1)	31.4 (4.1)
Tumor FSR	86.0 (7.4)	85.0 (9.4)	92.5 (3.6)
WPBR	341.1 (24.0)^a	125.6 (33.6)^b	159.8 (35.6)^b

We concluded that IGF-1 is beneficial to enhance leucine incorporation into muscle compared to that of insulin in tumor bearing rats wlthout stimulation of tumor FSR.

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Changes in protein synthesis and S6 kinase activity in rat kidney and in hepatoma cells in hypoxic conditions

Regulation of protein synthesis is primarily controlled at the level of initiation by phosphorylation/dephosphorylation reactions. Phosphorylation of the S6 ribosomal protein of the small subunit of the eukaryotic ribosome is a proposed regulatory event of translational initiation (Jeno P. et al. Proc. Natl. Acad. Sci. USA 85:406, 1988). This phosphorylation is regulated by a family of specific serine/threonine kinases called S6 kinases (Ahn N. G. et al. J. Biol. Chem. 265: 11487, 1990).

In this work we tried to evaluate the role of S6 kinase(s) activity in conditions of impaired protein synthetic activity such as i) interruption of blood supply in rat kidney, and ii) subcutaneous in vivo growth of Yoshida ascites (AH-130) hepatoma cells in respect to the normal intraperitoneal growth. In both conditions a reduction in total protein synthetic activity was observed, being 40% of control values after 30 min ischemia in rat kidney and 30% in Yoshida ascites cells grown subcutaneously. The S6 kinase activity, measured in vitro using total cell extracts as previously described (Novak-Hofer I. and Thomas G., J. Biol. Chem. 259:5995, 1984), was, however, significantly increased in both conditions. In the ischemic kidney it was 2-fold over that of control kidney, while in Yoshida ascites cells it was 3-fold higher in cells growing subcutaneously than in cells growing intraperitoneally. These findings suggest that an increased proteolytic activity, already observed in tissues and cells subjected to hypoxic conditions (Bronk S. F. and Gores G. J. Am. J. Physiol. 264:G744, 1993), as well as an increase in intracellular free Ca^{++} (Larsson R. and Cerutti P. J. Biol. Chem. 263:1745, 1988) could be responsible for the activation of a specific S6 kinase(s), in cells and tissues subjected to stressful conditions. The activation of this enzymatic activity may be important for the prompt recovery of protein synthesis where the normal environmental conditions are restored.

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The role of amino acid metabolism in photosynthesis

The original Benson-Calvin Cycle of photosynthesis does not involve any molecules containing nitrogen. However it is now well established that for higher plants grown in air, amino acid metabolism is an integral part of the photosynthetic process. In C_3 plants a major portion of the carbon initially fixed by RuBP carboxylase/oxygenase is released during the process of photorespiration as CO_2, which is lost to the atmosphere. The photorespiratory carbon and nitrogen cycle involves transport of metabolites between the chloroplast, peroxisome and mitochondria and carries out the conversion of two molecules of phosphoglycollate to one of phosphoglycerate.

During the cycle two molecules ol glycine are converted to one of serine with the subsequent release of one molecule of ammonia and one of CO_2. Initially the cycle was proposed on evidence derived from ^{14}C, ^{18}O and ^{15}N-labelling data. The absolute confirmation that the photorespiration pathway involved amino acid metabolism was obtained followed the isolation of mutants of *Arabidopsis thaliana* and *Hordeum vulgare* that lacked key enzymes of the cycle. A full description of the characteristics of the mutant lines will be provided.

The C_4 photosynthetic mechanism requires in some species, that there is a transport of aspartate from the mesophyll cells to the bundle sheath cells with a concurrent exchange for alanine. Glutamate is also involved in a number of transamination reactions. Until recently no mutants of C_4 plants had been isolated. However we have now characterised mutants of *Amaranthus edulis* that lack PEP carboxylase, and others that contain elevated levels of glutamine and glycine. The consequences of these mutations for C_4 photosynthesis will be discussed.

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N-Myristoyl transferase activity in diabetic rats

N-Myristoyl transferase (NMT) is the enzyme that catalyses the transfer of myristate from myristoyl-CoA to the NH_2-terminal glycine residue of a number a proteins of diverse function. Many of the known myristoylated proteins are important in signal transduction. We have compared the activity of rat liver NMT from normal and from two rat models of diabetes mellitus. Streptozotocin (STZ) induced diabetes is a model for insulin-dependent diabetes mellitus (IDDM), or juvenile onset diabetes. STZ treatment resulted in a 1.5–2.7 fold increase in the rat liver NMT activity with the cAMP-dependent protein kinase derived peptide (1.5–1.7) or the pp60^{src} derived peptide (1.6–2.7) in all fractions of the cell. In each case addition of vanadate (an insulin-like agent) to the diet of the STZ-induced diabetic rats reduced

the activity of the NMT to 75–120% of the untreated control NMT values. The changes in NMT activity were not due to any variation in the apparent K_m for the peptide substrates. Non-insulin dependent diabetes mellitus (NIDDM) is a more common form of diabetes mellitus evolving from different metabolic failures, initiated by insulin insensitivity. NMT activity isolated from the particulate fraction of obese (fa/fa) Zucker rat (NIDDM model) liver was 4.7-fold lower than the corresponding activity observed in either the controls or the vanadate treated obese rat livers. This pattern was only observed in the particulate fraction; the homogenate and soluble NMT activities were not significantly different to the control levels. NMT activity isolated from the brain of the four groups showed no significant variations. Therefore, these results would indicate that the rat liver particulate NMT activity appears to be inversely proportional to the level of plasma insulin. Subcellular distribution of rat liver NMT activity was markedly different to that observed in rat or bovine brain. Briefly, brain contained more activity in the soluble (cytosolic) fraction, whereas overall, the liver contained equal amounts of NMT in the soluble and particulate fraction. However, the specific activity of the particulate liver NMT was approximately 4.7-fold higher than that of the soluble liver NMT, raising the possibility that NMT exists in 2 populations, with the active form of NMT residing in the membranous fraction. This situation could provide a system whereby myristoylation is regulated by the translocation of NMT from the cytosol to its active site in the membranes.

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Total plasma homocysteine levels in young patients with venous thromboembolism

Mild elevated homocysteine (HCYS) plasma levels are considered to be an indipendent risk factor for arterial occlusive vascular disease (AOD). The role of elevated HCYS in venous thromboembolism pathologies (VTD) has not been well established, although recent studies in vitro have suggested that HCYS may interfere with protein C activation by inhibiting thrombomodulin expression in human umbelical vein endothelial cells In relation to this requirement, we have developed a sensitive and accurate high performance liquid chromatographic (HPLC) method to quantify total plasma homocyst(e)ine [H(e)], characterized by a within-day and between-day CV% of 2% and 5%, respectively.

Mean plasma H(e) in 59 young patients (41 females and 16 males, mean age 33.5 ± 10.3 yrs) with a history of VTD and normal coagulation profile (normal PT, APTT, fibrinogen, antithrombin III, protein C, protein S, plasminogen and absence of lupus anticoagulant) was not significantly different than in 55 age-matched controls (12.4 ± 4.1 μM vs 11.3 ± 2.4 μM) In both VTD patients and controls, plasma H(e) was higher in males than in females (15.1 ± 4.2 μM vs 11.1 ± 2.4 μM, $p < 0.006$ and 12.7 ± 2.3 μM vs 10.2 ± 1.8 μM, $p < 0.001$). Eleven VTD patients (6 M, 5 F, 18%) and one control (2%) had plasma H(e) levels persistently higher than the 95th percentile of the normal distribution. Moderately increased plasma H(e) was also detected in the relatives of two VTD patients.

Serum vitamin B12 and folate were normal in all subjects with increased plasma H(e) levels.

Plasma H(e) levels were determined 8 h after methionine loading (0.1 g/Kg b.w.) in 18 controls and 24 VTD patients. The diagnostic value of this procedure was not superior to the measurement of baseline H(e) levels.

Thus, moderately elevated plasma H(e) levels, unrelated to nutritional deficiencies, may be observed in a substantial proportion of patients with VTD normal coagulation profile.

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Evaluation of muscle amino acids balance under clamp administration of fatty free acids and triglycerides

Skeletal muscle in man, because of its large mass and high protein content, represents the principal reserve of amino acids (AA). Aim of this study was to investigate the influence of an acute increase of free fatty acids (FFA) and triglycerides (TGR), under strict conditions of hyperinsulinemia, on muscle balance of 5 AA: alanine (ALA), glutamine (GLN), leucine (LEU), isoleucine (ILE) and phenylalanine (PHE).

Six normal subjects underwent three tests in random order. Each test consisted in a euglycemic hyperinsulinemic (25 mU/Kg/h) clamp (120–240 min) combined with: I Test) Intralipid 20% (2.5 ml/min) infusion in order to increase both FFA and TGR levels II Test) heparin (30 mU/min) during the basal period, and plus Intralipid 20% (0.5 ml/min) infusion during clamp to increase FFA levels. During both tests bolus of heparin (250 U) was administered at 0 min and subsequently at the beginning of the clamp period (120 min). The III test was carried out with physiological FFA and TGR levels.

After two hours of Intralipid and heparin infusions, a remarkable FFA level increase was observed during test I and II in comparison with test III (1.76 ± 0.36 and 1.84 ± 0.25 *vs* 0.47 ± 0.04 mM, $p < 0.01$). TGR level, increase was observed only in test I in comparison with test II and III (340 ± 42.2 *vs* 74.2 ± 15.8 and 70.4 ± 10.1 mg/dl; $p < 0.01$).

Amino acids serum levels were assayed with a o-phthaldialdehyde (OPA) pre-column derivatization reaction followed by HPLC analysis on RP-C18 column and fluorometric detection. During the three tests in the clamp period, arterialized levels (AL; μM) and muscle forearm release (MFR; μM/L/min) were (mean ± SE):

	TEST I		TEST II		TEST III	
	AL	MFR	AL	MFR	AL	MFR
ALA	196 ± 27§	23 ± 48§	205 ± 19§	66.1 ± 40.4	268 ± 29§	130 ± 17.1
GLN	552 ± 27§	99 ± 45	539 ± 34§	141 ± 24§	586 ± 31.3	192 ± 41.3
LEU	79 ± 5.5§*	20 ± 14	79 ± 5.4§*	18 ± 12.8	106 ± 7.8§	12.2 ± 12.6
ILE	38 ± 2.7§*	2.6 ± 3.5*	35 ± 2.8*#	17.4 ± 2	48.5 ± 1.8§	4.4 ± 5.2
PHE	34 ± 2.7§*	6.6 ± 3.6	36 ± 3.2§*	13 ± 7.6	49 ± 3.5	14.4 ± 5.1

§: $p < 0.01$ *vs* Control Period (0–120 min); *: $p < 0.02$ *vs* test III; #: $p < 0.05$ *vs* test I and III

These data suggest that while FFA levels in hyperinsulinemic conditions are able to decrease arterial branched AA and PHE levels, TGR seem not to play any role on the regulation of AA muscle metabolism.

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D-amino acids in chronic renal failure and the effects of dialysis

D-amino acids in humans are derived mainly from microorganisms, fermented foods (e.g. cheese) and beverages, and also shellfish. Their role in metabolism is not fully understood but their incorporation into proteins is probably confined to the lens, the teeth and the brain (i.e. d-aspartate). Despite the presence of d-amino acid oxidase in the liver, kidney, brain and plasma, most d-amino acids are excreted in the urine. D-alanine and d-phenylalanine can be metabolised relatively well. A previous study has shown that d-amino acids are increased significantly in patients with chronic renal insufficiency. The aim of this study was to measure total d-amino acids, d-tyrosine and d-phenylalanine in non-dialysed and dialysed patients.

80 individuals were investigated: 20 normals, 20 non-dialysed (creatinine clearance <12 ml/min), 20 on CAPD (continuous ambulatory peritoneal dialysis) and 20 on HD (haemodialysis pre and post), with equal numbers of men and women in each group. D-amino acids were measured using d-amino acid oxidase. D-tyrosine and d-phenylalanine were measured in 8 individuals in each group using a modified HPLC method with a C18 column and a Crownpack CR (+) chiral column, both at 10°C, using 100% pH 2.0 $HClO_4$ and 95/5% $HClO_4$/methanol. 2 μl of plasma ultrafiltrate was injected.

Total d-amino acids and d-tyrosine were significantly greater than controls in all three groups. Highest concentrations were in non-dialysed and HD patients with threefold increases of normal means which were: d-amino acids; 9.2 μmol/l and d-tyrosine; 0.9 μmol/l. Both d-amino acids and d-tyrosine were decreased significantly after HD. D-phenylalanine was also significantly greater for non-dialysed and HD patients than the normals (0.5 μmol/l). It was not decreased during haemodialysis. Plasma creatinine correlated with d-amino acids ($p < 0.001$, $N = 88$) and also d-tyrosine ($p - 0.003$, $N = 38$).

These results indicate that d-amino acids accumulate in chronic renal failure and they are only partially removed by dialysis. The most effective clearance was achieved during CAPD, particularly for d-phenylalanine. Total d-amino acids and d-tyrosine were related to the degree of uraemia. Some d-amino acids may have adverse effects such as immunosuppression, nephrotoxicity, growth inhibition etc. However, the possible toxicity of d-amino acids has yet to be investigated.

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Retinylidene amino acids as water-soluble forms of vitamine A

To obtain water-soluble preparations of vitamine A we synthesized a set of N-retinylidene amines, including polar amino acids derivatives (retinylidene-lysine, -taurine, -glutamic and aspartic acids). Retinylidene amino acids were synthesized via condensation of amino component with 3 M excess of retinaldehyde (*all-trans* and *11-cis* isomers). All the compounds were characterized both in forms of base and conjugated acid (hydrochloride).

The solubility of the aldimines was determined in water, water-ethanol mixture and physiological solution. The kinetic of aldimine bond hydrolysis in water based solutions was measured using UV-spectrometry and reverse-phase high performance liquid chromatography. According to the data obtained hydrolysis of C=N bond depends on retinylidene amine structure and water content in media. For the investigated compounds $t_{1/2}$ varies from 9.8 min (in water) till 61.5 min (in 15 M water-ethanol solution) for retinylidene lysine and from 5.0 min (in water) till 4,6 hours in (15 M water-ethanol solution) for retihylidene taurine.

Chemical searching of water-soluble retinylidene amino acids with vitamine A activity and various stability will be shown in this presentation.

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Isolation and characterization of the most antimutagenic Maillard reaction products derived from xylose and lysine

The Maillard reaction products were prepared by refluxing D-xylose and L-lysine at 100°C and pH 9.0 for 10 h and then fractionated into various molecular weight (MW) ranges by membrane filters. The most antimutagenic effect toward 2-amino-3-methylimidazo[4,5-f]quinoline was observed in the fractions of MW 30,000–50,000 and MW 50,000–100,000 toward TA98 and MW 50,000–100,000 toward TA100, whereas the fraction of MW 50,000–100,000 showed the strongest activity against 2-amino-6-methyldiprido[1,2-a:3′,2′-d]imidazole either toward TA98 or TA100 at the dose of 0.8 mg/plate. Fractions with MW above 30,000 showed inhibitory effect to 4-nitroquinoline-N-oxide and N-methyl-N′-nitxo-N-nitrosoguanidine on TA100. No significant correlations ($P > 0.05$) were observed between the antimutagenicity of each fraction and the browning intensity, reducing power and antioxidative activity. The fraction with MW 50,000–100,000 was further separated by Sephadex G-100 and HPLC, and the peak GIIa exhibited the strongest antimutagenic effect with molecular weight about 63,000.

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The regulation of chicken gastrin secretion

Chicken gastrin has a structure resembling mammalian cholecystokinin, but biological properties resembling mammalian gastrin. The mechanisms controlling chicken gastrin release are poorly understood. We have investigated the factors which influence chicken gastrin secretion. Tissue gastrin concentrations were not significantly changed after 24 hr of food deprivation, although plasma gastrin concentration was decreased within 12 hr of fasting. However, in birds fasted for 24 hr and treated with the H^+/K^+ ATPase inhibitor, omeprazole, plasma gastrin concentration was greatly enhanced. It is well established that amino

acids stimulate gastrin release in mammals and in most cases aromatic amino acids such as phenylalanine and tryptophan are good stimulants. In chicken, however, methionine was the strongest stimulant amongst the essential amino acids investigated. Aromatic amino acids did not strongly influence gastrin release. Medium chain triacylglycerol (MCT) is rapidly hydrolyzed to fatty acids in the lumen. MCT strongly stimulated gastrin secretion but long chain triacylglycerol had no effect. This suggests that fatty acids, but not triacylglycerol, are gastrin releasing factors in birds; acid inhibits secretion and methionine stimulates.

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Plasma and humor amino acids in senile cataract patients

We determined the concentrations of 24 free amino acids in aqueous humor and plasma of 12 patients with senile cataract and 12 healthy subjects. Amino acid analysis was carried out by reversed-phase high performance liquid chromatography after pre-column derivatization with O-pathalaldehyde. Concentrations of 19 amino acids in aqueous humor of the senile cataract patients were lower than those in their plasma. Among them 12 being lower at $P < 0.01$: Asp, Cit, 3-MHis, Tau, Try, Orn, Glu, Ser, Gly, Ala and Lys. The majority of them are hydrophilic amino acids. Whilst 7 other amino acids, most of them hydrophobic and including sulphur-containing amino acids have concentrations higher in humor than in plasma at $P < 0.01$: Cys, Tyr, Met, Gln, Val Phe and Leu. Possible relationship of these differences in amino acid concentrations with nutrition, blood-aqueous barrier and primary structure of proteins present in humor were discussed. Based on these findings a hypothesis on the pathogeneis of senile cataract was proposed.

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Profiling of plasma amino acids in cancer patients

We used HPLC to determine 24 free amino acids in fasting plasma from 63 patients with different types of cancer. Plasma amino acid of healthy adults were assayed as controls. The cancer patients have significantly increased ($P < 0.001$) in the concentrations of aspartic acid, glutamic acid, taurine, phenylalanine and ornithine relative to the controls. Our result suggest that they might be as tumor indicator once they were all in high contents simultaneously. It is also noted that the amino acid levels in lung cancer patients were greatly decreased ($P < 0.001$ or 0.01), except the above mentioned 5 amino acids. And amino acid concentrations in lung cancer patients were much lower than other cancer patients [except 3-methylhistidine, which was increased ($P < 0.05$)]. 10 amino acids in liver patients were higher than in the controls ($P < 0.001$ to 0.05), while citrulline and valine are below the controls. $\bar{X}$-values of the glutamic acid, tyrosine, phenylalanine and ornithine were excess normal ranges.

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Plasma alanine and lactate levels in obesity

Fasting plasma alanine and lactate levels in 13 obese subjects with normal glucose tolerance (NGT) test (0.56 ± 0.04 mmol/l and 1.42 ± 0.24 mmol/l, respectively) and in 12 diabetic obese subjects (0.68 ± 0.09 mmol/l and 1.50 ± 0.15 mmol/l, respectively) were similar although significantly higher than those observed in 19 control subjects (0.39 ± 0.03 mmol/l and 0.73 ± 0.08 mmol/l, respectively; $p < 0.05$). Because body mass index (BMI) was well correlated with insulin, lactate and alanine, a foreward stepwise multiple regression analysis was performed considering BMI (index of obesity) as dependent variable. The results show that for BMI, plasma insulin accounted for 48 per cent of the variance ($p < 0.001$), lactate added an additional significant contribution of about 12 per cent of the total variance ($p < 0.001$) and alanine added an additional significant contribution of about 6 per cent of the total variance ($p < 0.05$), whereas plasma glucose did not contribute significantly to the model. It is concluded that in obesity, independent of glucose tolerance, gluconeogenesis from alanine and lactate is increased in the fasting state in spite of high circulating insulin levels.

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Role of the ubiquitin system in human B-lymphocyte differentiation

We have previously demonstrated that the phorbol ester TPA induces the acute lymphoblastic pre-B leukemia REH cells, to differentiate to monocytoid B lymphocyte stage (MBL) *in vitro* (Am J Hematol 33: 153, 1990). The differentiated cells have monocytoid morphology, adhere to plastic, lose CD10, acquire CD22, CD11c, and tartrate sensitive acid phosphatase, and lose their proliferative capacity. To investigate the mechanism(s) of such differentiation-induction, total cellular proteins from untreated REH (control) and after 72h of TPA treatment were mapped using standard 2-dimensional polyacrylamide gel electrophoresis. Gels were stained by silver or Coomassie blue and analyzed by the PDQUEST computer system. Similarity was noticed in the great majority of the approximately 1100 protein spots resolved on each map. However, a spot with a molecular mass of ~33,700 dalton and a PI of ~5.38 (P34-5.4) was clearly detected in the TPA-treated cells but was below threshold of detection in the uninduced cells. To identify this polypeptide, cellular proteins were transferred from the 2D-gel to polyvinylidine difluoride (PVDF) membrane and stained with Coomassie blue. The P34-5.4 spot was then cut and the amino acid sequence determined on a 470A gas phase sequenator. The N-terminal of P34-5.4 showed the following sequence: MQLKPMEINPEM. Search of the Protein Identification Resource (PIR) database showed 100% homology of this sequence with that of UCH-isozyme L1 described by Day et al (Biochem J 268: 521, 1990). We have cloned the gene encoding for P34-5.4 from TPA treated REH cells using RNA-PCR. Sequence analysis shows it to be highly homologous and possibly identical to published UCH-L1 sequence. UCH-L1 is among a family of enzymes required for the generation of active monomeric ubiquitin from a polypeptide

pre-cursor and various ubiquitin fusion proteins. Ubiquitin was first described as a polypeptide that induces T- and B-cell differentiation in mice (PNAS 72:11, 1975). It is involved in the degradation of many proteins, including nuclear oncoproteins (PNAS 88:139, 1991), and may be involved in lymphocyte homing receptors. It is possible that ubiquitin regulation is one mechanism by which the phorbol esters induce B cell differentiation.

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The use of non-native and modified amino acids for defining collagen-mediated biological activities

Basement membrane (type IV) collagen can directly promote the adhesion and migration of various cells. Two sequences from the triple-helical domain of the α1 chain of human type IV collagen have been identified as cell adhesion promotion sites: residues 531-543 (Gly-Glu-Phe-Tyr-Phe-Asp-Leu-Arg-Leu-Lys-Gly-Asp-Lys) and 1263-1277 (Gly-Val-Lys-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Cly-Ala-Pro). By applying chemical mutagenesis via solid-phase synthesis to these sequences, the effects of collagen primary structure on cell adhesion and migration can be better defined. To allow for specific correlations of biological activity with protein sequence, analogs used for the substitution of amino acids should alter only a single property of the native amino acid, *i.e.* charge or steric effects. Ideally, isosteric amino acids would be substituted for charged residues to examine charge effects while conservative amino acid substitutions would examine steric effects. For example, the incorporation of a non-native amino acid such as norleucine (Nle) for Lys specifically determines charge effects, while steric effects are examined by a substitution such as ornithine (Orn) for Lys. The contribution of Pro toward secondary structural stabilization is evaluated by substitution of norvaline (Nva). In addition, the chiral preferences of cell surface receptors for these sequences are defined by utilizing D-amino acid analogs. This "chemical mutagenesis" approach was applied to linear (single-stranded) analogs of the α1(IV) 531-543 and α1(IV) 1263-1277 sequences. To ascertain the importance of collagen tertiary structure for cell adhesion, triple-helical 'mini-collagen' polypeptides [C. G. Fields, D. J. Mickelson, S. L. Drake, J. B. McCarthy & G. B. Fields, *J. Biol. Chem.* **268**, in press (1993)] incorporating the α1(IV) 531-543 and α1(IV) 1263-1277 sequences were synthesized. Quantitation of the cell adhesion promoting activity of greater than 30 collagen-model peptides and peptide analogs has led us to conclude that, in general: (i) cell adhesion is dependent on both the *N*- and *C*-terminal regions of the α1(IV) 531-543 and α1(IV) 1263-1277 sequences, and is *not* solely mediated by charge, (ii) the effects of amino acid substitutions on adhesion to the α1(IV) 531-543 sequence differ based on the cell type, and (iii) collagen primary, secondary, and tertiary structural contributions are all important for cellular activities. By further defining the mechanisms of cell adhesion to type IV collagen it may be possible to more fully understand tumor cell invasion and metastasis and allow for the development of novel therapeutics for diseases, wound healing, or biomaterials.

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Effect of cold exposure on rat hind leg muscle amino acid balance and pool composition

Female Wistar rats weighting 240-250 9, fed standard rat chow and maintained at 22°C, were chronically cannulated under ether anaesthesia in the muscular branch of the left iliac vein and in the right carotid artery, in order to draw hind leg venous and arterial blood. The cannulas were threaded under the skin and exited the rat through the back using a Harvard jacket. The animals were left to recover for two days and then exposed to the cold (4°C) for up to 2 hours. A first group of animals was used for the estimation of hind leg muscle and skin flows by injecting ^{45}Sc microspheres at 0, 15, 30, 60, 90 and 120 minutes. Other animals were used to draw blood, and a third group were sacrificed at the stated times and samples of hind leg muscle and skin were obtained, frozen and later used for measurement of free amino acid pools. Blood and tissue amino acids were estimated with an amino acid analyzer/ninhydrin method. During the first hour there was an increase in tissue pools, with a net uptake of amino acids peaking at 15–30 min.; muscle pool size decreased thereafter, coinciding with increased amino acid efflux from hind leg. The data for essential amino acids (excluding branched chain) followed this same pattern, allowing to establish the net minimal contribution of protein amino acids to the maintenance of free amino acid pools and amino acid export from hind leg. Hind leg contained about 0.17 mmol free amino acids, which changed less than 6 % with cold exposure. The net hind leg/blood balance was -2 nmol·s^{-1} for unexposed animals (0 time), and -11, -19, $+31$, $+15$ and $+11$ for 15, 30, 60, 90 and 120 min. respectively (positive values indicate net efflux, negative net uptake). Cold exposure implies the net release of amino acids from hind leg protein in the first 30–60 minutes. Since the pool size increased slightly, this efflux must be maintained though increased proteolysis (or decreased protein synthesis). This trend is afterwards reversed thanks to increased blood amino acid uptake and relative decrease in tissue pool size. This implies a net synthesis of protein. The results indicate that upon cold exposure, rat muscle net proteolysis increases initially to provide substrates for thermal homeostasis; when ample fat is made available, however, with the onset of cold acclimation, the trend is reversed and the muscle recovers its protein through increased uptake and synthesis.

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Cross-links and racemization in human and rat cataractous lenses

Water-soluble and insoluble fractions were prepared from the nuclear region of human senile nuclear cataract lenses (age >65 y) and spontaneous (Shumiya) cataract rat (SCR) lenses (age 33 w) to analyze the contents of tyrosine-derived cross-links (dityrosine, trityrosine and pulcherosine) and the isopeptide cross-link (γ-Glu-ε-Lys, catalyzed by transglutaminase) as well as the extent of racemization, D/(D + L) of each amino acid.

Each sample was divided into two portions, and one was hydrolyzed with 6N HCI at 110°C for 20 h and the other was exhaustively digested by successive treatment with pronase E, leucine aminopeptidase, carboxypeptidase Y and prolidase. The HCI hydrolysates were analyzed by reverse-phase HPLC with UV and fluorescence detectors connected in tandem, and by a ninhydrin-type amino acid analyzer. The protease digests were analyzed for the isopeptide cross-links by amino acid analysis with an elution program modified for better resolution of the cross-link. The HCI hydrolysates were also analyzed for racemization on the apparatus AminoMate that automatically derivatizes the amino acids to fluorescent diastereomer FLEC-derivatives, which were then separated and quantitated by reverse-phase HPLC.

The human and SCR lenses turned out to have very little, if any, tyrosine-cross-links and isopeptide cross-link, in contrast to the fertilization envelopes (FEs) of sea urchin and medaka fish, respectively. The dityrosine content in sea urchin FE was 2.1 mol/10^4 mol total amino acid, and the content of γ-Glu-ε-Lys in medaka FE was 80 mol/10^4 mol total amino acid. Both water-soluble and insoluble fractions of human senile nuclear cataract lens exhibited rather high extent of racemization: Asp (20–23%), Tyr (15–20%) and Met (10–17%). The extent of other amino acids ranged 2–9%. In SCR lenses racemization was not so high: Asp (7–10%), Tyr(8–10%), and Met (7–9%) with other amino acids ranging 2–8%.

The results suggest that the above cross-links are not the major cause of opacity and insolubility, but rather, the possibility of other types of cross-link, if any . The analysis of racemization revealed that not only Asp but Tyr and Met are also racemized to high extent, and the extent seems to mainly depend on the age of the specimen.

In conclusion the opacity and insolubility of cataract lenses seem to be originated from variety of causes and to be of various chemical nature.

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Release, conversion and transport of phenylalanine and related substances using in vitro models: effects of proteases, pepitdases and phenylalanine ammonia lyase

In phenylketonuria (PKU) mutations of hepatic phenylalanine hydroxylase necessitates a lifelong diet and control in order to maintain the low phenylalanine (Phe) levels known to be essential for normal development of PKU patients and fetuses of PKU mothers. Reduction of Phe in the intestine by oral intake of the yeast enzyme phenylalanine ammonia layse (PAL) has been shown to be a possible alternative to the strict diet. PAL converts free Phe to cinnamic acid and, at a lower rate, tyrosine (Tyr) to p-coumaric acid. One of the questions in relation to the use of PAL in PKU treatment, is how much peptide-bound Phe is absorbed directly from the intestine and possibly by-passed from PAL, in case di- and tripeptides are absorbed faster than free amino acids.

Methods: In order to establish some of the criteria for efficient Phe reduction, we have used in vitro models to investigate (1) release of free Phe and Tyr from casein and di-/tripeptides by pepsin, chymotrypsin (CT) and carboxypeptidase A (CPA) and (2) transport and conversion of Phe/cinnamic acid, Tyr, and some di-/tripeptides containing these amino acids using CACO-2 monolayers as intestinal cell model. For analysis, a new reverse phase HPLC-method based on a C-18 column, gradient elution and single wavelength UV-detection of aromatic amino acids and peptides, cinnamic and p-coumaric acids has been used.

Results: CT does not accept tripeptides with Phe or Tyr in the mid-position, but CPA can release these amino acids rapidly from the C-terminal. Dipeptides are not substrates for CT and CPA. Following a simulated casein hydrolysis (pepsin/HCl followed by CT/CPA) Phe/Tyr were equally distributed as free amino acids and in smaller peptides. In a subsequent incubation with PAL, conversion of Phe was significant but not complete. Using the CACO-2 monolayers, efflux and conversion of a number of peptides, free Phe and Tyr, and cinnamic acid—incubated in different concentrations on both sides of the cells—have been investiaged. Phe at 3.5 mM showed an efflux from the apical side of 2.5% over 2 hours, whereas cinnamic acid under similar conditions showed an efflux of 42%. It was shown that a high aminopeptidase activity was present on the apical side, forming Phe-Y from X-Phe-Y. Carboxypeptidase activity could not be detected on this side with Y = Tyr. No tripeptide hydrolysis was observed during incubation on the basolateral side.

Conclusions: Simple in vitro experiments have pointed out some of the substrate and enzyme requirements for conversion of Phe by PAL. A supplementary, more advanced model is the CACO-2 monolayers, which include at least some of the intestinal peptidases and transport mechanisms. The rapid efflux of cinnamic acid compared to free Phe may be favourable for the in vivo performance of PAL.

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BCAA metabolism in whole body irradiated rats. Effect of essential phospholipids

Introduction: Elevated levels of branched chain amino acids (BCAA) that are released from injured cells are a common finding in blood plasma after irradiation. It has been proved that the administration of essential phospholipids (EPL) protects the cell membranes. For this reason we can expect EPL to have some effect on BCAA metabolism in radiation sickness.

Experimental procedures: The adult male Wistar rats were injected with EPL in a dose of 125 mg EPL/kg b.w. (Essentiale, RPR Natterman) or saline solution. Three hours later whole-body gamma-irradiation with a single dose of 10 Gy was performed. The rats were killed 2 days after irradiation.

Results: In irradiated rats we found a decrease in body weight, an increase in plasma BCAA, higher icorporation of labeled leucine into liver proteins and higher oxidation of branched chain keto acids in liver mitochondria. In rats treated by EPL before irradiation we found a higher body weight, lower levels of BCAA in blood plasma and a higher proteinsynthesis compared with irradiated, saline treated rats. We did not find significant changes in BCAA utilization measured by means of labeled ketoisocaproate in liver mitochondria.

Conclusions: BCAA released from injured cells after irradiation are utilized primarily in proteinsynthesis in the liver. EPL injection prevents muscle wasting and derangements in BCAA metabolism in whole body irradiated rats.

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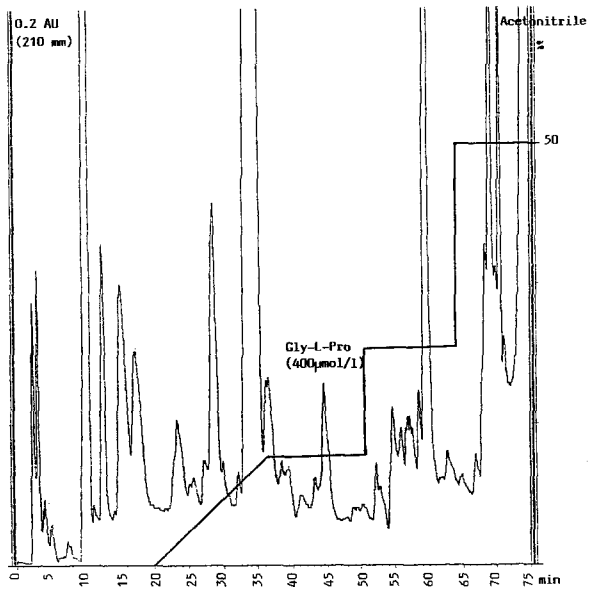
Prolidase deficiency and skin ageing

Prolidase deficiency is a rare inherited disease characterized by leg ulcers and an increase of a final product of the collagen degradation: the dipeptide Glycyl-L-Proline (Gly-L-Pro). We have observed in fibroblast cell cultures a correlation between the increase of Gly-L-Pro and the degradation of the collagen, mainly the newly synthesized collagen. Moreover, Gly-L-Pro could be measured in urine of patients (4 mmol/l) using an ion-paired reversed-phase HPLC and direct UV detection at 210 nm.



Ion-paired reversed-phase HPLC (ultrasphere ODS 5 μ-4.6 mm × 250 mm) Heptane sulfonate 19.8 mmol/l pH 2.3

Leg ulcers and collagen degradation are two manifestations of ageing. So we have tried to observe if a prolidase deficiency would appear during the evolution of ageing process using urine Gly-L-Pro as a marker.

With this purpose, the conditions of urine collection were defined to avoid Gly-L-Pro degradation. The addition of mercury-[(O-carboxyphenyl)thio] ethyl sodium salt (Thimerosal-Sigma) at 4.9 mmol/l final concentration was used for 24 hour urine collection. The sensitivity of the method was checked to detect a concentration as low as 50 μmol/l of Gly-L-Pro.

In these conditions, the urines from old people with trophic ulcers without vascular origin were studied. The appearance of an acquired prolidase deficiency during ageing will be discussed.

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Substrate specificity of immobilized *Pseudouonas* L-phenyalanine oxidase

Analysis of amino acids is important in clinical and industrial fields. An amperometric determination of various amino acids has been reported, but only L-Lys could be measured with relatively high specificity. *Pseudomonas* L-Phe oxidase catalyzes the oxidation of L-Phe, L-Tyr, L-Met, and L-Trp, but Km for L-Phe was much smaller than that for other amino acids. In this work, we immobilized the enzyme on nylon membranes covalently or noncovalently, and compared their substrate specificity, stability, and etc. By the immobilization of the enzyme, the substrate specificity became wider, especially by the chemical methods. Though the native enzyme showed high activity with L-Phe, the immobilized enzyme showed the activity similarly to L-Phe, L-Tyr and L-Met. Lowering the pH of the reaction medium, the covalently immobilized enzyme was active with L-Phe, L-Tyr, L-Trp and L-Met, but the noncovalently immobilized enzyme was active only for L-Phe and L-Tyr. The immobilized enzymes, especially the covalently immobilized, were stable over 40 days at room temperature at pH 7.0.

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Non-protein amino acids in *Canavalia ensiformis* and hepatic ornithine decarboxylase

The non-protein amino acid, canavanine, is ubiquitous in leguminous plants, occurring in concentrations of up to 51 g/kg dry weight in the seed of *Canavalia ensiforms* (jack bean; D'Mello, 1991). The toxicity of canavanine resides in its structural analogy with arginine, an indispensable amino acid for certain classes of animals. Toxicity may also result from the arginase-dependent degradation of canavanine to canaline, a structural analogue of ornithine and inhibitor of enzymes requiring pyridoxal phosphate.

Diets containing jack beans (JB) are particularly toxic to young chicks even after thermal inactivation of the deleterious lectin, concanavalin A, present in these seeds. Dietary supplementation with arginine only partially, alleviates the adverse effects on growth and food intake suggesting that residual toxicity may be associated with the ability of chicks to synthesise canaline from canavanine. The effect of canaline on the activity of ornithine decarboxylase (ODC), a pyridoxal phosphate-dependent enzyme, has not been reported. Accordingly, an experiment was conducted in which chicks were fed a control maize-soyabean diet or a similar diet containing JB included at 200 g/kg diet. Liver ODC activities were determined by the method of Bulfield *et al.* (1988). In chicks fed the JB diet, hepatic ODC activity declined to only 19% of that observed in control chicks. In a separate experiment, chicks were fed on a diet containing 150 g JB/kg. Analysis by HPLC of serum indicated a marked accumulation of ornithine with concentrations rising from zero in control chicks fed a maize-soyabean diet to 16 μm/100 ml in JB-fed chicks. Serum 3-methyl histidine (3-MH) levels rose from 1.7 μM/100 ml in control chicks to 6.7 μM/100 ml in JB-fed chicks. However, the latter results are tentative.

It is suggested that the adverse effects of JB emanate from at least two sources: inhibition of ODC activity, presumably arising from arginase-mediated catabolism of canavanine to canaline; secondly if the 3-MH results are confirmed, it may be possible that canavanine incorporation at the expense of arginine leads to the synthesis of aberrant tissue proteins with their subsequent degradation precipitating an increase in protein turnover rates.

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Interrelationships between rumen protozoa and bacteria in the metabolism of tryptophan

An *in vitro* study was conducted to examine the interrelationships between rumen protozoa and bacteria in the synthesis of tryptophan (Trp) and its degradation, especially to skatole (Skt). Skt is the causative agent of bovine pulmonary edema and emphysema. Rumen microorganisms were collected from fistulated goats. Suspensions of mixed rumen protozoa (P), mixed rumen bacteria (B) and their combinations (BP) were incubated with Trp, indolepyruvate (IPA) or indoleacetate (IAA; non-radiolabelled and [1-^{14}C]IAA) as substrates (1 mM each) at 39°C for up to 12 h and the concentrations of Trp, IAA, Skt and indole (Ind) in the incubation supernatants were measured by HPLC. When [1-^{14}C]IAA was added as substrate, however, radioactivities of Trp fractions in the microbial protein hydrolysates were also determined by a liquid scintillation counter.

The major metabolic pathways of Trp by rumen microorganisms were considered to be: Ind ← Trp ↔ IPA ↔ IAA → Skt. Skt production from Trp was hardly detectable in B and P suspensions, but was equivalent to 32% of the added substrate in BP suspensions. With IAA as the substrate, Skt was produced in suspensions of both B (20%) and BP (30%), but not in P. However, when IPA was the substrate, Skt production was limited to only 13% in BP suspensions and 0% in B and P suspensions. Furthermore, Skt production from IAA in B and BP suspensions was severely inhibited by the addition of IPA. Therefore, IPA, or its unidentified derivatives, appear to inhibit Skt production from IAA in B and BP suspensions.

In P suspensions, Trp production from IPA was equivalent to 12.5% of the added substrate, but Trp was not produced from IAA. Trp production from IPA indicates that the rumen protozoa have a high Trp aminotransferase (EC 2.6.1.27) activity. Thus the protozoa can rapidly metabolise IPA, if it is present, and convert it to Trp. It may be speculated that in BP suspensions, protozoa could decrease IPA concentrations produced from Trp by bacteria, and under such conditions Skt production from IAA by bacteria would not be inhibited. This may be the reason why Skt production from Trp was especially high in BP suspensions.

Trp production from [1-^{14}C]IAA in BP suspension was 1.81 times higher than that in B suspension when expressed per ml of the suspensions, but 1.13 times when expressed on a microbial nitrogen basis. This means that the ability of rumen protozoa to synthesise Trp from IAA is almost similar to that of the rumen bacteria when they coexis within the rumen.

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Effect of amino alcohols on stress tolerance in plants

Various crops (e.g.: cereals, potatoes) respond to leaf and root treatment with 2-aminoethanol or choline in field and greenhouse experiments with an increased stress tolerance.

Using 0.25–0.75 mg aminoethanol or choline per cereal plant under dry stress conditions the stress sensitivity of tillers (young shoots) was reduced.For this reason the number of fertile halms rose by 10–30%. An elevated content of ^{14}C labeled assimilates and an increased ^{15}N incorporation after amino alcohol application reflects the improved stress tolerance of the tillers. The influence of amino alcohols on the amino acid pattern in grain proteins of barley is subject of present investigations. Furthermore, amino alcohol treatment reduced the formation of the stress metabolite glycine betaine and trigonelline under water deficit and heavy metal influence. Under weak light conditions aminoethanol stabilized the chlorophyll content in Lemnaceae. The chlorophyll content in treated plants was about 50% higher than in untreated plants. We found a good uptake and translocation of ^{14}C aminoethanol and ^{14}C choline applied on crops. Two weeks after treatment with ^{14}C labeled amino alcohols about 20% of the starting compound were detectable in the cereal plants. Up to 24% of the ^{14}C label were found in the lipid fraction whereas 20–30% of the ^{14}C were detected as glycine betaine.

In field experiments over several years the efficency and economical importance of aminoethanol and choline was proved. Under dry conditions both amino alcohols (1.5 kg/ha) stabilized the biomass formation significantly. An official certification of these natural compounds in practical use is expected soon.

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Effect of amino alcohols on the betaine content in stressed plants

Stress is disturbance of the metabolic homeostasis and causes a switch of the metabolism towards stress metabolism. Hereby the plant aquires new opposed and better adjusted conditions which allow it better to get over subsequent stress periods. Considering the correlation between stress, stressor and plant the primary (physical) damage effect of the stressor (e.g.: to the plant water status) has to be seen separately from metabolic reactions of the plant which is in fact the adaption syndrome.This stress induced switch of the metabolism is energy costly, has a destabilizing effect, stimulates the senescence and reduces the gain of assimilates in the plant drastically. As a surviving reaction the adaption syndrome is in opposit to a high productivity, especially if the start is in the product formation stage. Therefore, measures reducing the adaption syndrome or delaying its start are interesting in particular for crops.

Indicators of stress induced disturbances of the metabolic homeostasis are stress metabolites. Especially the betains glycine betaine (GB) and trigonelline,good detectable by cationic HPLC, are signalising a stress status in the agricultural important Gramineae, Chenopodiaceae and Limnaceae. In summer barley we found a highly significant linear correlation ($r = 0.967^{***}$)

between the stress level induced by polyethyleneglycol and the GB-accumulation. Low soil water contents lead to a drastic GB-accumulation ($r = 0.861^{***}$) supporting the indicative value of these compound. The detectable missing parallelism between GB-accumulation and leaf water content indicates that wilting does not signalize a stress status for sure. Therefore, parameters of the plant water status can only be used limited as stress indicators.

By pre-treatment of plants with the toxicologic safe amino alcohols (2-aminoethanol and choline), occuring in plants natively, the start of the adaption syndrome can be reduced significantly. Summer barley plants treated with 2-aminoethanol or choline and subsequently exposed to dry stress showed a drastic reduced GB-accumulation. The GB-content rose in the untreated control plants from 35 μmol/g dry weight to 70 μmol/g dry weight ,whereas in the plants pre-treated with aminoalcohols only a gb-content of 50 μmol/g dry weight was detectable. Similar results were found in flax using trigonelline as stress indicator. Therefore, amino alcohols are able to change the stress disposition of the plant to the effect that the stressor is not recognized as stress, that means the acting stressor does not or only diminished start the adaption syndrome. These partly toleration of the stressor has a productivity stimulating effect, because each disturbing of the metabolic homeostasis is destabilizing and reduces the productivity. Pot and field experiments over several years under controlled stress or natural conditions showed that water deficit related yield losses can be reduced significantly by application of amino alcohols.Clear recognizable analogies to the action of gibberellins and cytokinins are subject of further investigations.

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Proline content and β-amylase activity as stress indicators in plant tissues

Barley chloroplasts are known to contain α-amylases, and phosphorylases capable of degrading starch. But at least, the major part of amylolytic activity in leaf cells (more than 95%) is located outside the chloroplast. It was found that the majority of this extrachloroplastic activity is exo-(β)-amylolytic. The purpose of this paper was to evaluate the function of the cytosolic amylases in green and white primary barley leaves of the "albostrians" mutant and also in Norflurazon-bleached leaves under stress conditions in relation to the free proline content as stress indicator.

In green control leaves under light (10 W/m^2) three fractions (I, II, III) with amylolytic activity could be resolved by anion exchange chromatography. Under the same conditions white leaves showed an activity in the three amylases 5 to 7 fold higher than in green leaves. In the dark the total amylolytic activity in this leaves is unchanged.

High light intensity (100 W/m^2), increase of growth temperatures and also a decrease of water potential by addition of sorbitol or NaCl caused a further increase on the total amylolytic activity in both white and green leaves. This activity increase was particularly due to an increase in fraction-III-amylase. Measuring the free proline content in different plant species the scale of salt tolerance can be characterized by a critical concentration of NaCl. At this NaCl concentration (in barley leaf cells at 150 mmol, in rye leaves at 95 mmol) the content of free proline rised dramatically. Under the same condition at this point the fraction-III-amylase activity is enhanced up to 60% of the total activity (normaly 35–40%) while the fraction-I-activity decreased at this salt concentration.

In white leaves the critical salt concentration is higher than in the control plants while the proline content increased moderately. In this case the fraction-III-amylase increased only at 350 mmol. Chlorophyll-less leaves are more resistent against a second stress than green control plants.

It was assumed that the increase of amylolytic activity (basing on increase of the β-amylase protein) and also of the free content of proline in the cytoplasmic compartment are a stress response to different stress factors both in white and green leaves. The inhibition of chloroplast differentiation by bleaching through Norflurazon or mutation may cause an internal stress leading to an increased activity of cytosolic enzymes such as β-amylases under light. Other stress factors caused a further increase of the amylolytic activity.

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Free amino acids determination in bile concrements treated with surface active substances

The file concrements are different from the kidney stones in their chemical composition and physical structure. Chemically they are generally cholesterol type, bilirubine type, and compound type. They have a specific structure without any expressed nucleus. The average content of albumins in their structure is approximately 1.5%.

We investigated the presence of free amino acids in 27 file concrements. The Thin Layer Chromatography, UV spectral analysis and electrophoresis were used. By means of Rf values comparison we determined the appearance of glycine, cysteine, phenylalanine, tryptophan and some lowmolecular albumins.

It is interesting to note that cystine was found in bilirubine concrements and never in cholesterol ones.

The resolving effect of non ionogenic surface active substances on file concrements was carefully studied with special attention to our originally developed medicine "*Pharmalit*" (USA Patent No. 4816483 from 1985; Canada Patent No. 214993; European Community Patient No. 0150248). In the case of *Pharmalit* application, which is also a surface active substance, a combined effect was observed: the formation of water soluble cholesterol complexes and solution of the free amino acids. Separately we have observed a surface tension reduction of the gall secretions treated with *Pharmalit*.

Obviously the presence of *Pharmalit* reduce substantially the probability of file concrement formation.

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Amino acids content in kidney concrement nucleus determined by thin layer chromatography

Within the matrix hypothesis the kidney concrement formation begins when certain glycoprotein sediments form a nucleus around different chemical salts.

To verify that, we investigated 36 kidney concrement nuclei, taken from kidney stones with different chemical compositions: oxalates, phosphates, urates, and others. The method of Thin Layer Chromatography and UV spectroscopy were used to find the appearance of amino acids and low molecular albumin in

these nuclei. The types of the amino acids were determined and compared with standard ones by means of their Rf values.

A dependence between the chemical composition of the kidney concrements and the amino acids in their nucleus was established.

The effect of our originally developed medicine "*Pharmalit*" (USA Patent No. 4816483 from 1985; Canada Patent No. 1214993; European Community Patent No. 0150248) on the kidney concrements and on the amino acids in their nucleus was investiaged. It was shown that not only the concrements themselves, but the amino acids in their nucleus are also soluble in *Pharmalit*.

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Why does cultured prawn, *Penaeus monodon*, grow faster in brackish water? – An approach from amino acid availability

Black tiger prawn, *Penaeus monodon*, is the most widely cultured marine shrimp. Farmers raise them in brackish water (<15 ‰) because they grow faster than those raised in sea water. Study on the free amino acid composition of the prawn showed that free amino acid in hemolymph varied greatly in different salinity. Yet the composition in muscle was quite stable despite the change of salinity. Detailed analysis revealed that free amino acids such as glycine, proline, taurine, alanine and arginine were used as major organic osmo-effector in the shrimp. They were transported from hemolymph to muscle when the environmental salinity was high (45 ‰). On the other hand, essential free amino acids for crustaceans such as leucine, lysine, phenylalanine, valine, histidine showed a much higher concentration in hemolymph of the shrimp under low salinity (15 ‰) than that under higher salinity. It is suggested that the availability of free amino acid for the growth of shrimp is not only a problem of feed formulation as in traditional aquaculture, but also worth to consider the nature of osmoregulation of aquatic invertebrates who use organic molecules as part of their osmo-effectors.

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The physiology of D-amino acids in marine organisms

At least three important criteria must be met to support a natural physiological role for unusual compounds such as D-amino acids: wide occurrence in a variety of species, transport across cell membranes and metabolism by specific enzymes. We have examined over 100 species of marine invertebrates from 9 phyla and have shown that free D-amino acids occur in the tissues of about 40% of them. The presence of D-amino acid was detected using an enzyme coupled assay employing D-amino acid oxidase (DAO). These data were confirmed for selected species from 8 phyla using chiral HPLC. For example, the gill tissue of the mollusk, *Mya arenaria*, contains 63 mM D-alanine and the hemoglobin containing coelomocytes (RBCs) of the annelid, *Glycera dibranchiata*, contain 1.4 mM D-alanine. D-Aspartate, D-glutamate, D-serine as well as D-alanine were detected in other species. We have also shown that ^{14}C-D-alanine is transported and metabolized in a variety of species. A comprehensive kinetic analysis of D-alanine and D-leucine transport by the RBCs of *Glycera dibranchiata* revealed that D-alanine is transported by a specific high affinity Cl-dependent D-stereoselective system (Kt = 0.17 mM, J_{max} = 203 μmol · min^{-1} · l · cell water^{-1}) and a low affinity (Kt = 9.9 mM, J_{max} = 1069 μmol · min^{-1} · l · cell water^{-1}) system. D-Leucino is transported with low affinity (Kt = 6.5 mM, J_{max} = 3230 μmol · min^{-1} · l · cell water^{-1}) by an L-amino acid preferring Na-independent system that resembles system "L" common in higher animals. Preliminary studies with other marine invertebrates suggest tht ability to transport D-amino acids is common in other organisms. Chromatography of ethanol extracts of animals incubated for 6 hr with ^{14}C-D-alanine in artificial seawater containing antibiotics showed that at least 60% of 44 species in 8 phyla were capable of metabolizing D-alanine. Assays for specific enzymes revealed that DAO, D-aspartate oxidase, D-alanine and D-aspartate aminotransferase activity was undetectable in most species. However, apparent racemase activity was detected in 12 species from 5 phyla using a coupled enzyme assay. Detailed studies using partially purified racemase from *Mya arenaria* gill showed that this enzyme was selective for alanine. Eight other neutral amino acids were poor substrates for this enzyme. For D-alanine the apparent Km (measured in the L-alanine → D-alanine direction) was about 9 mM. In summary, these data strongly support the conclusion that D-amino acids have a natural physiological role in transport and metabolism by marine invertebrates.

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Chemosensory abilities and pathways in the nudibranch gastropod, *Phestilla sibogae*

The larvae of the nudibranch gastropod, *Phestilla sibogae* hatch from eggs in about 5 to 7 days; these larvae are small (200 microns), shelled, and planktonic. Two to four days after hatching, when they become capable of metamorphosis into juveniles, they are referred to as competent larvae. The stimulation required to initiate metamorphosis is provided by the presence of an inducer substance that is produced and released by the prey organism of the adult animal, species of the coral genus *Porites*. The inducer substance is known to be a small (less than 500 MW) polar organic compound. It is apparently detected by the larvae using receptor cells located in the cephalic sensory organ located medially within the velar lobes. Unfortunately, exploration of the larval chemosensory system has proven to be extremely difficult due to the small size and active nature of the larvae. So we have moved on to the adult nudibranch with the hope that, since the adult animal has to find its food, it might have similar receptors and chemosensory abilities. However, despite the extensive use of opisthobranch molluscs over many years as neurobiological models, the chemosensory structures and abilities of these animals is largely unexplored, particularly for nudibranchs. The adult *Phestilla sibogae* is 2–3 centimeters long and its chemosensory structures probably include both the paired oral tentacles, which extend laterally from the plane of the animal's mouth, or the paired rhinophores, which extend upward from the dorsal surface of the head. These structures were tested for their sensitivity to coral extract and amino acids with the use of extracellular recordings from the cut ends of oral tentacular and rhinophoral nerves. The oral tentacles showed no response to perfusion with *Porites* coral extract or high concentrations of aspartic acid.

Similar recordings from rhinophoral nerves showed a positive response to coral extract as well as amino acids, with the greatest sensitivity is aspartic acid, but with some sensitivity to glutamic acid, arginine, and GABA. Serial sections of the rhinophore and rhinophoral nerve revealed the presence of likely receptor cells on the surface of the rhinophore, as well as other cell bodies just underneath the epithelial surface. The latter may synapse with the receptors and form the axons that travel down the length of the rhinophore. Serial sections have also revealed the existence of rhinophoral ganglia imbedded in the paired rhinophoral nerves near their attachment with the cerebral ganglia. Extracellular recordings from the rhinophore ganglion cells indicate that they receive information from rhinophoral receptors. Intracellular recordings provide evidence that they have inexcitable cell bodies, and are sensitive to aspartic acid and coral extract. When filled with Lucifer Yellow axons can be seen extending from the ganglion cells to the cerebral ganglia where they make connections with cells on the anterior aspect of the cerebral ganglia. This region also contains cells sensitive to aspartic acid. Similar peripheral ganglia have not been located for the oral tentacles.

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Glucose- and heparin- induced alterations of human α1 antitrypsin

It has been observed that in insulin-dependent diabetes mellitus (IDDM) both functional activity and the plasma concentration of the main protease inhibitor α1 antitrypsin (α1AT) is reduced whereas plasma serine protease activity is increased. These alterations have variously been associated with increased susceptibility to vascular complications in IDDM independently of metabolic control. An anomalous α1AT has recently been purified from plasma of IDD patients which shares the properties of a serine protease. Since the plasma was purified by heparin affinity chromatography, an attempt was made to test whether glucose and heparin may directly affect the structural and functional properties of human α1AT. The addition of 15 mM glucose (a concentration often found in the plasma of diabetic subjects) to a 250 μg/ml (3.85 μM) α1AT solution yielded a significant increase in fluorescence emission at 330 nm (exc = 295 nm) compared to the control (α1AT alone). An even more significant increase in peak fluorescence emission was observed after the addition of either heparin (5 μg/ml) or heparin plus glucose (15 mM). The functional activity of α1AT measured by the ability to inhibit the esterolytic activity of trypsin on Tos-Arg-OMe, showed that the trypsin inhibition by α1AT was markedly reduced by the addition of heparin plus glucose at trypsin concentrations ranging from 0.5 to 2.0 μg/ml. The immunological properties of α1AT were also found to be altered mostly in the presence of heparin plus glucose, the ring of precipitation between α1AT and its antibody being decreased by 30% and 46%, respectively, compared to the control, in the presence of either glucose or glucose plus heparin. SDS gel electrophoresis confirmed that both number and mobility of α1AT bands were altered after the treatment which also caused changes in the amino acid ratios for Gly, Leu and Pro.

It is concluded that the binding of glucose and heparin to α1AT causes significant and rapid structural alteration of the molecule, involving both the reactive center and antigenic sites on the inhibitor. The results pose the question as to whether heparin can further impair functioning of the protease inhibitor in hyperglycaemic conditions.

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. – Herausgeber: Prof. Dr. Gert Lubec, Universitäts-Kinderklinik, Währinger Gürtel 18–20, A-1090 Wien. – Redaktion: Sachsenplatz 4–6, A-1201 Wien. – Satz und Umbruch: Asco Trade Typesetting Limited, Hong Kong; Reproduktion und Offsetdruck: Novographic, Ing. W. Schmid, Maurer-Lange-Gasse 64, A-1238 Wien. – Verlagsort: Wien – Herstellungsort: Wien.
Printed in Austria.