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Abstracts

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Analysis

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Rapid hydrolytic techniques for food and feedstuff amino acid analysis

Analysis of amino acids in foods and feedstuffs is essential for commercial production of infant foods, dietary foods of defined chemical composition, animal feeds and petfoods. Also in other areas amino acid data is required to support nutritional labelling claims.

The analysis of free amino acids in solution is carried out accurately, rapidly and routinely by ion exchange chromatography using a system such as the Pharmacia Biochrom 20. However, the hydrolysis step is severely rate-determining requiring 20–24 hours to complete even using streamlined methods developed by collaborating laboratories within the EC.

Acceleration of this step using microwave heating would appear to be an attractive possibility but there is little evidence of application to protein mixtures. Therefore we have laid the foundations for a systematic study on feed ingredients containing a range of protein contents in the presence of different levels of carbohydrate and lipids. The materials were hydrolysed in screw top pressure containers of polymeric construction for 5–45 minutes. The samples were then neutralised and filtered and analysed on the Biochrom 20 analyser without further preparation. The yields of amino acids were comparable to those after 23 hours hydrolysis, indicating that this could be the basis of a method for obtaining a complete amino acid profile for urgent protein samples.

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Identification and quantitation of cysteine residues in proteins and peptides using volatile reducing and alkylating reagents

Identification or quantitation of cysteine residues in proteins and peptides requires stabilization of these residues. The most common technique used to stabilize cysteines is alkylation of the sulfhydryl group. However, alkylation requires repurification or desalting of the modified protein or peptide prior to analysis or sequencing to remove excess reagents. Repurification steps are frequently time consuming and lead to losses of sample. We describe here reagents for the alkylation of cysteine that do not require repurification or desalting. By use of triethylphosphine as reductant, bromopropane as alkylating reagent and triethylamine as base, we have alkylated cysteines in peptides and proteins. Since all of these reagents are volatile, alkylated peptides and proteins need only be vacuum dried prior to subsequent analysis steps. Peptides and proteins have been acid hydrolyzed and analyzed on an amino acid analyzer with recoveries of cysteine within 10% of the expected values. Peptides and proteins have been alkylated with bromopropane and the alkylation mixture applied directly to sequencer membranes. Excess alkylating reagent is removed simply by normal drying of the sample. The ease of this procedure allows the routine analysis of cysteine in peptides without additional, time consuming repurification or dialysis steps.

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Oxidation of cysteines and methionines following acid hydrolysis in the presence of sodium azide. A method for quantitation of cysteine residues in proteins

Sodium azide is widely used as a bacteriostatic agent during down-stream processing of proteins. Amino acid composition analysis of protein samples hydrolyzed with hydrochloric acid in a buffer containing sodium azide revealed the presence of cysteic acid, methionine sulfoxide and methionine sulfone in addition to the expected reaction products. Hydrolysis with methanesulfonic acid in the presence of sodium azide resulted in detection of only methionine sulfoxide in addition to the expected products. When sodium azide was absent or removed by dialysis, no oxidation products were detected after hydrolysis (except for minor amounts of methionine sulfoxide). The generation of each oxidation product was affected by the concentration of sodium azide in the protein solution. Hydrolysis with hydrochloric acid in the presence of 0.2% sodium azide resulted in 87 to 100% oxidation of the cysteines to cysteic acid. The results were reproducible so that protein hydrolysis in 0.2% sodium azide can be applied as a quantitation method of cysteine residues. The sodium azide based oxidation is superior to oxidation by performic acid in that (i) it is simpler as it is performed in solution not requiring protein lyophilization; (ii) it is

faster, as it is accomplished in approximately half of the time; (iii) it delivers slightly higher yields in cysteic acid and (iv) it does not affect tyrosine residues, which are almost completely destroyed during the performic acid treatment.

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Amino acid analysis for determination of peptidoglycan types of Gram-positive bacteria

The structure of cell wall peptidoglycan is of high significance for classification and identification of Gram-positive bacteria. Peptidoglycan types of actinomycetes are in good agreement with the phylogenetic relationship as determined by 16S rRNA sequence comparison. Structure elucidation of peptidoglycan is mostly accomplished by qualitative and quantitative analysis of amino acids and peptides in cell wall hydrolysates. False identifications of peptidoglycan types reported in the literature are mainly due to incorrect or insufficient amino acid analyses.

Whereas cell wall amino acids have been analyzed earlier predominantly by paper or thin layer chromatography, the application of gas chromatography (GC), high performance liquid chromatography (HPLC) and mass spectrometry (MS) opens up new possibilities for the determination of peptidoglycan types. As unusual non-proteinogenic amino acids like i.e. homoserine, 2,6-diaminopimelic, 2,4-diaminobutyric and 3-hydroxyglutamic acid as well as hitherto not observed components may occur in bacterial cell walls MS methods are very helpful for identification. By electrospray ionization MS amino acids in cell wall hydrolysates can be identified without any derivatization. N-Heptafluorobutyryl amino acid isobutyl esters are suitable derivatives for the quantification of cell wall amino acids by GC and for the identification of unknown components by GC/MS. The discrimination between certain peptidoglycan types necessitates the derivatization of enantiomeric amino acids with chiral reagents and separation of the diastereomeric products by reversed phase HPLC. Because the crosslinkage of peptidoglycan is usually incomplete the N-terminal amino acid of the interpeptide bridge can be determined by 2,4-dinitrophenylation. The sequence of the interpeptide bridge is concluded from the occurrence of characteristic peptides in the partial hydrolysate of peptidoglycan.

By combination of analytical approaches for identification and quantification of amino acids and their stereoisomers and for determination of their sequence peptidoglycan types of Gram-positive bacteria can be reliably determined and novel peptidoglycan structures can be elucidated.

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Capillary gas chromatography in multicomponent amino acid analysis. Evaluation and comparison of tert.butyldimethylsilyl and alkoxycarbonyl alkyl ester derivatives

Derivatizations with N-methyl-(tert.butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) [1] and alkylchloroformates [2] have been reported to be two particularly potent approaches to the multicomponent gas chromatographic analysis of amino acids and related classes such as hydroxycarboxylic and fatty acids and amino alcohols. A set of more than seventy protein and nonprotein amino acids and related compounds has been subjected to the tert.butyldimethylsilylation and chloroformate derivatization procedure. Factors influencing the reaction yields such as effect of reagents, solvents, catalysts and reaction temperature have been investigated. Particular attention has been devoted to the amino acids being "troublesome" from the view of the GC analysis, namely those possessing guanidino, amido, ureido and N-methyl moiety. Chromatographic behaviour and mass spectral data of the studied compounds have also been reviewed and the two derivatization approaches compared.

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Automatic derivatisation and determination of amino acids

Many years of experience have demonstrated the importance of the right amino acid profile for optimum animal performances. Corn, soybean meal, alfalfa cubes and barley protein samples were acidhydrolyzed and analyzed for amino acid content by reverse phase liquid chromatography (LC) and by conventional ion-exchange chromatography (IEC) using an amino acid analyzer. The concentration of amino acids in animal feeds was determined using an automated data acquisition system and pre-column o-phtaldialdehyde derivatization and fluorescence detection.

In general, comparing the LC and IEC methods, amino acid contents of the different feedstuffs were close to each other and both agreed well with published amino acid composition data.

The LC method showed the following advantages: shorter retention times, narrower and better resolved peaks, more stable baseline and a lower detection limit, but a slightly lower repeatability for some amino acids.

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Development of a routine method for selenomethionine determination in selenium yeasts

There exists an increasing interest to determine selenomethionine (SeMet) contents routinely in selenium yeasts which are commercially available from different pharmaceutical companies as effective agents for selenium supplementation and as potential anticarcinogens. First, chromatograms of SeMet samples from different suppliers were performed with an Eppendorf/Biotronik (Maintal, Germany) LC 3000 IIPLC system, equipped with a BT 2410 ion exchange column (125 x 4 mm), a gradient former using 5 different buffers [A to E, pH 2.85 (A),

3.30 (B), 4.25 (C), 8.00 (D), 10.30 (E)], a ninhydrin post-column derivatization unit and a two-channel filter photometer (440 and 570 nm). From the SeMet samples of the different sources investigated, only one was found to cause a single peak in the chromatogram which then was used as a standard for the following investigations. It could be demonstrated that almost no decomposition of the pure SeMet takes place treating the sample under conditions used for protein hydrolysis of different selenium yeasts (6N, HCl, 110 °C, 24 h, anaerobic conditions).

For the determination of SeMet in animo acid mixtures received after hydrolysis of different proteins, the standard buffer elution program used for conventional amino acid analysis had to be optimized using the following buffer sequence: C (24 min), D (18 min), E (6 min), C (13 min). Using this program, a concentration/peak area linearity curve was achieved for quantitative determination of SeMet concentrations with a detection limit of about 10 nmol/mL. Based on these results in hydrolysates of selenium-enriched yeast samples up to 2 µg SeMet/mg yeast could be determined.

Basic Chemistry

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Albumin release from thermoplastic biodegradable hydrogel composed of poly(γ-benzyl L-glutamate) and poly(ethylene oxide)

Multi-type block copolymers composed of poly(γ-benzyl L-glutamate) (PBLG) as the hard block clusters of crystalline nature and poly(ethylene oxide) (PEO) as the soft hydrophilic blocks of random coils were synthesized by polymerization of γ-benzyl L-glutamate N-carboxyanhydride initiated by primary amino groups located at tetravalent PEO chain. From infrared spectra measurement, it was found that the polypeptide block exists in the α-helical conformation, as in PBLG homopolymer. The intensity of wide-angle X-ray diffraction patterns of the block copolymers depends on the PEO content and shows basically similar reflections as the PBLG homopolymer. The morphology examined by transmission electron microscopy and differential scanning calorimetry revealed microphase-separated structure. Water content of the block copolymer increases with increasing content of PEO in the block copolymer due to the hydrophilicity of PEO. The released amount of albumin from the block copolymer in the phosphate buffered saline (pH 7.4) increased with increasing content of PEO in the block copolymer. Albumin release from the block copolymer was governed by the pore diffusion mechanism.

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The behaviour of asparagine as ligands of cations

The here described investigation reports the behaviour of asparagine as ligand towards cadmium(II), cobalt(II), iron(II), lead(II), nickel(II), zinc(II), calcium(II) and magnesium(II). Such cations are frequently present in food and are considered dangerous under the nutritional point of view, but in many cases even playing a therapeutical role.

The equilibria taking place in solutions containing asparagine and the above reported cations are studied at 25 °C and in 1.00 M NaCl.

In the case of cobalt(II), iron(II), nickel(II), calcium(II) and magnesium(II), the electromotive force (e. m. f.) measurements of galvanic cells with a glass electrode were performed.

The free concentration of cadmium(II), lead(II), and zinc(II) was measured by means of suitable amalgam electrodes and on the same solutions the free hydrogen ion concentration was also measured by using a glass electrode.

Experimental data could be explained by assuming the presence of mononuclear complexes in the cation, of the type ML_n. However in the case of lead(II), complexes with participation of protons ($PbHL$ and PbH_2L_2) were also formed.

Calcium(II) and magnesium(II) form only the species CaL and MgL and the latter is more stable than the former.

All the other cations from complexes in the ratio 1:1 and 1:2, between cation and asparagine. Cadmium(II) and zinc(II) form complexes 1:3 too.

Species and relative stability constant values found for asparagine can be compared with those proposed for glycine and other amino acids.

From the stability constant values obtained for cobalt(II), nickel(II) and zinc(II) the formation of a five membered ring can be suggested.

It seems reasonable to suppose the participation of aminic nitrogen and carboxylic oxygen to bond the cation. On the contrary the amidic group of asparagine does not participate to the complex formation. It represents even a steric impediment responsible of the less stability of the complexes with respect to the corresponding species formed by glycine.

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Specific phosphonamidate hapten-recombinant antibody recognition

Our goal is to produce antibodies displaying catalytic activities in peptide synthesis. A selection of antibodies was performed using different structures mimicking the transition state of the target reaction (coupling of peptide fragments). These analogues with a phosphonamidate moiety are specifically recognized by antibodies fragments (Fab) originated from a naive library.

Binding studies have shown that the recognition could occur very specifically in localized regions of the different haptens. More precisely, 2 Fab's are able to recognize specifically the tetrahedral phosphorus atom present in the hapten structure.

This study has shown the strong potential for recombinant antibodies to discriminate within the molecular structure.

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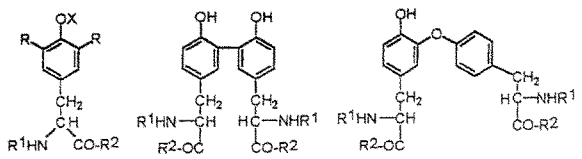
Oxidative coupling of tyrosine derivatives

The phenolic amino acid tyrosine as well as its (ring) substituted derivatives **1** can be oxidized at the phenolic OH to give phenoxenium ions **2** (two-electron oxidation) or phenoxy radicals **3** (one-electron oxidation) [1].



1 : X = H
2 : X = +
3 : X = •

4

5

For R = *t*Bu stable phenoxy radicals have been produced (oxidation by PbO_2, phenyliodosobis(trifluoroacetate) (PIFA) and investigated by ESR-spectroscopy. These free radicals are potential spin-labels.

For R = H phenoxy radicals are not persistent. They can dimerize to dityrosines **4** or isodityrosines **5**, structures which occur in several natural products, e.g. vancomycine antibiotics [2] and some antitumor hexapeptides [3]. Dityrosines also result from radiation of proteins [4].

The dimerizations can be performed electrochemically (carbon anode, acetonitrile, E = 1300–1400 mV vs Ag/0.01 M Ag^+) or chemically (PIFA, VOF_3, $K_3[Fe(CN)_6]$, cerium(IV)-ammoniumnitrate). Also oxidations with horseradish peroxidase/H_2O_2 (borate buffer, pH = 9.4) [5] have been carried out (90% yield of **4** with R^1 = Ac, R^2 = NH_2); peptide dimers could be produced from tyrosyl peptides.

Most of the oxidations generate both types of dimers **4** and **5**, but the main products depend on the reaction method, e.g. dityrosines are formed preferentially by horseradish peroxidase and anodic oxidation, isodityrosines by $K_3[Fe(CN)_6]$. Formation of trimers and oligomers is influenced by the reaction time and the concentration of the tyrosine derivatives. The dimers **4** and **5** have been analyzed by analytical HPLC, electrospray mass spectrometry and NMR-spectroscopy.

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Effects of diagenesis on the stable isotope composition of amino acids in fossils and sediments

The stable isotope compositions of fossil proteinaceous materials are used to reconstruct paleodiet, ancient food webs and paleobiochemistry, the assumption being that the fossil materials faithfully preserve the isotope compositions of the organisms. However, diagenetic reactions such as hydrolysis, cyclization and condensation may alter the stable isotope compositions of proteins, peptides and amino acids. Laboratory simulation experiments were performed to begin to assess the magnitude of stable isotope fractionations associated with these reactions. Aliquots of a 0.02 M unbuffered aqueous solution of the peptide glycylglycine (GlyGly) were heated for up to 800 hours at 120 °C. The residual dipeptide, free glycine (resulting from hydrolysis) and diketopiperazine (resulting from cyclization of the peptide) were isolated for stable carbon isotope analysis. Although the cyclization of dipeptides is a reversible reaction, the rate of formation of diketopiperazine (DKP) exceeds the rate of decyclization to the original dipeptide during the earlier stages of heating. A kinetic isotope effect is observed in which DKP is up to 5 ‰ depleted relative to the initial $\delta^{13}C$ value of GlyGly. Glycine released via hydrolysis was depleted by up to 7 ‰ relative to GlyGly. Additional experiments were performed in which aqueous solutions of alanine and sugars were heated (100 °C) for up to 80 days. Stable carbon and nitrogen isotope fractionations were observed in which the residual alanine remaining in solution was enriched by several per mil relative to its initial $\delta^{15}C$ and $\delta^{15}N$ values. In contrast, the insoluble condensation products (melanoidins) were depleted in ^{13}C and ^{15}N relative to the starting values. Given that hydrolysis, cyclization and condensation are common diagenetic pathways and that isotope fractionations associated with them appear to be significant (and may be greater at ambient temperatures), it is important that these processes be considered when attempting to interpret the stable isotope record of proteins, peptides and amino acids in fossils.

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Pulse radiolysis study of homocysteine thiolactones in aqueous solution

It has been reported [1, 2] that homocysteine thiolactone (HCTL) as well as its alpha-alkylated derivative, alpha-methyl-homocysteine thiolactone (α-MHCTL) are strongly reactive towards the primary products of water radiolysis (OH, e-aq) and are acting as radiation protective agents. Comparative studies on the survival curves of E. coli bacteria AB 1157 in a very broad range of radiation dose (up to 1.5 kGy) using α-MHCTL, HCTL and cysteamine in the presence of air showed that α-MHCTL is the most efficient radiation protector. This effect was also confirmed in the frame of experiments with animals [3].

Pulse radiolysis studies have been now performed in order to learn more about the reaction mechanisms of the transients resulting from HCTL and α-MHCTL by the attack of e-aq (solvated electrons) and OH radicals. Both, HCTL and α-MHCTL react with e-aq at a rate constant in the order of $k = (3 \pm 0.2) \times 10^{10}$ dm^3 mol^{-1} s^{-1}.

A careful analysis of the kinetics showed that e-aq deposits in the first few microseconds preferentially on the sulfur atom (lambda max around 500 nm) and to a lower extent on the carbonyl group (lambda max 270 nm). Subsequently an electron transfer takes place from the S-atom to the oxygen on the carbonyl function with $k = 1.4 \times 10^5 s^{-1}$. This process is completed in about 1 millisecond. A similar process was also observed with α-MHCTL.

The OH radicals are scavenged very efficiently by both substrates. HCTL reacts with OH at $k = 2.2 \times 10^9$ dm^3 mol^{-1} s^{-1} and the resulting transients decay with $2k = 8.3 \times 10^9$ dm^3 mol^{-1} s^{-1}. α-MHCTL undergoes similar reactions. The absorption spectra and the kinetics of the transients resulting from the reaction of HCTL and α-MHCTL with e-aq and OH species as well as probable reaction mechanisms will be presented. The knowledge of these data are of importance for the explanation of the radiation protection abilities of both substances.

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Relationship between amino acid sequence and secondary structures of plant proteins

Comparison in peptide sequences of globulin storage and membrane proteins from different plants were identified. Secondary structure of proteins was computerized by dot matrix and hydropathy analyses.

Using the FASTA and TFASTA computer programmes, homolgy was detected between a short region of 29 residues of legumes and other plants. Sequences of 12S globulin from *Glycine max* (soybean) showed identity of 93.1% and 89.7% with chains of gy(2) and A2B1a; and Ala and Gy(3), respectively. It was found through the sequence analyses that soybean legumin (100% identity), whose sequence is available, has some homology with other legume and cereal globulins.

Based on sequence of 32K thylakoid membrane protein precursor from chloroplast of *Amaranthus hybridus* (A.), the search showed 93.5% identity in 475 overlaps of *Pisum sativum, Gossypium hirsutum* and Oryza sativa. The BLAST Search (99–119 amino acids) showed some matching only with 11S globulin from Cucurbita maxima (pumpkin) in 161–181 amino acids. In the length of 21 amino acids a peptide plot was obtained for 11S globulin β-subunit precursor from Cucurbita maxima. Peptide structures were calculated for amaranth and pumpkin proteins and presented plot structures, according to Chou-Fasman prediction. Secondary structure predictions for 32K thylakoid membrane protein precursor of amaranth chloroplast differed from 11S pumpkin globulin β-subunit. A countour plot of the helical hydrophobic moment and a helical wheel recognized amphiphilic regions, and showed 14 hydrophobic regions in amaranth in comparison with that of pumpkin which exhibited 13. Two sequences from amaranth membrane and pumpkin globulin proteins were also compared and their secondary structures were predicted.

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Study of the copper(II)-L-prolylglycine system by NMR

Carbon-13 relaxation in paramagnetic systems is widely used to obtain structural and dynamical informations on complexes of transition metal ions. The selective line-broadening is often utilized to characterize the presumed binding sites. This method should be exercised with some caution if it is used to determine the accurate geometry. This paper is devoted to a study of the Cu(II)-L-Prolylglycine complex (noted Cu-PG) in aqueous solution by measuring carbon-13 longitudinal relaxation times at 62,86 MHz and two temperatures 37° et 61 °C. Values of T_{1obs} are obtained from partially relaxed Fourier Transform, 180° – τ – 90° pulse sequences with fast inversion recovery variant for aqueous solution at pH = 9, a fixed concentration PG = 1 $mol.l^{-1}$ and a variable concentration of Cu^{2+} from 0 to 40×10^{-5} $mol.l^{-1}$. Under these conditions, the copper(II) cation is completely bound to the peptide under the form of Cu-$(PG)_2$ complex [1]. The measured longitudinal relaxation times T_1 of the ^{13}C nuclei depend on their values in the paramagnetic sites T_{1M} (PG in the bound site) and in the diamagnetic site T_{1D} (PG free)

$1/T_{1obs} \approx P_M/T_{1M} + 1/T_{1D}$ with $[PG]/[Cu^{2+}] >> 1$ and fast exchange.

The specific relaxation ratio $1/T_{1M}$ in case of a fast exchange ($\tau_M << T_{1M}$) is obtained from the slope of the least squares lines representing the relaxation rates $1/T_{1obs}$ measured as function of the concentration of copper(II). $1/T_{1M}$ is the sum of two contributions scalar and diploar. The scalar contribution is negligible because the electronic relaxation time T_{1e} is long (10^{-9} s). The longitudinal relaxation rate is therefore purely dipolar.

$$1/T_{1M} = K_{DD}\, \tau_c / r^6$$

K_{DD} is the dipolar constant, r_C is the distance between the copper(II) cation and the examined carbon nucleus, τ_c is the correlation time (in this case equal to the reorientation time of the complex).

For each carbon, the values of the ratio $a = \tau_c/r^6$ are determined by $1/T_{1M}$ measurements. A computing procedure fully described in a previous work [2], allows to determine the geometry of the complex. The prolyglycine atomic coordinates which serve as a basis for the computation of these distances are taken from crystallographic data relative to the prolylglycine structure [3]. Values of the specific $1/T_{1M}$ (s^{-1}) of ^{13}C in bound prolylglycine at 37 °C, together with the Cu^{2+}-^{13}C distances (Å) in the Cu$(PG)_2$ complex are displayed in Table 1.

Table 1

	Cα	C_β	C_γ	C_δ	C = 0	CH_2	CO^-_2
$1/T_{1M}$	395	64	55	360	306	360	65
$r_{Cu^{2+}-{}^{13}C}$	2.99	4.04	4.06	3.06	3.12	3.09	4.05

The $1/T_{1M}$ values at 61 °C are equal about to 70% of the values at 37° C. Two values of the rotational correlation time $\tau_R = 4.86 \times 10^{-11}$ and 3.4×10^{-11} s at 37 and 61 °C allow us in turn to compute mean Arrhenius parameters $\tau_R° = 3.4 \times 10^{-13}$ s and E_R = 12.8 kJ with a large uncertainty. The value of the magnetic moment μ_M = 1.93 B. M. is in agreement with similar complexes of copper(II). The UV-visible spectra shows a maximum at λ_{max} = 630 nm and EPR measurement yield the following parameters: $g_{//}$ = 2.234, $g_\perp$ = 2.046, $A_{//}$ = 155 G, A_{iso} = 60.8, g_{iso} = 2.109 at pH = 9).

We can therefore propose a planar tetracoordinated structure for the Cu$(PG)_2$ by assembling two prolylglycine molecules where the four coordination sites are two amino nitrogens and two deprotonated peptide nitrogens, the carboxylate group being free. The internuclear distances N(amino)-Cu^{2+} = 1.99 Å and N(peptide)-Cu^{2+} = 1.97 Å were computed from the program.

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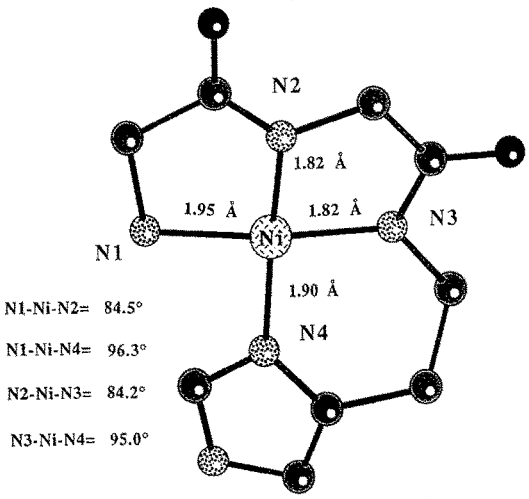
Structure of Ni(II)-glycylglycylhistamine by X-ray and NMR

The tripseudopeptide glycylglycylhistamine is of particular interest as it can serve as a model for human serum albumin and as it is able to form transition metal complexes.

Yellow crystals of Ni(II)-GGHist were obtained by slow evaporation of an aqueous solution containing nickel(II) and glycylglycylhistamine (GGHist) in a 1:1 mol ratio at pH ≅ 10. The crystals of Ni(II)-GGHist cristallize in the triclinic P1 space group with 6 molecules of the complex and 11 water molecules per asymetric unit. The 6 complex molecules have almost the same conformation with a square-plane for four coordinated nitrogen atoms. The cell parameters are a = 10.035 (1), b = 10.092 (1), c = 19.209 (3) Å, α = 86.54 (2), β = 89.48 (2), γ = 77.74 (2)°. The mean values of bond lengths and bond angles for the Ni coordination environment are indicated on the figure.

The ^{1}H NMR spectra of GGHist shows, that at pH ≅ 10, all proton peaks of the bonded ligand are significantly shifted upfield; this is in agreement with an expected 4N coordination.



Ni(II)-GlycylGlycylHistamine

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Utilization of some non coded amino acids as isosters of peptide building blocks

In our study on utilization of non coded amino acids in peptide chemistry we synthesized methylene-thio [CH_2-S] and methylene-oxy [CH_2-O] group containing amino acids and pseudodipeptides which could be used as building blocks in a construction of peptide hormone analogues. The [CH_2-S] isoster of peptide bond exhibits increased flexibility, lipophility and resistance to proteolytic enzymes. The similar properties this group exhibits as the isoster of disulfide bond in the side chain of cystine residue. The [CH_2-O] isoster is moreover similar in its geometry to extended conformation of peptide bond. As a consequence, the changed profile of biological activities could be expected in corresponding peptide hormone analogues containing such isosteric alterations. The [CH_2-S] isosters of the peptide bond were prepared by alkylation of thiolates of 2-mercaptocarboxylic acids, those of the disulfide bond by alkylation of cysteine or homocysteine. The [CH_2-O] isosters were prepared by $(AcO)_4Rh_2$ catalyzed addition of carbens generated from corresponding alkyl-diazocarboxylates to N-protected amino alcohols. The pseudodipeptides Z-Leu-ψ(CH_2-S)-Gly-NH_2 and Z-Leu-ψ(CH_2-O)-Gly-NH_2 were inserted in the carboxyterminal part of the oxytocin molecule using solution methods of peptide chemistry. The both isosteric bonds were resistant against proteolytic degradation, the first one decreased enzymic cleavage of the distant Tyr^2-Ile^3 bond in the corresponding analogue, too. In general, the oxytocin analogues exhibited dissociated biological activities, more decreased at the [CH_2-S] isoster. The [CH_2-S] isosters of the disulfide bond orthogonally protected at their α-amino (Fmoc) and α- (OAll, OH) or ω-(OBu^+, OPac, OEt, OH) carboxylic groups were applied in the solid phase synthesis of the carba-analogues of the strong vasoactive peptide endothelin. The peptides synthesis was carried out either by the step-wise procedure or by the fragment condensation.

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Proofreading in *trans* by an aminoacyl-tRNA synthetase

The non-protein amino acid homocysteine (Hcy), an obligatory precursor of Met in all cells, poses an accuracy problem for the protein biosynthetic apparatus. Hcy is misactivated *in vitro* by three aminoacyl-tRNA synthetases, MetRS, IleRS, and LeuRS, at a frequency exceeding the frequency of translational errors *in vivo*. Two other synthetases, ValRS and LysRS, misactivate Hcy less efficiently. These five enzymes possess an efficient editing mechanism which prevents misincorporation of Hcy into tRNA by destroying the Hcy-AMP intermediate. The editing pathway involves reaction of the side chain sulfhydryl group of Hcy with its activated carboxyl group yielding a cyclic thioester, Hcy thiolactone [Microbiol. Rev. 56: 412 (1992)]. The editing reaction occurs in the same active site that carries out the synthetic reaction, as demonstrated by our structure-function studies of MetRS [Kim et al., PNAS 90: 11553 (1993)].

A pivotal element in editing is the side chain -SH group of Hcy. When Hcy is in the active site, its -SH group occupies a subsite (an -SH subsite) next to its carboxyl carbon. This subsite is vacant when a cognate amino acid is in the active site. If our

model of the synthetic/editing site of an aminoacyl-tRNA synthetase is correct, filling an empty -SH subsite by providing the -SH function in *trans*, i. e., on another molecule, should lead to editing of a cognate amino acid. Such editing reaction would lead to the formation of a thioester of a cognate amino acid. With cysteine donating the -SH function in trans, the subsequent transacylation from the sulfur to the α-amino group of Cys would afford a dipeptide, Cys-AA (where AA is an amino acid cognate to a synthetase being tested).

As predicted, IleRS catalyzes novel reactions of Ile-tRNA with thiols. A product of IleRS-dependent reaction of Ile-tRNA with Cys was indeed Cys-Ile, whereas with N-acetyl-Cys, 3-mercaptopropionate, and dithiothreitol, corresponding thioesters of Ile were formed. Enzymatic reactions of Ile-tRNA with thiols exhibited thiol specificity; L-Cys and dithiothreitol were the most efficient and stimulated enzymatic deacylation of Ile-tRNA with saturation kinetics. Control experiments showed that hydroxylamine, a potent nucleophile, did not affect the rate of enzymatic deacylation of Ile-tRNA. In contrast, nonenzymatic deacylation of Ile-tRNA was stimulated by hydroxylamine but not by thiols. Thus, a model of a single synthetic/editing site applies also to physiologically important editing of Hcy by IleRS.

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Proofreading *in vivo*: Editing of homocysteine by isoleucyl- and leucyl-tRNA synthetases in *Escherichia coli*

Homocysteine (Hcy), an obligatory precursor of methionine in all cells, is misactivated *in vitro* by three aminoacyl-tRNA synthetases, MetRS, IleRS, and LeuRS, at a frequency exceeding the frequency of translational erros *in vivo*. Two other synthetases, ValRS and LysRS, misactivate Hcy less efficiently. These five enzymes possess an efficient editing mechanism which destroys Hcy-AMP and prevents misincorporation of Hcy into tRNA. A cyclic thioester, Hcy thiolactone, is a product of these editing reactions [Microbiol Rev 56: 412 (1992)]. So far, only editing of Hcy by MetRS has been shown to be a physiologically important process which prevents misincorporation of Hcy into tRNA and protein in *E. coli* [PNAS 87: 4504 (1990)], yeast [EMBO J. 10: 593 (1991)], and some mammalian cells [FEBS Lett. 317: 237 (1993)].

It is unclear whether other editing reactions which have been demonstrated *in vitro* are physiologically relevant. Our previous data indicated that, because of compartmentation of Hcy metabolism in *E. Coli* [Biochemistry 33: 11528 (1994)] *endogenous* homocysteine (formed in the methionine biosynthetic pathway) is edited exclusively by MetRS. To test whether *exogenous* Hcy (taken up for the medium) can be edited by other aminoacyl-tRNA synthetases, cultures of *E. coli* cells that overproduce aminoacyl-tRNA synthetases have been incubated with Hcy and assayed for Hcy thiolactone by UV spectrometry and by TLC. These assays showed that Hcy thiolactone was formed in cultures overproducing IleRS and LeuRS, in addition to MetRS which is known to edit both *endogenous* and *exogenous* Hcy *in vivo* from our previous studies. Overproducers of other aminoacyl-tRNA synthetases, such as CysRS, ArgRS, AspRS, and SerRS, or JM101 *E. coli* host strain produced Hcy thiolactone > 10 times less efficiently. No detectable Hcy thiolactone was formed in the absence of bacterial cells or exogenous Hcy. Other experiments have shown that IleRS-, LeuRS-, and MetRS-dependent synthesis of Hcy thiolactone was abolished only by a corresponding cognate amino acid. Similarly, the low level of Hcy thiolactone produced by JM101 cells was abolished by the presence of Ile, Leu, and Met, but not by any other amino acid(s), in culture media. These results directly demonstrate that editing of errors in amino acid selection by IleRS and LeuRS, in addition to MetRS, operates *in vivo* in *E. coli*.

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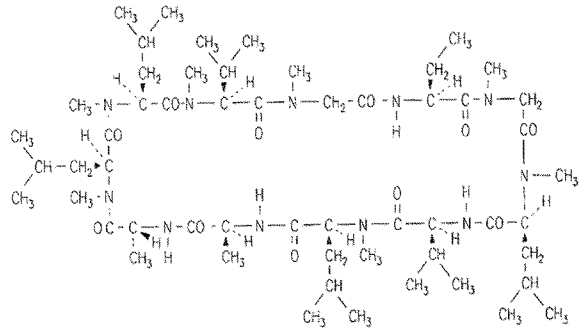
An unusual side chain C-C cleavage on the MeBmt amino acid in cyclosporin A

Treatment of the cyclic undecapeptide cyclosporin A in alkaline media causes changes on the amino acid MeBmt (MeBmt = (4R)-4-((E)-2-butenyl)-4, ↑N-dimethyl-L-threonine) without splitting of the cyclopeptide structure. The mixture of the decomposition products was analysed by HPLC-continuous-flow-FAB-MS. Dehydratation of MeBmt leading to a mixture of cyclosporins with anhydro-Me-Bmt isomers was identified as the main degradation pathway. Besides this reaction, an unusual side chain C-C cleavage on the MeBmt has been detected. Cyclsporin [Sar[1]]CS was isolated as the product and was characterized by MS and NMR spectra.



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Study of XRCC1 analogues in radiation-resistant and radiation-sensitive CHO cell lines

CHO (Chinese Hamster Ovary) cells represent an excellent model of human cells in terms of mechanism of radiosensitivity including DNA repair processes. Since 1980 several different radiation-sensitive CHO mutant cell lines were obtained. Human XRCC1 gene, which affects the cellular sensitivity to ionizing radiation, corrects the DNA repair deficiency of the CHO cell mutant EM9 [1].

EM9 [2] is sensitive to both, X- and UV-radiation. Another mutant, UV5 [2], derived from the same parental strain AA8, is sensitive to UV-irradiation only. However, these strains have never been characterized genetically.

Studying the differences between the XRCC1 analogues of these CHO cell lines at the molecular level, we started to genetically characterize the XRCC1 analogues in AA8, UV5, and EM9 CHO cell lines. Using the different sensitivity to X- and UV-radiation of these cell lines we tried to characterize the overlap

of the radiation resistance mechanisms and to find out whether the resistance to X- and UV-radiation of these cell lines reside in the same group of genes.

In order to identify XRCCl analogue genes of CHO cells PCR experiments have been performed. The PCR primers were designed from the highly conserved regions of known XRCC1 and RAD4 genes. From the whole genomic DNA of EM9 a single fragment of the expected 95 bp length was amplified under highly stringent conditions yielding no background. From the whole genomic DNA of AA8 and UV5 under the same conditions two fragments of the same majority were amplified: one of the expected 95 bp length, the other one of 130 bp length. Here we report the determined sequences and homologies of the amplified fragments.

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DNA-binding and DNA-modifying activity of thioredoxins from *Streptomycetes*

Thioredoxin is a low molecular weight ubiquitous ditiol protein with a highly conserved structure. It has been suggested to play a vast variety of biological functions.

Thioredoxin (Trx) and thioredoxin reductase are non-essential thiols present in the cell in perplexingly high concentrations. Among the functions proposed are the antioxidant effects and the radioprotective effect. These functions are currently under intensive study.

We focussed on the study of the effects of Trx and DNA replication and modification:

We found that Trx bound to DNA as evaluated by gel shift assay in the presence and in the absence of ATP. Furthermore, nick removing activity was detected by incubating Trx with several forms of supercoiled plasmid DNA (pUC 18) and subsequent electrophoresis:

In analogy with other nick removing structures involved in DNa replication we suggest an implication for Trx in this process.

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Authentication of indigeneity of amino acids in fossil and extraterrestrial materials using GC/C/IRMS

Amino acids are major components of fossil materials and of carbonaceous meteorites; their occurrence in the former reflecting ancient biosynthetic pathways whereas, in the latter, reflects abiotic synthesis at the time of and perhaps prior to the formation of our solar system. The fact that amino acids are ubiquitous to all living organisms and that ancient fossils and meteorites are not closed systems poses a serious challenge with respect to determining the extent to which amino acids in a given sample reflect their original mode of formation. Because amino acids retain their stable carbon and nitrogen isotopic integrity during racemization, it is suggested that a comparison of the stable isotope compositions of the D- and L-enantiomers of amino acids in fossil systems can be used to assess indigeneity. Exogeneous amino acids introduced into fossils subsequent to burial are likely to have different isotopic compositions and stereochemistry; these differences allow for resolution of original organic components from recent contaminants. The isotopic composition of D- and L-enantiomers of individual amino acids in carbonaceous meteorites should be identical, owing to their common synthetic pathways. Differences in isotopic composition may reflect contamination subsequent to impact. The development of a method for the stable isotope analysis of nanomole levels of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) enables us to test this hypothesis. The results of applications of this method with respect to establishing the indigeneity of amino acids in fossil shells and in a carbonaceous meteorite are presented.

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A kinetic and thermodynamic investigation of ostrich pepsins I and II

Pepsins are gastric proteases present in the stomachs of many species, including those of mammalian and avian origin. Pepsins are active in the acidic pH region, and are capable of catalyzing transpeptidations of both the amino- and carboxyl-transfer type.

Ostrich pepsins I and II have been purified from the proventriculus, and the effect of pH and temperature on their action has been investigated. The pH optima of the ostrich enzymes with haemoglobin, the synthetic hexapeptide Leu-Ser-Phe-(NO_2)-Nle-Ala-Leu-OMe, and N-acetyl-L-phenylalanyl-L-diiodotyrosine (APDT) were determined. An extensive study was conducted to determine the alkaline stability of ostrich pepsins and pepsinogens in the pH region 6 to 12, and ostrich pepsins were found to be stable up to pH 7.5.

The temperature optima and thermal stability of ostrich pepsins were determined at the optimum pH of 2 in the temperature range 10 to 70 °C. Energies of activation (E_a) and free energies of activation ($\Delta G^{\ddagger}$) were obtained. Ostrich pepsins I and II revealed similar pH optima, pH stability, temperature optima and thermal stability profiles to those of avian pepsins. Ostrich pepsins were, however, more resistant to alkaline denaturation than porcine pepsin and other pepsins of mammalian origin.

Both ostrich pepsins showed a 7 to 27-fold lower catalytic activity (k_{cat}) with the substrate haemoglobin when compared to porcine pepsin. Ostrich pepsin I showed a lower Michaelis constant (K_m) than porcine pepsin (2.43 and 9.94 mM, respectively), while the K_m value for ostrich pepsin II was found to be 16.06 mM. The hexapeptide substrate Leu-Ser-Phe-(NO_2)-Nle-Ala-Leu-OMe was hydrolyzed more efficiently by porcine pepsin than by ostrich pepsin II (k_{cat}/K_m values of 356.41 and 136 $sec^{-1}.mM^{-1}$, respectively). Ostrich pepsin I exhibited no activity with the hexapeptide substrate. Ostrich pepsins I and II showed very low specific activities with short synthetic substrates, the one exception being APDT.

The inhibitory effect of pepstatin A, 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), diazoacetyl-DL-norleucine methyl ester (DAN) and p-bromophenacyl bromide on ostrich pepsin

action was investigated. Inhibition constants (K_i) of pepstatin A with ostrich pepsins I and II were 2.14×10^{-8} and 2.2×10^{-8} M, respectively. DAN required the presence of cupric ions to exert its inhibitory effect. Kinetic data obtained for ostrich pepsins are in agreement with those documented for other avian pepsins.

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A chemical and kinetic characterization of ostrich pancreatic secretory trypsin inhibitor

Pancreatic secretory trypsin inhibitors (PSTIs) in mammals are single polypeptide chains comprised of 50–60 amino acid residues (M_r of 6500) and are secreted with all the zymogens into the pancreatic juice where it accounts for 1% of the total potential trypsin activity. The main physiological role of PSTI has been thought of as the prevention of premature activation of zymogens by trypsin while they are in the pancreatic duct and the other function of PSTI as a growth-stimulating factor in the human body has also been suggested.

PSTIs from animals have been purified and characterized thoroughly, but data on the chemical and kinetic characteristics of avian PSTIs is relatively meagre in comparison.

PSTI was isolated and purified from ostrich pancreases by sulphuric acid extraction, followed by ammonium sulfate precipitation and various column chromatographic steps, namely SP-Sephadex C-50, QAE-Sephadex A-25 and RP-HPLC. All fractions were assayed for PSTI activity and the active fraction was characterized by SDS-PAGE under reducing conditions, PAG-isoelectric focusing and amino acid sequencing.

Ostrich PSTI revealed an M_r of 9000 and an pI of 5.7. The complete amino acid sequence of ostrich PSTI showed some homology to PSTIs from other species. The inhibition constants (K_i) of ostrich PSTI with ostrich and commercial bovine trypsin were 7.2×10^{-9} and 1.53×10^{-7} M, respectively. The K_i values of commercial bovine PSTI with ostrich and commercial bovine trypsin were 1.66×10^{-8} and 1.45×10^{-7} M, respectively. Ostrich PSTI showed no inhibitory effect with chymotrypsin, elastase and plasmin.

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A pulse radiolysis study on redox reactivities of carbon- and nitrogen-centered amino acid radicals produced by OH radical reaction in aqueous solution

Pulse radiolyses of amino acids ($RCH(NH_3^+)COO^-$) and their analogs in aqueous solution were carried out to characterize various types of amino acid radicals that are produced via hydrogen abstraction by hydroxyl radical ($^\bullet OH$). A redox titration method demonstrated the formation of oxidizing radicals which caused one-electron oxidation of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and reducing radicals which caused one-electron reduction of tetranitromethane (TNM) or p-nitroacetophenone (PNAP). Carbon-centered radicals produced by α-hydrogen abstraction, such as $RC^\bullet(NH_3^+)COO^-$ and the deprotonated form $RC^\bullet(NH_2)COO^-$, were identified as reducing radicals. Similar carbon-centered radicals $R^\bullet CH(NH_3^+)COO^-$ due to $^\bullet OH$ attack on C-H in the substituent R were of neutral reactivity, which could reduce neither TNM nor PNAP. Hydrogen abstraction from protonated amino group occurred more efficiently in acidic solution to produce cation radical intermediates $RCH(NH_2^{+\bullet})COO^-$, as in the case of one-electron oxidation by $SO_4^{-\bullet}$. Strongly reducing carbon-centered radicals $RC^\bullet H(NH_2)$ that are derived from decarboxylation of $RCH(NH_2^{+\bullet})COO^-$ were produced in higher yield upon increasing pH. Nitrogen-centered radicals $RCH(^\bullet NH)COO^-$ produced via deprotonation of $RCH(NH_2^{+\bullet})COO^-$ were identified as oxidizing radicals. For comparison, $^\bullet OH$ reactions of basic amino acids such as L-lysine and L-arginine bearing ε-amino and ε-guanidino groups, respectively, were also investigated. The mechanism of $^\bullet OH$ reaction of amino acids is discussed on the basis of pH-dependent G-values of the total reducing and oxidizing radicals.

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Information and coding in genome sequences

The mutual entropy in information theory is applied to introduce a new measure indicating the difference between two sequences, which enables us to construct a genetic matrix writing philogenetic trees.

The above measure is used to study the coding structure of genes by comparing genome sequences with those coded due to artificial coding methods.

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On multiple alignment of amino acid sequences

According to accumulation of data of amino acid sequences, the molecular evolution and mathematical explanation of structure of amino acid sequences have been discussed by these data. It is expected to analyze many sequences of genes simultaneously, in particular, the alignment of such sequences is necessary to study the evolution of species. In this talk, we perform the alignment of n amino acid sequences by the method of "Simulated Annealing" which is one of methods for solving some combinational optimization problem. The alignment of n amino acid sequences is shown to be particularly useful compared with the pairwise alignment method. We call the alignment of n amino acid sequences the multiple alignment. After giving short mathematical explanation of this method, we construct genetic distance and matrix corresponding to the object function in the annealing theory for the multiple alignment. Our method is better than other alignment in the sense that we obtain a result having a smaller value for the genetic distance. We discuss further development along on new method.

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Modeling the solubility and activity of amino acids with the LCCI method

The method of linear combinations of connectivity indices used to model the physicochemical properties of a wide range of compounds is here applied to model the solubility and activity of 19 natural amino acids. The modeling starts with a restricted set of molecular connectivity indices from which reciprocal molecular connectivity indices and supra molecular recprocal connectivity indices are derived. While linear combinations of reciprocal connectivty indices achieve an optimal description of the solubilty and activity of 16 amino acids, linear combinations of reciprocal connectivity indices, that include supra connectivity reciprocal indices for the amino acids Pro, Ser and Arg, are optimal descriptors of the full solubility and activity space of the 19 amino acids.

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Interaction between amino acid and poly(ethylene glycol) in aqueous solution

The equilibrium constants of the binding of amino acids to PEG chain were estimated from the solubility data. The interaction between amino acid and PEG in aqueous solution can be explained by the cosphere concept of Gurney. The amino acids having the hydrophilic hydration structure by polar groups are excluded from the hydration layer around PEG chain in terms of the structural incompatibility of hydration cospheres. With increasing the size of hydrophobic group of the amino acid, the hydrophobic interaction between the methylene groups of PEG and the side chain of amino acid becomes stronger than the exclusion effect by polar groups. Therefore, the side chain of Phe and Trp are strongly bound to the PEG chain. The equilibrium constants showed a correlation with the dynamic hydration number (n_{DHN}) which expresses the hydration properties of amino acids in aqueous solution. This result suggests that the details of the motion and structure of the hydration water of amino acid can play an important role in the hydrophobic interaction between the methylene groups of PEG and amino acid side chain.

The volume changes on mixing aqueous solutions of amino acid-PEG were measured as functions of concentration and temperature. The volume changes of different total molality for the Gly-PEG-H_2O system almost linearly decreased with an increase in temperature and converged around 110 °C by extrapolation. On the other hand, those for the Ala-PEG-H_2O system were almost zero and independent of temperature. It is considered that the influence of hydration cospheres on Gly-PEG interaction disappears around 110 °C.

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Oxidation of free and peptide bound tryptophan

The formation of primary oxidation compounds of free and peptide bound tryptophan (Trp) was investigated in order to find tracer compounds for the oxidative damage of food during processing.

The oxidation of free and peptide pound Trp was performed in a model system using aqueous solutions of hydrogen peroxide (H_2O_2) at pH 8.3 and 40 °C. The reaction was arrested by catalase. The oxidation rate of Trp and the formation of 16 possible degradation compounds were examined using RP-HPLC and UV, fluorescence and DAD detection.

Oxindolylalanine (Oia) (Fig. 1), N-formylkynurenine (NFK) (Fig. 2), 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrroloindol-2-carboxylic acid (PIC) (Fig.3), kynurenine (Kyn) (Fig. 2), dioxindolylalanine (DiOia) (Fig. 1) and 5-hydroxytryptophan (5-OH-Trp) were identified in this order of quantity as degradation products of free Trp.

Short peptides like H-Ala-Trp-Ala-OH were used as model substances for peptide bound Trp. After hydrolysis by immobilized Pronase E Oia, NFK and Kyn could be identified as main degradation compounds, as could traces of DiOia, PIC and 5-OH-Trp. After total enzymatic or acid (6 N HCl) hydrolysis of lysozyme after H_2O_2-treatment Kyn could be determined. Oia could be detected after acid respectively alkaline (4,2 N NaOH) hydrolysis, however, it proved to be not stable under these conditions. Further investigations for a non-destructive hydrolysis of Oia are required.

In conclusion Oia and Kyn were elucidated as suitable tracer substances for oxidative degradation of Trp-containing proteins.

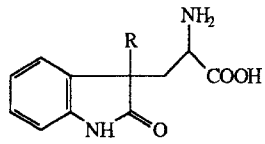

Fig. 1. R =H oxindolylalanine
R =OH dioxindolylalanine

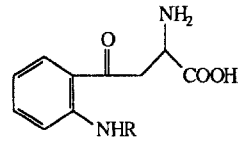

Fig. 2. R =H kynurenine
R =CHO N-formylkynurenine

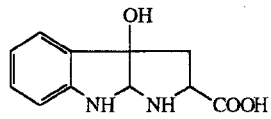

Fig. 3. 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrroloindole -2-carboxylic acid

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The use of sodium hypochlorite to remove allochthonous organic matter in fossil mollusca

A number of studies have suggested that treatment of fossils with sodium hypochlorite removes allochthonous organic matter. Fossil Cepaea sp. from Tattershall thorpe contain unusually high concentrations of amino acids and D-alloisoleucine/L-isoleucine ratios that are scattered and lower than expected for oxygen isotope stage 7 (210ka). Treatment with sodium hypochlorite reduces the concentrations of amino acids to that expected in fossils, reduces the scatter of D-alloisoleucine/L-isoleucine ratios and increases the value of the ratio (0.142 ± 0.042 –4 shells to 0.178 ± 0.014 –5 shells). It is concluded that pre-treatment with sodium hypochlorite has removed "contaminating" amino acids from the fossils and we recommend further investigation of the pre-treatment.

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Amino acid stratigraphy of British Pleistocene deposits

Epimerization of L-isoleucine to D-alloisoleucine has been used to correlate marine, freshwater and terrestrial deposits in the British Pleistocene with the global climatic signals preserved in the deep oceans. This has revealed greater complexity in the British Pleistocene record. Amino acid stratigraphy has also helped improve our understanding of some of the earliest Homonid remains at Boxgrove in Sussex, suggesting an age of Oxygen isotiope stage 11 rather than an age of stage 13 based on mammal palaeontology.

Medicine

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Syntheses and effects of bursin and its analogs on the reduced B-lymphocytes of uremic patients

A selective B-lymphocyte-differentiating tripeptide, bursin, H-Lys-His-Gly-NH_2, and its ten analogs were synthesized by a solid-phase method and were tested their effect on reduced B-lymphocytes of uremic patients. Incubation of peripheral lymphocytes isolated from uremic patients with the synthetic bursin showed an enhancing effect on the reduced B-lymphocytes. Of the synthetic analogs, [Sar^3]bursin exhibited the most potent effect. The structure-activity relationship of bursin is to be discussed.

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Glycinemia and glutaminemia in arthroscopic shoulder surgery using glycine

The Trans Urethral Prostate Resection or TURP syndrome has been attributed to the intravascular resorption of the glycine contained in the irrigating fluid (IF). It may include neurologic, cardiovascular and sensorial disturbances. This is well known in urologic and gynecologic endoscopy, but not in arthroscopic surgery. We carried a prospective study to measure the glycine resorption in arthroscopic shoulder surgery (AS), following a postoperative death caused by an unexplained cerebral oedema.

This study included 17 patients (53 ± 2.6 years; mean ± SEM). The IF was 1.5% glycine. Glycinemia, ammoniemia, glutamate+glutamine sum, alanine and serine rates, natremia and measured plasma osmolarity (Osm) were measured before the operation (T0), right after (T1), then 3 hrs (T2), 6 hrs (T3), 9 hrs (T4), 12 hrs (T5), 18 hrs (T6) and 24 hrs (T7) later. The total volume of IF used was recorded, and the statistical analysis used a repeated measures variance analysis. Glycinemia increases at T1 ($p < 0.01$) (620–11 100µmol.L^{-1}), then quickly goes back to the basic rate (fig). Natremia starts decreasing at T1, down to a minimum at T3 (4% less, $p < 0.001$). Osm decreases significantly ($p < 0.01$) and its evolution could match the one of natremia ($p < 0.05$). The peak in hyperglycinemia is significantly correlated with the largest decrease in natremia ($p < 0.05$) and with the total volume of IF ($p < 0.01$). The glutamate+glutamine rate, ammoniemia and alanine don't change significantly, but the serine rate rises significantly from T1 ($p < 0.01$) and back to the basic rate at T7.

This study confirms the existence of an intravascular glycine resorption during AS. Just like during the TURP syndrome, it comes with a hyponatremia linked to the quantity of glycine resorbed. Some authors have attributed the neurological disturbances to the cerebral oedema caused by hyponatremia and plasma hypoosmolarity. Others have regarded them as a direct toxicity of the glycine and of some of its metabolites – normally detoxified mostly by the liver. The accumulation of these substances would be derived from a hepatic metabolism failure (in the urea cycle) [2]. In our study, the lack of increase in the glutamate+glutamine sum and in ammoniemia has shown a normal ureogenesis in all our patients even when mild troubles were present. In conclusion, the TURP syndrome can happen during AS, and there is a chance of complications, particularly cerebral ones. The action of the liver in this process remains unclear and has to be specified.

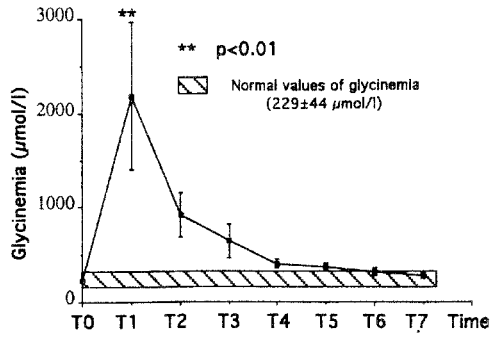

Fig. 1. Evolution of glycinemia (mean ± SEM)

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The use of synthetic peptides for studies of gliadin toxicity in coeliac disease

Wheat gliadin contains proteins which are causative agents in coeliac disease. This project is attempting to define structures in A-gliadin which are responsible for the damage to the small intestinal mucosa seen in this disease.

Synthetic peptides based on certain sequences in A-gliadin were tested in an "in vitro" animal model of coeliac disease in

which foetal chick small intestinal mucosa was cultured in the presence of the peptides. Activity was measured by the ratio of sucrase production by the tissue compared with controls without peptide, expressed as a percentage.

The peptides tested corresponded to the three main peaks from HPLC of the toxic Fraction 9, obtained from a peptic-tryptic-pancreatinic digest of wheat gliadin. They were

(a) The principal peptide present, a tyrosine-containing dodecapeptide matched to residues 78–86 of A-gliadin

(b) A peptide corresponding to residues 213–227, and

(c) Peptide 8–19, suggested by amino acid analysis of a purified subfraction containing serine and asparagine. Several smaller peptides contained within this latter sequence were also evaluated.

The results show that peptide 75–86 (activity 72%) does not, by itself, account for the high activity of Fraction 9 (37%) and peptide 213–227 was only weakly active. However peptide 8–19 was quite active (60%), peptide 9–19 was even more so (– 6%) and peptide 11–19 was extremely active (– 33%). Peptide 12–19 was quite active (68%) while peptides 11–18 and 11–17 displayed very little activity (95%, 91% respectively).

We conclude that peptide 11–19 is the smallest and most active peptide that we have sofar tested and that there is an optimal size for this activity. One or more of the key sequences PSQQ, QQQP and QQPY are present in active peptides but the activity is governed by the types and numbers of amino acids which flank these motifs.

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D-Amino acids in normal and dysfunctional tissues

In the late 1970s and early 1980s D'Aniello et al. reported the presence of free D-aspartate (D-Asp) in the nervous system of cephalopods (octopus and squid) and the presence of D-alanine (D-Ala) in crustacea muscle and hepatopancreas and in developing embryos of sea urchin. In the mid 1980s – early 1990s Fisher and Man et al. reported finding protein-bound D-Asp in normal human brain white matter, in myelin basic protein, and in Alzheimer neurofibrillary tangles. Other researchers have found free D-Asp as well as other free D-AAs in chickens, rodents, and human plasma. D-Asp has also been reported in proteins of human teeth and normal and cataractous human eye lens. In 1990 Fisher and D'Aniello began further collaborative studies on the role of D-amino acids (D-AAs) and D-amino acid oxidase/D-aspartate oxidase in normal and dysfunctional tissues, such as normal and Alzheimer human brain and cerebrospinal fluid (CSF). Free D-Asp and D-Ala occur at about twice the concentration in Alzheimer brain as in normal brain. Alzheimer brains also show a significantly higher content of protein-bound D-Asp and D-Ala in both gray and white matter. Free D-AAs (in particular D-Asp) are present at significantly higher levels in Alzheimer CSF than in normal CSF, whereas in the CSF of patients affected by multiple sclerosis, D-AAs occur at the same level as in normal CSF. The results and significance of these collaborative studies will be discussed.

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Effect of glutamine on intestinal adaptation after 70% small bowel resection in rats

The aim of this study was to determine whether the presence of *Glutamine* in an amino acid chemically defined oral diet interfer with intestinal adaptation of rats submitted to 70% small bowel resection. Eighty-two Wistar rats (203 g bw) were divided into 3 groups: Group GLN (n = 36) – enterectomy + amino acid diet with glutamine; Group A (n = 36) – enterectomy + amino acid glutamine free diet; Group SO (n = 10) – sham-operated + casein diet. The diet were isocaloric and isonitrogenous, and glutamine represented 30% of the dietary nitrogen. At post-operative days 4, 14 and 30 twelve animals of each experimental groups were sacrificed for mucosal Alkaline Phosphatase activity (APa) determination and morpho-kinetic study of remaining small bowel. APa of Group GLN was higher ($p < 0.05$) than Group A in days 14 and 30. The crypt and villous cells populations of the jejunun were higher ($p < 0.05$) in 4 and 14 post-operative days in Group GLN; ileal segment not showed differences. The crypt cell production rate was higher ($p < 0.05$) in Group A in day 30. We conclude that *Glutamine* exerted a positive influence on intestinal adaptation after extensive small bowel resection.

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Change in the concentrations of amino acids in CSF and serum of patients with essential tremor

The concentrations of amino acids in the cerebrospinal fluid (CSF) (n = 20) and serum (n = 20) taken from patients with essential tremor were measured by HPLC and compared with those of controls (n = 10), Reduced concentrations of some amino acids (asparagine, glutamine, glycine, threonine, isoleucine, leucine) were observed in serum taken from patients with tremor. Significant increases were detected in the concentrations of glutamate ($p < 0.001$) and aspartate ($p < 0.01$). The general tendency of the changes in CSF and serum was similar; although the highest differences were observed in amino acid concentrations in the serum of patients with essential tremor. Opposite shifts of some amino acids were detected, in the concentrations of aspartate, serine, tyrosine, leucine, and isoleucine, which may indicate the independence of the changes in the serum from those in the CSF. This study raises the possibility that a genetically determined metabolic disorder is involved in the etiology of essential tremor that appears peripherally and, partly, centrally. The slight increase in the concentration of glutamate together with the reduced levels of GABA, glycine, and serine in CSF may form the neurochemical basis of the central oscillation observed in essential tremor.

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Effect of aspartate on ethanol oxidation and ethanol-induced oxidative stress in the perfused rat liver

Ethanol-induced tissue damages are closely related with the metabolic sequelae of ethanol oxidation, such as the increased amount of ethanol, acetaldehyde and high NADH/NAD ratio. The deranged cellular NADH/NAD ratio would be mainly responsible for a variety of ethanol-induced metabolic dysfunctions. Therefore, in the present study, the correction of the deranged cellular NADH/NAD ratio has been attempted in order to prevent the ethanol-induced tissue damages. The basic assumption for the study is that aspartate supplementation might provide the regeneration of NAD from NADH via coupled reactions of aspartate aminotransferase and malate dehydrogenase, which would be enforced by the efficiency of malate-aspartate shuttle. Aspartate was infused into the ethanol-perfused rat liver (0.1%), resulting in the maximum effect at 2 mM for ethanol oxidation. The derangement of cellular NADH/NAD ratio by ethanol oxidation, represented by the increased lactate/pyruvate ratio in the perfusate, was ameliorated by aspartate. The aspartate effect was blocked by the simultaneous infusion of transaminase inhibitor, aminooxyacetate, suggesting the active participation of aspartate on ethanol oxidation. Moreover, aspartate supplementation caused the decrease in ethanol-induced lipid peroxidation and protein carbonylation, which might be explained by the normalization of ethanol-induced cellular XDH/XO ratio through modulation of cellular NADH/NAD ratio with aspartate. From these data, it is suggested that aspartate can be an antidote to ethanol toxicity by its effect on metabolic facilitation of ethanol oxidation and metabolic regulation of radical generation through the correction of cellular NADH/NAD ratio deranged by ethanol oxidation.

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Proton magnetic resonance spectroscopy of biological fluids: a powerful tool in the diagnosis of inherited metabolic diseases

The use of Nuclear Magnetic Resonance (NMR) spectroscopy of the hydrogen has been proposed in clinical chemistry during the last few years for the analysis of biological fluids such as serum, urine, cerebrospinal and other fluids. Among the advantages of NMR technique, the possibility of a screening for many different metabolites by means of a single multicomponental analysis makes this method helpful in diagnosing particularly inborn errors of metabolism, where the improved prognosis depends on early diagnosis and treatment. As a rule, hydrogen containing analytes whose concentration is higher than 10 μmol/l has a chance to be identified in the NMR spectrum of the fluid.

The present communication represents the result of several years of fruitful cooperation with a number of clinical centers within the Naples area, mostly involved in the diagnosis and care of inherited diseases. Cases of phenylketonuria, hypertyrosinemia, cystinuria, hyperornitinemia, maple syrup urine disease (MSUD), lysinuria were unambiguously and rapidly diagnosed by means of the appearance in the urine and/or serum spectra of increased peaks of the relevant amino acid. At the same time, a number of organic acids are routinely detected in normal urine spectra, such as formic, acetic, lactic, pyruvic, acetoacetic, β-hydroxybutyric, β-hydroxyisovaleric, citric acids, while others (such as ketoisovaleric, ketomethylvaleric and ketoisocaproic acids in MSUD) were evidenced only when their concentration increases as a consequence of metabolic disorders such as lactacidemias, propionic and methylmalonic acidemias. Some of these organic acids are also evidenced in serum and in cerebrospinal fluid.

The possibility of making routinely available multicomponental analysis of several biological fluids, together with the nowadays more affordable costs of medium-low magnetic field instrumentation makes this technique an essential tool in the future of clinical biochemistry.

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Detection of amino acids in biological fluids using nuclear magnetic resonance spectroscopy

The use of nuclear magnetic resonance (NMR) spectroscopy of the hydrogen has been recently proposed in clinical chemistry for the analysis of biological fluids such as serum, urine and cerebrospinal fluid.

A NMR method will be presented for the detection of most of naturally occurring amino acids in any biological fluid, provided that their concentration is above 100 μM. At the same time, other metabolites can be detected as well, if over the above stated concentration; in this way the method provides a multicomponent analysis of the fluid. Consequently, the NMR spectra offer a simultaneous screening of the most abundant fluid components, thus producing specific patterns for each pathology (fingerprint). Moreover, the method requires very small specimens (300 μl) and a very short time (few minutes per analysis are usually enough).

The method has been successfully employed on urine, as a diagnostic tool in several inherited diseases related to the metabolism of amino acids such as phenylketonuria, maple syrup urine disease, ipertyrosinemia, lysinuria. The technical features of the method are suitable for a wide screening of such pathologies that can be monitored simultaneously by a single NMR test.

Attempts to evaluate urinary content of several amino acids and other related metabolites in renal failure are currently under investigation.

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Proton magnetic resonance spectroscopy of biological fluids in the diagnosis and follow-up of branched chain ketoacidurias

The use of proton Nuclear Magnetic Resonance (NMR) spectroscopy in the diagnosis of maple syrup urine disease

(MSUD) has been proposed during the last few years. The technique allows the simultaneous quantitation of many metabolites that accumulate when an altered metabolism of branched chain is evidenced. Valine, leucine and isoleucine among the amino acids as well as the corresponding keto acids brought about by transamination, namely ketoisovaleric, ketoisocaproic and ketomethylvaleric acids, respectively and hydroxy acids such as hydroxyisovaleric acid can be evidenced in a single multicomponental analysis of urine, that involves very simple manipulations, in less than 20 min. Previous techniques involved instead the use of gas-chromatography after a cumbersome and time consuming manipulation of specimens for the detection of keto acids and an amino acid analyzer for quantitation of amino acids, thus allowing only occasional monitoring.

Based on the literature reports and by simple comparison of the NMR spectrum of an urine specimen of a 10 days old newborn with those of other diagnosed MSUD patients, an altered metabolism of branched chain amino acids was unambiguously diagnosed in one hour after collection of an urine sample. Exploiting the rapidity and completeness of NMR analysis, the amounts of the mentioned amino, keto and hydroxy acids were monitored at least twice in a week in patient's serum, by means of a newly developped quantitative analysis. A dramatic decrease of relevant metabolites was registered in this way, following a diet in which intake of valine, leucine and isoleucine was reduced, in this way confirming *ex adiuvantibus* the diagnosis. The possibility of counting on fast analyses allowed rapid adjustment of diet. Increased levels of the above mentioned amino, keto and hydroxy acids were also detected for the first time in samples of the cerebrospinal fluid, thus explaining the effects of mental retardation in untreated MSUD patients.

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Proton magnetic resonance spectroscopy of urine in the diagnosis and follow-up of urolithiasis

Urolithiasis is characterized by accumulation in urine of high concentrations of metabolites (oxalates, cystine, urates etc) above the saturation threshold; the high concentrations of the precipitating metabolites are then present in the urine samples of the patients. A single multicomponental analysis by proton Nuclear Magnetic Resonance (NMR) spectroscopy can easily detect the increased metabolites, provided that these molecules contain unexchangeable hydrogens when in aqueous solution.

Several urine samples from cystinuric children have been analyzed by means of NMR spectroscopy, that clearly evidenced accumulation of cystine, ornithine, arginine and particularly lysine up to millimolar levels. The quantitation of cystine by NMR is one order of magnitude more sensitive than the Brandt test, whose positives were always confirmed by NMR. Cystine quantitation by NMR was also quantitatively validated by control high pressure liquid chromatography analyses of amino acids.

Conversely, direct detection of oxalic acid in urine cannot be performed owing to the lack of unexchangeable hydrogens within the molecule; nevertheless, increased levels of oxalic acid precursors such as glycolic and/or glyceric acids have been observed in the NMR spectrum of urine of iperoxaluric patients.

Citraturia is also of relevance in urolithiasis in that citric acid plays an important role in regulating urine pH, and hence the precipitation conditions as well. In a follow-up study citraturia was monitored by NMR spectroscopy in 44 children for several years with normo- and ipercalciuria. Ipocitraturia was found to increase the risk of urolithiasis, particularly when associated with ipercalciuria.

In conclusion, a single NMR spectrum of urine was found to provide many of the data of relevance in urolithiasis, such as the levels of cystine, lysine, glycolic and citric acids and other metabolites as well as creatinine in urine. The method can thus be proposed in both diagnosis and follow-up of the disease.

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Elevation of plasma amino acid concentrations by cigarette smoking in women

The uptake of amino acids (AA) by cells is an energy-dependent active process and is one of the critical steps for their metabolism in the cell. It has been demonstrated that several components of cigarette smoke and hypoxia depress AA uptake using placental villus cells as a model system. If the cellular uptake of plasma AA are depressed by cigarette smoking, their plasma concentrations will be elevated. Elevation of plasma AA concentrations is a risk factor for humans with inborn errors of AA metabolism. Therefore, the plasma concentrations of AA in women smokers (N, 10; 19 ± 3 cigarettes/day) and nonsmokers (N, 11) were measured. This study was approved by the Institutional Review Board for Clinical Investigations. The venous plasma samples were extracted with acetonitrile for endogenous AA. The AA in these extracts were separated by ion exchange chromatography and derivatized using *o*-phtalalaldehyde and 2-mercaptoethanol. The resulting fluorescent derivatives (excitation 340 nm, emission 410 mn) were assayed using a Waters 840 HPLC AA system. Plasma concentrations of several essential (thr, val, met, ilue, leu, tyr, phe, his) and nonessential (ser, gly, arg) AA in smokers were significantly higher by about 45–88% and 54–89% respectively in smokers than nonsmokers. There were no significant differences in total hemoglobin levels (15.6 ± 0.2 g/dl) in smokers and nonsmokers. Smokers had higher levels of COHgb (6.5 ± 0.4%) in their Hgb than nonsmokers (2.3 ± 0.2%). The plasma cotinine level in smokers was about 230 ± 98 ng/ml while it was not detectable in nonsmokers. These observations suggest that smoking increases plasma AA concentrations in women and may enhance the effects of the inborn errors of AA metabolism. (Supported by United States HHS – NIH DA-06207, The Council for Tobacco Research, U.S.A., Inc. 2835 and The Study Center for Anesthesia Toxicology of Vanderbilt University.)

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Information processing in individuals heterozygous for phenylketonuria, given the sweetener aspartame which contains phenylalanine

Individuals heterozygous for the genetic disease phenylketonuria, have a somewhat impaired ability to metabolize the amino acid phenylalanine. It has been suggested that consumption of aspartame (L-aspartyl-L-phenylalanine methylester)

might increase brain phenylalanine concentrations and thereby alter brain chemistry and brain function. This speculation has been evaluated in 48 adult phenylketonuric heterozygotes. Subjects received aspartame (either 15 or 45 mg/kg/day) and placebo in a randomized, double-blind crossover study for 12 weeks on each treatment. Information processing tasks from the SVAT [1] were administered at weeks –2, –1, 6, 12, 18, and 24, and standard EEGs were also recorded. The SVAT evaluates attentional control in two peripheral (sensory input, response selection) and two central processing stages (memory search, decision), sustained and focused attention, and memory capacity. The SVAT hat proven to be a sensitive detector of information processing dysfunctions in treated homozygotes for PKU, with outcome measures correlating up to r = .75 with concurrent plasma phenylalanine level [2, 3].

The current study did not reveal any significant differences between placebo and treatment conditions on any of the information processing measures as well as in the EEG. These results refute speculations that aspartame may affect information processing or the EEG, and reaffirms the safety of aspartame in subjects heterozygous for PKU.

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Amino acids and keto acids in plasma of patients after orthotopic liver transplantation and the *in vivo* and *in vitro* effects of C-galactose

The immediate postoperative period after surgical trauma in general is characterized by protein catabolism, including increased proteolysis, enhanced liberation of amino acids from muscle and increased amino acid uptake into the liver. Expecially in liver transplantation, there is a drastic decrease of plasma arginine, possibly due to decreased urea cycle activity. Even in the presence of sufficient plasma concentrations of insulin, glucose utilization is impaired due to insulin resistance. In this phase, D-galactose, as an insulin-independent monosaccharide, may be suitable for accelerating the restoration of anabolic metabolism.

In this study, both, the *in vivo* and the *in vitro* effect of D-galactose on the concentration of amino acids and keto acids in the plasma of liver recipients and in primary hepatocyte cultures was investigated. A group of 9 patients was given 100 ml D-galactose (40%) daily in addition to standard parenteral nutrition during the posttransplant period. Postoperative plasma concentrations of amino acids and keto acids were determined by precolumn fluorescence HPLC using and compared to those of control patients (n = 30) receiving standard parenteral nutrition.

Distinct differences were observed between the galactose group and the control group: in plasma of patients receiving galactose, the ratio of glutamate and α-ketoglutarate to glutamine was markedly increased in comparison to control patients. The normalization of arginine and ornithine plasma levels was accelerated in the galactose group. Plasma concentrations of lysine in patients receiving galactose were within the normal range of healthy persons, while in control patients, lysine levels were increased up to day 15.

By *in vitro* incubation of primary hepatocyte cultures with D-[^{14}C]galactose, radiolabelling of arginine concurrently with an increase in intracellular arginine concentrations in comparison to control cultures incubated with D-[^{14}C]glucose was shown.

The results show a favourous effect of D-galactose on nitrogen metabolism after liver transplantation. Increased concentrations of glutamate and α-ketoglutarate facilitate ammonia fixation. A fast recuperation of arginine is important for protein, NO and urea synthesis. The increased concentrations of branched chain keto acids suggest an accelerated restoration of protein anabolism. Further studies are necessary to investigate the influence of D-galactose on glycoprotein and glycolipid metabolism.

Metabolism and Transport

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Transport properties of mouse and human cationic amino acid transporters are determined by a protein domain of 40 amino acids

Three related transporters for cationic amino acids (CAA) encoded by two different genes (mCAT-1 and mCAT-2) have been identified in mouse cells (mCAT for mouse cationic amino acid transporter) [2, 6, 5, 3, 4]. The mCAT proteins share the same substrate specificity and their transport activity is independent of the presence of sodium ions. However, the three carrier proteins differ in their transport properties and tissue distribution. MCAT-1 and mCAT-2B demonstrate high substrate affinity and sensitivity to trans-stimulation. These properties are consistent with y^+, the principal mechanism for cellular uptake of cationic amino acids. The third transporter, mCAT-2A, differs from mCAT-2B only in a stretch of forty-one residues. However, it has ten-fold lower substrate affinity, greater apparent maximal velocity and is much less sensitive to trans-stimulation. This suggests that the region divergent between mCAT-2A and mCAT-2B determines the transport properties of the mCAT-2 proteins. In addition, chimeric transporters between mCAT-1 and mCAT-2A/B demonstrate transport properties identical to the donor of the corresponding protein domain.

To determine if CAA transporters in human cells show similar structure and function, we cloned two cDNAs encoding the human homologues to mCAT-2A and mCAT-2B, designated HCAT-2A and HCAT-2B, respectively. Analysis of these cDNAs together with the cDNA encoding HCAT-1 [1] show an overall 87–90% identity between the deduced amino acid sequences of the murine and human proteins. In the "critical" protein domain, the sequences of the human and mouse proteins differ in seven (CAT-1) and one (CAT-2A and CAT-2B) amino acids, respectively. Transport assays performed with the human carrier proteins expressed in Xenopus laevis oocytes demonstrate that HCAT-1 and HCAT-2B are "y^+"-like carrier proteins with K_ms for CAA of about 0.2 mM. In contrast, HCAT-2A has a ten times lower substrate affinity. These data suggest that the transport properties of human and mouse CAT-proteins is determined by the same protein domain and that this domain might contain the substrate binding site.

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Amino acid transmembranar transport study in human red cell

A method for amino acid transmembranar transport study in human red cells, using isotope dilution GC/MS technique, is presented. Efflux of ^{14}N-glycine was calculated by using a stable isotopic labeled amino acid, ^{15}N-glycine, as internal standard. ^{15}N-glycine was also used to estimate the influx value. The quantitation of glycine, as trifluoroacetyl butyl ester derivative, was performed on a 1% EGA packed cromatographic column, at 140 °C, coupled to a double focusing mass spectrometer. The validation of the method in the range 0–70 µg/ml gave good linearity and reproducibility. The amino acid influx study in the red cell was related to temperature, time, amino acid concentration, presence of some ingredients (NaCl, drugs). An increasing amino acid influx in the red cell was detected. The influx was measured at 37 °C for 10 minutes to the concentrations of ^{15}N-glycine of 0.1, 1 and 5 mmoles/ml. Similar results were obtained by stable isotopic and radioisotopic measurements. Small influxes were detected for glycine. Incubation in the presence of some ingredients showed an increasing amino acid transport in the cell. The presence of an electric field gave increased values for glycine transport. The method is applied in glycine transport studies during electroporation.

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Emerging roles for sodium-dependent amino acid transport in mesenchymal cells

The major components of the intracellular amino acid pool in mesenchymal cells are L-glutamine and L-glutamate. The pools of these compounds are easily exchangeable; therefore, in glutamine-rich media both pools are maintained by membrane transport of this amino acid from the extracellular compartment. Through its interaction with the main transport systems for neutral amino acids (systems A, ASC, L, and N), L-glutamine plays a pivotal role in the exchanges of amino acids across the cell membrane thus influencing the intracellular concentrations of most amino acids. System A is the only transport system whose capability of accumulate glutamine into the intracellular compartment has been definitely ascertained. Therefore, the intracellular pool of glutamine (and, hence, of most amino acids) is modulated through changes in the activity of the system. The activity of system A is sensitive to a variety of conditions. An example of regulatory mechanism is offered by changes in the cell content of amino acids associated with volume-perturbing conditions. In hypertonic media the activity of the system is upregulated while it is depressed under hypotonic conditions. The mechanisms underlying these changes are only partially understood.

Due to the low affinity of system A for its substrates, all these mechanisms require the presence of fairly high concentrations of glutamine and neutral amino acids in the extracellular compartment. However, in several conditions glutamine avail-

ability is reduced. Unter these conditions cell glutamine rapidly falls and the maintenance of cell glutamate depends essentially upon the membrane transport of the anionic amino acid from the extracellular compartment. Three systems transport glutamate into mesenchymal cells, systems X^-_{AG}, ASC, and x^-_c; among these, only the activity of system X^-_{AG} appears to be responsible for the maintenance of cell glutamate. In mesenchymal cells system X^-_{AG} presents the same operational features described for the Na,K-dependent transporters cloned in nervous and epithelial tissues. Recently, its activity has been found to be completely suppressed in NIH3T3 cells expressing activated ras oncogenes. Moreover it is markedly sensitive to the proliferative status of the cell population, being 10-fold enhanced in cells maintained quiescent for several days. Also in cells incubated in the absence of glutamine or treated with asparaginase (an antitumor enzyme that rapidly converts glutamine to glutamate) the activity of the system is markedly upregulated through a slow mechanism that requires several days for a full expression. Thus, in mesenchymal cells system X^-_{AG} may represent a device to maintain a high transmembrane gradient of glutamate even under conditions of relative shortage of extracellular glutamine.

These results suggest that the physiological roles of sodium dependent transport systems for amino acids are not restricted to nutritional homeostasis but are involved in complex adaptive mechanisms such as cell volume regulation and cell response to environmental stress of various origin. (Aided by CNR, Target Project ACRO, Rome).

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Metabolite-activated protein kinase regulates catabolite repression in Bacillus

Two proteins, **CcpA** and **HPr**, have been identified to play a role in catabolite repression in *Bacillus subtilis*. Mutations affecting **CcpA** cause a pleiotropic relief from catabolite repression. **CcpA**, a repressor exhibiting homology to the LacI-GalR family, was suggested to bind to the catabolite responsive element present in most of the catabolite sensitive operons in Gram-positive bacteria. **HPr** is a phosphocarrier protein of the PEP:glycose phosphotransferase system (PTS). It can be phosphorylated at two different sites: a) at His-15 in a PEP-dependent reaction catalyzed by enzyme I of the PTS and b) at Ser-46 in an ATP-dependent reaction catayzed by the metabolite-activated **HPr** kinase. Mutations preventing the phosphorylation at Ser-46 of **HPr** lead to a pleiotropic relief from catabolite repression. These mutants exhibited a phenotype identical to ccpA mutants, thus suggesting a crosstalk between **CcpA** and P-ser-**HPr** during catabolite repression. Indeed, P-ser-**HPr** and **CcpA** were found to specifically interact with each other in a metabolite-dependent manner. **HPr**, P-his-**HPr** and the doubly phosphorylated P-his,P-ser-**HPr** did not interact with **CcpA**. Mutations affecting His-15 of **HPr** caused also a relief from catabolite repression. In contrast to wild-type P-ser-**HPr**, the serylphosphorylated His-15 mutant **HPr** did not interact with **CcpA**. This suggested that His-15 of **HPr** is involved in binding of P-ser-**HPr** to **CcpA** and that mutation or phosphorylation at this residue prevents the interaction of P-ser-**HPr** with **CcpA**.

The results summarized above suggest that catabolite repression in Gram-positive bacteria is triggered by a metabolite activated protein kinase catalyzing the formation of P-ser-**HPr** which in turn interacts in a metabolite-dependent manner with the repressor **CcpA** allowing its binding to the catabolite responsive elements present in catabolite sensitive operons. The sensitivity of this process towards phosphorylation of **HPr** at His-15 provides a link between catabolite repression and PTS function.

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Comparison different methods used to determine endogenous amino acid losses in chicken

True digestibility, unlike apparent digestibility of amino acids, appears to be independent of dietary protein level and may allow feed ingredients to be compared accurately even if they are ingested in different quantities. However they are not sufficient information about the level and the effects of different factors which influence endogenous amino acid losses (EAAL). Therefore different methods for the measurement of EAAL were compared.

During the first experiment EAAL were determined using 9 week old fasted chicks, after force feeding of N-free diet or using the regression method. During a second experiment 3 and 11 week old chicks were used to determine the EAAL at different ages. In this case N-free diet, containing TiO_2 as a marker was fed ad libitum and excreta samples were taken for 48 hours after an 48 hour long adaptation period. In order to investigate the effect of microbial activity in the lower gut digesta samples were also taken immediately after killing birds with carbon dioxide.

Using different methods for determination resulted different values for EAAL.

As expected the lowest value was measured with fasted chicks and dry matter intake increased EAAL. Since the highest dry matter intake was registered during the regression method the daily EAAL was the highest in this case. However when the daily EAAL was divided by the daily dry matter intake (EAAL expressed as mg EAAL/g d. m. intake) the highest value was registered during the method when chicks were force fed N-free diet.

There were significant differences in the endogenous amino acid contents of ileal digesta and excreta when they were measured at different ages. EAAL (the sum of AA) measured from digesta was significantly higher ($p < 0.05$) in young chicks, than in older animals. On the other hand EAAL determined from excreta was slightly higher in the older group, but this difference was not statistically significant.

Measuring with older chicks the EAAL content of excreta was significantly higher ($p < 0.05$) than the ileal amino acid flow. On the other hand in young chicks the EAAL measured from digesta was significantly higher ($p < 0.05$).

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A study of the status of threonine biosynthesis system in animals

Threonine is a member of the aspartate group "essential" amino acids which are not synthesized in animals. In this study we show that no activity of enzymes of threonine synthesis pathway is detectable in the tissues of invertebrate (Drosophila, Vermes) and vertebrate (fish, mouse, rabbit, mouse embryos) animals. Among the substrates of the pathway, only homoserine is present in high amounts in the animal tissues, as well as in human urine. In mice the highest concentration of homoserine is in liver (60 nmol per gram of organ). Also liver accumulated more exogeneous ^{14}C-homoserine compared to kidney, spleen and blood. It follows from the concentration of homoserine in the urine (6–10 μmol per ml) that about 1.5 mg of homoserine is removed from human organism daily at norm. Its amount rised more than 10 fold in urine of hepatite B patients, and decreased again to norm upon successful treatment.

We suggest that homoserine is not an active metabolite of animal and human cells but a final product of the methionine catabolic (transsulforation) process, and is extensively removed from the organism.

Experiments on transferring of E. coli genes for threonine synthesis into mice are in progress in this laboratory, with expectation that transgenic animals will utilize endogenous homoserine for threonine synthesis.

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Correaltion between nitric oxide synthase activity and reduced glutathione level in human and murine endothelial cells

A possible correlation between intracellular reduced glutathione (GSH) and sytheses of nitric oxide (NO) has been studied in human endothelial cells cultured from umbilical vein (HUVEC) and murine endothelial cells transformed by middle T antigen (tEnd.1). The synthesis of NO (detected as citrulline, which is produced stoichiometrically with NO from arginine) in HUVEC and tEnd.1 cells correlated with intracellular GSH. In HUVEC GSH level was 22.29 ± 1.6 nmol/mg protein and citrulline production was 1.24 ± 0.18 (basal) and 7.07 ± 1.14 (after ionomycin stimulation) pmol/min/mg protein; in tEnd.1 cells both GSH and citrulline levels were significantly higher: GSH = 77.57 ± 12.28 nmol/mg protein and citrulline = 9.9 ± 0.45 (basal) and 38.2 ± 1.3 (after ionomycin stimulation) pmol (min/mg protein. When tEnd.1 cells were loaded with GSH in order to double its intracellular level, ionomycin-elicited citrulline synthesis increased 2.4-fold. Similarly to what we have already observed in HUVEC, in tEnd.1 GSH depletion by incubation with 1-chloro-2,4-dinitrobenzene (CDNB) caused a dose-dependent decrease of citrulline synthesis, especially after ionomycin treatment: when GSH was lowered by 64% or 79%, ionomycin-elicited citrulline synthesis was lowered by 61% and 85%, respectively. Loading of cells with the permeating thiol N-(2-mercaptopropionyl)-glycine (MPG) did not significantly increase citrulline production, both basally and after ionomycin stimulation. Cell loading with GSH (but not with MPG) relieved the block of citrulline synthesis elicited by CDNB. These results suggest that GSH is necessary in transformed murine as well as in human endothelial cells for NO synthesis.

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Early destruction of tryptophan residues of apolipoprotein B is a vitamin E independent process during copper mediated oxidation of Low Density Lipoprotein (LDL)

The decrease of the tryptophan fluorescence (Ex/Em = 282/331 nm) was used to monitor the kinetics of copper mediated LDL oxidation. Cu^{++} causes a concentration dependant quenching of the LDL Trp-fluorescence, the maximum of about 22% suggests that 8–9 Trp residues (out of a total of 37) are accessible for Cu^{++} ions. Decomposition of LDL tryptophan commences immediately after addition of Cu^{++} and proceeds in two stages with quite different rates. At a molar ratio of Cu^{++}/LDL = 33:1 the LDL particle looses 1 Trp every 13.5 minutes in the initial slow phase and every 4.1 minutes in the subsequent rapid phase. The second, stage temporarily coincides with the propagating lipid peroxidation. In the initial phase loss of Trp proceeds with a constant rate for up to 200 minutes depending on the copper concentration. Whereas lipid peroxidation accelerates after consumption of vitamin E, rate of Trp loss does not increase. Loading of LDL with vitamin E has also no effect on the initial rate of Trp loss. During the initial phase a loss of one Trp residue/LDL is accompanied by the loss of two α-tocopherols and the generation of two conjugated lipid-hydroperoxides. The results suggest Trp residues play a role in initiating the lipid peroxidation process in the LDL particle. In such kinetic studies, precautions must be taken to avoid photodecomposition of LDL-Trp. The LDL vitamin E fluorescence (Ex/Em = 290/323 nm) does not interfere with the Trp fluorescence even at high concentrations.

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Mechanism of protein modifications by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction

Long term incubations of glucose with proteins lead to crosslinking and modifications like N-ε-(carboxymethyl)lysine (CML) and pentosidine. Both structures have been established in vivo and quantitatively correlated with aging and diabetes. The formation of CML is so far explained by oxidative cleavage of the Amadori product, the first more stable modification in the reaction of proteins with reducing sugars, also called Maillard reaction.

As CML incorporates a C2-fragment of the original sugar this suggests an alternative reaction pathway including glyoxal and glycolaldehyde. These carbonyl structures represent highly reactive intermediates of the Maillard reaction, the formation of which so far has been only established in heated reaction systems. Indeed, both structures could be identified as immediate precursors of CML. The nature and importance of this reaction pathway under physiological conditions was elucidated by incubating amines with higher sugars in the presence of amino-

guanidine and boric acid, as well as with radioactive labelled sugars.

Both, glyoxal and glycolaldehyde lead to extensive crosslinking of proteins. Crosslinking via imine bonds could be established starting from both carbonyl compounds as well as from higher sugars by monitoring a common reduction product. C2-iminecrosslinks are strictly depend on the amount of glyoxal/glycolaldehyde present in the system and after initial rapid formation are fast degraded to more stable modifications.

To gain other independent evidence for glyoxal protein modifications, the reaction with the guanidino group of arginine was studied. After acid hydrolysis the modification can be followed as an imidazolinon derivative not only from glyoxal but also from glycolaldehyde and higher sugars. Studies are in progress to verify glyoxal-arginine modifications in vivo.

The results of this study establish unequivocally the formation of glyoxal and glycolaldehyde during the Maillard reaction of higher sugars under physiological conditions and the formation of resulting lysine and arginine modifications. Glyoxal and glycolaldehyde could therefore play an important role in modification and crosslinking of proteins in vivo.

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Glutaminase and glutamine sythetase activities in rat and human hepatic materials

Glutaminase and glutamine synthetase, the major enzymes involved in glutamine metabolism, are heterogeneously distributed in liver. The aim of our work was to compare glutaminase and glutamine synthetase activities in rat and human liver and in the human hepatoma cells Hep G2 in culture. Our technique is based on aqueous chromatographic separation of [U $^{-14}$C] glutamic acid from [U $^{-14}$C] glutamine on DEAE-cellulose paper, adapted for simultaneous enzymatic determinations. Activities [expressed as nmoles of product formed per minute per mg of protein, mean ± SD] were 28.5 ± 2.6 (n = 13) for glutamine synthetase and 58.0 ± 3.9 (n = 10) for glutaminase in rat liver homogenates, in agreement with previous reported values. Glutamine synthetase (7.1 ± 1.3, n = 8) and glutaminase (17.3 ± 1.2, n = 9) were lower in normal human liver than in rat liver. When we compared normal human liver and Hep G2 cells enzymatic activities, we observed similar values for glutamine synthetase (7.6 ± 1.4, n=4) and an increased glutaminase activity (62.2 ± 5.8, n = 6). Since glutaminase and glutamine synthetase activities are both present in Hep G2 cells, this cell line provides a uesefull tool to investigate the parameters involved in the coordinated regulation of these two major enzymes of glutamine metabolism.

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A novel study on retinal histamine biosynthesis

Histamine (HI) is a polyhedric functional compound and there is increasing interest in studying its metabolism in various tissues and biological fluids. It has been associated with mast cell and blood vessels; it has been localized in many brain areas and recent biochemical assays have also indicated its presence and biosynthesis in the vertebrate retina (Nowak et al., 1984).

Recently, a possible addition of histamine to the list of neurotransmitters and/or neuromodulatory agents has been suggested as a result of evidence involving histamine as the neurotransmitter released by a variety of arthropod photoreceptors.

The vertebrate retinal rod photoreceptor cell contains a specialized organelle called the rod outer segment (ROS) which functions in phototransduction.

Aim. To describe for the first time the presence of histamine and histidine decarboxylase activity (HDC) in rod outer segments from bovine retina.

Methods. Rod outer segment preparation was performed as reported by Nicotra et al. 1982.

Histamine analysis. HI was separated by high performance liquid chromatography (HPLC) using a pre-column derivatisation with Shore's o-phthalaldehyde (OPA) reaction (1959). The liquid chromatographic system consisted of a model 510 pump Waters, equipped with a Theodyne 0193 injection valve with 20 µl sample loop and a Waters 420-AC fluorescence detector. The wavelengths were set at 450 nm for emission and 350 nm for excitation. Chromatograms and all calculations were performed on an Waters model-730 Data Module. The reversed phase column used was an analytical Spherisorb column, particle size 5 µm, 5 cm × 4.6 mm, 83 ODS 2 with a mobile phase of methanol, 20 mmol/L sodium acetate in distilled water, acetic acid (55 : 43 : 2 : v/v) and 0.33 mmol/L 1-octanesulfonic acid sodium salt, as the ion-pairing agent. The flow rate was 1.0 ml/min. The system was operated at room light and one temperature. The retention time for HI was 2.5 min.

HDC assays. HDC was assyed with 8 mg of ROS proteins in 1.5 ml of reaction mixture (final concentrations) als follows: 100 mM sodium phosphate buffer (pH 6.1), 100 nM L-histidine, 100 µM PLP, 100 µM aminoguanidine and 0.1% Triton X-100. In blanks, the substrate was replaced by an identical volume of buffer during incubation and L-histidine was added after protein precipitation. Incubation was carried for 2 h at 37 °C, and the reaction was terminated by addition of 0.5 ml of 1.6 M HClO4. Histamine formed during incubation was separated as reported above.

Results and conclusions. HI content of photoreceptor rod outer segments of bovine retina was determined by HPLC chromatography with o-phtalaldehyde pre-derivatisation. In our previous study HI was separated by ion exchange chromatography and evidenced with OPA. Using this new sensitive method of determination of histamine, HDC activity from ROS was assayed, determining a reaction rate of 9 pmol/min/mg protein. Our previous observation (Gueli et al., 1990, 1991) that retina photoreceptor membranes are a site of histamine formation was confirmed. We are about to study this biosynthesis regulation.

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Blood to brain transport of basic and acidic amino acids following toxic liver damage and treatment with ornithine aspartate

The present study investigated the brain uptake index (BUI) for basic amino acids ornithine (Orn), arginine (Arg) and lysine (Lys) and an acidic amino acid aspartate (Asp) in control rats and in rats treated with a hepatotoxin, thioacetamide (TAA) to induce hepatic encephalopathy (HE). Some of the animals in either group received i. v. ornithine aspartate (OA), a drug structurally related to the γ^+ substrates that ameliorates neurological symptoms following liver damage by improving detoxi-

cation of ammonia in peripheral tissues: The compound was administered either by continuous infusion for 6 h at a routine therapeutic concentration of 2 g/kg (final blood concentration ranging from 0.19–0.5 mM), or as a 15 sec. bolus together with the radiolabelled amino acids, at a concentration of 0.35 mM. In control animals, the BUI values for Arg, Orn, Lys and Asp were on average 16.05, 10.26, 8.56, and 2.07 respectively. In consistence with earlier data (Albrecht et al., Neuroreport, 5, 671, 1994), TAA treatment resulted in a delayed and progressive increase of BUI for ORN, to 186% of control at 7 d posttreatment and to 345% of control at 21 d posttreatment, when despite sustained liver damage the HE symptoms were already absent. By contrast, the BUI for Arg decreased to 30% of control at 7 d posttreatment and remained low (42% of control) at 21 d posttreatment. A 6 h infusion of OA to untreated rats resulted in a reduction of the BUI for Arg and Orn to 51% and 62% of the control level, respectively. Reductions of a similar magnitude were noted with both amino acids following the 15 sec OA bolus, indicating direct interaction of OA with the transport site in either case. OA administered by either route abolished the enhancement of BUI for ORN, but did not further inhibit the BUI for ARG in the TAA-treated animals. The BUI for Lys was unaffected by TAA and/or OA treatment in all the groups of animals, and the BUI for Asp was slightly increased in a 21 d group treated with OA only. The results indicate that some as yet unspecified factors released from damaged liver either modify the structure or conformation of the γ^+ transporter at the BBB from the normally Arg-preferring to the Orn-preferring state, or activate (induce) a different transporter specific for Orn which is normally latent. The potential pathophysiological (and in the case of OA therapeutic) implications of the changes in Arg and Orn transport are discussed in view of the role of the two amino acids as substrates for several metabolic events associated with neurotransmission. (Supported by SCSR grant n. 6 P207 059 05, and Merz & Co, GmBH & Co. (G. Q.))

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Retarded proteolysis of oxidized myosin relative to the native protein

Oxidized proteins have been documented to be prone to proteolysis as do denatured proteins in general. We found that when myosin, the main skeletal muscle protein is peroxidized by H_2O_2/Hemoglobin – it forms covalent myosin aggregates. Since skeletal muscles provide an important dietary protein source, it was of interest to study proteolysis of the peroxidated and native myosins by representative digestive enzymes of the mammalian digestive tract, pepsin, trypsin and chymotrypsin under conditions prevailing at their loci of action. Thus 1 mg/ml of either myosin forms was incubated for 30 min. At 37 °C with 400 unit/ml pepsin at gastric pH of 1.85 followed by 10 min incubation at duodenal pH of 8.0 with 10 unit/ml trypsin and 1 unit/ml chymotrypsin. Reactions supernatants as well as SDS-solubilized supernatants of reactions pellets were analyzed by both SDS-PAGE and 280 nm absorption. Analysis of pepsin treated myosins revealed that myosin monomer was more susceptible to degradation than the crosslinked form. Despite resolubility of the crosslinked, but not the native protein, upon elevation of the solutions' pH from 1.85 to 8.0, the combined enzymatic digestion by trypsin and chymotrypsin of the crosslinked form was still retarded relative to the native protein. We concluded that unlike protein oxidations which confer enhanced proteolysis, oxidatively crosslinked myosin is relatively proteolysis resistant.

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Glutathione: The essential factor for live functions

The inhibition of mitochondrial DNP- or phosphate transport by the thiol reagent NSPM (N' [N"-n-nonyl-4-sulfamoylphenyl]-malei-mid) suggests the involvement of a regulatory factor in this transport activities [1, 2]. This regulatory factor proved to be glutathione, meanly by its reactivity with the sulfenyl reagent NTU (n-Nonylthiouracil) and the modulation of free and bound glutathione concentrations by phosphate [1, 2]. A mechanism for mitochondrial ATP synthesis on the 30 kDa P_i/H^+-symport system with oxidized glutathione as catalyst has been presented. Sulfenylphosphate participation in this mechanism should be proven by the sulfur analog of phosphate, namely thiophosphate, which presumably functions as suicide inhibitor for ATP synthesis [1, 2].

Mitochondrial K^+/H^+-antiport, K^+-pump and P_i/H^+-symport/ATP synthase are linked in versatile energy driven K^+/H^+-cycling and oscillations. The whole system is dependent on oxidized and reduced glutathione. Many diseases are due to perturbation of this system [1, 2]. The described system is also responsible for thermoregulation of our body. Acetylsalicylic acid presumably acts via nervous system (brain) on the center responsible for thermoregulation. Antipyretic malaria therapeutics like chinin may change Ca^{2+}/Mg^{2+}-binding to mitochondria and by this way regulate the body temperature [1–3].

The functions of glutathione in the cells were extensively discussed and described [4]. The functions in mitochondria, besides the involvement in ATP synthesis, are not as clear. Detoxification of mitochondria results in their "evolutionary development" with raised glutathione concentrations (relation to Moris hepatoma). A high turnover rate of chemicals (plasma–cell–mitochondria–cell–plasma) sharply rises glycolysis rates, which finally lead to enhanced proliferation of cells. Mitochondria are able to be inhibited by Ca^{2+} (via – for instance – complexation of glutathione) and this inhibition may lead to certain types of tumors (high rates of glycolytic formation of ATP).

Glutathione seems not to be directly involved in the redox potentials responsible for development of atopic eczema or AIDS and change in proliferation rates [5].

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Enzymes of tryptophan catabolism

The oxidative transformation of tryptophan into kynurenine take significant place in the tryptophan utilization in animals. The first and rate-limiting reaction of this process is catalysed by the tryptophan pyrrolase (TP). The TP realizes the detoxication of tryptophan, the regulation of liver haem metabolism and the amount of biologically active amins in the organism. The

present communication gives the results of isolation and study of TP properties from the white rat liver and the influence of the high external temperature and insolation on its activity. The mechanism of enzyme induction by hydrocortizone under these conditions was also studied. The TP was isolated from crude liver homogenate by solt fractionation with different concentrations of ammonium sulfate. The 8-fold enzyme purifying was obtained.

The study show that enzyme is the highest active at pH 8.8 with wide activity interval (from pH 8.5 to pH 9.5). The enzyme activity depends on the nature of buffer used: TRIS-buffer, carbonate buffer and phosphate buffer. The enzyme temperature dependence determination reveals that optimal temperature for TP holoenzyme is 45 degree of C and for TP apoenzyme – 35–40 degree of C. TP heating at 55 degree of C for 5 min only leads to full pyrrolase activity loss. On the other hand, the enzyme is high stable to temperature effects in the phosphate buffer, pH 7.4. The presence of monovalent ions in incubation media causes the TP activity decreasing more expressed from 0.2 M concentration. The Natrium more strongly depresses the TP holoenzyme activity, and potassium – on the apoenzymy one. The TP kinetics was investigated and Mihaelis constants (Km) were measured. Obtained values of Km are for tryptophan 1500 mkM and for haem – 0.1 mkM. These results reflect the high TP affinity to substrate and haem. We also studied the TP activity induction by high temperature and insolation exposure. The results of these experiments show that there are two different mechanisms involved into increasing of tryptophan pyrrolase activity.

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Regulation of the β-system in Ehrlich ascites tumor cells

The β-system transports β-amino acids and tolerates, in contrast to other amino acid transporting systems, a sulphonate and a sulphinate group. Taurine influx via the β-system in the Ehrlich cells is characterized as an appartently electroneutral $2Na^+$, $1Cl^-$, 1 taurine cotransport, which is driven by the transmembrane electrochemical Na^+ gradient [1, 2]. However, taurine influx in Ehrlich cells is stimulated by alkalinization and by hyperpolarization of the cell membrane [3]. The isoelectric point pI of a taurine/β-alanine transporter, cloned from a mouse brain cDNA library, is estimated at 5.98 [4]. Treating the taurine carrier as a protein with a single dissociable group, an alkalinization would increase the fraction of taurine carrier on the anionic form. The cell membrane in Ehrlich cells is also hyperpolarized at alkaline pH [3]. Thus, an accelerated return of unloaded negatively charged carrier to the outside of the cell membrane at alkaline pH could explain the concomitant increase in the taurine influx. Taurine efflux from Ehrlich cells suspended in isotonic standard medium is mediated by a Na^+-independent system, which resembles the β-system with respect to pH and potential sensitivity [3].

Molecular cloning of the β-system from other mammalian cell lines unveils potential intracellular sites for phosphorylation by protein kinase A (PKA) and by protein kinase C (PKC) [5]. The intracellular content of 3',5'-cyclic adenosine monophosphate (cAMP) and the rate constant for taurine influx in Ehrlich cells are both increased by addition of forskolin, and the effect is potentiated by preincubation with the phophodiesterase inhibitor theophylline. The rate constant for taurine influx is also increased by addition of the membrane permeable dibutyryl cAMP, which is resistant to breakdown by the phosphodiesterases. In contrast, exposing the Ehrlich cells to phorbol 12-myristate 13-acetate (PMA), results in a significant reduction in the rate constant for taurine influx. Since cAMP and PMA are known to stimulate PKA and PKC, respectively, it is suggested that activity of the β-system in Ehrlich cells is increased when the β-system is phosphorylated by PKA and decreased when it is phosphorylated by PKC.

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Regulation of amino acid transport in renal epithelial cells

The bovine renal epithelial cell line NBL-1 has proved a useful system to study the regulation of amino acid transport by amino deprivation-related stress. These cells express a single Na^+-dependent transport system of unusually broad specificity for neutral amino acids, similar to the broad specificity system termed System B° previously described in renal brush border membrane vesicles. System A is absent in confluent cultures of these cells. The cells also express high activities of the high affinity Na^+-dependent glutamate transporter System X_{AG}^-. Total amino acid deprivation of these cells over a period of hours causes induction of System X_{AG}^- and simultaneously causes the emergence of System A activity. System B° is not induced under these conditions, but is specifically induced if the cells are further stressed by addition of high concentrations of phenylalanine to the amino acid-free medium. Neither System B° nor System A have yet been cloned. Induction of System B° is accompanied by the appearance of a new mRNA transcript which hybridises with the cDNA probe known as SAAT1 which encodes a low affinity glucose transporter, suggesting the possibility that SAAT1 and System B° may be related. Induction of System A is accompanied by the increased glycosylation of a membrane glycoprotein of novel amino acid sequence with a Mr of 110 kD. Some properties of this protein will be described and its possible relationship with the System A transporter discussed.

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Complex regulation of the cationic amino acid transporter, mCAT-2

The *m*CAT-2 gene encodes two distinct proteins, mCAT-2 and mCAT-2a that mediate the flux of arginine, lysine and ornithine in a Na^+ independent manner [1–3]. When expressed in Xenopus oocytes, mCAT-2 protein mediates a high affinity transport, whereas the mCAT-2a isoform appears to mediate both high and low affinity arginine transport [4]. Another gene, *m*CAT-1 encodes a protein that is functionally indistinguishable from mCAT-2 [3]. We have examined their expression patterns and the molecular mechanism of *m*CAT-2 gene regulation to address the reason for the apparent functional redundancy of

these genes. The expression of mCAT-1, mCAT-2 and mCAT-2a was examined by reverse transcriptase/polymerase chain reaction assays in quiescent and activated lymphocytes, normal and regenerating liver, skeletal muscle from fasting and fed animals and in kidney epithelia with and without cytokines. The results demonstrate that mCAT-2a transcritps predominate in liver, a finding consistent with the reported low affinity arginine uptake in hepatic tissue. The *m*CAT-2 gene gives rise to transcripts that show five distinct 5' untranslated regions (UTRs). The exons encoding those UTRs are dispersed over 36 kilobases of DNA (illustrated below). Some of them are preceded by classical TATA-less, G rich promoter sequences with a variety of potential hormone binding regions, high affinity SP1 of AP1 consenus sequences and other regulatory sites. One of the exons contains a classical TATA promoter located 26 bp upstream of the start sequence. The complex promoter structure of the *m*CAT-2 gene permits highly regulated expresion under a variety of physiological conditions. The *m*CAT-2 gene is induced during the maturation of monocytes to macrophages following activation. This induction is coordinate with an increase in arginine transport. Since arginine is the immediate and exclusive precursor of nitric oxide, the control of arginine transport may play a pivotal role in macrophage cytotoxic function in response to pathogens and tumor cells.

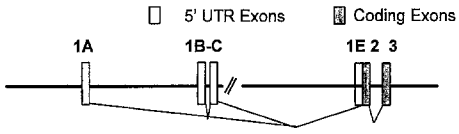

Fig. 1

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Intramitochondrial localization of alanine aminotransferase in rat-liver mitochondria: comparison with glutaminase and aspartate aminotransferase

The removal of the outer mitochondrial membrane and hence of constituents of the intermembrane space in rat-liver mitochondria using digitonin showed that phosphate-dependent glutaminase, alanine and aspartate aminotransferase were localized in the mitoplasts. Further fractionation of mitoplasts following their sonication resulted in 90% of glutaminase, 98% of alanine aminotransferase and 48% of aspartate aminotransferase being recovered in the soluble fraction while the remainder of each enzyme was recovered in the sonicated vesicles fraction. These results indicated that glutaminase and alanine aminotransferase were soluble matrix enzymes, the little of each enzyme recovered in the sonicated vesicles fraction being probably due to entrapment in the vesicles. On the other hand aspartate aminotransferase had dual localization, in the inner membrane and matrix. In contrast to the other two enyzmes, aspartate aminotransferase had a high specific activity in sonicated vesicles indicating its association with the membrane. Comparison of its relative activation by triton X-100 in mitoplasts and sonicated vesicles suggested that the membrane-bound enzyme was localized on the inner side of the inner mitochondrial membrane.

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Expression of human glutamate transporter subtype mRNAs in central nervous system and peripheral tissues

Excitatory amino acid transporters play an important role in regulating synaptic and extracellular concentrations of glutamate. We compared the expression of mRNAs of three glutamate transporter subtypes, hEAAC1, hGLT-1 and hGluT-1, in human central nervous system (CNS) and peripheral tissues by Northern blot analysis. Each subtype mRNA expression showed a different distribution pattern among various human tissues. Hybridization with hGLT-1 revealed that 10.0 kb mRNA was abundant in the brain and slight in the pancreas. The expression of hGluT-1 mRNA (4.2 kb) was the highest in the brain and low in the placenta, skeletal muscle or heart, and also detectable in the lung, liver, or kidney. cDNA probe of hEAAC1 hybridized multiple bands (3.8 kb and 2.4 kb) in almost all tissues and the proportion of these two bands was different among various peripheral tissues.

We compared the amount of subtype mRNA expression in 16 human CNS regions. All three subtype mRNAs were expressed in every region examined. hGLT-1 mRNA was abundant in the cerebral cortex, limbic system, including amygdara and hippocampus, or caudate nucleus, but scarce in the spinal cord. The expression of hGluT-1 was high in all regions examined, but the highest in the cerebellum where that of two other subtype mRNAs was low. hEAAC1 messages, both 3.8 kb and 2.4 kb was distributed similarly; rich in the thalamus, cerebral cortex and caudate nucleus, but poor in the cerebellum, corpus callosum and subthalamic nucleus. These studies provide useful informations to understand the role of glutamate transporters in human excitatory neurotransmission and in the pathology of CNS degenerative diseases.

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Some unique effects of proline on glycogenesis, glyconeogenesis, and gluconeogenesis in perfused rat livers

The hepatic enzyme glucose-6-phosphatase (EC 3.1.3.9) occupies a critical position in glucose homeostasis. The enzyme catalyzes several hydrolytic and biosynthetic activities. It functions as a system consisting of at least six components. The objective of these studies was to investigate possible directive interactions of various metabolites with several components and activities of this enzyme system. A combination of enzyme kinetic and perfusion techniques with livers of 48-h fasted rats was employed.

It was found that: a) the complexities of the glucose-6-phosphatase system provide for activity-discriminant interactions with various inhibitors and substrates, b) 3-mercaptopicolinate specifically inhibits the glucose-6-P phosphohydrolase activity of the enzyme and thus favors glycogen production over glucose formation from gluconeogenic substrates, c) a proline-derived metabolite, in situ, similarly favors glyconeogenesis, d) an enhanced availability of carbamyl-P for non-urea cycle functions with added proline in contrast with glutamine promotes glycogenesis from glucose initiated via carbamyl-P:glucose phosphotransferase activity of the glucose-6-phosphatase system, e) ammonium ion favors ureagenesis

over glycogenesis; *nor*valine (an inhibitor of ornithine transcarbamylase) with ammonium ion inhibits ureagenesis and stimulates glycogenesis from glucose; the glycogenic effect of *nor*valine is greater with 34 mM than with 9 mM glucose; and ethoxyzolamide (an inhibitor of carbonic anhydrase) inhibits both ureagenesis and glycogenesis, f) glycogenesis correlates with enhanced availability of carbamyl-P for non-ureagenic functions, g) a Km,glc for glycogenesis from glucose of 31 mM with isolated livers perfused with glucose, ammonium ion, and *nor*valine, obtains h) an increase in general ionic strength between 0.0025 and 0.05 favors biosynthetic activity of the glucose-6-phosphatase system over its glucose-6-P phosphohydrolase activity with intact microsmomes, i) Cl^- specifically and selectively inhibits phosphotransferase over glucose-6-P phosphohydrolase, and thus loss of Cl^- deinhibits the former more profoundly than the latter.

These observations identify the hepatic glucose-6-phosphatase system as a focus for metabolic regulation, in situ, both through inhibitions specific for glucose-6-P phosphohydrolase activity, and via activation of biosynthetic function(s) of this system (e.g., carbamyl-P:glucose phosphotransferase activity) under appropriate metabolic conditions. (This work was supported in part by research grants DK07141 from the National Institutes of Health and EHR 9108770 from the National Science Foundation).

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Long term osmotic regulation of amino acid transport systems

Several amino acid transport systems are up-regulated after hypertonic shock in mammalian cells. In the renal epithelia cell line NBL-1, the Na-dependent amino acid transport systems A and Xag- (EAAC1) are induced under specific culture conditions. This is accompanied by an increase in Na, K-ATPase activity and a 3-4fold increase in the mRNAs for the α1 subunit of the pump and for the EAAC1. The major transport system in this cell type, B^0, is not up-regulated after hypertonic treatment. Induction of system A is independent of the known adaptive response, because it is additive to the amino acid starvation effect and insensitive to tunicamycin. Furthermore, the increase of system A activity also occurs in fibrolasts. CHO-K1 cells do also show this response in hypertonic stress and the alanine resistant mutant CHO-K1 ala^r4, which has lost its ability to further respond to amino acid starvation, is still sensitive to anisotonic media. We provide evidence for the existence of a putative system A activating protein(s) (SAAPs), which may be under osmotic control at the gene level and activate system A carriers previously inserted into the plasma membrane. The characteristics of this response regarding the specificity and requirement of cytoskeletal elements, has been studied in both NBL-1 (epithelial polarized cells) and CHO-K1 cells (non polarized cells). Although the biological effect is similar, the microtubule and microfilament network is not equally contributing to the increase of system A activity in mammalian cells depending on whether they are polarized or not.

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Uncharged tRNA-phosphofructokinase interaction in amino acid deficiency

When the tRNA of mammalian cells is incompletely charged due to an amino acid deficiency or by analogs which cannot be activated, many metabolic events become limited. This rapid demise of cell function appears to be because of the inhibition of phosphofructokinase (PFK) by uncharged tRNA (FEBS Lett. 302: 113 (1992)). Charged tRNA has been shown to be "sequestered within the protein synthetic machiner", (Negrutskii, B. S. and Deutscher, M. P., Proc. Natl. Acad. Sci. U. S. A. 89: 3601 (1992)) and would therefore be removed from an inhibitory role. Besides the direct demonstration that tRNA inhibits PFK in an assay regarded as indicative of its control mechanism, several reports in the literature support this model. These include 1) The rapid onset of inhibition of glycolysis and glucose uptake by intact cells upon amino acid deficiency and the similar lesion at the 43S ribosomal subunit on glucose or amino acid deprivation. 2) The recognition that unusually high concentrations of cAMP required to stimulate protein synthesis in energy depleted or gel filtered lysates correlates with its action on PFK as an analog of the positive effector, adenosine-5'-monophosphate. 3) The often repeated observation that the product of PFK activity, fructose-1,6-diphosphate, is a stimulant of protein synthesis (see Jackson, R. J., et al., Eur. J. Biochem. 131: 289-313 (1983)). This diphosphate has been shown to be the proximate effector, binding to eIF-2B, the guanine nucleotide exchange factor (Singh, L. P., Arror, A. R. and Wahba, A. J., FASEB J. 8: 279 (1994)) which by releasing GDP bound to the inactive GDP:eIF-2 complex, permits the factor to initiate a new peptide chain.

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Characterization of l-arginine transport in smooth muscle cells (SMC) and its regulation by Angiotensin II (Ang II)

In view of the fact the Ang II and nitric oxide (NO) exert contrasting effects on SMC contraction, growth and proliferation, we evaluated the possibility that Ang II modulates NO synthesis through the regulation of l-arginine transport in SMC. To accomplish this objective, the uptake of [^{3}H]-arginine was measured in cultured aortic SMC derived from Long Evans rats.

Arginine uptake in these cells occurs through Na^+-independent (55%) and Na^+-dependent (45%) pathways. The Na^+-independent arginine transport occurs through system y^+, whereas the Na^+-dependent route is similar to system $B^{+,0}$, because it is inhibited by neutral and cationic amino acids. Arginine uptake through system $B^{+,0}$ is inhibited (45–90%) by 100 nM All with a Kd = 8.9 ± 0.9 nM, a value similar to the Kd (2.4 ± 0.2 nM) of the Ang II-induced calcium mobilization in this preparation. The inhibition of arginine uptake by Ang II is reversed by DUP 753, a specific inhibitor of the Ang II type-1 receptor. This inhibition is specific for system $B^{+,0}$ because systems A and L are not affected by this hormone. The effect of Ang II is inhibited by Pertussis toxin and by staurosporine, suggesting a role for G-Proteins and Protein Kinase C. The latter agrees with inhibitory effects of phorbol esters on arginine transport.

To determine whether the effect of Ang II on arginine transport is correlated with NO synthesis, the IL-1β-induced nitrite

production was measured in the presence and absence of this hormone. The results indicate that Ang II substantially reduced nitrite synthesis as did N-monomethyl-arginine and aminoguanidine, which are inhibitors of NO synthase. These results suggest that Ang II regulates NO synthesis through alterations in arginine transport in vascular SMC. (Supported by NIH grant RR-08224 Sharp and Dohme Corp.)

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Involvement of CFTR protein in the efflux of neutral amino acids

Recent data indicate that CFTR may behave as a multifunctional protein, endowed with both channel and transporter capabilities. To identify possible organic substrates of the protein we have determined the *trans*-membrane fluxes of leucine in mouse C127i cells transfected with the wild type CF gene (C127 CFTRw/t), the ΔF508 CF gene (C127 CFTRΔF508) or the vector only (C127 mock). Leucine influx was comparable in all the cell lines and referable to a "L-type" transport system. On the contrary, leucine efflux was significantly faster in C127 CFTRw/t. No significant differences in leucine content were detected among the three cell lines when maintained in complete growth medium; in contrast, after a prolonged incubation in amino-acid-free saline solution, the amount of intracellular leucine was significantly smaller in C127 CFTRw/t than in both C127 CFTRΔF508 and C127 mock cells. Leucine behavior was shared by valine, isoleucine, glutamine but not by glycine or threonine. Phosphorylation of CFTRw/t upon PKA activation and orthotopic expression of ΔF508-CFTR on cell membrane (obtained through a prolonged incubation at 30 °C) produced the expected changes in Cl^- fluxes, as indicated by modifications in the membrane potential detected by non invasive methods. However, neither treatment significantly affected leucine efflux, as indicated by determinations performed in parallel cultures under the same experimental conditions. These results suggest that normal CFTR is involved in the efflux of a group of neutral amino acids; the relationship existing between this effect and the chloride fluxes mediated by the protein remains, however, to be defined.

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Murine liver homogentisate, 1,2-dioxygenase (HGO): Biochemical properties of the key enzyme of alkaptonuria and its cDNA

HGO catalyses an irreversible key reaction in the catabolic pathway of aromatic amino acids. Complete deficiency of HGO activity has been shown to cause alkaptonuria. In 1994 an animal mutant for alkaptonuria, the aku mouse, was described.

We purified HGO from normal mice to homogeneity. It is a homomultimer consisting of subunits of about 49 kDa.

We used the purified protein as antigen for the production of polyclonal rabbit antibodies. The rabbit serum detects the mouse HGO as well as rat and human HGO as a single band of almost identical molecular mass on Western blots. No such band could be identified on Western blots of aku mouse livers. HGO protein seems to be completely absent in aku mice.

After tryptic digestion, a partial sequence of altogether 45 amino acids could be determined from 4 different peptides. We designed 3 degenerated oligonucleotide primers for RT-PCR and were able to amplify 3 specific products from $polyA^+$-mRNA of mouse liver. A PCR-fragment of 200 bp containing 2 primers from different peptides was cloned and sequenced. Its DNA sequence and the amino acid sequence showed no significant homology to proteins in the GENBANK and SWISSPROT databases. PCR with the same primers on rat and human mRNA resulted in amplification of an analogous fragment. The 200 bp fragment was used to screen a murine liver cDNA-bank to get the full length cDNA of about 1.5 kb.

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Reaction mechanism to D-tryptophan in tryptophanase

Chiral breaking in amino acids is very great riddle in origins of life. We are interested in the selection mechanism of L-amino acids in enzymes, and we assume that the high specificity for L-amino acids is partly responsibility for environment around enzyme. We succeed in breaking the very high specificity for optical isomers by enhancing ion strength of enzyme solution. Tryptophanase which we choose as the subject of our study is enzyme that can rigidly discriminate L-isomer form D-isomer. It can react with L-tryptophan only under ordinary condition. It becomes to react with D-tryptophan in the solution of highly concentrated diammonium hydrogen phosphate, $(NH_4)_2HPO_4$. This reaction rises with saturation concentration of $(NH_4)_2HPO_4$ in the range from 0% to 50%. On the other hand, it falls over 50% saturation. According to kinetical analyses, the reaction can be expressed conveniently in terms of the sequential Bi-Bi reaction model in the former concentration, while in therms of noncompetitive inhibition in the latter concentration. Reaction model from kinetics shows that complex of tryptophanase · D-tryptophan · $(NH_4)_2HPO_4$ will be formed. Ion exchange column chromatography is used to establish the complex from the following viewpoint. Fraction number of tryptophanase, which is eluted over the ion exchange column chromatography, treated with $(NH_4)_2HPO_4$ will differ from one of tryptophanase untreated with $(NH_4)_2HPO_4$ because of different electrical charge on tryptophanase surface. Experiments support this description. Our results show that kinetical analysis is very effective to elucidate the reaction mechanism, and propose that higher-order conformation of tryptophanase relates to the selectivity of L-tryptophan.

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Changes in gene expression and protein localization of glutamate transporter (GLAST/GLUT-1) following transient retinal ischemia

We have demonstrated cellular localization of glutamate-aspartate transporter (GLAST/GluT-1) in the rat retina and its induction after transient ischemia using in situ hybridization and immunohistochemistry.

GLAST mRNA was expressed in the inner two-thirds of the inner nuclear layer (INL) and sparse small cells in the inner portion of the ganglion cell layer (GCL) of the rat retina. GLAST-like immunoreactivity was predominantly localized in

Müller fibers in the outer plexiform layer, the inner plexiform layer, and the Müller end feet in the retina. Experimental occlusion of the central retinal artery followed by reperfusion for 48 h resulted in degeneration of neurons in the INL and the GCL, a marked increase in GLAST mRNA expression in the INL and the GCL, and emergence of intense GLAST-like immunoreactivity in INL. At 168 h of recirculation, GLAST mRNA levels returned to control levels, but intense GLAST-like immunoreactivities were found in the Müller fibers in the outer nuclear layer.

These findings suggest that GLAST may play an important role in regulation of extracellular glutamate concentration especially under ischemic conditions by changing the gene expression and protein localization.

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Regulation of amino acid transport in intestinal epithelia

Amino acids are transported by small intestinal cell plasma membranes via systems unique to epithelia plus systems found throughout the body. With evidence from cell lines as well as the intact organism, a picture has evolved whereby intestinal amino acid transport adctivites are independently regulated. Adaptive regulation can be triggered by individual amino acid substrates specific for a particular transporter, by certain peptide growth factors (EGF, TGFα), or by enterocytic differentiation. We compare and contrast the differential regulation of intestinal amino acid absorption using the examples of sodium-activated neutral amino acid transport system B and sodium-independent cationic amino acid system y^+. For each system, growth factor-induced and specific substrate-induced chronic upregulation of uptake activities are prevented by cycloheximide or actinomycin D. Transport capacities (Vmax, but not Km) are stimulated by phorbol esters, while growth factor-induced uptakes are blocked by chelerythrine or photoactivated calphostin C. Neither forskolin nor dbcAMP influence amino acid transport. Thus, intestinal amino acid uptake regulation involves *de novo* protein synthesis under the control of protein kinase C. Intestinal system y^+ activity is associated with membrane expression of the mCAT isoforms, as evidenced using anti-peptide polyclonal antiserum generated against 11-mer sequences from the N- and C-termini of the predicted sequences of the cloned mCAT isoforms. Western analysis of human intestinal cell protein identifies a 75 kDa polypeptide band representing the mCAT expression product. Immunofluorescent labeling of adult small intestinal sections stains only crypt cells, with little or no staining in villus tip cells. The y^+ capacity (Vmax) decreases when cultured human intestinal cells advance from the undifferentiated state to differentiated state. Northern analysis of enterocyte RNA probed using mCAT-1 gives a 7.9 kb band; density of this band remains relatively constant in spite of modulations in system y^- transport capacity (Vmax). A single 90 kDa protein responsible for system B transport activity has been purified, although this neutral amino acid transport system remains uncloned. In concert recent data suggest that the intestinal epithelial transport capacity may be regulated by post-translational modification events yet to be discovered.

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Functional organization of a renal cystine transporter

Recently three laboratories, including ours, cloned homologous proteins from rat, rabbit, and human kidneys which, when expressed in *Xenopus* oocytes induce Na^+-independent transport of neutral as well as basic amino acids (AAs) and cystine (system $b^{0,+}$-like). The various acronyms used for these proteins include NAA-Tr, NBAT, D2, and rBAT. In this paper I will refer to it as NBAT (for neutral and basic AA transporter). Immunocytochemical studies show that NBAT is primarily localized in the brush border membranes (BBMs) of renal tubular and jejunal epithelial cells, and NBAT appears to be the major cystine transporter in these cells. Recent studies provide strong evidence that mutations in the human NBAT gene are responsible for defects in cystine and basic AA reabsorption associated with the inherited disease, cystinuria.

Two contrasting topological models for NBAT were proposed: one based on 4 membrane-spanning domains (MSDs) and another on a single N-terminal MSD. Experimental data are consistent with at least 2, and possibly 4, MSDs. Irrespective of which topological model eventually turns out to be correct, NBAT does not resemble other well-characterized metabolite transporters which, in general, have been proposed to contain from 8 to as many as 12 MSDs. Indeed, questions were raised as to whether NBAT itself is a transporter or is, in fact, a regulator subunit of a larger transporter complex. In this context, we have investigated the oligomeric organization of NBAT in BBMs and have found that in the rat kidney and jejunal BBMs, as well as in the rabbit kidney BBMs, NBAT (glycosylated subunit in rat is approx. 85 kDa and in rabbit, 90 kDa) is associated with another protein, approx. 50 kDa. The association involves one or more interprotein disulfide bonds. Our data suggest that such a heterodimer is most likely the minimal functional unit of NBAT-associated AA transport. Induction of AA transport activity in *Xenopus* oocytes from NBAT/cRNA apparently involves a complex formation between newly synthesized NBAT and endogenous oocyte protein(s).

Although various observations indicate that NBAT is probably the cystinuria gene, the possibility that other genes might also have a role in cystinuria must be considered in view of the fact that at least 3 types of cystinurias have been described with different clinical and biochemical manifestations. Our findings that NBAT-mediated transport might require another protein (50 kDa), therefore, raises tantalizing questions regarding its role in cystine transport and in cystinuria.

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Renal excretion rates of γ-carboxyglutamic acid correlate with resting metabolic rates in children and adolescents (3–18 years old)

γ-Carboxyglutamic acid (Gla) is formed posttranslationally by carboxylation of some glutamic acid residues in various proteins like prothrombin, coagulation factors (VII, IX, X), plasma proteins (C, S, Z), osteocalcin, matrix Gla-protein [1]. Urinary Gla can be used as a general marker for whole body degradation of these proteins because it is quantitatively excreted as has been shown in rats [2]. In the present study, we investigated in 3–18 years old humans whether the excretion

rates of Gla per kg body weight (BW) are correlated with the resting metabolic rates (RMR) per kg BW.

Urine samples (n = 288) from healthy female (n = 149) and male (n = 139) human subjects were quantitatively collected for 16–32 h. 16 age groups (mean BW: from 15.5 ± 2.2 up to 72.0 ± 10.9 kg) with 16–20 urine samples each were formed. Urinary Gla was determined by HPLC after derivatization of Gla with o-phthalaldehyde. RMR was calculated via BW using published formulas [3].

Mean Gla excretion rate (μmol/kg BW/d) was 2.4 times higher in the youngest group (1.0 ± 0.25) compared to the oldest group (0.42 ± 0.11); respective values of RMR (kJ/kg BW/d) differ by a factor of 2.1 (225 ± 19.4 vs. 107 ± 7.9). We found a high correlation (r = 0.94; n = 16) between the mean Gla excretion rates and the mean RMR of all age groups.

In different mammalian species with various BW we found previously that whole body degradation rates of proteins (including Gla excretion rates) as well as of RNAs are highly correlated with RMR [4]. Our findings in humans of different age groups also indicate a relation between the degradation of Gla containing proteins and the energy turnover, i.e. the oxygen consumption. We hypothesize that proportional to the oxygen consumed reactive oxygen species are formed which initiate the degradation of proteins as well as of RNAs.

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Neurobiology

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Effect of valproic acid (VA) on brain amino acids and in vitro glutamic acid decarboxylase activity

The best known effect of VA, an antiepileptic drug, is to increase GABA endogenous levels via an enhancement of GAD activity, which was demonstrated in *in vivo* experiments. We studied 1) the effect of acute and chronic (10 days) administration of 200 mg/kg (i. p.) of VA on endogenous levels of aspartate, glutamate, alanine, glycine and taurine in the cerebral frontal cortex and corpus striatum and 2) the *in vitro* effect of 10^{-6} to 10^{-3} M of the drug on GAD activity in homogenates of cerebral cortex and subcortical (rest of the brain) areas.

Methodology and results: 1) Quantification of the amino acid levels was performed by HPLC. In cerebral frontal cortex there was not found any change neither under acute nor under chronic treatment. VA did not either induce changes on these neurotransmitters contents in corpus striatum after acute treatment but after chronic administration we found a decrease on the endogenous levels of glutamic acid (24%, $p < 0.05$) and taurine (32%, $p < 0.05$) which was not related to an increase in the KCl 30 mM evoked release of such amino acids in animals chronically treated. 2) VA did not produce any change on GAD activity neither in cerebral cortex nor in the rest of the brain.

Conclusions: 1) VA increases endogenous levels of glutamate and taurine in corpus striatum only after chronic treatment. 2) VA does not increase GAD activity by a direct mechanism and the increments in this parameter reported by other authors may be produced by an indirect mechanism.

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Kynurenic acid and kynurenine aminotransferases KAT I and KAT II in the brains of patients with Down syndrome and Alzheimer's disease

The present study was designed to examine the biosynthetic machinery of the excitatory amino acid receptor antagonist kynurenic acid (KYNA) in frontal cortex of patients with Down syndrome (DS) and Alzheimer's disease (AD). We measured the content of KYNA, determined by HPLC and activities of enzymes synthesizing KYNA, kynurenine aminotransferases I and II (KAT I and KAT II) in the presence of the 1 mM cosubstrates 2-oxoglutarate, 2-oxoisocaproate and pyruvate at their pH optima of 10.0 for KAT I and 7.4 for KAT II. KYNA content was normal in frontal cortex from AD patients (0.28 ± 0.05 pmol/mg tissue) and increased in DS (205%) as compared with control (0.22 ± 0.05 pmol/mg tissue). In DS and AD the activities of KAT's were remarkably altered. KAT I was significantly reduced (75-86%) in the presence of all three 2-oxo acids in both diseases. Significantly lowered KAT II activity was observed only in the presence of 2-oxoglutarate (34%) in frontal cortex from AD. Reduction of KAT I and KAT II activities in frontal cortex did not correlate with the decline of choline acetyltransferase in frontal cortex from both diseases. Measurement of amino acids revealed no changes in the levels of γ-aminobutyric acid, taurine, glycine, aspartate, glutamate and arginine in frontal cortex from DS and AD patients. Our data demonstrated changes in the parameters of kynurenine metabolism and suggest the involvement of KAT's deficit in the brain in DS and AD patients. As KYNA antagonizes competitively the N-methyl-D-aspartate (NMDA) receptors, a system responsible for memory, learning and cognitive functions, our present data may suggest a link to the neuronal degeneration mediated by overstimulation of NMDA receptors. (Supported by grants from Red Bull and Austria Science Research Fund [No H0043-MED to H. B.]).

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Modulation of serotonin (5-HT)$_{1A}$ receptor-mediated spontaneous tail-flicks (STFs) by inhibitory and excitatory amino acid receptors in the dorsal horn of the spinal cord: Relationship to antinociceptive mechanisms

Activation of 5-HT$_{1A}$ receptors in the dorsal horn of the spinal cord enhances nociception, an effect reflected in the induction of STFs in the rat (that is, flicks in the absence of noxious stimulation) by the 5-HT$_{1A}$ agonist, 8-OH-DPAT. Here, we examined the role of inhibitory and excitatory amino acid receptors in their modulation. STFs were measured 10–15 min after administration of 8-OH-DPAT (0.63 mg/kg, s.c.) to Male Wistar rats (225–275 g) (Bervoets, K. et al., J. Pharmacol. Exp. Ther., 269, 110–120, 1994). Other drugs were given s.c. or i.p. 30 min before 8-OH-DPAT, or i.t. onto lumbar spinal cord immediately before 8-OH-DPAT. For the rotarod test of ataxia in male NMRI mice, drugs were given 30 min pre-testing.

The GABA$_A$ agonist, muscimol, the GABA$_B$ agonist, baclofen, the NMDA competitive antagonist, CPP, the partial agonist and antagonist at the NMDA receptor coupled glycine B site, (+)-HA 966 and L 701,324, respectively, and the AMPA antagonists, YM 90K and NBQX, all dose-dependently and completely blocked STFs. They were also active upon i.t. administration at lower doses, consistent with a spinal site of action. Only at higher doses did they elicit ataxia in the rotarod test indicating that their actions do not reflect motor disruption. Further, at doses active in the STF model, these drugs are little active in the tail-flick test to noxiuos heat in rats (not shown) revealing that the capacity to emit this behaviour is not compromised by these drugs. The high sensitivity of STFs suggests that this response may not simply relate to processes of acute, nociceptive pain but, rather, neuropathic mechanical hyperalgesia/allodynia. Indeed, aspirin did not affect STFs (up to 1,0 g/kg, p.o.), and morphine, which only modifies neuropathic pain at high doses, was less potent in inhibiting STFs (ID_{50}, C.L = 8.2, 5.8–16.9 mg/kg, s.c.) than tail-flicks to heat (ID_{50}, C.L = 1.1, 0.3–3.2 mg/kg, s.c.). Further, the anticonvulsant, carbemazepine, which is clinically used to treat neuropathic pain, blocked STFs (ID_{50}, C.L = 11.2, 5.9–21.1 mg/kg, i. p.). These data show that mechanisms employing GABA$_A$ and GABA$_B$ sites, as well as NMDA and AMPA receptors, play a role in the modulation of 5-HT$_{1A}$ receptor-mediated STFs. In addition, they suggest that activation of GABAergic receptors, or blockade of glycine B and AMPA receptors, in the dorsal horn, may be of utility in the management of neuropathic pain. Finally, this paradigm offers a simple and sensitive screen for the detection of novel drugs acting at glycine B and AMPA receptors.

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Patch-clamp studies of forward and reversed glutamate uptake

The glutamate (Glu^-) uptake carrier in salamander retinal glia (Müller cells) may transport 2 Na^+ and a Glu^- into the cell, and a K^+ and an OH^- out of the cell (Nature 360, p 471). When $[K]_o$ rises, as in brain ischaemia, this mechanism runs backwards, pumping Glu^- into the extracellular space where it triggers neuronal death. Reversed uptake can be detected as an outward current evoked by a rise in $[K]_o$ when cells are clamped with electrodes containing Na^+ and Glu^- (Nature 348, p 443).

In ischaemia the brain goes acid, and for the stoichiometry mentioned above a decrease of extracellular $[OH^-]$ might inhibit reversed uptake, by depriving the carrier of external OH^- substrate. To test this we monitored reversed uptake electrically (as described above) and also by detecting Glu^- release from the opening of AMPA receptor channels in rat Purkinje cell bodies positioned near a salamander glial cell. Acidifying the external solution decreased both the reversed uptake current, and the release of glutamate produced by Müller cell depolarization in raised $[K]_o$. These data suggest that the acidification occurring in ischaemia could slow the release of glutamate occurring by reversed uptake, and thus protect neurons against transient (but not sustained) ischaemia.

Glutamate uptake is often thought of as being presynaptic or in glial cells, but immunostaining suggests that rat cerebellar Purkinje cells express EAAC1 carriers (Neuron 13, p 713). By applying glutamate analogues iontophoretically to Purkinje cells in cerebellar slices, we detected a membrane current component attributable to glutamate uptake. Postsynaptic EAAC1 carriers could contribute to terminating the EPSC at climbing and parallel fibre to Purkinje cell synapses. (Supported by the Wellcome Trust and M. R. C.).

	Baclofen	Muscimol	(+)-HA 966	L 701,324	CPP	YM 90K	NBQX
8-OH-DPAT STFs (mg/kg)	0.4 (0.1–1.3) s.c.	0.10 (0.05–0.30) s.c.	14.8 (9.0–24.2) s.c.	2.6 (1.3–4.8) i.p.	0.3 (0.1–1.0) s.c.	0.3 (0.1–1.7) i.p.	8.2 (5.0–13.6) i.p.
8-OH-DPAT STFs µg/rat	0.03 (0.01–0.09) i.t.	0.11 (0.01–2.00) i.t.	7.0 (1.4–34.2) i.t.	N.T.	0.0007 i.t.	0.5 (0.2–1.2) i.t.	N.T.
Rotarod/Ataxia (mg/kg)	9.7 (2.3–40.0) s.c.	0.4 (0.2–0.5) s.c.	> 80.0 s.c.	16.5 (7.9–34.2) i.p.	2.9 (0.9–8.9) s.c.	6.3 (2.3–17.7) i.p.	40.0 17.0–94.8) i.p.

Doses are Inhibitory $Dose_{50}$s (95% Confidence Limits). N. T. = not tested.

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Metabotropic glutamate receptors and neuronal death

We have studied the influence of metabotropic glutamate receptors (mGluRs) on the excitotoxic neuronal degeneration in mixed cultures of murine cortical neurons and glia. MGluRs have been classified in three different classes, based on the signal transduction mechanism. MGluR2 and 3 (class II) and mGluR4, 6 and 7 (class III) are negatively linked to adenylyl cyclase, whereas mGluR1 and 5 (class I) are positively coupled to polyphosphoinositide hydrolysis. Selective agonists of subtypes which belong to class II or III of mGluRs, such as $(2_S,1'_R,2'_R,3'_R)$-2-(2,3-dicarboxicypropyl)glycine (DCG-IV) (selective for subtypes mGluR2 and 3) and L-serine-O-phosphate (selective for subtypes mGluR4, 6 and 7), were highly potent and efficacious in protecting cortical neurons against toxicity induced either by a brief exposure to NMDA or prolonged exposure to kainate. DCG-IV was still neuroprotective when applied to cultures after the NMDA pulse and this effect was associated with a reduction in release of endogenous glutamate. In contrast, preferential agonists of class I mGluRs, such as quisqualate, 3,5-dihydroxyphenylglycine (DHPG) and trans-azetidine-2,4-dicarboxylic acid (t-ADA), substantially enhanced the potency of NMDA in inducing neuronal degeneration. The amplification of NMDA toxicity by DHPG or quisqualate was attenuated by a series of protein kinase C (PKC) inhibitors, suggesting that class I mGluRs operate, at least in part, through activation of PKC. Quisqualate and, in particular, DHPG enhanced neuronal degeneration even when applied after the toxic pulse with NMDA.

These results suggest that individual mGluR subtypes differentially affect excitotoxic neuronal degeneration, as activation of class II and III mGluRs is involved in neuroprotective mechanisms, whereas activation of class I mGluRs is likely to contribute to the maturation of the excitotoxic damage.

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Role of dopamine in striatal synaptic long-term depression

Repetitive activation of excitatory pathways in some brain areas causes different forms of synaptic plasticity such als long-term potentiation (LTP) and long-term depression (LTD). These long-term changes in synaptic efficacy have been considered as cellular correlates of memory and learning. Since the striatum has been involved in the formation of motor memory, recently our group has investigated the long-term changes of striatal excitatory transmission caused by tetanic stimulation of the glutamatergic corticostriatal fibres in a brain slice preparation. Repetitive activation of the corticostriatal pathway induced a LTD of both extracellularly and intracellularly recorded synaptic potentials. Two main physiological conditions are required for the induction of LTD: 1) membrane depolarization and 2) activation of metabotropic glutamate receptors. Striatal LTD was blocked either by SCH 23390, a D1 dopamine (DA) receptor antagonist, or by 1-sulpiride, a D2 DA receptor antagonist suggesting that coactivation of both DA and D2 receptors is required for this phenomenon. Slices obtained from DA-denervated rats (unilateral nigral injection of 6OH-DA) did not express striatal LTD. However, in DA-denervated slices, LTD could be restored when both D1 and D2 receptor agonists were coadministered, but not by the application of a single class of receptor agonists alone. This findings demonstrate that endogenous DA participates to the formation of this form of synaptic plasticity. Since it has been hypothesized that motor memory involves both DAergic and glutamatergic systems within the striatum, we propose that striatal LTD might be involved in the storage of motor skills.

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Energy metabolism, *N*-methyl-D-aspartic acid receptor antagonism and anoxic damage in hippocampal slices

We have investigated the relationship between energy metabolism, *N*-methyl-D-aspartic acid (NMDA)-receptor antagonism and anoxic damage *in vitro*. Anoxic damage was assessed by measuring protein synthesis, defined as the incorporation of [^{14}C]-lysine into perchloric acid insoluble tissue extracts. The concentrations of energy metabolites were measured by ion-exchange high-performance liquid chromatography. Anoxia caused an inhibition of protein synthesis, a reduction in phosphocreatine and adenosine triphosphate, and extensive neuronal damage. The reduction of protein synthesis depended on the duration of anoxia and the time allowed for recovery. There was a similar impairment of protein synthesis under normoxic conditions when the NMDA receptor-channel complex was activated by removing Mg^{2+} and adding NMDA. This impairment could be prevented by the addition of various NMDA receptor antagonists (MK-801, BIII 277 CL, phencyclidine or CGP 37849). In contrast, incubation with NMDA antagonists failed to prevent the anoxia-induced inhibition of protein synthesis, although it moderately facilitated the postanoxic recovery. Preincubation with the creatine dose-dependently (0.03–3 mmol/L) increased baseline levels of phosphocreatine, reduced the anoxia-induced fall in phosphocreatine and adenosine triphosphate, prevented the impairment of protein synthesis and reduced neuronal death. Incubation with (*R,S*)-3-guanidinobutyric acid, a synthetic analogue of creatine that cannot be phosphorylated, did not prevent the anoxia-induced impairment of protein synthesis and did not enhance the levels of phosphocreatine and adenosine triphosphate. Incubation with a combination of both creatine and the noncompetitive NMDA antagonist MK-801 provided complete protection. These results indicate that energy status is a major factor controlling anoxic damage in the rat hippocampal slice.

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Neuroactive non-protein amino acids in *Ephedra* affecting glutamatergic signalling

Tissues of *Ephedra* spp. poor in ephedrine alkaloids contain substantial amounts of several pharmacologically-active cyclic mimics of the neurotransmitters L-glutamate and glycine. Two *cis*-diastereoisomers and one *trans*-diastereoisomer of the L-glutamate analogue L-2-(carboxycyclopropyl)glycine, namely

(2S,3S,4R)-, (2S,3R,4S)- and (2S,3S,4S)-(carboxycyclopropyl)-glycine (L-CCGIII, L-CCGIV and L-CCGI respectively) occur in the seeds and stems of *Ephedra foeminea* and *E. altissima.* Many species of *Ephedra*, including the two above, contain large amounts of another cyclopropyl amino acid, *cis*-2,3-methano-L-proline, and a quinoline-2-carboxylic acid, 6-hydroxykynurenic acid. Two other quinoline-2-carboxylic acids, kynurenic acid (KYNA) and 6-methoxykynurenic acid, are found in the stems of *E. pachyclada* ssp. *sinaica*, a species rich in ephedrine alkaloids. Because of their restricted stereochemistry, cyclic neurotransmitter analogues act selectively at different glutamate receptors and transporters and disrupt their normal function. In mammalian neurons, L-CCGIII is a high-affinity glutamate transporter substrate and potentiates neuronal responsiveness to L-glutamate, L-CCGIV activates the N-methyl-D-aspartate (NMDA) subtype of L-glutamate receptor, and L-CCGI is a metabotropic glutamate receptor agonist. KYNA is an endogenous non-selective antagonist of glutamate receptors, while 6-methoxy-KYNA (and possibly 6-hydroxy-KYNA) is a selective non-NMDA receptor antagonist. In insects, both L-CCGIII and L-CCGIV are actively accumulated by the epidermis *in vitro*, and L-CCGIII and L-CCGI, but not L-CCGIV and 6-hydroxy-KYNA, have myotropic activity on visceral muscle. The cyclopropyl and quinoline compounds found in *Ephedra* may act as insect antifeedants.

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Modulation by nitric oxide of rat brain $GABA_A$ receptors

Nitric oxide (NO) is an important intercellular messenger in the mammalian central nervous system. It has been discussed as possibly involved in events such as Long Term Potentiation.

Referring to its action on neurotransmitter activated ionic channels, it modulates negatively the function of glutamate NMDA-type receptors.

We have studied whether rat brain $GABA_A$ receptors are modulated by this messenger. Our data show that such receptors are negatively modulated by NO in both rat cerebral cortex microsacs (biochemical approach involving the study of $^{36}Cl^-$ accumulation) and neonatal rat cerebellar granule cells (patch clamp approach). In particular, in the first approach there was a significant reduction in the accumulation V_{Max} with no change in the k value.

In further studies in the cerebellar granules model we investigated the mechanism of the nitric oxide effect. In the first place, the effect of chemically supplied NO was matched by the NO biological precursor L-Arginine, indicating that endogenously produced NO can modulate $GABA_A$ receptors. In further experiments we collected evidence that the nitric oxide effect involves activation of Protein Kinase G in the granule cells and its action on $GABA_A$ receptors.

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The role of the entorhinal-hippocampal system in spatial memory

The key position of the entorhinal cortex (EC) between the hippocampus and the neocortex as well as the importance of the hippocampus in spatial memory suggest that the EC is also involved in spatial memory. We partially lesioned the EC of the rat with the NMDA-agonist and excitotoxin quinolinic acid. Postoperatively we tested the animals in a spatial learning paradigm in an eight-arm radial maze. Animals had to learn to navigate to four baited arms of the maze. During several trials the position of the baited arms was not changed (acquisition phase). If a reversal phase was given, unbaited arms were changed to baited arms and vice versa.

Animals with a lesion restricted to the medial part of the EC had only a small memory deficit mainly due to a reference memory (RM) deficit. If the lesion affected also parts of the lateral EC, subiculum and dentate gyrus, the deficit was more prominent. The working memory (WM) deficit was compensated throughout the experiment, but the RM deficit became more evident towards the end. In a second experiment, animals with a small lesion were challenged in the reversal phase with a low dose of the noncompetitive NMDA-antagonist MK-801 (0.04 mg/kg). The extent of the RM deficit was comparable to that of the large lesion-group. Thus, there is an additive effect of small lesion and challenge. In a third experiment, the RM deficit of the lesioned animals was antagonized by the partial glycine agonist D-Cycloserine (DCS, 12 mg/kg) whereas DCS had no influence on unoperated control animals. The positive effect of DCS was thus restricted to animals with a lesioned glutamatergic system.

In summary, lesion of the EC affects mainly, in contrast to hippocampal lesions, the RM component in a spatial learning task. The RM deficit is exacerbated by the NMDA-antagonist NM-801 and antagonized by the glycine agonist DCS.

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The mechanism of glutamate uptake into synaptic vesicles

Glutamate is taken up into synaptic vesicles by a Mg-ATP dependent process. The uptake is inhibited by several different types of inhibitors. Inhibitors of Mg-ATPases such as CCCD and bafilomycin, but not other ATPase inhibitors such as ouabain inhibit all vesicular uptakes.

Ionophores such as gramicidine and nigericine also inhibit uptake. The uptake of glutamate in contrast to that of other transmitters are greatly stimulated by low conc of chloride and bromide ions, but inhibited by fluoride ions. Compounds known to inhibited chloride channels such as SITS, DIDS, furosamide are specific inhibitors of glutamate uptake. To our surprise they were competitive inhibitors of glutamate and noncompetitive inhibitors of chloride.

A series of excellent inhibitors such as Evans Blues, and Chicago Sky Blue has recently been identified.

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Distribution and characterization of NO synthase activity in the primate brain

The distribution of nitric oxide synthase (NOS) within the brain of the common marmoset *(Callithrix jacchus)*, a non-human primate species, was investigated using the [^{3}H]-L-citrulline formation assay and Western blot analysis. No hemispheric asymmetry of specific NOS activity was shown. The highest levels of NOS are found in the putamen and caudate nucleus, which are more than two times greater than in the cortex and the cerebellum, the brain regions with the lowest activities. The regional distribution pattern is similar to that in the ferret brain and contrasts to that in the mouse and bovine brain. However, analysis of NOS catalytic activities in subcellular fractions reveals marked differences in the subcellular localization. Neuronal NOS accounts mainly for the measured catalytic activity in the brain. Therefore, the differences in the regional distribution pattern of brain NOS activity among species may be indicative of diversities in the functional role of nitric oxide and NOS in mammals. (Supported by grants from the Bundesministerium für Forschung und Technologie [01 EB94-10]).

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Behavioral effects induced by systemic NMDA in the rat. Changes in brain putrescine concentration

The effect of 150 mg/kg of NMDA i. p. on the behavior of male Wistar rats was evaluated by both direct observation and image dynamic analysis (Videotrack) for 1 h. NMDA-induced behavioral response was classified as convulsant and non-convulsant. In a previous work, we observed that at this dose, the convulsant episode begins very fast after injection and animals only survive during a short time (few minutes). For this reason, in this study convulsant rats were killed immediately after convulsion. Non-convulsant animals exhibited abnormal behaviour consisting in stereotypic movements and were killed at 8 or 24 h after NMDA administration. The concentration of polyamines in blood plasma and brain (frontal cortex and hippocampus) was determined by HPLC. In convulsant rats, the plasma putrescine concentration was increased 55% over the saline controls, but there were no modifications of polyamines content in brain. In non-convulsant animals, the increase of putrescine was found in the brain: In frontal cortex, was of 170% at 8 h and of 91% at 24 h and in hippocampus the increase was of 40% at 24 h. Moreover, there is a correlation between the concentration of putrescine in brain and the developed motor activity evaluated with Videotrack at 8 ($r = 0.8194$, $p < 0.01$ for frontal cortex; $r = 0.8563$, $p < 0.001$ for hippocampus) and 24 h ($r = 0.7397$, $p < 0.01$ for frontal cortex; $r = 0.8073$, $p < 0.01$ for hippocampus). These results are discussed in relationship to the behavioral response. (Work supported by the FIS 93-0350 and SAF 92-0913 grants.)

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The role of striatal transmitter systems in the control of motor behaviour

The striatum is the main input structure of the basal ganglia receiving glutamatergic afferents from the cerebral cortex and dopaminergic afferents from the midbrain. The striatum projects via two different pathways to the basal ganglia output nuclei. A direct pathway involves direct striatal projections to the substantia nigra pars reticulata (SNpr) and the entopeduncular nucleus (EP). An indirect pathway projects from the striatum via the globus pallidus to the SNpr/EP. Little is known about the relative contribution of these two pathways to the control of motor behaviour. Recent studies revealed that striatal projection neurons of the indirect pathway almost exclusively express adenosine receptors of the A_{2a} subtype. Furthermore there exist antagonistic interactions between striatal adenosine A_{2a} and dopamine D_2 receptors.

Our present studies show that intrastriatal administration of the A_{2a} receptor agonist CGS 21680C induced profound catalepsy suggesting a motor inhibitory role of the indirect pathway. Systemically administered glutamate antagonists of the N-methyl-D-asparte (NMDA) subtype completely reversed CGS 21680C induced catalepsy. This anticataleptic effect seems to be brought about by a blockade of NMDA receptors located within the indirect loop. Systemic administration of the nonselective adenosine receptor antagonist theophylline reversed catalepsy induced by D_2 antagonists, but not by D_1 antagonists. The anticataleptic effect of theophylline is most probably mediated by an A_{2a} receptor blockade, but not by an A_1 receptor blockade. In addition theophylline potentiated the anticataleptic effects of NMDA antagonists.

In conclusion, striatal NMDA, D_2 and A_{2a} receptors seem to regulate the activity of striatal projection neurons of the indirect pathway which substantially contributes to the control of motor behaviour.

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On the origin of extracellular glutamate levels monitored in peripheral and brain tissues with in vivo microdialysis

Using highly sensitive analytic procedures, several putative neurotransmitter and metabolism systems have been monitored simultaneously in the extracellular space of neostriatum, substantia nigra, fronto-parietal cortex, as well as subcutaneous tissue of the rat by in vivo microdialysis.

The excitatory amino acids, glutamate (Glu) and aspartate (Asp) are found at μM cncentrations, in all investigated brain regions (≈ 0.8 μM and ≈ 0.1 μM, for Glu and Asp, respectively), as well as in subcutaneous tissues (≈ 50 μM and ≈ 4 μM, for Glu and Asp, respectively). The inhibitory amino acid, GABA is found at nM concentrations, with levels ≈ 2 fold higher in both neostriatum and fronto-parietal cortex (≈ 25 nM), than in the substantia nigra (≈ 10 nM). The highest concentration of the monoamine dopamine (DA) is found in the neostriatum (≈ 5 nM), followed by the fronto-parietal cortex (≈ 1 nM) and the substantia nigra (≈ 0.5 nM). The neuropeptides dynorphin B (Dyn B) and cholecystokinin (CCK) are found in a pM range

(≈ 25 pM and ≈ 3 pM, respectively) in all investigated brain regions. No GABA, DA, Dyn B or CCK levels are detected in subcutaneous tissue. The metabolism products, pyruvate (Pyr) and lactate (Lact) are, however, found at ≈ 200 μM and ≈ 10 μM ranges, respectively, in all examinated regions.

The following criteria have been applied to assess the neuronal origin of substances measured by microdialysis: sensitivity to (i) K^+-depolarization, (ii) Na^+-channel blockage, and (iii) removal of extracellular Ca^{2+}. It has been found that DA, Dyn B, CCK and GABA largely satisfied these criteria. In contrast, Glu and Asp levels respond in a paradoxical manner, despite being monitored under the same conditions. These levels are poorly affected by K^+-depolarization and are increased by perfusing with the Na^+-channel blocker, tetrodotoxin, or with a Ca^{2+}-free medium including 2 mM EGTA.

In order to further investigate the origin of these amino acids, the selective neurotoxin, α-latrotoxin was unilaterally injected into the neostriatum, before bilateral microdialysis implantation, in order to block vesicular neurotransmitter release from surrounding synapses. It was found, compared to the saline injected control side, that Glu and Asp extracellular levels were decreased by > 50%, under both basal and K^+-depolarizing conditions, suggesting that, in the neostriatum of the rat, there is a pool of releasable Glu and Asp. Similar effects were obtained for GABA and DA, which were simultaneously monitored.

Some of the paradoxical results presented here can be explained by methodological considerations, but it appears that they point out to exclusive mechanisms by which extracellular glutamate and aspartate levels are regulated under in vivo conditions.

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Role of GABAergic neuron on effects of the interactions between dopaminergic and glutamatergic neurons

The interactions between glutamate and dopamine (DA) transmissions by role of the GABAergic neuron action on this mechanism were studied using in vivo microdialysis in awake and freely moving rats. Infusion of a high concentration of NMDA (10 mM) via dialysis probe into the striatum (Str) and the nucleus accumbens (NuAc) increases the release of DA in the perfusate of both areas. In contrast, infusion of a low concentration of NMDA (10 μM) decreases the release of DA. GABA (100 mM) did not influence the release of DA in both areas; however beclofen (100 mM), a GABA-B agonist, decrease DA release in the NuAc without any affect in the Str. Bicuculline (100 mM), a GABA-A antagonist, and saclofen (100 mM), a GABA-B antagonist, all can reverse the inhibitory action on DA release by effect of a low concentration of NMDA. These results suggest that: (1) high concentration of NMDA may increase the DA release by directly depolarizing the terminal of DA neurons, whereas low concentration of NMDA may decrease the DA release via GABA interneurons, (2) the distribution and kinetic properties of GABA-A and GABA-B receptors in the NuAc and the Str might be different.

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Effects of amphetamine and MK-801 on spontaneous alternation in the eight-arm radial maze as an indicator of their abuse potential

The induction of psychomotor activation, behavioural sensitization and the reduction of behavioural variability have been proposed to be common properties of drugs of abuse. The present investigation tested whether all of these drug effects could be measured using spontaneous alternation in an eight-arm radial maze. Behavioural effects of repeated treatment with amphetamine and the non-competitive NMDA receptor antagonist MK-801 on spontaneous alternation were evaluated in this paradigm. Both, amphetamine (2 mg/kg, i. p.) and MK-801 (0.1 and 0.2 mg/kg, i. p.) stimulated locomotor activity. Analysis of the sequences of arm entries revealed that amphetamine and the higher dose of MK-801 induced different perseverative locomotor patterns. Amphetamine treatment resulted in a significant arm bias, indicating decreased behavioural variability due to perseverative sequences of arm entries. Repeated treatment with the higher dose of dizocilpine led to the development of an angle bias, as well indicating decreased behavioural variability but due to a different kind of perseverative locomotor pattern. These findings might reflect differences in dopaminergic and glutamatergic influence on sensory-motor integration. Furthermore, repeated treatment with both substances produced behavioural sensitization to a subsequent drug challenge that persisted for at least one month. Taken together, these results support earlier findings suggesting a possible abuse potential of MK-801.

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Presynaptic substance P receptor sites in the rodent and primate neostriatum and prefrontal cortex: Morphological evidence for peptidergic modulation of excitatory neurotransmission

The neuropeptide substance P (SP) and its receptor in the brain have been implicated in stress-induced (McLean et al., 1993) and defensive rage behaviors (Shaikh et al., 1993), and in conditions such as normal aging and Alzheimer's disease (Kowall et al., 1993), Parkinsons's disease (Gai et al., 1991), Huntington's disease (Gale et al., 1978; Kowall et al., 1993) and schizophrenia (Michelot et al., 1978; Toru et al., 1988). To examine the synaptic circuitry underlying these functions, we studied the subcellular distribution of the SP receptor protein in rat and primate striatal and prefrontal cortical tissue, employing antibodies raised against SP receptor (Shigemoto et al., 1993).

SP receptor immunoreactivity has been found in postsynaptic sites in the rodent striatum and cortex (Shigemoto et al., 1993; Kaneko et al., 1994; Liu et al., 1994). In the present study, electron microscopic analysis revealed that SP receptor immunoreactivity, besides being present in the somatodendritic domains of the cholinergic and somatostatinergic interneurons of the striatum, and in a subgroup of GABAergic non-pyramidal cells of the cortex, was also localized to presynaptic profiles in both species. SP receptor immunoreactivity was usually restricted to a portion of the axoplasm both in the cortex and striatum. The majority of SP receptor immunoreactive axons

formed asymmetric synapses with dendrites and dendritic spines in both structures.

Since the overwhelming majority of asymmetric synapses are formed by glutamate/aspartatergic pyramidal neurons in the cortex, and most of such synapses in the striatum are established by corticostriatal projection of pyramidal neurons, the present results suggest that presynaptic SP receptors are generally associated with glutamatergic cortical projections. The association of SP receptors with asymmetric synapses which use excitatory amino acids can serve as a morphological substrate for peptidergic modulation of excitatory neurotransmission.

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The role of amino acids in sensitization to cocaine

Repeated administration of cocaine in rats results in a progressive augmentation of its acute motor stimulant effects, and this phenomenon has been termed behavioral sensitization. An action by cocaine in the region of dopamine cell bodies in the ventral tegmental area (VTA) is especially important in developing sensitization. In contrast, drug action in the nucleus accumbens (a dopamine terminal field) appears most critical in the actual expression of the behavioral response. Although cocaine binds the dopamine transporter, a variety of data show that alterations in glutamate and GABA transmission may be critical in both the initiation and expression of sensitization to cocaine. In vivo microdialysis was conducted in both the VTA and nucleus accumbens of rats behaviorally sensitized to cocaine and changes in the extracellular content of GABA and glutamate were measured. Two general conclusions have been derived from these data. 1) D_1 receptor mediated increases in glutamate release and NMDA receptor stimulation in the VTA are important in initiating behavioral sensitization. 2) Enhanced glutamate release and postsynaptic responsiveness to glutamate in the nucleus accumbens is associated with the expression of sensitization.

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Affinity of the antiparkinsonian drug budipine for NMDA and sigma binding sites in postmortem human brain tissue

Budipine (1-t-butyl-4,4-diphenylpiperidine) was developed for clinical use in Parkinson's disease more than 10 years ago and has been shown to improve parkinsonian and neuroleptic-induced extrapyramidal symptoms. Of the three main symptoms of Parkinson's disease, tremor was improved more effectively than rigidity and bradykinesia. We have estimated the affinity of the antiparkinsonian drug budipine to the PCP binding site of the NMDA receptor and to $sigma_1$ binding sites in membranes from postmortem human brain frontal cortex using [^{3}H]MK-801 and [^{3}H]pentazocine as ligands. We found K_i-values of about 12 and 2 µM at the PCP and $sigma_1$ binding site, respectively. The affinity of budipine to both binding sites is in a concentration range that may be reached under therapeutic conditions.

The antiparkinsonian effects of amantadine and memantine are thought to be mainly due to their uncompetitive antagonism at the NMDA receptor (Kornhuber et al., 1994). Memantine at therapeutic concentrations appears to specifically interact with the NMDA receptor channel. Amantadine, on the other hand, has an additional action on the $sigma_1$ binding site at therapeutic concentrations. When compared to amantadine, budipine has a similar affinity to the PCP binding site and a higher affinity to the $sigma_1$ binding site. We have recently hypothesized that, in addition to the PCP binding site, the $sigma_1$ binding site may also be involved in the antiparkinsonian effects of amantadine. This hypothesis might explain better antiparkinsonian effects of amantadine and budipine compared to memantine and is supported by effects of sigma ligands on the behavior of experimental animals. Interactions with NMDA receptors as well as $sigma_1$ binding sites may contribute to the antiparkinsonian effects of budipine.

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Are the striatum and the nucleus accumbens modulated by strychnine-insensitive glycine binding sites?

The striatum and the nucleus accumbens (NAC) are the main input structures of the basal ganglia (BG). They are innervated by glutamatergic (GLU) efferents from the cortex and dopaminergic (DA) efferents from the midbrain. The DA/GLU transmitter balance in the striatum and NAC differently contributes to motor behavior. Blockade of the NMDA receptor in the striatum induces sniffing stereotypy and antagonizes DA D2 receptor mediated catalepsy. Blockade of the NMDA receptor in the NAC induces locomotion but has no effect on catalepsy. The NMDA receptor is modulated by glycine via a strychnine-insensitive receptor. To test in which way the glycine receptor modulates the behavioral variables, the effects of intrastriatal and intraaccumbal injections of the glycine receptor antagonist 7-chlorokynurenate (7-CLKYN) on sniffing stereotypy, catalepsy and locomotion were investigated in the rat.

Injection of 7-CLKYN (2.5–20 nmol/0.5 µl bilaterally) into the striatum induced a dose-dependent sniffing stereotypy and reduced catalepsy mediated by a DA D2 receptor antagonist (haloperidol 0.5 mg/kg i. p.). However, intrastriatally given 7-CLKYN had no effects on locomotion. Injection of 7-CLKYN (2.5–10 nmol/0.5 µl bilaterally) into the NAC had also no effect on locomotion, but elicited a mild sniffing stereotyped.

Thus, some aspects of motor behavior mediated by the striatum and the NAC are controlled in a glycine receptor independent way. While behavior mediated by striatopallidal efferents are modulated by the glycine receptor, that mediated by accumbopallidal efferents are not. The heterogeneous nature of the NMDA receptor complex may explain these findings. (Supported by the DFB [SFB 307].)

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Differential effects of competitive and non-competitive NMDA-receptor antagonists on amphetamine induced behaviour of rats

Numerous studies have demonstrated a close relationship of the two afferent systems to the input structures of basal ganglia, which are dopaminergic (DAergic) projections from the midbrain and glutamatergic (GLUergic) projections from cortical areas. Pharmacological manipulations influencing the DAergic or GLUergic receptors within basal ganglia can generate a variety of motor and cognitive effects. Competitive and non-

competitive antagonists acting at the NMDA-receptor subtype of GLU-receptors have some actions (anticataleptic, anticonvulsive and neuroprotective properties) in common, whereas having contrasting effects too. Non-competitive NMDA-receptor antagonists induce a stable behavioural stimulation, whereas competitive antagonists are approximately ineffective at this point, except for high dosages.

As we have shown, that 6-hydroxydopamine lesions can diminish the stimulant effects non-competitive NMDA-receptor antagonists by reducing DAergic activity, we examined the effects of reduced GLUergic activity on the stimulant effects of amphetamine (AMPH). Spontaneous and drug induced behaviour was analysed in an open field equipped with a hole-board and a sniffing-box for analysis of sniffing stereotypies.

AMPH and Dextrorphan (non-competitive NMDA-receptor antagonist) induced a robust locomotion. Dextrorphan pretreatment *elicited more line-crossings* than AMPH alone (Fig. 1). CGP37849, CGP40116 and CPPene (competitive NMDA-receptor antagonists) pretreatment however *reduced the number of line-crossings* as compared to AMPH administration alone. Moreover even a high dosage of CPPene, inducing stimulant effects on its own, reduced the AMPH-induced stimulation (Fig. 2). This contrasting effect of competitive and non-competitive NMDA-receptor antagonists was observed for the stereotypy too.

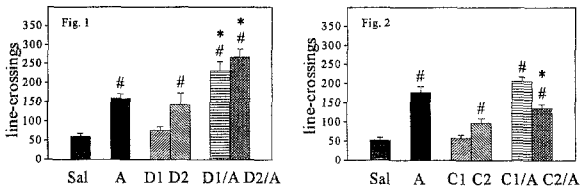

Fig. 1 and **2.** Locomotion/5 min in an open field (MEAN + S. E. M.); (# indicates a significant difference of $p < 0.05$ as compared to saline, * of $p < 0.05$ as compared to AMPH treatment; *A* AMPH 2.0 mg/kg; *D1* Dextrorphan 20.0 mg/kg; *D2* 40.0 mg/kg; *C1* CPPene 4.0 mg/kg; *C2* 8.0 mg/kg)

This experiment emphasizes the differential effects of competitive and non-competitive NMDA-receptor antagonists. In summary these data show, that as the DAergic system is involved in the stimulatory effects of non-competitive NMDA-receptor antagonist, the GLUergic system is involved in the stimulatory effects of amphetamine. (Supported by DFG SFB 307/A4 and BMFT 01 KL 9008/0.)

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Alterations of N-methyl-D-aspartate receptors in the brains of patients with Parkinson's disease

Animal experiments suggest that in Parkinson's disease there exists a glutamatergic overactivity within the basal ganglia and that a selective reduction of glutamatergic function may be of therapeutic benefit in parkinsonian patients. We have studied the alterations of brain N-methyl-D-aspartate (NMDA) receptors in Parkinson's disease. Brain tissue was obtained from patients with neuropathologically confirmed Parkinson's disease and from matched control subjects without neurological or psychiatric diseases. Using washed membrane homogenates, receptor binding was performed in various brain regions with the NMDA antagonist [^{3}H]-CGP 39653. In comparison with control tissues, [^{3}H]-CGP 39653 binding was reduced in the medial globus pallidus and unchanged in the other cortical and subcortical brain regions examined. The present results suggest that NMDA receptors are downregulated in the medial globus pallidus of parkinsonian patients as a result of increased neuronal activity in the subthalamic nucleus. An increase in subthalamic activity is a prominent feature of the neural mechanisms of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism in monkeys. Subthalamic nucleotomy or pallidotomy may therefore be effective lesions for the neurosurgical treatment of Parkinson's disease.

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Effect of excitatory amino acid receptor agonists on GABA release from female rat median eminence

In order to test the hypothesis that neuroexcitatory amino acids (EAA) interact with the tuberoinfundibular GABAergic system to influence GnRH/LH and prolactin secretion, we have investigated the direct effects of EAA receptor agonists on GABA release from hypothalamic fragments containing the arcuate nucleus and the median eminence (ARC/ME) of regularly cycling female rats on the first day of diestrus.

ARC/ME were incubated with N-methyl-D-aspartate (NMDA), kainate (KA) and quisqualate (QUIS) in the absence of MG 2+. GABA was assayed by 3H-muscimol radioreceptor assay in incubation media and tissue.

NMDA did not modify GABA release or content in the ARC/ME, KA decreased basal (control: 0.550 ± 0.101 nmol/mg protein; KA 10 uM: 0.283 ± 0.04; KA 100 uM: 0.236 ± 0.04; KA 1000 uM: 0.379 ± 0.057, n = 6, $p < 0.01$) and K+ evoked-GABA release (control: 2.83 ± 0.05, KA 10 uM: 1.91 ± 0.12, KA 100 uM: 1.63 ± 0.10, KA 1000 uM: 1.66 ± 0.10, n = 6, $p < 0.05$). However, KA increased GABA content in the ARC/ME (control: 46.64 ± 5.25, KA 10 uM: 56.82 ± 2.32, KA 100 uM: 75.12 ± 4.29, KA 1000 uM: 77.91 ± 3.38, n = 7, $p < 0.01$). QUIS stimulated evoked-GABA release only at the highest concentration tested (control: 1.63 ± 0.05; QUIS 1000 uM: 3.66 ± 0.24, n = 6, $p < 0.01$).

These results show that KA decreases GABA release from ARC/ME of intact female rats. This decrease may positively contribute to the stimulatory action of KA on GnRH/LH secretion.

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Phencyclidine (PCP)-induced hyperlocomotion reflects release of serotonin (5-HT) onto 5-HT$_{2A}$ receptors in nucleus accumbens: Potent blockade by atypical antipsychotics and glycine B receptor antagonists

PCP reproduces both the positive and negative symptoms of schizophrenia in man. Further, in contrast to amphetamine, 5-HT may be involved in its functional actions. Here, we analysed the mechanisms underlying PCP (20.0 mg/kg, s.c.) – as compared to amphetamine (2.5 mg/kg, i.p.) – induced hyperlocomotion in male Wistar rats. Except S 17828 (p.o.), all drugs were given

	Haloperidol	Clozapine	Ziprasidone	S 16924	S 17828	Eticlopride	MDL 100,907
Inhibition PCP	0.09	0.04	0.03	0.02	0.07	0.07	0.001
	(0.06–0.14)	(0.02–0.08)	(0.016–0.08)	(0.01–0.04)	(0.03–0.18)	(0.007–0.66)	(0.0003–0.003)
Inhibition AMPH	0.04	8.8	0.6	2.3	0.4	0.01	> 10.0
	(0.03–0.06)	(6.4–12.0)	(0.1–1.1)	(1.5–3.6)	(0.2–0.7)	(0.004–0.03)	–
PCP : AMPH ratio	2.3	0.005	0.05	0.009	0.2	7.0	0.0001
D_2, K_i	1	186	3.5	54	1.9	1.0	> 1.000
5-HT_{2A}, K_i	83	23	1.6	2.4	3.3	100.0	0.6
5-HT_{2A} : D_2 ratio	83	0.1	0.5	0.04	1.7	100.0	0.001

Data are Inhibitory $Dose_{50}$s (95% Confidence Limits) in mg/kg. K_is are in nM and were determined as previously (Millan et al., J. Pharmacol. Exp. Ther., 268, 337–351, 1994).

s.c. 30 min before PCP or amphetamine, and locomotor activity was measured automatically for 60 min thereafter.

In contrast to haloperidol, the atypical antipsychotics, clozapine, ziprasidone, S 16925 and S 17828, preferentially blocked PCP as compared to amphetamine. Whereas the selective D_2 antagonist, eticlopride, preferentially blocked amphetamine, the selective 5-HT_{2A} antagonist, MDL 100,907, selectively blocked PCP. Affinity at 5-HT_{2A} sites correlated highly with potency for PCP blockade (0.97, $p < 0.01$), whereas the coefficient between affinity at D2 sites (0.57) and blockade of PCP was not significant ($p > 0.05$). Contrariwise, coefficients for inhibition of amphetamine were 0.38 ($p > 0.05$) and 0.94 ($p < 0.01$) for 5-HT_{2A} and D_2 sites, respectively. Inasmuch as PCP showed only low affinity for 5-HT_{2A} sites ($pK_i < 5.0$), and did not occupy central 5-HT_{2A} sites under ex vivo conditions, a direct action at 5-HT_{2A} sites is unlikely to be involved. In freely-moving rats with dialysis probes, PCP increased extracellular levels of 5-HT_{2A} by 450, 250 and 370% in frontal cortex, accumbens and striatum, respectively. However, locomotion was elicited only when PCP (20 μg) – or amphetamine (10 μg) – were microinjected into the accumbens. Microinjection of 5,7-dihydroxytryptamine (5 μg) into the accumbens, which markedly depleted levels of 5-HT by 85%, abolished the action of PCP. In contrast, the action of amphetamine was not affected. This was, however, abolished by 6-hydrosytryptamine (2 μg), which decreased accumbens DA levels by 85%: these lesions only partially (45%) reduced the action of PCP. Finally, both PCP and amphetamine were blocked by the weak partial agonist at the glycine B site coupled to NMDA receptors, (+)-HA 966 (ID_{50}s, C.L. = 13.9, 6.5–29.7 and 22.6, 11.2–44.6 mg/kg, s.c. respectively and by an antagonist at this site, L 701,324 (12.8, 8.4–19.3 and 18.0, 9.9–32.8 mg/kg, i.p., respectively). In contrast, the glycine B agonist, D-cycloserine (40.0 mg/kg, s.c.) was inactive. It is concluded that, first, PCP- and amphetamine-induced locomotion involve, respectively, activation of accumbens 5-HT_{2A} receptors by the release of 5-HT and activation of accumbens D_2 sites by release of DA. Second, blockade of PCP-induced hyperlocomotion by atypical antipsychotics reflects antagonist properties at 5-HT_{2A} receptors. Third, both PCP- and amphetamine-induced locomotion are inhibited by antagonism of the glycine B site coupled to NMDA receptors, suggesting that such ligands may possess distinctive antipsychotic properties.

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Role of NMDA receptors in sensitization to dopamine D_1 receptors in dopamine denervated rats

Our group have developed a model of behavioral sensitization (priming) in rats with a unilateral 6-hydroxydopamine (6-OHDA) lesion of the dopaminergic nigro-striatal pathway, which is induced by a single exposure to D_1/D_2 receptor agonists like L-dopa and apomorphine or to selective D_1 (SKF 38393) or D_2 (quinpirole) agonists. Priming is expressed after administration (3 days apart), of a dose of SKF 38393 which is effective in inducing turning behavior in primed rats but not in drug-naive 6-OHDA lesioned rats. N-methyl-D-aspartate (NMDA) receptors play an important role in priming since administration of MK 801 during the induction of priming with apomorphine, blocks the subsequent turning behavior response to SKF 38393. An important candidate mechanism for drug-induced sensitization might be the activation of cellular immediate early-genes, since their protein products activate or repress gene transcription. Here we report that in the striatum of 6-OHDA lesioned rats the activation of the early-gene c-fos, stimulated by apomorphine during the induction of priming, is partially repressed by the combined administration of MK 801 although turning behavior is potentiated. In these rats administration of SKF 38393, three days after priming, does not induce any turning behavior and this effect is accompanied by a reduced activation of c-fos by SKF 38393, as compared to rats primed with apomorphine alone. At variance, in rats primed with SKF 38393 instead of apomorphine, MK 801 does not influence the activation of c-fos during the induction of priming and does not block the turning behavior and the striatal c-fos activation induced by the subsequent administration of SKF 38393. The results suggest that repression of c-fos activation during the induction of priming by MK 801 might be part of the mechanisms responsible for the blockade of priming and consequently early-genes might mediate the long-lasting changes taking place in the process of behavioral sensitization.

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Regulation of tyrosine hydroxylase gene expression by membrane depolarization is mediated by multiple signalling pathways

Membrane depolarization is associated with the induction of tyrosine hydroxylase (TH) and TH mRNA. Using rat pheochromocytoma PC12 cells as a model system, we have shown that the TH gene proximal promoter region from –272 to +27 is activated by depolarization with 50 mM KCl and that this activation is dependent on the influx of Ca^{2+} via L-type voltage-gated Ca^{2+} channels. Using site-directed mutagenesis, our studies show that three promoter elements within this proximal 5' flanking region of the TH gene are responsive to membrane depolarization. These three elements are the canonical cAMP response element (CRE, position within the TH gene from –45 to –38), the activating protein-1 element (AP1, –205 to –199), and the newly identified THCRE2 (–97 to –90). The use of minimal promoter constructs demonstrates that the TH gene CRE and AP1 sites are independently responsive to depolarization. Depolarization with 50 mM KCl does not activate cAMP-dependent protein kinase in PC12 cells. Treatment of the cells with the selective Ca^{2+}/calmodulin-dependent protein kinase (CamK) inhibitor, KN62, totally inhibits the stimulation of TH gene promoter activity by 50 mM KCl; this effect is largely due to its ability to block the high K^+-mediated elevation of intracellular Ca^{2+}. In contrast, KN62 (10 uM) only slightly inhibits the stimulation of TH gene promoter activity elicited by the Ca^{2+} ionophore, A23187. Our results suggest that multiple signal transduction pathways acting via multiple transcription factors mediate the stimulation of TH gene transcription by membrane depolarization. (Supported by DA05014 and STRC grant 0481.)

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Human glutamate transporters and neurological diseases

Glutamate is the major neurotransmitter in the brain. It is involved in complex physiological processes like learning. On the contrary, the elevation of extracellular glutamate concentrations causes neuronal death in the case of ischemic states and neurodegenerative diseases. The glutamate transporters rapidly remove glutamate from the synaptic clefts and prevent increased extracellular glutamate. Since neurodegenerative diseases, such as amyotrophic lateral sclerosis and Alzheimer's disease, show a reduction of glutamate transporter, it is believed that the glutamate transporter may play an important role in the onset and progress of neurodegeneration.

Recently, we have cloned and characterized a human glutamate transporter (hGluT-1) to analyse on the relation between the glutamate transporters and neurological diseases. Injection of RNA transcribed from cDNA encoding hGluT-1 into Xenopus oocytes resulted in expression of a transport activity with a high affinity for glutamate and a dependency on external Na^+. The cDNA is highly homologous to congeneric proteins from rat and mouse brains. Chromosomal location of hGluT-1 gene was determined by fluorescence in situ hybridization as chromosome 5p13. Isomers of 2-(carboxycyclopropyl)glycine (CCG), L-, D-aspartate, threo-β-hydroxyaspartate, or L-cystein sulfinate markedly inhibited glutamate uptake through hGluT-1.

We have confirmed a release of glutamate by reversal uptake in the presence of hGluT-1. Since a significant fraction of the glutamate release in ischemia is expected to occur via reversed operation of the transporter, the regulation of hGluT-1 seems important for the prevention of neurological diseases, including cerebrovascular diseases.

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The effects of *N*-methyl-D-aspartate (NMDA) and its competitive antagonist, 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP), injected into the caudate/putamen on kindled amygdaloid seizures in rats

Effects of injections of NMDA and CPP into the caudate/putamen (CP) were examined in amygdaloid (AM)-kindled rats. NMDA is an NMDA receptor agonist, and CPP is a competitive NMDA receptor antagonist. NMDA was bilaterally injected into the CP at 1 (n = 8) or 2 (n = 9) nmol/0.5 μl per side. CPP was injected bilaterally into the CP at 2.5 (n = 8) or 10 (n = 7) nmol/0.5 μl per side. Also, 10 nmol/0.5 μl of CPP was ipsilaterally (n = 7) or contralaterally (n = 7) given into the unilateral CP. Either 20 min after NMDA, or 60 min after CPP, the kindled AM was stimulated at the previously established generalized seizure triggering threshold. Bilateral intra-CP injections of NMDA (1 and 2 nmol) slightly and insignificantly suppressed kindled seizures. However, bilateral intra-CP injections of CPP (2.5 and 10 nmol) significantly suppressed kindled seizures. Unilateral intra-CP CPP (10 nmol), regardless of the side of the injection, had no apparent effect.

The results suggest: (1) NMDA receptors in the CP exert an excitatory action on kindled AM seizures, although they have an inhibitory action to a lesser extent; (2) NMDA receptors in the CP regulate the cortical motor mechanisms responsible for bilateralization and expression of the kindled seizures. However, they do not participate in the linkage between the AM and cortical motor mechanisms during the kindled AM seizures.

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The influence of chronic haloperidol treatment on the striatal glutamatergic receptors

The aim of the present study was to assess the influence of chronic treatment with haloperidol on (1) the contralateral turning behavior induced by intrastriatal injections of agonists of NMDA and non-NMDA receptors, and (2) the density of striatal NMDA and non-NMDA receptors measured by an autoradiography.

Haloperidol was given to animals in a dose of ca. 1 mg/kg/day in drinking water for 6 weeks or 3 months. Experiments were carried out on day 5 of withdrawal.

N-methyl-D-aspartate (NMDA, 500 ng/0.5 μl), α-amino-3-hydroxy-5-methyl-4-isoxasole-propionic acid (AMPA, 1000 ng/0.5 μl) or kainic acid (50 ng/0.5 μl), injected into the intermediate and caudal parts of the caudate-putamen, induced contralateral head turns and rotations. Chronic haloperidol (6 weeks) decreased the number of contralateral head turns induced by NMDA, AMPA and kainic acid. 3-month haloperidol administration decreased also the binding of [3H]AMPA to the intermediate-caudal caudate-putamen. In contrast, chronic haloperi-

dol did not influence the binding of [3H]CGP 39653 or [3H]MK-801 to the NMDA receptors in the striatal tissue. At the same time, chronic haloperidol enhanced the stereotypy induced by apomorphine (0.25 mg/kg sc) and [3H]spiperone binding to D2 striatal receptors.

The present results suggest that chronic treatment with haloperidol induces supersensitivity of D2 dopamine receptors and subsensitivity of NMDA, AMPA and kainate receptors. The subsensitivity of AMPA but not NMDA receptors was related to the decrease in the receptor density.

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Synaptic localization and handling of glutamate and precursor glutamine

Quantitative, postembedding immunogold cytochemistry at the electron microscopic level has provided a detailed picture of the cellular and subcellular distribution of glutamate and glutamine in the brain and has also served to elucidate the metabolic and transmembrane fluxes of these amino acids.

Terminals of a variety of presumed glutamatergic fiber systems have been shown to display severalfold higher levels of glutamate immunoreactivity in vesicle clusters than in the surrounding cytoplasmic matrix, attesting to the ability of the vesicles to accumulate this amino acid. There is also a high level of glutamate immunolabelling in mitochondria; this probably reflects the mitochondrial localization of phosphate activated glutaminase.

Glutamine immunolabelling predominates in glial processes including those abutting on presumed glutamatergic synapses, and inhibition of the glial synthesis of glutamine (by use of methionine sulfoximine, an inhibitor of glutamine synthetase) causes a reduction of the glutamate immunolabelling intensity in nerve terminals. The latter studies, which were performed in organotypical slice cultures, suggest that the maintenance of the glutamate transmitter pool is contingent on a continuous supply of precursor glutamine from glia.

Quantitative immunogold labelling of exogenous D-aspartate has been used to identify, at high anatomical resolution in brain slices, the cellular compartments that are capable of L-glutamate uptake (the transporters do not differentiate between L-glutamate and D-aspartate). The data indicate that L-glutamate uptake occurs primarily in glial processes and in putative glutamatergic nerve terminals, and that there is little uptake in postsynaptic elements and in nerve terminals not believed to use glutamate as transmitter.

The above studies and similar studies of brain slices subjected to depolarizing stimuli have amply confirmed the notion, originally gained from biochemical studies, that glial processes play an active role in the handling of synaptically released glutamate: they take up glutamate and convert it to glutamine, which is then transferred to the nerve terminals as a substrate for de novo synthesis of glutamate. The transporter molecules responsible for the glial uptake of glutamate have been identified and localized by immunocytochemistry (Danbolt, this symposium).

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Excitatory amino acids, the limbic system and drug dependence

Experimental models of intravenous drug self-administration in rodents have allowed the characterization of a neural circuit within the limbic system which seems to be critically involved in the drug-seeking behavior. Specifically, the nucleus accumbens of the ventral striatum (NAC) is a key structure within this neural network and appears to play an important role in motivated behavior and in mediating the reinforcing properties of psychostimulants, opiates and ethanol.

Excitatory amino acids (EAA) are the neurotransmitter of the major neural afferents to the NAC from the limbic cortex and prolonged EAA synaptic activity produces long-term changes of synaptic efficacy. A heuristic hypothesis is therefore the possible involvement of EAA neurotransmission in the neural plasticity associated with the development of drug dependence and in the adaptive changes which represent an integral part of the addictive process.

In a series of behavioral studies using microinfusion of EAA receptor antagonists, we have shown that intact NAC EAA neurotransmission is essential for the full expression of the acute locomotor activating and reinforcing properties of psychostimulant drugs and for the maintenance of ethanol self-administration.

Also, EAA neurotransmission within two recently identified substructures of the NAC, the core and the shell, seems to differentially regulate the integrated function of the NAC in naive animals and following pharmacological activation with psychostimulant drugs. These observations are of importance since a differential regulation of dopamine-dependent behavioral activation by core and shell EAA neurotransmission may suggest the possibility that different NAC subregions may modulate separate behavioral functions associated with motivation, action and reinforcement under the control of functionally separate EAA afferents of allocortical origin.

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A GABA operated Cl^- extrusion mechanism on the Deiters' neuron membrane

Microdissection of single giant Deiters' neurons from the rabbit allows the preparation of their plasma membranes and their use for the study of Cl^- permeation with solutions of exactly known composition on both sides of the membranes.

In this way, it was demonstrated in earlier studies the presence, on the extracellular side of the Deiters' neurons, of the classical $GABA_A$ receptors. This is consistent with the powerful continuous GABA-ergic inhibition these neurons receive from cerebellar Purkinje cells as demonstrated by electrophsiological studies.

Using the same procedure, GABA was put on the cytoplasmic side of the Deiters' membrane: in this way, Cl^- permeation was markedly affected in the in->out direction at a GABA concentration of 1 μM. This effect was blocked by the classical antagonists bicuculline and picrotoxin on the membrane cytoplasmic side.

From these pharmacological studies it was argued that structures acting as $GABA_A$ receptors are on the inner side of the membrane. Moreover the GABA effect displayed a desensitization phenomenon.

Further studies showed that it was blocked increasing the ionic strength and pH. The same result was obtained activating endogenous PKC which suggests that phosphorylation is involved in the desensitization step.

Overall, we suggest a mechanism by which cytoplasmic GABA, interacting with structures behaving like $GABA_A$ receptors with the GABA recognition site towards the neuronal membrane interior, extrudes Cl^- ions from the nerve cell. The Cl^- electrochemical gradient so created and maintained is the basis of the hyperpolarizing inhibitory potentials under the release of GABA by the cerebellar inputs.

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Neuroexcitatory amino acids: 4-Methylene glutamic acid derivatives

Glutamate (Glu), the main brain excitatory neurotransmitter, acts on two classes of receptors. The first class includes glutamate ion channel-receptors, which are subdivided into three subtypes: NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and KA (kainate). Glu also activates metabotropic receptors (mGluR) linked via a G-protein, to phospholipase C (PLC), or adenylate cyclase. Molecular cloning has revealed the existence of at least 8 subtypes of mGluRs, mGluR2, mGluR3, mGluR4, mGluR6, mGluR7 and mGluR8 are coupled to adenylate cyclase inhibition, while mGlulRlα, mGluRlβ, mGluRlγ, mGluR5a and mGluR5b are linked to PLC stimulation. The activation of PLC-coupled mGluRs leads to the hydrolysis of membrane phosphatidylinositol bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3). Diacylglycerol is an activator of protein kinase C (PKC), whereas IP3 mobilizes calcium from intracellular stores. IP3 is then catabolyzed into various inositol phosphates (IP).

PLC-coupled mGluRs are likely involved in the molecular mechanisms underlying brain synaptic plasticity phenomena such as those occuring in learning and memory processes, in postlesional compensatory events and in nervous system development. These receptors could also serve to prevent neuronal apoptosis in granule cell cultures. Despite this tremendous potential implications in brain physiology and pathophysiology, the precise role of these PLC-linked mGluR has not yet been clearly elucidated, largely because of the lack of specific agonists or antagonists.

Our aim was to develop new substances to obtain pharmacological tools for studying mGluRs. We will describe the synthesis and pharmacology of 4-methylene glutamic acid derivatives.

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Amantadine, memantine and L-DOPA mediated behavior in the rat is affected by 6-OHDA lesion of locus coeruleus neurons

The degeneration of dopaminergic neurons in the substantia nigra pars compacta and the loss of noradrenergic cells up to 96% in the locus coeruleus (LC) are main findings in parkinsonian brains. We have examined behavioral and neurochemical effects in rats after 6-OHDA lesion (2 × 2 µl, 6 µg/µl) of the LC. AP = –2.6 mm (IA 0); L = ± 0.9 mm (bregma); V = +3.4 mm (IA 0); [Paxinos and Watson, 1986], under an angle of 30° in rostro-caudal direction. Starting one week after surgery, rats were tested in an experimental chamber after single injections of saline (spontaneous behavior), amantadine (50 mg/kg), memantine (10 mg/kg) and L-DOPA (100 mg/kg free base, + benserazidhydrochloride 50 mg/kg) and after combined injections with haloperidol (0.5 mg/kg) respectively. Lesion of LC neurons reduced sniffing in spontaneous behavior, after injection of L-DOPA, haloperidol and haloperidol + memantine respectively. Number of turns was not affected. Compared to sham-lesioned rats, time of inactivity was prolonged after injection of haloperidol. Following a drug-free period of one week, rats were decapitated and their brains were dissected for neurochemical analysis. In lesioned rats the levels of noradrenaline were reduced in the prefrontal cortex and posterior striatum, whereas the levels of dopamine, DOPAC, HVA, serotonin and 5-HIAA were unchanged. The results show, that a central noradrenergic depletion lowers motor activity and intensifies parkinsonian symptoms. Amantadine, memantine and L-DOPA are unable to completely reverse the behavioral deficits in LC-lesioned rats. (Supported by the BMFT [01KL9008/0 and 01KL9305], Merz+Co. GmBH&Co., and Hoffmann-La Roche AG.)

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Modulation of glutamate release by presynaptic metabotropic glutamate receptors

In cerebrocortical nerve terminals the activation of the metabotropic glutamate receptors, mGluRs, by ACPD enhances the release of glutamate, only if arachidonic acid, AA, is coapplied. The unsaturated fatty acid is necessary to synergistically activate protein kinase C, PKC, together with the diacylglycerol generated by the agonist ACPD. The facilitation of release is observed under 4-aminopyridine, 4AP-, but not KCl-induced depolarization and is sensitive to protein kinases inhibitors. Apart from these facilitatory mGluRs, nerve terminals also exhibit inhibitory mGluRs. The addition of L-AP4 and ACPD reduces the release of glutamate observed both under 4AP- and KCl-induced depolarization. The L-AP4 inhibition does not require arachidonic acid, is insensitive to protein kinases inhibitors and is consistent with a reduction in the cytoplasmin free Ca^{2+} concentration, $[Ca^{2+}]_c$, but not with the decrease in the cAMP levels which is also observed after the L-AP4 receptor activation. Developmental studies indicate that the effects of inhibitory mGluRs are observed within a narrow developmental window (1–3 weeks) but not in adult rats, while the facilitation of release is seen after week 3 remaining in adult rats. In nerve terminals from young (3 weeks) rats interaction studies between the facilitatory and inhibitory pathways have shown that the L-AP4 inhibition is suppressed by the activation of protein kinase C both with phorbol esters or with the metabotropic glutamate receptor agonist ACPD in the presence of arachidonic acid (AA). Thus, these results suggest a dominance of the ACPD and AA-dependent facilitatory pathway of glutamate release due to the heterologous desensitization of the L-AP4 inhibitory pathway.

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Absence of neuropsychological and neurophysiological effects from chronic and acute aspartame (a source of phenylalanine) consumption at high and low doses

The safety of aspartame, whose principle constituent of interest neurologically is phenylalanine, has been widely debated. We designed a study to determine whether aspartame adversely affects cognitive, neuropsychological or behavioral functioning in normal individuals. This was based primarily on preliminary work from our group that suggested that there was an adverse interaction.

We studied fifty-one normal, healthy volunteers in a randomized, double-blinded, crossover experiment that also used sucrose as a comparison condition. Subjects participated for four consecutive months. The first month was a treatment free baseline. Subjects then consumed sodas and capsules containing placebo, aspartame or sucrose for 20 days, followed by a washout period. Treatment order was randomized. Subjects were randomly assigned either to high (45 mg/kg) or low (15 mg/kg) dose aspartame groups. Neuropsychological testing and blood measurements of phenylalanine and other neutral amino acids were obtained on the 10th and 20th days of treatment. Acute effects were assessed on Day 10, 1.5 hours after ingesting the afternoon dose. Chronic effects were assessed on Day 20 prior to the morning dosing. Electroencephalograms were obtained on Day 20.

Statistical analyses compared neuropsychological results, adverse experiences, laboratory values for amino acids, insulin and glucose, and Day 20 EEGs by gender and by treatment condition. Plasma phenylalanine concentrations showed significant increases with aspartame consumption. All other dependent analyses failed to show either an acute or chronic effect of aspartame or sucrose on neuropsychological, cognitive or behavioral functioning.

We concluded that daily consumption of large amounts of aspartame in the context of a normal diet had only small effects on plasma phenylalanine and no demonstrated effects on cognitive or behavioral functions in this healthy, young adult sample.

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p-Chlorophenylalanine, a serotonin synthesis inhibitor, modulates glutamate and GABA levels in the injured spinal cord (A study in the rat using HPLC and immunocytochemistry)

The involvement of the excitatory amino acid, glutamate and the inhibitory amino acid, gamma-amino butyric acid (GABA) in the pathophysiology of spinal cord trauma is not known in details [1]. Increased levels of glutamate in various models of cerebral ischemia are associated with cell injury indicating a role of this amino acid neurotransmitter in excitotoxic cell damage [2].

This investigation is focused on the involvement of glutamate and GABA in a model of spinal cord injury induced by an incision into the right dorsal horn of the T10-11 segments and a 5 h survival period [3]. This will result in profound cell injuries and edema in the perifocal T9 and T12 segments. These effects are reduced by pretreatment with p-chlorophenylalanine (p-CPA), a potent inhibitor of serotonin synthesis indicating a role of serotonin in the production of perifocal secondary injuries following trauma to the cord [3]. However, no single compound is responsible for all the secondary injuries.

We investigated alterations of glutamate and GABA levels in the perifocal spinal cord segments at 5 h. In addition, we studied the influence of serotonin synthesis inhibition on the glutamate and GABA levels after trauma to the cord.

HPLC determinations showed that trauma to the spinal cord resulted in a 6–8 fold increase of glutamate in the perifocal T9 and T12 segments compared with controls. In these segments, the GABA levels were significantly reduced from the control value. Pretreatment with p-CPA significantly thwarted the increase in glutamate. The decrease in GABA was less pronounced in the drug treated rats compared with untreated injured rats. Immunohistochemical investigation of GABA showed a reduction in the number of GABAergic neurons in the T9 and T12 segments. This decrease was most prominent in the ventral gray matter. Pretreatment with p-CPA significantly reduced the changes of GABA immunostaining.

These results show that glutamate and GABA levels are altered around a primary injury to the cord indicating their involvement in the pathophysiology of spinal cord injury. The results obtained with p-CPA pretreatment indicate that inhibition of serotonin synthesis influences the glutamate and GABA levels after trauma to the cord indicating a functional interrelationship between these neurotransmitters, not reported earlier.

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Alteration of amino acid neurotransmitters in the central nervous system following heat stress (An experimental study in conscious young rats)

Heat stress (HS) and associated hyperthermia can induce mental abnormalities and brain damage [1, 2]. The probable mechanisms are unclear. Previous reports from our laboratory suggest that serotonin [1], prostaglandins [2] and opioid peptides [3] are involved in the pathophysiology of brain damage in a rat model of HS. This indicates that neurochemicals play an important role in brain pathology of HS. The central nervous system (CNS) is very rich in various amino acid neurotransmitters. However their involvement in the pathophysiology of brain injury following HS is largely unknown. This investigation was undertaken to examine the possible alteration in two excitatory amino acids glutamate and aspartate as well as two inhibitory amino acids γ-amino butyric acid (GABA) and glycine in the CNS of heat stressed rats. In addition, changes in these amino acids are examined following HS in rats pretreated with drugs modifying serotonin, prostaglandin or opioid peptide neurotransmission. Subjection of conscious young rats (9–10 weeks

old) to HS at 38 °C in a biological oxygen demand (BOD) incubator [1–3] resulted in marked alteration of these amino acids in the brain and spinal cord. After 1 h HS gutamate, aspartate, GABA and glycine showed a marked increase (6–10 fold) in cerebral cortex, cerebellum and brain stem compared with controls. Hippocampus and spinal cord (C1-5) showed a 2–4 fold decrease in glutamate and GABA, whereas aspartate and glycine levels were unchanged. Two hours after HS hippocampus and spinal cord exhibited a mild increase (150–240%) in glutamate and aspartate compared to the control group. These amino acids levels continue to increase in the cortex, cerebellum and brain stem. GABA and glycine were elevated in all the samples examined. At 4 h HS, all the CNS regions exhibited a marked decrease in GABA and glycine levels (4–6 fold). Cerebral cortex and cerebellum showed a decrease in glutamate and aspartate levels, whereas hippocampus and brain stem showed a mild increase in these amino acids. Pretreatment with p-CPA (a serotonin synthesis inhibitor), indomethacin (a prostaglandin synthase inhibitor) or naloxone (an opioid receptor antagonist) resulted in a profound elevation of GABA and glycine levels (4–8 fold) in many brain regions after 4 h HS. The levels of glutamate and aspartate remained low in the CNS of these rats. These results suggest that HS has the capacity to induce a widespread alteration in excitatory and inhibitory amino acids in the CNS. This effect of HS can be influenced by drugs modifying serotonin, prostaglandin and opioid neurotransmission. Our results in HS for the first time provide experimental evidence indicating a functional relationship between serotonin, prostaglandins and opioids on one hand and amino acid neurotransmitters on the other, such a relationship has not been reported earlier.

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Anticonvulsant effect of (1S, 3R)-1-aminocyclopentane-1, 3-dicarboxylic acid (1S, 3R-ACPD), a selective metabotropic glutamate receptor agonist, in kindled amygdala of rats

In order to elucidate the functional roles of metabotropic glutamate receptors (mGluRs) in kindling model of epilepsy, we examined the EEG and behavioral responses after intra-amygdaloid (AM) injection of (1S, 3R)-1-aminocyclopentane-1, 3-dicarboxylic acid (1S, 3R-ACPD), a selective agonist of mGluRs, in the AM-kindled and non-kindled rats. In addition, the effect of intra-AM injection of 1S, 3R-ACPD on kindled seizures was examined. In non-kindled rats (n = 5) receiving intra-AM injection of 200 nmol of 1S, 3R-ACPD, all the animals showed spiking activities associated with limbic behavioral seizures, including immobility, searching, and wet-dog shakes. However, the same dose of 1S, 3R-ACPD injected into the kindled AM of kindled rats (n = 6) caused neither EEG nor behavioral changes. When kindled AM was stimulated after receiving 40 nmol of 1S, 3R-ACPD (n = 6), kindled seizures were markedly suppressed 3 days after the injection. With the 200 nmol dose (n = 6), a similar and significant anticonvulsant effect was obtained 24 h after the injection. Our results suggest that kindling manipulation reduces excitatory action of mGluRs in the kindled AM and that, rather, mGluRs in the kindled AM may exert an inhibitory action against kindled seizures.

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NMDA receptor-antagonists in animal models of drug-seeking behaviour

In a first set of experiments, the effects of the non-competitive NMDA-antagonist MK-801 and of the competitive NMDA-antagonist CGP 37849 on morphine- and amphetamine-induced conditioned place preference (CPP) were examined. When given alone, both NMDA-antagonists produced CPPs which, however, were very unreliable across different experiments. Morphine-CPP was completely blocked by coadministration of MK-801 during conditioning sessions and significantly attenuated by CGP 37849. In contrast to this, amphetamine-CPP was not affected by MK-801 whereas it was completely blocked by CGP 37849. Since neither MK-801 nor CGP 37849 produced a place aversion when given alone, the results cannot be due to a simple additive effect of drug actions. The detrimetral results for morphine- and amphetamine-induced CPP suggest substantial differences in the mechanisms underlying morphine- and amphetamine-induced reward and/or conditioning, and a differential involvement of the different binding sites of the NMDA receptor-complex.

In a second experiment we looked at the effect of MK-801 on morphine-induced behavioural sensitization. An open field (OF), a sniffing box (SN) and an eight-arm-maze were used to examine the behavioural effects of repeated administration of morphine (10 mg/kg) alone and in combination with MK-801 (0.1 mg/kg). Rats received drug injections for 10 days and were tested on days 1, 4, 7 and 10. A saline challenge was given to all animals on day 11, a morphine challenge on day 12 and a MK-801 challenge on day 13. Tests were conducted at 30 min and 2 h after drug injection for 5 min.

The test on day 11 showed conditioned responses in the SB in all treatment groups except control, but not in the OF and in the EAM. The morphine challenge on day 12 showed a sensitization in the morphine group as well as in the morphine+MK-801 group, indicating that coadministration of MK-801 was unable to block sensitization to morphine. Sensitized effects were most evident in locomotion and rearing in the OF, in upward sniffing and turns in the SB, and in the number of arm entries in the EAM. No sensitization occurred in exploratory behaviour (head dips) in the OF (in fact, a profound reduction was seen in all treatment groups across days) and in downward sniffing in the SB. No changes were seen in arm bias and directional bias in the EAM. No sensitization was seen on day 13 after challenge with MK-801. Thus, MK-801 was unable to block morphine sensitization.

There are two other implications of our results. First, they give is further evidence for a dissociation of locomotion and exploration in the open field. Second, the fact that there is a clear sensitization in (upward) sniffing, a behaviour commonly associated with DA-transmission in the ventrolateral striatum, indicates that sensitization is not a phenomenon that occurs exclusively in the mesolimbic DA-system, as most commonly discussed, but also in the nigrostriatal DA-system.

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Behavioural sensitization to morphine: failure of blockade by MK-801, and evidence for sensitization within the nigrostriatal DA-system

An open field (OF), a sniffing box (SN) and an eight-arm-maze were used to look at the behavioural effects of repeated administration of morphin (10 mg/kg) and to examine whether these effects could be blocked by MK-801 (0.1 mg/kg). Rats received drug injections for 10 days and were tested on days 1, 4, 7 and 10. A saline challenge was given to all animals on day 11, a morphine challenge on day 12 and a MK-801 challenge on day 13. Tests were conducted at 30 min and 2 h after drug injection for 5 min.

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The role of dopaminergic and glutamatergic interaction in regulation of the muscle tone in rats

An increase in the muscle tone is one of the main symptoms of Parkinson's disease. In the present study alterations in the muscle tone (MMG) during passive bending and stretching of the hind leg in the ankle joint and its electromyographic (EMG) correlates (stretch reflex) were recorded in rats treated with reserpine (10 mg/kg, 1–27 h after ip injection) or haloperidol (0.5–10 mg/kg ip), or in rats which had the substantia nigra lesioned with 6-hydroxydopamine (6-OHDA, 6.5 μ). The obtained results demonstrate that all the 3 methods, which lead to hypofunction of the dopaminergic system, i. e. dopamine depletion from the presynaptic neuron, lesion of the presynaptic nigrostriatal neurons and blockade of the postsynaptic dopamine receptors, evoke muscular rigidity (MMG) and an increase in the late supraspinal components of the stretch reflex-induced EMG activity. These symptoms seem to be similar to those seen in Parkinson's disease. The early spinal component of the stretch reflex and the resting EMG activity were influenced differently by these substances. A non-competitive antagonist of NMDA receptors – MK-801 (0.32, 0.64 and 1.28 mg/kg) inhibited the reserpine-induced muscle rigidity, as well as the late supraspinal components of the reserpine-increased stretch reflex. The early spinal EMG component increased by reserpine was not diminished by MK-801. The present results suggest that (1) an increase in the muscle tone and late supraspinal EMG components evoked by reserpine, 6-OHDA or haloperidol is a good model of parkinsonian rigidity; (2) a disturbed dopaminergic-glutamatergic balance is responsible for the parkinsonian rigidity and (3) non-competitive antagonists of NMDA receptors can be useful as antiparkinsonian drugs.

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Demographic study of the effect of old age and Alzheimer's disease on enzymes forming kynurenate and aminoadipate

Among the most pressing health care problems is the care of old people with Alzheimer's disease (AD) which afflicts at least 2.5 million and perhaps as many as 4 million Americans, most over the age of 65. Our most recent findings in autopsy brain tissues of patients with senile dementia of the Alzheimer type (SDAT) showed a significantly higher L-α-aminoadipate δ-semialdehyde oxidoreductase (ASO) activity when compared to normal controls. ASO is the enzyme that synthesizes the neurotoxic and gliotoxic compound L-α-aminoadipic acid (L-α-AAA) (Yang and Chang, 1995). Our earlier study in rat astrocyte cultures, showed the presence of L-α-AAA inhibited the synthesis of kynurenic acid (KYNA) significantly. It is therefore possible that the higher ASO activity in the Alzheimer's brian may increase the L-α-AAA concentration which decreases the kynurenine aminotransferase (KAT) activity and the kynurenic acid concentration. It has been reported that in human CNS neurons in vitro, brain affected with SDAT contains a factor that induces paired helical filaments (PHF) similar to those occurring in Alzheimer's disease (De Boni and Crapper, 1978). Glutamate and aspartate were also shown to have the same results (De Boni and McLachlan, 1985). In this project we will also examine the demographic changes in ASO and KAT activities. In the future the L-α-AAA and KYNA concentrations will be examined. After this has been done we will analyze in human CNS neurons in vitro for the possibility of the effect of AAA and KYNA on induction of PHF. Although it is known that L-AAA plays a role in the synthesis of KYNA in the brain (Gramsbergen et al., 1989; Okuno et al., 1991), the interaction and interrelationship of these two compunds and their synthesizing enzymes are not known at the present. With this discovery I would like to propose a research project to investigate the KAT that synthesizes KYNA (Okuno et al., 1991) and ASO that synthesizes L-AAA (Chang et al., 1990) in the autopsy brain tissues of Alzheimer's disease patients. In order to find out how wide spread this phenomenon is in the people of old age and how it relates to the Alzheimer's disease pathology, a long term systematic study is also proposed to investigate the change of these two enzymes in the autopsy human brains and their reaction products in the cerebral spinal fluid according to age and sex.

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Phenytoin protected mouse cortex cells against kainate-induced neurotoxicity

The protective effect of phenytoin (PHT) against the excitotoxicity was investigated in cultured mouse cerebral cortex neurons by monitoring the activities of lactate dehydrogenase (LDH) leaked from the cultured cells. The suppressive effect of PHT on the responses of the cultured neurons to kainate and N-methyl-D-aspartate (NMDA) was also examined under voltage-clamp conditions.

1) Both kainate and NMDA elicited neuronal death at 10 μM or above. Kainate was slightly more potent than NMDA in the range of concentrations examined, 10 μM to 1 mM.

2) PHT significantly protected against the excitotoxicity induced by kainate; 100 μM PHT decreased the LDH activity induced by 100 μM and 1 mM kainate by ~ 70% and 35% respectively. In contrast 100 μM PHT failed to protect against the excitotoxicity induced by NMDA at the same concentration range.

3) The suppression curve for PHT against 100 μM kainate-induced excitotoxicity was examined. The protective effect of PHT appeared at ~ 1 μM and was increased with increasing PHT concentration. The IC50 was ~ 30 μM.

4) Preincubation with 100 μM PHT significantly protected against kainate-induced excitotoxicity whereas failed to protect against NMDA-induced excitotoxicity.

5) The voltage-clamp experiments showed that PHT suppressed the responses to kainate and NMDA non-selectively.

The present results suggest that PHT has clinical efficacy against non-NMDA receptor dependent neuronal death. The mechanism of the protective effect will be discussed.

Nutrition

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Free amino acid profile in the jejunal mucosa of aged rats submitted to starvation. Correlation with amino acid inflow and protein sythesis rates

Aim: To our knowledge there is no available information on the regulation of prolonged fasting in the aged rat. The present study was undertaken to measure the effect of prolonged starvation on jejunal mucosa amino acid (AA) inflow, free AA levels and protein synthesis rates in old rats.

Methods: 100 2-yr-old male Wistar rats were randomly allocated into four groups to be submitted to 1 (Group 1), 5 (Group 2), 9 (Group 3) or 15 (Group 4) days of fasting. Jejunal mucosa was obtained by removing the proximal 30 cm of the jejunum, which was flushed with cold 0.9% NaCl, everted and scraped with a microscope slide. Protein content was determined according to Lowry. Protein synthesis was calculated by the flooding dose method (L-[1-^{14}C]-Leucine) after Ketoisocaproate correction. Blood flows were measured using ^{57}Co labelled microspheres. AA concentrations were analyzed by HPLC (Pico-Tag^{r}). Results are expressed as mean ± SD. Simple linear regression was applied to infer the relationship between days of starvation and AA levels (inflow and intraorgan concentrations) (* $p < 0.05$). Statistics: One-way ANOVA+Tuckey test. Values in a row with different superscripts are significantly different ($p < 0.05$).

Results: a. Organ Weight (g): 1.: 0.57 ± 0.2^{a}; 2.: 0.35 ± 0.01^{b}; 3.: 0.36 ± 0.01^{b}; 4.: 0.40 ± 0.19^{a,b}; *b.* Protein Content. (mg.organ): 1.: 63,0 ± 11,5^{a}; 2.: 39,8 ± 7,7^{b}; 3.: 41,1 ± 4,7$^{b;}$ 4.: 32,7 ± 2,8^{c}; *c.* AA inflow (Correlation Coefficient) and *d.* Free AA levels (Correlation Coefficient):

AA	AA Inflow	Free AA Levels
ASP	0.6238 *	–0.1777
GLU	0.8169 *	0.5037 *
SER	0.5215 *	0.1161
GLI	0.7927 *	0.4379 *
GLN	0.8875 *	0.4633 *
TAU	–0.6387 *	0.1897
HIS	–0.6198 *	0.0159
THR	0.8084 *	–0.1297
ALA	0.3753 *	–0.2016
ARG	–0.7049 *	
PRO	0.3965 *	–0.1031
TYR	–0.6618 *	0.0182
VAL	0.7976 *	0.2292
MET	–0.6612 *	–0.2001
ILE	0.7689 *	0.0317
LEU	0.7716 *	0.4735 *
PHE	–0.6655 *	0.0495
TRP	–0.8987 *	0.1151
ORN	0.7288 *	0.1017
LYS	0.7734 *	–0.1160

e. Fractional Synthesis Rates (Ks%) 1.: 239 ± 73^{a}; 2.: 256 ± 88^{a}; 3.: 264 ± 72 ^{a}; 4.: 225 ± 24^{b}

Conclusions: In aged rats the intestine responds to starvation with a marked loss of mucosal protein. However, both AA supply and free AA levels, as well as fractional protein synthesis rates, are well preserved practically all during the period studied, suggesting basically an enhanced protein breakdown. Further studies are still necessary to establish its practical effects on protein metabolism.

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Does supplemental arginine in total parenteral nutrition (TPN) improve liver protein metabolism in aged rats? Correlation with arginine blood inflow and arginine intraorgan concentration

Aim: The present study was undertaken to evaluate the impact of arginine (Arg) supplementation to TPN on liver protein synthesis rates and on proteolytic enzymes (activities and gene expression) in old rats. To assess the actual liver disposal of

administered Arg, hepatic Arg inflow and its intraorgan concentration were measured.

Methods: 120 2-yr-old Wistar rats were divided into four groups (n = 30 each): 1.: Non-operated control rats; 2.: Sham-operated control rats; 3.: Conventional TPN ([Arg] = 0.44g gN_2^{-1}, 0.75% total energy) fed rats; 4.: Supplemented Arg-TPN ([Arg] = 0.76g gN_2^{-1}, 3,32% total energy) fed rats. TPN solutions – isocaloric, isovolemic and near isonitrogenous – provided: 0.59 gN_2, 114 np kcal kg^{-1} day^{-1} and were administered during four days. Liver protein synthesis was calculated by the flooding dose method (L-[1-^{14}C]-Leucine) after ketoisocaproate correction. Cathepsin D (Cat D) activity was determined by the method of Nenurkar and Cathepsin B (Cat B) according to Barret using specific substrates. Cat D and B mRNA levels were measured by Dot Blot. Blood flows were measured using ^{57}Co labelled microspheres. Arginine concentrations were analyzed by HPLC (Pico-Tag®). Statistics: Mean ± SD, One-way ANOVA+Tuckey test. Values in a row with different superscripts are significantly different ($p < 0.05$).

Results:

	Group 1	Group 2
Arg Inflow (μMol ml^{-1} min^{-1} g^{-1})	0.13 ± 0.02^a	0.16 ± 0.04^b
Free Arg Levels (μMol g^{-1} ww)	0.62 ± 0.06^a	0.79 ± 0.13^b
Fractional Synthesis Rate (Ks%)	60 ± 7^a	$69\pm14^{a,b}$
Cat D (ugTyr 10 $min^{-1}mg^{-1}$pt)	4.3 ± 0.2^a	6.5 ± 0.4^b
Cat B (mU mg^{-1} protein)	159 ± 15^a	270 ± 59^a
mRNA levels		
Cat D (Relative units)	1.0 ± 0.2^a	2.1 ± 0.3^b
Cat B (Relative units)	1.0 ± 0.2^a	1.8 ± 0.3^b

	Group 3	Group 4
Arg Inflow (μMol ml^{-1} min^{-1} g^{-1})	0.09 ± 0.03^c	0.15 ± 0.04^a
Free Arg Levels (μMol g^{-1} ww)	0.50 ± 0.05^c	0.65 ± 0.18^a
Fractional Synthesis Rate (Ks%)	96 ± 17^c	76 ± 8^b
Cat D (ugTyr 10 $min^{-1}mg^{-1}$pt)	4.5 ± 0.4^a	4.1 ± 0.5^a
Cat B (mU mg^{-1} protein)	221 ± 54^a	164 ± 46^a
mRNA levels		
Cat D (Relative units)	1.2 ± 0.2^a	1.0 ± 0.1^a
Cat B (Relative units)	1.2 ± 0.2^a	0.9 ± 0.3^a

Conclusions: 1. Levels of dietary arginine supplementation, that significantly increased blood and tissue arginine concentrations, with respect to conventional TPN, did not imply any additional benefit on liver protein metabolism. In contrast protein synthesis rates were inhibited supporting the concept that availability of supplementary arginine is not always beneficial. *2.* Additionally, our results demonstrate that nutritional manipulations can stimulate liver protein synthesis in old rats, without altering proteolytic activity, which could be useful in certain situations. (Grants: ESPEN R.F. 1993; FIS-93/1256).

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Postprandial concentrations of plasma amino acids in very low birth weight infants during the first weeks of life: Influence of dietary protein source

Objective: Preprandial plasma amino acid concentrations have extensively been used as marker of the nutritional value of dietary proteins in very low birth weight (VLBW) infants. The present study was designed to investigate the postprandial plasma essential amino acid (EEA) concentrations in VLBW infants fed different dietary proteins with nearly similar amounts of EEA during the first weeks of life.

Patients and methods: In VLBW infants, pre- and postprandial plasma amino acid concentrations were measured when 100 ml/kg/day of enteral nutrition was tolerated. All infants were enterally fed with human milk until the time of study. At day of study, five infants received as a test meal 10 ml/kg human milk fortified with human milk protein (HMP) (0.22 g HMP/meal) and five infants were fed with 10 ml/kg standard preterm formula (0.2 g bovine milk protein (BMP)/meal). The plasma amino acid concentrations were measured preprandial and 15, 30, 45 and 60 min after the test meal. The preprandial blood sample was taken 2.5 hours after the last meal.

For each measurement 200 µl blood was obtained via a cenral venous catheter which was placed due to the supplemenatry parenteral nutrition. The plasma amino acids were determined by reversed-phase high-performance liquid chromatography based on o-phthaldialdehyde/2-mercaptoethanol precolumn derivatization.

Results: The intakes of EEA with the two test meals were nearly similar in both groups. Only the intakes of THR, VAL, and MET were higher in the group fed BMP than in the group fed HMP. There was an increase of the concentrations of all EEA within the first 15 min after the test meal in both groups. In the group fed BMP, the concentrations of all EEA increased more than in the group fed HMP. In the HMP group, the concentrations change only marginally between 15 and 60 min postprandial and in the BMP group the concentrations remained nearly constant between 15 and 45 min postprandial. Between 45 and 60 min after the meal the concentrations of all EEA decreased in the group fed BMP, so that 60 min postprandial, the concentrations of most of the EAA were similar in both groups.

Conclusion: The postprandial concentrations of plasma amino acids depend on the dietary intake but also significantly on the amino acid source. The data of the present study suggest that in VLBW infants the 30 min postprandial amino acid concentrations should be included in the evaluation of the nutritional value of dietary proteins.

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A reduction in the concentration of dietary methionine lowers protein quality and increases hepatic and renal purine enzymes and uric acid in growing chickens

Previous studies have shown that in chickens, the hepatic levels of the purine enzymes xanthine dehydrogenase and nucleoside phosphorylase as well as the concentration of uric acid

in liver and excreta in growing chickens increase when the quality of the dietary protein decreases. To further study this relationship, in the present experiment, one day old chickens were fed diets which provided 0, 5, 10, 25, 50 and 100 of their methionine requirement. The results showed that decreasing dietary methionine, the growth of the chickens as well as the quality of the dietary protein, measured by PER and NPU decreased precipitously. In contrast, the levels of the purine enzymes and uric acid in liver and kidney increased by several fold. This increment was also observed in the concentration of uric acid in the excreta. A correlation analysis of PER or NPU on the purine enzymes or the concentration of uric acid showed a high and negative correlation, indicating that the quality of the protein consumed can be estimated from the purin enzymes or the uric acid data. These results together with the result of other experiments which showed that the effect of the quality of the dietary protein on the purine enzymes and uric acid occurred within the first day after initiating the consumption of the diet suggests that these data could be very useful in the rapid estimation of protein quality. (Grant CONICIT S1-1242.)

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Assessment of amino acid nutritional status in young Belgian blue bulls at different growth rates

Optimizing muscle protein (MP) deposition, i.e. meat production in farm animals aims at correctly matching amino acid (AA) supply and utilisation. To do so, a valid estimate of the AA nutritional status is needed. In this communication 3 approaches e.g. plasma concentration of free amino acids (FAA) (=extracellular) [AA]e, intracellular FAA concentration in muscle (needle biopsy), [AA]i, and measurement of AA flux (uptake) in a defined muscle system, e.g. the catheterised hindlimb were compared in young bulls of the Belgian Blue breed on two different growth rates. The first two approaches were measured in either a control (4 bulls) or a group with initially a slow growth (0.5 kg/d) (SG, 4 mo) followed by a 6 mo fattening period (RG, 4 bulls). Blood and muscle biopsy samples were taken in both periods. The third approach was more limited in time due to the short time effectiveness of the catheters. Hindleg AA arterio-venous differences (AVD) and uptakes were measured on 4 animals during 3 days in slow growth and on 4 others in rapid growth. In approach 1 & 2, FAA concentrations (F) were expressed as % of protein bound (B) AA concentrations. In approach 3, uptakes in µmol/min were related on the same base: µmol/min/µmol/gDM, as %. Due to high demands on analytical accuracy for AVD estimates, discussion on essential AA is limited to Val, Thr, Leu, Ile, Phe, Lys and Arg.

[AA]e, were about 20 times lower than [AA]i. Mean F/B% ratio increased from 0.017 during SG to 0.021 in RG, while in control 0.022 and 0.018 were noted during the corresponding periods. Large differences were observed between individual AA. Arg was lowest in SG and increased most in RG. Leu was lowest during RG (0.011).

Mean [AA]i increased from 0.324 to 0.434 % from SG to RG, while in the control group an inverse tendency appeared 0.446 & 0.327. Leu was lowest in both periods and even decreased in RG.

Supposing that the AA with the lowest F/B ratio could be considered as the limiting one, both [AA]e and [AA]i point in the direction of Leu.

Comparing AA uptake rates across the perfused hindlimb as related to MP AA composition, a significant mean increase was noted between SG and RG, e.g. 0.434 to 3.345. However, this increase is for a large part due to Lys which rose from 0.11 to 8.63, although the other AA increased also. Surprisingly, a numerical negative uptake was observed for Arg, which became however less negative in SG. The lowest absolute increase in relative uptake was thus observed for Arg. So, postulating that the AA with the smalles increase could be the limiting AA, Arg could be the candidate. On the other hand the large increase for Lys could indicate an increased need for this AA in conditions of rapid growth.

In conclusion, depending on the approach used, different AA could be regarded als limiting for meat production in young beef bulls. (Research sponsored by I.R.S.I.A. Brussels).

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Pyrraline and pentosidine: two post-translational lysine derivatives in food proteins

The well-known Maillard reaction is an important post-translational modification of food proteins, resulting predominantly from the reaction between reducing carbohydrates and the ε-amino groups of lysine residues. Analogous reactions in vivo have generated considerable interest in view of their contribution to the development of long-term complications of diabetes and aging.

After total enzymic hydrolysis of food proteins, quantification of pyrraline, an acid labile pyrrole derivative of lysine with mutagenic and antiproteolytic properties, was achieved using either ion-exchange chromatography with photodiode array measurement or isocratic ion-pair RP-HPLC with direct UV-detection. Values for pyrraline in milk products ranged from not detectable (lower than 1 mg/kg of protein) up to 150 mg/kg of protein. Increased values were found for "overprocessed" samples of sterilized milk (260 mg/kg of protein) and milk powder or whey powder, respectively (up to 3100 mg/kg of protein). Between 10 and 130 mg pyrraline per kg of protein were determined for pasta products, correlating with heat intensity during pasta drying. Considerable higher amounts could be found in bakery products (200 to 3700 mg/kg of protein). Pyrraline proved to be a suitable indicator for the advanced Maillard reaction.

In acid hydrolysates, sensitive determination of pentosidine, a fluorescent cross-link amino acid in which one lysine and one arginine residue is linked together by a ribose, in addition to common amino acids was possible using ion-exchange chromatography with direct fluorescence detection and subsequent ninhydrin derivatization. The detection limit was lower than 10 µg/kg protein. Levels of pentosidine in food were very low (between not detectable and 2 to 5 mg/kg of protein; some bakery products up to 35 mg/kg of protein), indicating that pentosidine does not play a major part in cross-linking of food proteins.

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Excessive dietary L-lysine fed to male young rats causes a fatty liver

L-lysine (5%) fed to young male rats causes, within three days, an accumulation of lipids in the liver. The fatty liver of L-lysine is only transitory since it is present after 15 days of feeding but not after one month. This fatty liver requires that lysine is offered with casein since the replacement of this protein by soybean protein results in normal livers. The factor in soybean protein which alleviates the fatty liver of L-lysine seems to be its high concentration of arginine since the addition of 1% Arg to the casein L-lysine mixture results in normal livers. Since both a deficiency of arginine and the mixture of casein L-lysine are associated in rats with orotic aciduria and a fatty liver and dietary orotic acid itself also causes a fatty liver, it is thought that the fatty liver of lysine may be caused by orotic acid. The lipids which accumulate in the fatty liver of lysine similarly to other fatty livers (orotic acid, ethionine) are triglycerides, cholesterol and phospholipids. Moreover, in rats fed 6.3 and 0.67% L-lysine or orotic acid respectively we found a progressive accumulation of hepatic palmitic, oleic, linoleic and linolenic acid and a reduction in stearic and arachidonic acid. Despite the similar composition of the fatty liver of lysine with other fatty livers we have found that the histological appearance, the transport of lipids from the liver to the circulation, the hepatic fatty acid synthesis as well as the concentrations of the circulating lipids and lipoproteins are unique in the fatty liver caused by L-lysine feeding.

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The effect of feeding a bolus of branched-chain amino acids on a battery of decision making tasks

Several studies have indicated an increase in the concentration of serotonin (5-HT) in the brain may contribute to the feeling of fatigue during sustained exercise (Blomstrand et al., 1988, 1989). The alteration in the concentration ratio of plasma free tryptophan (free tryp) to branched-chain acids (BCAA) is thought to be associated with the alteration in brain 5-HT level. Some of these amino acids are transported into the brain on the same carrier and competition for entry can therefore occur. Hence raising the level of plasma BCAA by ingestion of a solution of these amino acids may prevent an increase in the brain level of 5-HT. Whilst evidence suggests BCAA supplementation may enhance the feeling of "well being" during endurance exercise, papers have suggested BCAA administration can increase cognitive function, post-exercise, especially on the more attention-demanding tasks (Blomstrand et al., 1991; Hassmén et al., 1994).

The study was designed to evaluate effects of supplementing BCAA on a battery of decision making tasks, in conjunction with evaluating the profile of amino acid uptake across the forearm muscle, with no prior exercise regime. Ten subjects were used in a double blind crossover trial, each subject receiving a placebo and BCAA drink on two separate visits. Two cannulae were inserted, both retrogradely, one in a vein draining a deep muscle at the anticubital foss, the other in the dorsum of the hand. The hand, with the cannula, was placed into a pre-heated box and warmed up to approximately 42 °C, from which "arterialized" samples were taken. Blood pressure and heart rate was monitored continuously throughout the duration of the trial. The BCAA was given as an isotonic drink (1 litre), adjusted to body weight, at 60 minutes from the start of the trial.

The test battery was split into three separate conditions, shape rotation, sentence picture and lexical decision making tasks, results were obtained for percentage errors and reaction time. Results from the shape rotation task did not yield any significant differences in either condition. The sentence picture and lexdec tasks showed no signifacant differences in reaction time and right and wrong decisions between the placebo and BCAA condition, although graphically, the reaction times for both trials were lower in the BCAA fed group. Plasma valine, leucine and isoleucine showed significant differences ($p < 0.0001$) between the fed and placebo group; also significant a-v differences in the BCAA fed group ($p < 0.001$). Plasma alanine and glutamine showed significant increases in a-v differences ($p < 0.005$) in the BCAA fed group. Venous plasma insulin increased in the BCAA fed group and correspondingly venous plasma glucose decreased ($p < 0.05$).

In conclusion, whilst it has been recognised that BCAA supplementation does improve cognitive performance, it has only been demonstrated after prolonged exercise. The evidence from this study suggests the maximum effect of BCAA supplementation, on decision making, may only be noticed after periods of prolonged exercise or perhaps prolonged psychological stress, neither condition was present in this study. In contrast, the results obtained from the physiological samples taken correspond with previous findings demonstrating BCAA feeding increases muscular output of alanine and glutamine considerably, increases plasma insulin level, decreases plasma glucose level and may have a hypervolaemic effect on the blood (by increasing the osmolarity), as indicated by the venous haematocrit samples.

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Protein synthesis and breakdown in cirrhotic rats. Effect of nutrition

Rats were injected intravenously with [^{14}C]leucine and then divided into three groups. Rat cirrhosis (group C), was induced by repeated intragastric administration of CCl_4 in oil. Ad-libitum (group AL) and pair-fed (group PF) of rats were treated with mineral oil alone and served as controls. Three days after the last treatment followed by an overnight fast rats were injected with [^{3}H]leucine and sacrificed 20 min afterwards.

In PF rats we observed: slower increase of body weight; slight decrease of blood plasma BCAA level; lower weight and protein content of visceral tissues; higher [^{3}H]leucine specific activity in blood plasma; lower leucine incorporation into proteins of plasma, liver, spleen and heart; higher [^{14}C]leucine radioactivity in proteins of gastrocnemius muscle and small intestine than in AL controls.

In C rats we observed: slower progression of increase of body weight, strong decrease of blood plasma BCAA level; lower protein content in liver and higher one in spleen, kidney and heart; lower leucine incorporation into proteins of liver and skeletal muscle and higher one into spleen proteins; higher [^{14}C]leucine radioactivity in liver and spleen than in PF and/or AL controls.

We conclude that: (a) decreased food intake and liver cirrhosis induced by CCl_4 resulted in the fall in protein turnover; (b) particular changes of protein metabolism in liver cirrhosis are not mediated by malnutrition (primarily increase of protein content in spleen and kidney); (c) decrease in BCAA plasma levels in liver cirrhosis is probably mediated both by malnutrition and by specific abnormalities elicited by liver disese; (d) mechanisms increasing protein content in spleen and kidneys of cirrhotic rats are probably higher protein synthesis and slower rate of protein breakdown than in pair-fed rats; (e) the higher decrease of protein synthesis in cirrhotic rats than in pair-fed rats is probably responsible for increased breakdown of muscle proteins; (f) cytokines are probably important mediators responsible for particular changes in protein metabolism in liver cirrhosis.

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Leucine metabolism in specific tissues of endotoxin treated rats.
Effect of nutritional status

Rats were injected with [^{14}C]leucine and then divided into four groups. Groups I and II, consisting of ad libitum fed rats were injected with saline or endotoxin of Salmonella enteritidis (5 mg/kg) eight and twenty two hours after [^{14}C]leucine treatment. Animals of Group III (saline) and Group IV (endotoxin) fasted after [^{14}C]leucine injection and were treated using the identical scheme as ad libitum fed animals. Twenty three hours after [^{14}C]leucine treatment rats were injected with [^{3}H]leucine and sacrificed 20 min afterwards.

Endotoxin administration resulted in a significant decrease of body weight in ad libitum fed rats, an increase of weight and protein content of spleen, and decrease of plasma levels of all amino acids (primarily glutamine, proline, glycine, alanine, citrulline, methionine, valine, leucine and isoleucine). [^{3}H]leucine specific activity in blood plasma was significantly higher and leucine incorporation into proteins of all observed tissues decreased after endotoxin treatment. The highest decrease was observed in skeletal muscle. [^{14}C]Leucine radioactivity was significantly higher in the spleen and lower in skeletal muscle of endotoxin treated rats. All changes were less expressed in fasted than in ad libitum fed animals.

Our results indicate that endotoxin treatment results in (a) changes in host metabolism that are not mediated solely by anorexia; (b) the loss of the normal protein-conserving responses associated with decreased food consumption; (c) both decrease of protein synthesis and increase of protein degradation in skeletal muscle; (d) reutilization of leucine released from skeletal muscle in viscera; (e) the slower disappearance rate of leucine from the blood and (f) increase of leucine oxidized fraction associated with a decrease of leucine turnover that is probably the mechanism decreasing branched-chain amino acid and likely of other amino acid levels in our model of endotoxemia.

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Intestinal absorptions changes in growing tumour-bearing rats

Cancer cachexia is characterized by a loss of appetite, generalized host wasting, and a variety of metabolic alterations. The Walker 256 carcinoma was considered to be an appropriated model of human cachexia where weight loss and reduction in food intake are originated from metabolic effects.

In the present study we evaluated the effects of Walker 256 carcinoma evolution in growing rats and its consequences on intestinal absorption and biochemical parameters. Weanning rats (25 days) received 0.25×10^6 neoplastic cells (YW) or saline solution (YC), s.c., and were housed in metabolic cases receiving control diet with 18% protein. Body weight, tumor weight, food intake, nitrogen balance, serum protein, glycemia, hepatic glycogen and muscle protein were measured daily until the 13th day. After this period animals are submitted to a 12 hours starvation and prepared for surgery. The small intestine was isolated in vivo and perfused with 2.77 mM glucose, 7.38 mM DL-methionine and 8.39 mM L-leucine at a flux rate of 0.5 to 1.0 ml/min. The growing tumour-bearing rats showed significant decrease in body weight (11.0%), food intake (61.8%), nitrogen balance (61.9%). The neoplastic growth was pronounced and the weight rate tumour/carcass was 11.0% in YW rats. The glycemia, hepatic glycogen, muscle protein reduction in YW suggest deviation of these substrats for cancer development. Slight decrease in glucose, methionine and leucine intestinal absorption were observed in YW rats. These intestinal absorption changes were more pronounced in the growing rats than adults tumour-bearing rats, as observed in our previous studies. These data showed that the host growing rats present elevated catabolic waste, decreased food intake and negative nitrogen balance which result in cachexia and these state could be aggravete by reduction of the intestinal absorption of some substrates. (Technical assistence: Geraldo, A. S.)

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Effects of feeding on leucine kinetics and protein turnover in healthy children and in children with cystic fibrosis (CF)

In healthy adults, enteral feeding, via the effects of insulin secretion, results in suppression of whole body protein breakdown. We hypothesized that there may be diminished suppression of protein breakdown (B) with feeding in patients with cystic fibrosis (CF) who generally have decreased insulin secretion. Using a 2 hr primed, constant [1-^{13}C]-leucine infusion, we measured rates of whole body protein synthesis (S) and B in nine clinically stable CF patients (6.4–11.4 yr) and in five healthy controls (7.8–9.9 yr) in both the fed and fasted states. In the CF patients, S and B ($X \pm SD$, $\mu mol \cdot kg^{-1} \cdot hr^{-1}$) in the fed state were 172 ± 61 and 157 ± 67, and in the fasting state were respectively, 140 ± 24 and 178 ± 26. In the controls, S and B were respectively 129 ± 27 and 114 ± 20 in the fed state and 136 ± 13 and 173 ± 18 in the fasting state. Mean leucine balance in either the fed or fasted state was nearly identical in the two groups. By analysis of variance, there was a significant effect of feeding on B but no significant interaction between subject group and this effect. However, using paired analysis of each group separately, feeding resulted in a 34% decrease in B in the controls ($P = 0.001$) and a 23% increase in S in the CF group ($P = 0.058$). This study did not identify an unequivocal abnormality in the effects of feeding on protein turnover in CF, but the data suggest that protein accretion during feeding may occur via different mechanisms in CF patients compared to healthy controls.

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Physical growth and nutritional status of children with phenylketonuria: Study with a new phenylalanine-free formula

Dietary management of phenylketonuria (PKU) centers on the controlled intake of low protein foods and of low phenylalanine (Phe) or Phe-free amino acid mixture formula. Due to elimination of high protein foods and inadequate intake of formula, protein intakes of phenylketonuric children are often low. Recently, a new Phe-free formula with an improved taste became available. A study was designed to eveluate the acceptance of this new formula and its effect on physical growth and blood Phe. Three male and three female children with PKU aged 3–16 years were subjects of this study. They were instructed to take the new formula for six months. Three-day food records, formula intakes, height and weight, and blood parameters are being periodically evaluated. Before the administration of the new formula, our initial observations were that these children had stunted physical growth. Five of the six children were below the 25th percentile for both height and weight for their ages and sex. Their protein intakes were also low; the mean protein intake was 62 ± 15% of their recommended levels. Other low intakes included calcium, phosphorus, and zinc. Blood analysis showed that these children hat abnormal values of serum albumin, blood urea nitrogen, creatinine, and cholesterol. Younger children, aged 3 and 4 years, had a better control of blood Phe levels (6 and 6.2 mg/dl) than did older children, aged 8, 9, 12 and 16 (9.5, 13.9, 16.9, 17.5 mg/dl). Currently, we are collecting midpoint data for height, weight, and blood parameters.

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The improvement of the sunflower protein nutritional value by adding L-lysine HCl

In our region, sunflower seed is the main source of oil and concentrated proteins for animal feed. In technological procedure for production of sunflower meal with 44% protein has been employed for more than twenty-five years. The great defect of this meal is the remarkably lower level of lysine in relation to soybean meal. This evident deficiency of lysine produces amino-acid debalance, that is, inadequate utilization of rich amino-acid potential of sunflower proteins. Some of the results obtained show that the diets containing sunflower meal with 44% protein with the addition of L-lysine HCl and no animal proteins may have the biological value some as the standard mashes based on corn, soybean meal and fish meal.

In this work, the nutritive value of sunflower meal with 44% protein with and without L-lysine HCl in swine feeding was investigated.

The experiment was carried out with swines of domestic meat race that were alloted into three groups equalized by origin, sex, age and the starting livebody mass.

The nutritional value of three alternative diets for fattening swine was determined: (1) basal diet based on corn and sunflower meal with 44% crude protein, (2) experimental diet based on corn and sunflower meal with 44% crude protein plus L-lysine HCl, (3) standard diet based on corn and sunflower meal. Compared to the basal diet, the experimental diet had a significant effect on live weight gain (616 vs. 728 g, $P < 0.01$), feed: conversion ratio (3.97 vs. 3.46 kg, $P < 0.05$), muscle tissue yield (39.7 vs. 36.4 kg/100 kg livebody mass, $P < 0.05$), adipose tissue yield (27.6 vs. 24.7 kg/100 livebody mass, $P < 0.05$). Consequently, L-lysine HCl supplementation resulted in a significant improvement in the nutritional value of diet based on corn and sunflower meal. On the other hand, live weight gain intensity and feed conversion were similary affected by the experimental and standard diets. However, compared to the standard diet, the experimental diet had a better effect on muscle tissue yield (38.2 vs. 39.7 kg/100 kg body mass, $P < 0.05$).

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Fasting plasma free amino acid of elderly men

Mean values for the fasting amino acid plasma levels of individual are given for eight men, 75 ± 7 yr (mean ± SD). All subjects were in good health. Three blood smaples were taken from them at one or two week intervals for three months. There were no statistically significant differences among the three samples for each subject for all amino acids (analysis of variance with repeated values). The plasma amino acid levels (mean ± SD) for 24 blood samples, or eight subjects, three samples each (μmol/L) are on the table. These results when compared with those of Armstrong and Stave [1], Hagenfeldt and Arvidsson [2], for younger men, and Young at al. [3] show the following differences: statistically, $p < 0.05$, lower values for the essential amino acids, but lysine is higher in the elderly. Serine and aspartic acid are also higher, but alanine and arginine are lower. For cystine/2, glycine, histidine, proline and tyrosine there are no statistically significant differences. The amino acid profile presented here, for these well fed elderly men can be related to aging process or due to lower protein/energy intake.

Fasting plasma free amino acid (μmol/L) of elderly men (75 ± 7 * yr).

	Present study	Armstrong and Stave [1]	Hagenfeldt and Arvidsson [2]	Young et al. [3]
n	**8**	83–91	20	4
Alanine	**312±58**	419±89†	320±67	
Arginine	**65±14**	89±26†	79±23	
Aspartic acid	**19±7**		7±3†	
Cystine/2	117±39	118±17	99±19	
Glycine	224±51	236±42	240±49	
Histidine	88±13	89±11	89±12	
Isoleucine	**67±15**	84±18†	62±11	116±17†
Leucine	**120±28**	160±27	132±15	202±19†
Lysine	**210±25**	198±31	167±25†	201±23
Methionine	**22±8**	32±6†	24±5	37±4†
Phenylalanine	**66±11**	65±9	53±8†	73±10
Proline	195±52	239±70	183±43	
Tryptophan	**29±6**	60±15†	45±9†	
Tyrosine	64±19	72±15	54±11	70±20
Valine	**202±35**	252±37†	235±31	326±45†

* Mean ± SD., † Two unpaired samples "t" test. Results from literature against present work, $p < 0.05$.

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Supplementation of α-ketoglutarate improves amino acid metabolism in malnourished hemodialysis patients

Hemodialysis patients show changed amino acid pattern compared to healthy persons, especially decreased concentrations in plasma of serine and essential amino acids.

α-Ketoglutarate is an intermediate in the tricarbonic acid cycle and possess regulatory functions in view of the concentrations of histidine, arginine and proline and due to the inhibition of the gluconeogenesis-catalysing enzym phosphoenolpyruvate carboxykinase. Arginine influences hormonal release and is essential for synthesis of nitric oxide.

After one year of administration of calcium-α-ketoglutarate (3 × 1.5 g per day) in 24 malnourished hemodialysis patients we could show a significant ($p < 0.05$) increase of concentrations [μmol/l] in plasma (before/after one year) of arginine (54 ± 18/71 ± 16), histidine (89 ± 21/103 ± 19) and proline (159 ± 48/228 ± 24). Furthermore, the concentrations of leucine (64 ± 28/81 ± 16) and the ketoanalogue α-ketoisocaproate (11 ± 3/14 ± 5) increased significantly in direction to normal levels of a healthy control group. Leucine and α-ketoisocaproate are regulating compounds of the proteolysis/proteinbiosynthesis balance. Most of the hemodialysis patients experienced a substantial weight gain (2.0 ± 0.3 kg).

Thus, α-ketoglutarate application improves the amino acid metabolism in malnourished hemodialysis patients.

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Effect of addition of lysine and histidine in the manufacture of fish sauce

Fish sauce is a hydrolysis product of salted fish and is commonly used as a condiment in Southeast Asia. It is also an important source of protein in the diets of some social classes in certain parts of the region. It contains about 20 g/L of nitrogen, of which 16 g is in the form of amino acids. It usually takes about a year to produce a good quality fish sauce. The long waiting is a limiting factor in the viability of fish sauce industry. The demand is more than the supply thus forcing the industry to produce low quality fish sauce. The effect of addition of histidine and lysine in the production of fish sauce was investigated. The amino acids of traditionally produced commercial fish sauces and the experimentally prepared sauces were analyzed. Addition of histidine to fish mixtures before fermentation accelerated the hydrolysis of fish protein and the nitrogen content of the sauce was higher than the control, and comparable to the traditionally produced sauce after 4 months fermentation. The histidine in the sauce was several times higher than the control. In a lysine fortified sauce, the amount of lysine also increased with the increasing amount of lysine added, and was higher than the control. Both histidine and lysine were higher than the control in the final products suggesting that they were not degraded during fermentation process in the manufacture of fish sauce.

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Synthesis and analysis of contaminants in EMS-related tryptophan

Several trace contaminants in tryptophan (Trp), among them 1,1'ethylidenebis-tryptophan (1,1'-EBT) and 3-anilinoalanine (AAL) were statistically associated with the occurrence of the eosinophilia-myalgia syndrome (EMS) [1]. The in-vitro formation conditions of 1,1'-EBT and AAL were compared with those to be found during the manufacturing process of the EMS implicated Trp lots. 1,1'-EBT is formed preferentially in slightly acidic, Trp saturated, acetaldehyde containing solutions at low temperatures. AAL is formed at slightly acidic or slightly basic conditions from the precursors aniline and dehydroalanine by Michael-type addition. In consequence both compounds re likely to be formed during the ion exchange purification process. Both contaminants were synthesized in good yields (> 70%) and at high purity (> 90%, HPLC/UV) and could serve as reference substances.

Most efforts in contaminant search in Trp was done by RP-HPLC. RP-HPLC separation of a solution of EMS related Trp that was first digested by Tryptophanase (EC 4.1.99.1), indicated that several unidentified peaks coelute with the large Trp peak.

Occurrence of EMS sets new demands for an analytical quality control of Trp raw material. Therefore an RP-HPLC separation of 19 Trp related substances and simultaneous UV and fluorescence detection is applied to biotechnically manufactured Trp. The presence of Trp-metabolites and non-physiological Trp oxidation and carbonyl condensation compounds serves as marker for the quality of the purification process after fermentation. Selectivity and sensitivity achieved by the presented method allow the detection of 1 ppm 1,1-EBT or 1 ppm AAL in Trp raw-material. Two different pharmaceutical grade Trp raw materials (one using glucose and ammonia; the other, serine and indole as substrates for fermentation) have been examined. The number and the amount of trace contaminants were significantly lower than in EMS-implicated Trp. Both materials contained less than 1 ppm AAL and 1,1'-EBT. In 8 Trp containing infusions 1,2,3,4-tetrahydro-β-carboline-3carboxylic acid (THCC) (0,04–0,66 mg/l) and 1-methyl-THCC (MeTHCC) (0,03–0,20 mg/l cis-MeTHCC and 0.01–0.04 mg/l tr-MeTHCC) could be determined.

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Recognition of deficient nutrient intake in the brain of rat with L-lysine deficiency monitored by functional magnetic resonance imaging, electrophysiologically and behaviorally

Each L-amino acid (AA) in plasma and brain remains unchanged all day long while normal diet is available. Once L-lysine (Lys) deficient diet was offered to rats, they selected the Lys solution quantitatively and both appetite and growth normalized. The recognition site for the deficit in rat's brain was identified by non-invasive magnetic resonance imaging (MRI 4.7 tesla, 40 cm bore in diameter). Brain oxygenation was monitored by T2* weighted image. Young Wistar strain male rats were fed a diet with or without Lys deficiency for 4 days. After Lys injection (0.2 M, 10 ml (kg BW, i. p.), higher signals in the ventromedial and lateral hypothalamus (VMH and LHA) appeared, reflecting the increased oxygenation of neurons at 30–50 min, and then recovered. There were no changes in controls. Degree of Lys hunger by bar pressing (50 mg normal diet/30 presses) was suppressed by micro-injection of Lys into LHA, similar to free Lys ingestion but any other AA never did. This fact supports that the lateral hypothalamus is a possible candidate for a recognition site for Lys deficit in rats with Lys deficiency. The single neuron activity in LHA of these Lys-deficient rats suggested that neural plasticity occurred, specifically responding to Lys, both by iontophoretic application and during ingestion of AA, as previously reported. The present results suggest that in essential AA deficiency, the lateral and probably ventromedial hypothalamus may play important roles in recognition responses specifically to particular deficient nutrients with some neural plasticity for the maintenance of AA homeostasis.

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Effects of phenylalanine supplementation on in vivo phenylalanine and tyrosine kinetics in humans

Some studies in rodents have suggested that consumption of excess dietary phenylalanine (Phe) may reduce the activity of liver Phe hydroxylase. This enzyme converts Phe into tyrosine (Tyr), which is an essential step for the complete oxidative disposal of Phe carbons. We studied the effects of a 3-week oral phenylalanine (Phe) supplementation on *in vivo* Phe and Tyr plasma fluxes and on the rate of Phe conversion to Tyr in 24 healthy adults (age 29.5 ± 5 yrs), using a double-blind, placebo-controlled randomized design. Subjects were allocated to receive either 22.5 or 7.5 mg/kg/day of Phe, or placebo. Phe and Tyr kinetics were determined before and after supplementation, in the postabsorptive state, by means of a 6-hour primed, constant infusion of [1-^{13}C]-Phe and [3,3-$^{2}H_2$]-Tyr. Prior to each infusion, all subjects received a balanced diet providing 1.2 g/kg of protein per day. Pre- and post-supplementation results are shown in $\mu mol.(kg.h)^{-1}$ (Table 1).

Rates of conversion of phenylanine to tyrosine accounted for 13-16% of the total Tyr flux. When groups were compared using standardized differences between baseline and post-supplementation values, no statistically significant differences were found in any of the variables.

These results indicate that consumption of additional dietary Phe, beyond the levels provided by a typical American diet, does not alter the rate of phenylalanine conversion to tyrosine nor the rate of oxidation of Phe-derived carbons in healthy individuals. (Supported by the Nutrasweet Co. and by NIH RR00035)

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Determination of γ-radiation induced products in free and peptide bound tryptophan

Conservation of food by ionizing radiation has become legally accepted in several countries. Although some methods for the identification of irradiated food have been developed, there is a deficit in the detection of the irradiation in food with a high protein content. One single method based on radiation induced hydroxylation of phenylalanine forming o-tyrosine and m-tyrosine in addition to that of p-tyrosine is known. Corresponding to this method we wished to find some radiation induced products of tryptophan which could serve as markers for irradiated protein containing food.

A solution of tryptophan was irradiated using a Co-60 radiation source at dose rates of 10 and 20 kGy. The identification of radiation products was carried out by RP-HPLC and UV, fluorescence and DAD detection. Apart from oxindolylalanine (OIA) and N-formylkynurenine (NFK), two hydroxylated tryptophan isomers, 5-hydroxytryptophan (5-OH-Trp) and 7-hydroxytryptophan (7-OH-Trp) were identified as the main irradiation products. Two further tryptophan isomers, probably 4-OH-Trp and 6-OH-Trp, were detected and chracterized by their fluorescence and UV-spectra.

Aqueous solutions of the tripeptides H-Ala-Trp-Ala-OH, H-Leu-Trp-Leu-OH and H-Leu-Trp-Met-OH were used as model substances for peptide bound Trp. The irradiation conditions were the same as described above. After enzymatic hydrolysis with Pronase E, similar irradiation products were identified.

As NFK and 5-OH-Trp are formed during normal biological processes, the occurence of the aphysiological 4-, 6-, and 7-OH-Trp could possibly be used as markers for irradiated protein containing food. Synthesis of OH-Trp isomers as reference substances and further investigations concerning the occurrence of Trp radiation products in protein containing food are in progress.

Table 1

	Phe flux		Tyr flux		Phe → Tyr	
	Pre	Post	Pre	Post	Pre	Post
Placebo	37.3 ± 3.3	37.7 ± 3.7	30.9 ± 2.4	29.8 ± 9.1	4.4 ± 1.1	3.7 ± 2.0
7.5 mg/kg/d	35.3 ± 7.5	34.0 ± 7.8	28.6 ±4.5	29.1 ± 3.6	3.1 ± 1.2	3.7 ± 1.7
22.5 mg/kg/d	42.7 ± 7.9	42.7 ± 6.9	29.0 ±3.4	31.2 ± 4.6	4.3 ± 0.9	4.8 ± 1.2

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Effect of methionine lacking diet on methionine incorporation into the tumor protein in nude mice

Our previous report showed that methionine lacking TPN slowed tumor growth, because tumor protein breakdown was increased. However, tumor protein synthesis was unaltered with methionine free diet. This is probably because endogenous methionine production was increased with methionine free diet. The aim of this study was to determine whether methionine lacking diet enhanced methionine incorporation into the tumor protein in nude mice. The human esophageal cancer cells (KE-3) were cultured in 10% FBS+PRMI 1640 medium and were injected into the back of nude mice (n = 24, BW:20 – 24g) subcutaneously on day 0. On day 15, mice were randomly assigned into 2 diets, a standard liquid diet (SLD) and a methionine lacking diet (MLD). The mice were maintained on each diet for 5 days. On day 19, L-(methyl-^{3}H)methionine (5uCi/mouse) was injected intraperitoneally and sacrifice animals 1 hour after the injection of isotope. The tumor and liver were collected, homogenized in the sulphosalicylic acid, and centrifuged to precipitate protein. The supernatant and protein were counted in LSC. Consumption of the diets was measured daily (kcal/day).

Tumor weight (TW, g) was measured at the sacrifice point. Results are mean (SEM), Stat. by paired t-test, *: $P < 0.05$ vs. SLD.

	diet consump.	TW	Liver pro. count.	Tumor pro. count.
SLD	22(3)	0.98(0.1)	65202(1383)	959(33)
MLD	20(3)	0.93(0.1)	39432(1916)*	4338(130)*

In conclusion, methionine lacking diet caused a decrease in uptake of methionine into the liver, but an increase of methionine uptake into the tumor.

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L-Arginine supplementation in young patients with renal transplantation: A double-blind, placebo-controlled study

It has been recently suggested that L-arginine (L-Arg) supplementation might have beneficial effects on progression of renal damage and cyclosporine nephrotoxicity.

We have studied the effect of oral L-Arg intake on and renal hemodynamics in renal allograft recipients. 11 patients, six males, (range of age 11–22 yrs), post-TX time: 2–7 yrs, with mild-to-moderate renal insufficiency (range of Cr.Cl. 30–70 ml (min/1.73 sqm) and with proteinuria (range 0.5–1.8 g/day), none of them treated with ACEI, were randomly assigned to: Group 1: a 6-week treatment period with placebo (Pla), followed by 2 subsequent six-week periods with L-Arg; Group 2: a 6-week treatment period with L-Arg, followed by 2 subsequent six-week periods with Pla. Arginine was administered as L-arginine hydrochloride at the dosage of 0.2 g/kg/day (max 8 g/day). At the end of each treatment period, GFR (Cl. Inutest), RPF (Cl. PAH) and urinary albumin excretion (Alb.u) were determined. Urinary excretion of cGMP and urea (U.u) were evaluated in order to study the metabolic pathway of L-Arg. Data are given as mean ± sd, * $p < 0.05$ Arg vs. P (ANOVA) (Table 1).

L-Arg supplementation did not affect proteinuria and renal hemodynamics. This is consistent with the lack of a significant increase in cGMP excretion. Supplemented L-Arg seems, therefore, mainly addressed to the urea cycle rather than inducing an increase of NO synthesis.

Table 1

	Group I			Group II		
	Pla	Arg	Arg	Arg	Pla	Pla
GFR (ml/min/1.73m2)	34 ± 11	29 ± 12	31 ± 8	46 ± 18	46 ± 20	41 ± 14
RPF (ml/min/1.73m2)	159 ± 53	145 ± 41	162 ± 52	251 ± 129	231 ± 104	208 ± 84
Alb.u (g/day/1.73m2)	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.4	0.2 ± 0.2	0.2 ± 0.2
cGMP (n mol/100GF)	2.4 ± 1.0	2.9 ± 1.3	3.1 ± 2.2	1.8 ± 1.6	1.7 ± 1.1	1.5 ± 0.5
U.u (g/day/1.73 m2)	25 ± 4	29 ± 5	27 ± 4*	24 ± 5	21 ± 5	23 ± 6*

Physiology and Pharmacology

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Absolute confirmation of 1,2-Benzo-8-(D,L alanyl)-3-phenoxazone as anti-viral/tumor agent by HMQC and HSQC experiments

In our previous study we have characterized 1,2-Benzo-8-(D,L alanyl)-3-phenoxazone (BLP Fig. 1) by ^{1}H-NMR, ^{1}H-COSY, J resolved 2D and ^{13}C-^{1}H heteronuclear correlation experiments (Fig. 2–7). Upon biological screening of BLP by National Institute of Cancer, Bethesda, MD, it has shown to possess activity against prostrate cancer. This compound has received a patent by the U. S. Patent Office. Because of the usefulness of this compound, it felt necessary to confirm its absolute configuration.

For this study we have used Heteronuclear Multiple Quantum Coherence (HMQC) and Heteronuclear Single Quantum Coherence (HSQC) experiments which were conducted on Bruker AMX 500.

Both HMQC and HSQC experiments optimize long range and direct H^{1}-C^{13} connections respectively rather than c^{13}-H^{1} connections. In this study HMQC experiments allows the assignement of quaternary carbons C-3 and C-17 (Fig. 8) with protons that are two and three bonds away ($^{2,3}J_{C\text{-}H}$). While by HSQC experiments we obtain the direct H^{1}-C^{13} connections for C-3 and C-17 by using C^{13} satellites in proton spectrum to acquire correlation (Fig. 9).

This study confirms the attachment of alanyl group at C-8 and position C-3 in the form of C=O from C-14 proton of BLP.

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Excitatory amino acid antagonists offer a protection against pesticide toxicity in mice

Excitatory amino acid antagonists have been proposed to possess neuroprotective, anticonvulsant, antiischemic and myorelaxant properties. Possible beneficial effects of a competitive NMDA antagonist D-CPPene ([3-(2-carboxypiperazine-4-yl)-1-phosphonic acid, Sandoz) and a non-competitive NMDA antagonist MK-801 (dizocilpine, RBI) upon toxicity induced by systemic exposure to hydrocarbon insecticides were studied in mice. Experimental groups consisted of 6–8 mice, weighing 20–30 g. All the drugs were administered intraperitoneally. Hydrocarbon pesticides: dieldrin (Sigma), α-endosulfane (Sigma) and lindane (hexachlorocyclohexane, Sigma) produced clonic-tonic convulsions and death of animals in a dose-dependent manner. D-CPPene and MK-801 reduced the lethality of animals following the pesticide administration. D-CPPene (20 mg/kg, 30 min) produced more than 2, 3 and 10-fold increase in the lethal doses of dieldrin, α-endosulfane and lindane, respectively. MK-801 (0.4 mg/kg, 30 min) elevated the lethal doses of α-endosulfane, lindane and dieldrin from 7.2 to more than 25 mg/kg, from 47.1 to 391 mg/kg and from 31.5 to more than 90 mg/kg, respectively. The excitatory amino acid antagonists blocked tonic convulsions induced by all the pesticides tested and elevated the threshold for clonic seizures evoked by dieldrin. It may be suggested that NMDA antagonists offer a novel possibility of the pesticide poisoning treatment.

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Relationships of plasma taurine (TAU) to other amino acid (AA) levels in trauma and sepsis

An adequate characterization of the properties of TAU involves the study of the relationships between plasma TAU and other AA levels. This issue is particularly relevant in trauma and sepsis, owing to the limited amount of existing information.

Data from 466 plasma AA-grams obtained in 16 patients with trauma complicated by sepsis have been processed by regression analysis. All patients were receiving total parenteral nutrition (TPN) with glucose, fat and a standard AA solution. Seventy-one measurements were performed before the onset of sepsis (Group A); the remainder after the onset of sepsis and randomization to continue TPN with the same AA solution (Group B, n = 228) or with a isonitrogenous branched chain-enriched AA solution (Group C, n = 167). Neither AA solution contained TAU, glutamate (GLU) or aspartate (ASP).

In the three groups, and in all measurements together, plasma TAU was directly related to GLU and ASP and unrelated, or very poorly related, to all the other AA. However in Group C, compared to Groups A and B, the levels of the three AA (TAU, GLU and ASP) were significantly higher. Changes in TAU (and in GLU and ASP) in all measurements were directly related to the branched chain AA dose and unrelated to the non-branched chain AA dose. The increases in TAU in Group C were not explained by differences in TAU precursor AA doses.

These results expand the available information on the links between plasma levels of TAU, GLU and ASP and suggest an increased availability of the three AA mediated by increasing exogenous supply of branched chain AA. The interdependency between TAU and GLU (and also ASP) may perhaps be related to the metabolism of common intermediates.

Mean ± SD of amino acid plasma levels and main overall regressions:

	Group A	Group B	Group C
TAU (μM/L)	38.8 ± 28.7	80.8 ± 53.4	121.0 ± 90.9
GLU (μM/L)	38.3 ± 25.3	73.1 ± 48.8	82.4 ± 67.2
ASP (μM/L)	5.0 ± 3.2	8.7 ± 5.6	10.6 ± 12.0

TAU = 0.85 (GLU) + 28.13 (n = 466) $r^2 = 0.43$ $p < 0.001$
TAU = 5.98 (ASP) + 36.04 $r^2 = 0.48$ $p < 0.001$
TAU = 126.02 (Branched-chain AA dose) + 39.29 $r^2 = 0.20$ $p < 0.001$

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Free proline content and beta-amylase activities in plant cells as a tool for stress indication

During hypertonic stress induced by ions or higher temperatures compatible solutes (betains, glycerol, proline) were formed in plants and generally localized in the cytoplasm. Under the same conditions hydrolyzing enzymes may participate in the osmotic adjustment. Primary leaves but also non-photosynthetic tissues of cereals contain higher activities of beta-amylases than alpha-amylases. The major part of amylolytic activities in leaf cells (more than 95%) is localized outside the chloroplasts.

The purpose of this investigation is to evaluate the function of these beta-amylases especially under stress conditions in leaves of different cereals in relation to the free proline content as stress indicator. Amylolytic activity was extracted from primary leaves of the Triticaceae family (barley, wheat, rye), of two oat species (Avena sativa, A. nudum) and further of the maize and sorghum group. In the first family the beat-amylases could be resolved into three isoenzyme fractions (I, II, III), in oat leaves into two and only into one in maize and sorghum by anion exchange chromatography on DEAE-fractogel. The beta-amylases of the mentioned last two groups have different exchange properties in relation to the Triticaceae group. An increase of the growth temperature over a critical value (wheat and oat at 35 °C, barley at 37,5 °C and maize at 43,5 °C) or growth in the presence of osmotic active substances (mannitol or sorbitol at 300 mmol) or NaCl (at 95 mmol in rye and at 150 mmol in barley) all stimulated the amylolytic activity due in particular to a selective increase of the fraction-III-activity up to 500%.

The degree of the salt tolerance can be characterized by this critical salt concentration. At these concentrations specific for each species also the endogenous sodium and free proline content rised dramatically.

It was proposed that the increase of the proline content and also the stimulation of the beta-amylase activity are general responses to stress at a species dependent critical salt concentration or a critical temperature, and that the two parameters can be used as a tool for determining the threshold value of the stressor.

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Oligopeptide fragments of aprotinin and lysozyme with antiviral activity

Digestion of aprotinin, a proteinase inhibitor and of chicken egg white lysozyme a muramidase with bactericidal properties, by clostripain, yielded oligopeptide fragments which exerted antiviral activity *in vitro*. Two fragments, one derived from aprotinin and the other from lysozyme were purified and their amino acid sequence determinated. The aprotinin fragment, a hexapeptide with the sequence **YFYNAK** corresponding to amino acids 21–26 of the intact aprotinin, showed antiviral activity against human herpes simplex virus-1 (HSV-1) and bovine parainfluenza virus 3 (PI-3). The lysozyme fragment with the sequence **IVSDGNGMNAWVAWR** corresponding to amino acids 98–112 of native lysozyme possessed antiviral and antibacterial activity. Its antiviral activity was directed against HSV-1. Synthetic oligopeptides with the sequence **YFYNAK** and **IVSDGNGMNAWVAWR** showed the same antiviral spectrum as the natural oligopeptides. Within the inhibitory concentration range of the two oligopeptides, aprotinin and lysozyme were ineffective against HSV-1 and PI-3. Both oligopeptides were not cytotoxic in the antiviral assay.

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Role of carboxy-terminal region in editing function of methionyl-tRNA synthetase in *Escherichia coli:* Channeling of amino acids from biosynthetic pathway to protein synthesis

E. coli MetRS is a dimer of two 676 amino acid subunits [Dardel et al., J. Bact. 160: 1115 (1984)]. Deletion of the C-terminal 129 amino acids produces biologically active monomer of 547 amino acids [Mellot et al., JMB 208: 429 (1989)]. The C-terminal region responsible for dimerization of *E. coli* MetRS is absent in yeast MetRS; yeast MetRS is in fact a monomer [Fasiolo et al., JBC 260: 15571 (1985)]. It is not known why MetRS exists as a dimer in *E. coli*, since it can function perfectly well as a monomer both in yeast and, in modified form, in *E. coli*. One possible reason for different forms of MetRS utilized by these two microorganisms is suggested by different needs for homocysteine (Hcy) editing in *E. coli* and yeast cells. In *E. coli* Hcy levels are high, which forced *E. coli* MetRS to evolve to efficiently edit Hcy. In yeast Hcy levels are low, therefore yeast MetRS does not have a need to edit; when forced to edit by exposure to excess Hcy yeast cells are less efficient than *E. coli* [Jakubowski, PNAS 87: 4504 (1990), EMBO J. 10: 593 (1991)]. Thus, a function of the C-terminal region of *E. coli* MetRS may be to enhance editing activity.

The synthetic and editing functions of three forms of *E. coli* MetRS with different C-terminal sequences have been compared. These include a full length wild type dimer (MRS676), a truncated monomer (MRS547), and a third form denoted MRS581*. DNA sequencing revealed that MRS581* is predicted to contain 18 additional amino acids from the wild type full length sequence at the carboxy-terminus of truncated form MRS547, and this is then fused to an additional 16 amino acids encoded by vector pBR322. Both MRS676 and MRS581* edit *endogenous* Hcy 20-fold more efficiently than MRS547 *in vivo*. However, the three MetRSs edit *exogenous* Hcy in bacterial cultures to similar extents. Purified proteins exhibited no significant differences in editing *in vitro*. Synthetic activity of MRS676 *in vitro* was 2.5 fold higher per subunit compared to the shorter forms of the enzyme. The C-terminal region in *E. coli* MetRS is thus suggested to play an important role in editing *in vivo*. These data support a model of channeling of at least some metabolites in bacterial protein synthesis.

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Effect of two fluid therapies on brain amino acids and carbohydrates during treatment of diabetic ketoacidemia

In order to understand the pathologic basis of brain swelling during treatment of diabetic ketoacidemia (DKA), we focused on the direct effects on the brain. The most severe complication in children during treatment of DKA is death from brain swelling. To study this phenomenon, we induced DKA in rabbits

with a combination of streptozocin and alloxan monohydrate. We measured intracranial pressure (ICP) in groups that included controls of non-diabetic animals (G_c), a non-treated DKA group, and two treatment groups, G_1 and G_2. G_1 received i. v. administration of 0.45% NaCl rapidly. G_2 received i. v. administration of 0.9% NaCl slowly. Both groups were given equivalent doses of continous i. v. regular insulin. Subsequent readings of ICP were recorded at half hour intervals. At six hours, the animals were euthanized, serum was collected and brain extracted. Brain water content, free amino acids and monosaccharides were measured.

DKA was corrected similarly in both treatment groups; however, ICP was significantly increasd in GT1 when compared with GT2, but not when G_2 was compared with G_c. Tissue analyses further revealed uniform depression of tissue pools of free amino acids while monosaccharide content appeared to remain constant. In addition, there was no significant difference in amino acids between G_1 and G_2. These compounds, therefore, do not appear to serve a role in brain osmoregulation, in contrast to previous studies that employed hypernatremia or hyperglycemia (without DKA) as models.

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Mitochondrial presequence peptides are bound to the mitochondrial surface through electrostatic interactions with the acidic import receptor Mas20p

Most mitochondrial precursor proteins are synthesized in the cytosol as precursor proteins with an amino-terminal, 20–80 amino acid extension peptide, the presequence. These presequences contain the information for targeting the precursor proteins to their correct location within the mitochondria. Presequences do not share primary structural homology, but are amphipathic and contain several positively charged and few, if any, negatively charged residues. Their common structural motif appears to be an amphiphilic α-helix [1, 2]. To determine the mechanism by which mitochondrial presequences are bound to the protein import receptors at the mitochondrial surface we have made use of a binding and chase assay which represents a partial reaction of the authentic import process [3]. Productive binding of mitochondrial precursors with a positively charged amino-terminal presequence, such as a fusion protein between the first 69 amino acids of the *Neurospora crassa* F_1F_0-ATPase subunit 9 precursor fused to mouse DHFR (SU9-DHFR), mitochondrial hsp60 and chaperonin10 (cpn10), was strongly inhibited by salt, by low concentrations of a mitochondrial presequence peptide, and by a deletion of the import receptor Mas20p, but was independent of the receptor heterodimer Mas37p/Mas70p. The productively bound precursor of cpn10 could be crosslinked to Mas20p [4]. We conclude that the import receptor Mas20p binds mitochondrial precursor proteins through electrostatic interactions with the positively charged presequence.

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Studies on the active sites of *Bacillus cereus* sphingomyelinase-substitution of some amino acids by site-directed mutagenesis

Sphingomyelinase (SMase) hydrolyzes sphingomyelin to ceramide and phosphorylcholine. We have purified *Bacillus cereus* SMase, cloned the gene and sequenced it. Various functional domains seem to exist in the enzyme, such as adsorptive, catalytic and metal ion-binding sites. Chemical modifications suggested that the acidic amino-acids such as Asp and Glu are involved in the catalytic and adsorptive activities of the enzyme. To elucidate the role(s) of the aspartic acid residues in the enzymatic and hemolytic functions, Asp-126, Asp-156, Asp-233 and Asp-295 in the conservative regions were converted to glycine by *in vitro* mutagenesis. Four mutant SMases, D126G, D156G, D233G and D295G, were produced in *Bacillus brevis* 47, a protein-hyperproducing strain. The sphingomyelin (SM)-hydrolyzing activity of D295G was completely abolished and those of D126G and D156G were reduced by more than 50%, while that of D233G was not so profoundly affected. The mutants, D126G and D156G, were purified and characterized further. The activity of D126G toward *p*-NPPC was comparable to that of the wild-type, while D156G catalyzed the hydrolysis of hydrophilic substrates such as HNP and *p*-NPPC more efficiently than the wild-type. Therefore, Asp-126 and ASP-156 may well be involved in a substrate recognition process rather than catalytic action. Hemolytic activities of the mutant enzymes were parallel to their SM-hydrolyzing activities.

According to the prediction on structural similarity to DNaseI, two histidines corresponding to those in the active center of DNaseI, were converted to alanine and expressed as H151A and H296A. Both mutants lost their catalytic activities, suggesting the evolutional relationship between the functional structures of these two phosphodiesterases.

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Function and role of amino acid sensors in physiological and pathological conditions

We have recently found vagal amino acid sensors (arginine, alanine and leucin) in the liver by using the methods of recording the hepatic nerve discharge, and the methods of hepatic vagotomy and/or celiac vagotomy (sectioning of the hepatic branch and/or the celiac branches of the vagus nerve) on the secretion of insulin and glucagon after ip injection of arginine, alanine and leucin. In normal rats, hepatic vagotomy enhanced plasma insulin and glucagon with reduced plasma glucose after ip arginine as compared to those in sham-vagotomized rats. Hepatic vagotomy enhanced plasma glucagon with enhanced plasma glucose after ip alanine, while it enhanced plasma insulin with reduced plasma glucose after ip leucin. These effects, following these amino acids administration, were blocked by celiac vagotomy, however, celiac vagotomy alone did not affect plasma insulin, glucagon and glucose. Injection of these amino acids into portal vein enhanced hepatic nerve discharge. In

diabetic rats, hepatic vagotomy enhanced plasma insulin and glucagon with reduced plasma glucose after ip arginine. Hepatic vagotomy enhanced plasma glucagon with enhanced plasma glucose after ip alanine, while it did not affect plasma insulin, glucagon and glucose after ip leucin. The effects induced by hepatic vagotomy after ip arginine and alanine were blocked by celiac vagotomy, however, celiac vagotomy alone did not affect plasma insulin, glucagon and glucose. In VMH lesioned rats, hepatic vagotomy did not affect plasma insulin, glucagon, and glucose after ip arginine, alanine and leucin. Celiac vagotomy alone reduced plasma insulin and glucose, but did not affect plasma glucagon after ip arginine and leucin. Hepatic vagotomy did not affect plasma glucagon, insulin and glucose after ip alanine. In cirrhotic rats, hepatic vagotomy increased plasma insulin with no charge of plasma glucose, which might be produced by insulin resistance. In summary, amino acid sensors may work to prevent amino acid-induced exaggerated pancreatic hormone, maintaining blood glucose homeostasis in physiological condition, but this system disturbs in pathological conditions, contributing to the disturbance in glucose homeostasis.

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The effect of glutamine supplementation on glutamine uptake in the liver following hepatectomy

The objective of this study was to determine whether supplemented glutamine caused an increase in hepatic glutamine uptake in rats following partial hepatectomy.

32 male Donryu rats (BW: 250-270 g) were assigned into 4 groups: 1) sham operation + standard TPN solution (C-STPN), 2) C + glutamine supplemented TPN (C-GTPN), 3) 70% partial hepatectomy + STPN (H-STPN), 4) H-GTPN. On day 0, rats were underwent either sham operation or 70% partial hepatectomy and concomitantly catheterized into jugular vein for receiving TPN. TPN was begun immediately after the surgery. GTPN was isocaloric and isonitrogenous with STPN and 25% of total nitrogen was given as glutamine. On day 2, the animals were sacrificed and the blood was taken from abdominal aorta, portal vein and hepatic vein.

Glutamine levels in the plasma was measured by HPLC, using pre-column derivatization with o-phthaldialdehyde. Glutamine uptake by the liver was calculated from differences in glutamine levels between artery and hepatic vein and between portal vein and hepatic vein. Extraction ratio in the liver was calculated as follows: Extraction ratio = (A–HV)/A + (PV–HV)/PV.

Data are mean (SEM). Stat. by paired t-test. Different superscripts indicate significant difference ($p < 0.05$).

	C-STPN	C-GTPN	H-STPN	H-GTPN
n	8	8	8	8
A-HV	–107.0(11.4)	–81.6(5.3)	24.9(8.9)	94.6(25.0)*
PV-HV	–121.0(18.2)	–113.3(17.9)	–18.7(12.7)	16.2(4.0)*
Ext. Ratio	–0.75(0.07)	–0.50(0.07)†	0.01(0.04)†	0.17(0.04)*

In conclusion, glutamine supplement and partial hepatectomy stimulated glutamine uptake in the liver.

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Effect of cystathionine as a scavenger of superoxide generated from human leucocytes in vitro

Cystathionine, a metabolic precursor of cysteine, is converted to cysteine by γ-cystathionase under the physiological conditions in liver. However, effects of cystathionine against reactive oxygen species such as superoxide radical have not been examined. Therefore we studied scavenging effects of cystathionine on human leucocyte-generated or xanthine-xanthine oxidase (X-XOD) derived superoxide radical in vitro. Washed leucocyte suspensions (10^6 cells/ml) prepared from healthy male volunteers were stimulated with phorbol myristate acetate (PMA, 3×10^{-8} M) or opsonized zymosan (OZ, 1 mg/ml). Generated superoxide radical was measured by MCLA-chemiluminescence method. Cystathionine (30 μM–3 mM) significantly reduced superoxide radical-dependent chemiluminescence on the washed leucocyte system with dose-dependent manner. Furthermore, significant scavenging effect of cystathionine on the superoxide radical was seen on the X-XOD system. On the other hand, cystathionine did not show strong scavenging effect against hydroxy radical derived from Fe^{2+}-H_2O_2 on erythrocyte membrane suspension. These results indicate that cystathionine may scavenge superoxide radical rather than hydroxy radical in vitro.

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A model of the synthetic/editing site of an aminoacyl-tRNA synthetase

A model of the active site of Escherichia coli methionyl-tRNA synthetase (MetRS) that partitions amino acid substrates between synthetic and editing pathways is described. The synthetic pathway (1) involves intermolecular reaction of the activated carboxyl group on methionine (Met) with the 2'-hydroxyl of the terminal adenosine of tRNA.

Met-AMP + tRNA ⇆ Met-tRNA + AMP (1)

The editing pathway (2) involves intramolecular reaction of the activated carboxyl group of homocysteine (Hcy) with the sulfur of its side chain.

Hcy-AMP → Hcy thiolactone + AMP (2)

Whether an amino acid completes the synthetic or editing pathway is determined by the competition for its activated carboxyl group between the side chain of the amino acid and the terminal adenosine of tRNA. Met completes the synthetic pathway because its side chain is firmly bound in the active site by hydrophobic and hydrogen bonding interactions with Trp305 and Tyr15, respectively, preventing the sulfur atom of Met from competing with the 3'-terminal adenosine of tRNA for the carboxyl carbon of Met. Hcy, missing the methyl group of Met on its side chain, cannot interact with Trp305 and Tyr15 as strongly as Met does. Therefore, the activated carboxyl of Hcy reacts intramolecularly with the sulfur of the side chain instead of the 3'-terminal adenosine of tRNA. Since intramolecular reactions are more favoured than intermolecular reactions, this explains why Hcy is not transferred to tRNA but is cyclized to Hcy thiolactone. The editing pathway is a default pathway. As expected from this model, Met enters the editing pathway when its side chain cannot firmly bind to the active site, as is the case with W305A, Y15F, and Y15A MetRSs. Residues R233 and

D52 of MetRS, essential for catalysis of the synthetic reaction, are also involved in catalysis of the cyclization reaction during editing [Kim et al., PNAS 90: 11553 (1993)].

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Synthesis of a peptide bond by an aminoacyl-tRNA synthetase

Most aminoacyl-tRNA synthetases exhibit a deacylase activity toward cognate aminoacyl-tRNAs. With synthetases that exhibit low initial selectivity (such as ValRS, IleRS, MetRS), this activity is a manifestation of an editing function which contributes to high accuracy of tRNA aminoacylation by destroying noncognate intermediates or products [Microbiol. Rev. 56: 412 (1992)]. Because of their high initial selectivity, some aminoacyl-tRNA synthetases, such as CysRS [Fersht and Dingwall, Biochemistry 18: 1245 (1979)], do not need editing function to reject noncognate amino acids. Yet, CysRS also possesses deacylase activity, which might be a remnant of a chemistry used by an ancestral synthetase [NAR 22: 1155 (1994)]. To shed more light on the nature of the deacylase activity, systematic studies of the deacylation reactions catalyzed by purified aminoacyl-tRNA synthetases were undertaken.

Arg-tRNA^{Arg} in a complex with ArgRS is deacylated slower ($k = 0.003$ min^{-1}) than free Arg-tRNA^{Arg} in solution ($k = 0.035$ min^{-1}) (pH 7.4, 37 °C). Arg, but not ATP nor tRNA^{Arg}, accelerated the deacylation of Arg-tRNA^{Arg} in the complex to a rate observed for free Arg-tRNA. Unexpectedly, whereas some amino acids (Lys, Ser) tested as controls did not affect the deacylation reaction, cysteine (Cys) and homocysteine (Hcy) accelerated enzymatic deacylation of Arg-tRNA^{Arg} over 3000- and 100-fold, respectively. Nonenzymatic deacylation of Arg-tRNA was not affected by Cys and Hcy. Thin layer chromatography revealed that new products formed in Cys- and Hcy-dependent deacylation reactions. Chemical reactivities of these products indicate that they are dipeptides ArgCys and ArgHcy, respectively. For example, the new products were sensitive to thiol reagents and insensitive to NaOH treatment. Treatment with Raney nickel of the product formed in Cys-dependent reaction resulted in the formation of the dipeptide ArgAla. The data indicate that ArgRS catalyses formation of a dipeptide bond in a reaction (equation 1) in which activated Arg in Arg-tRNA^{Arg} is subjected to a nucleophilic attack by Cys or Hcy.

$$\text{Arg-tRNA}^{Arg} + \text{Cys} \rightarrow \text{ArgCys} + \text{tRNA}^{Arg} \qquad (1)$$

Similar reactions are catalyzed by ValRS and IleRS, leading to the formation of dipeptides Val-X and Ile-X (X = Cys, Hcy), respectively, from the cognate aminoacyl-tRNAs.

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A mechanistic role for polypeptide hormone receptor lateral mobility in signal transduction

Lateral diffusion of membrane-integral receptors within the plane of the membrane has been postulated to be mechanistically important for signal transduction. Direct measurement of polypeptide hormone receptor lateral mobility using fluorescence photobleaching recovery indicates that tyrosine kinase receptors such as that for insulin are largely immobile at physiological temperatures. This relates to their signal transduction mechanism which requires intermolecular autophosphorylation (receptor aggregration/immobilisation) for activation. In contrast, G-protein coupled receptors must interact with other membrane components to effect signal transduction, and consistently, the phospholipase C-activating vasopressin (VP) V_1- and adenylate cyclase activating V_2-receptors are highly laterally mobile at 37 °C. Modulation of the V_2-receptor mobile fraction (f) has demonstrated a direct correlation between f and maximal cAMP production *in vivo* at 37 °C, indicating that f is a key parameter in hormone signal transduction especially at physiological hormone concentrations, consistent with mobile recptors being required to effect G-protein activation. Measurements using a V_2-specific antagonist show that antagonist-occupied receptors are highly mobile at 37 °C, indicating that receptor immobilisation is not the basis of antagonism. In contrast to agonist-occupied receptor however, antagonist-occupied receptors are not down-regulated (immobilised prior to endocytosis). This implies that receptors are freely mobile in the absence of ligand and that agonist induces receptor complexation with other membrane proteins and possibly the cytoskeleton, which is critical for G-protein-mediated signal transduction, leading to receptor immobilisation. Receptor lateral movement is required to bring about this complexation with other membrane components.

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Free and bonded homo-isoleucine in sclerotia of parasitic fungus *Claviceps purpurea*

Homo-isoleucine, an unique amino acid recently discovered in the structure of ergot alkaloid ergogaline [1], was determined by gas chromatography-mass spectrometry (GC-MS) in the parasitic fungus *Claviceps purpurea* Fr. (Tul.), growing on rye *Secale cereale* (L.) and in its host plant. Free homo-isoleucine was detected in the amino acid pool of sclerotia at most the fungal strains examined. In the acidic hydrolysates of sclerotia, increased amount of homo-isoleucine was found at some strains which accounts for its incorporation into ergogaline, a homo-isoleucine-containing alkaloid (see Fig. 1). Since homo-isoleucine has not been detected in rye, it seems likely that the amino acid is synthetized by the fungus.

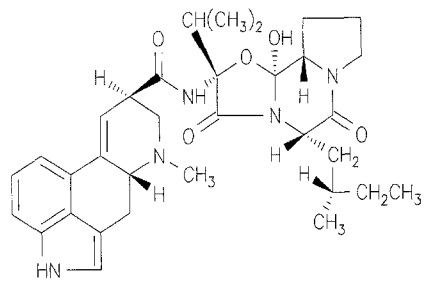

Fig. 1

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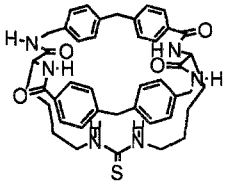
Synthetic receptors for amino acids and peptides



1

The synthesis and binding properties of a C_2 symmetric macrobicyclic receptor **1** will be described [1]. The receptor incorporates a thiourea moiety designed to serve as a carboxylate binding site [2, 3], and additional amide functionality to provide hydrogen bonding interactions with appropriate amino acid guests. The receptor was found with to bind a range of N-acetyl amino acids as the tetrabutyl ammonium salts, with seemingly little sidechain selectivity and little enantioselectivity. However, we have determined (from detailed NMR and modelling work – using nOe based distance constraints) that the receptor binds L-amino acid derivatives, such as *N*-acetyl *L*-phenylalanine tetrabutyl ammonium salt and *N*-acetyl*L*-alanine tetrabutyl ammonium salt, deep in the cavity of the macrobicycle, placing the benzyl or methyl side chain of the guest and the acetyl methyl group of the guest, close up to the biaryl methane units of the receptors (resulting in dramatic upfield shifts of the relevant guest protons in the NMR). As well as placing the substrate in the cavity of the macrocycle, the study has unambiguously shown that the substrate is bound with the acetyl amide in the cis conformation, allowing it to establish two hydrogen bond interactions with the rim of the macrobicyclic receptor. The corresponding *D*-amino acid derivatives, however, are bound simply by a carboxylate-thiourea interaction, outside the macrobicycle.

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Regulation of voltage-gated K^+-channel transcription in *Schizosaccharomyces pombe*

Living cells, both excitable and nonexcitable, have to maintain a tight control of their electrical responsiveness by regulating the quality and quantity of channels expressed in the cell membrane. Regulation of the transcription of voltage-gated ion channels is an important part of the molecular basis of charge translocation in cells. However, the factors which control the expression of voltage-gated channels are so far poorly understood. Because K'-channels have been shown to play an important role in both the energization of cell membranes and the excitability of differentiated cells of higher organisms, we have begun to study the effects of physico-chemical properties and ion specificity of the channels on their transcription of the voltage-gated potassium channels in the yeast *Schizosaccharomyces pombe* chosen as a model organism. K^+-channels of S. pombe have already been characterized in our laboratory by the patch-clamp technique [1]. Their conductivity of 153 pS was considerably higher than the 50 pS of their equivalents in Saccharomyces cerevisiae. Voltage-gated K^+-channels are highly conserved polytopic membrane proteins. In order to prepare a specific probe for the Northern blot hybridization and RNase protection analysis we have amplified, cloned and sequenced 122 bp fragment of voltage-gated K^+-channel from chromosomal DNA of *S. pombe* by PCR. The PCR primers were designed on the basis of highly homologous protein sequences between S4 and H5 domains of known K^+-channels [2, 3, 4]. The difference in potassium channel transcription was quantified densitometrically from Northern blots. The half-life of K^+-channels was also determined to estimate its excitable properties.

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Free amino acid pools in medicinal plants. Seasonally accumulated concentrations of the smart drug arginine in *Valeriana officinalis* L.

Strategies of perennial plants to storage free amino acids beside proteins as nitrogen compounds during winter time refer especially to aspartate/asparagine, glutamate/glutamine and/or arginine.

In mammalian metabolism arginine plays an important role in the synthesis of urea, creatine, cratinine and nitric oxide, which regulates heart muscle activity and erythropoietin synthesis, as well as hormon releasing compound.

We studied free amino acid concentrations in roots of the perennial *Valeriana officinalis* in comparison to the annual *Valerianella coronata* L. From June to October in roots of *Valeriana officinalis* a striking increase is found of the concentrations of arginine and glutamine. In comparison, in *Valerianella coronata* arginine and glutamine believe unchanged, but glutamate, asparagine, τ-aminobutyric acid (GABA) and alanine are accumulated (Table 1).

Table 1

Free amino acid	*Valeriana officinalis* June	*Valeriana officinalis* October	*Valerianella coronata* June	*Valerianella coronata* October
arginine	2.4 (16%)	97.9 (46%)	0.2 (2%)	0.2 (1%)
glutamine	0.5 (3%)	49.7 (23%)	1.3 (12%)	2.0 (5%)
glutamate	4.3 (28%)	15.2 (7%)	2.4 (22%)	9.0 (25%)
asparagine	0.2 (2%)	3.6 (2%)	0.2 (2%)	8.4 (23%)
GABA	0.2 (1%)	2.4 (1%)	0.9 (8%)	5.2 (14%)
alanine	2.1 (13%)	8.8 (4%)	1.9 (18%)	4.9 (13%)
total free amino acids	16	215	11	37
concentrations in [μmol/g dry weight] (% of total free amino acids)				

Possibly, the nearly fortyfold increased content of arginine in *Valeriana officinalis* roots in the harvest time is to take in consideration as a resource of a smart drug or a synergistic compound with regard to the pharmacological properties of *Valeriana officinalis.*

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Changes in phenolic-, di/polyamin- and amino acid contents and in peroxidase activity in crops during stress periods by treatment with amino alcohols

During the ontogenesis plants synthesize various secondary metabolites. Often these substances are produced as a result of abiotic and biotic stress. Secondary metabolites (e.g. phenolics) act as defence or protective substances and allow the plant a better adaption to stress and the survival. Abiotic stress very often results in an increase of the level of di/polyamines and free amino acid. Peroxidases (POD) are important enzymes in the formation of secondary metabolites of the phenolic type. The activity of these enzymes as well as the amounts of the primary and secondary "stress" metabolites are useful stress indicators in plants. Various crops (e.g. cereals) respond to leaf and root treatment with the toxicologic safe amino alcohols (e.g. 2-amino ethanol, choline) in field and greenhouse experiments with an increased stress tolerance. Therefore, the objective of our work was to determine the levels of selected phenolics (lignin precursors), di/polyamines, free essential amino acids and the activity of soluble POD in plants cultivated under drought and heavy metal stress with and without treatment with stress tolerance inducing amino alcohols.

Barley, maize, soybean and pearl millet plants were cultivated in Mitscherlich pots under drought and/or heavy metal stress. At different stages of the ontogenesis plants were harvested. Using HPLC and UV/VIS-spectrophotometry the concentrations of phenolics, di/polyamines and the activity of soluble POD (using guaiacol as substrate) were determined in plant extracts. After seperation of protein extracts by native polyacrylamide gel electrophoresis and staining in substrate solution POD-isoforms in treated and not treated plant species were detected.

The amount of the phenolic compounds ferulic acid, coumaric acid and cinamic acid (lignin precursors) was 5–60% higher in leaves and shoots of drought stressed barley, soybean and pearl millet than in control plants. A significant higher level of di/polyamines putrescine and spermidine (increase of 74% and 66%, respectively) was found in barley when cultivated under water shortage compared to plants grown under well watered conditions. As a biochemical response to heavy metal and/or drought stress an increase of the pools of the amino acids arginine (precursor of the diamine putrescine), prolin and phenylalanin (precursor of various phenolic compounds) by 9–22% compared to control plants was found in barley and maize. Pretreatments of the plants with 2-amino ethanol and/or choline and subsequent exposure to drought and/or heavy metal stress reduced the amount of the "stress"-indicators (putrescine, cinnamic acid, coumaric acid, arginine, proline) significantly compared to not-treated stressed plants. In leaves and shoots of drought stressed barley plants a 50% higher POD-activity was found compared to well watered controls. Pre-treatment of barley plants with choline resulted in a reduction of the POD-activity to the level of well watered control plants. Therefore, treatment of plants with amino alcohols may be a useful way for inducing stress tolerance adaption mechanisms in crop plants.

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Some antiviral mechanisms of action and effectiveness of proteolytic inhibitor E-aminocaproic acid

The reuslts of our own investigations have demonstrated that proteolytic systems play an important role during interactions of various viruses with their hosts.

We have studied some mechanisms of etiotropic and pathogenetic action and we have shown an antiviral effectiveness of synthetic inhibitor of proteolysis E-aminocaproic acid (E-ACA). E-ACA inhibits replication of A and B influenza viruses in chorio-allantoic membranes tissue culture of chick embryos. It was shown that E-ACA decreases the proteolytic activity: (1) of surface membranes of sensitive cells; (2) of concentrated and purified A and B influenza viruses; (3) of the virus-membrane complexes. It was discovered by means of electron microscopy that E-ACA hinders virus deproteinization and its penetration through the surface of cell membranes. Inhibitors of proteolysis, including E-ACA, prevent the proteolytic activation of viral infectivity.

E-ACA when used in the treatment or the prevent of an experimental influenza in mice decreased virus replication in lungs and also enhanced the humorel immune responce. E-ACA: (1) promotes preservation of a lung tissue airness, structure and blood supply of lung vascular; (2) prevents development of perivascular oedema and hemorrhagic syndrome; (3) preservates of air-haematic barrier damages; (4) induces a marked tendency to normalization of elevated alkaline protease activity in lung tissue and in the blood in infected mice. Lowering the death rate of infected animals was the result of E-ACA administration.

The results of clinic researches witness that the proteolytic inhibitor together with antiviral action improves some indices of non-specific humoral and cellular defence and have the pathogenetic efficacy which lead to decreasing duration of catarrhs, intoxication, decreasing of quantity of bacterial complications and shortening of time of the patients beeing at a hospital in complicated forms of influenza and other acute respiratory viral infections in babies.

The Ministry of Public Health of Ukraine was decided about employment of E-ACA for treatment of influenza and other acute respiratory viral infections in children in virtue of our investigations.

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Chemical modification and inactivation of human tyrosine hydroxylase by diethylpyrocarbonate

Tyrosine hydroxylase (TH) catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) which is the first step in the biosynthesis of catecholamine neurotransmitters. TH is a non-heme iron and tetrahydrobiopterin dependent enzyme and the four human isoforms (hTH1-hTH4) are all overexpressed in *E. coli* as tetrameric apoenzymes (apo-hTH1 to hTH4) which are rapidly activated (up to 40 fold) by the incorporation of one equivalent of Fe(II) per subunit. In the present

work it is shown that hTH1 shows a time- and concentration-dependent loss of catalytic activity when incubated with diethylpyrocarbonate (DEP) after reconstitution with Fe(II). The inactivation follows pseudo-first order kinetics with a second order rate constant of 300 $M^{-1} \cdot min^{-1}$ at pH 6.8 and 20 °C and is partially reversed by hydroxylamine. The difference absorption spectrum of the DEP-modified vs. native enzyme shows a peak at 244 nm, characteristic of mono-N-carbethoxyhistidine. Up to five histidine residues are modified per enzyme subunit by a five-fold excess of the reagent, but derivatization of only one residue appears to be responsible for the inactivation. Thus, no inactivation by DEP was found when the apoenzyme was preincubated with this reagent prior to its reconstitution with Fe(II), modifying four histidine residues. The essential histidine appears to become accessible to modification by DEP by a structural change accompanying the binding of Fe(II) to the apoenzyme. Possible catalytic roles of this histidine in the hydroxylation mechanism will be discussed.

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Effect of aspartate on ethanol oxidation and ethanol-induced oxidative stress in the perfused rat liver

Ethanol-induced tissue damages are closely related with the metabolic sequelae of ethanol oxidation, such as the increased amount of ethanol, acetaldehyde and high NADH/NAD ratio. The deranged cellular NADH/NAD ratio would be mainly responsible for a variety of ethanol-induced metabolic dysfunctions. Therefore, in the present study, the correction of the deranged cellular NADH/NAD ratio has been attempted in order to prevent the ethanol-induced tissue damages. The basic assumption for the study is that aspartate supplementation might provide the regeneration of NAD from NADH via coupled reactions of aspartate aminotransferase and malate dehydrogenase, which would be enforced by the efficiency of malate-aspartate shuttle. Aspartate was infused into the ethanol-perfused rat liver (0.1%), resulting in the maximum effect at 2 mM for ethanol oxidation. the derangement of cellular NADH/NAD ratio by ethanol oxidation, represented by the increased lactate/pyruvate ratio in the perfusate, was ameliorated by aspartate. The aspartate effect was blocked by the simultaneous infusion of transaminase inhibitor, aminooxyacetate, suggesting the active participation of aspartate on ethanol oxidation. Moreover, aspartate supplementation caused the decrease in ethanol-induced lipid peroxidation and protein carbonylation, which might be explained by the normalization of ethanol-induced cellular XDH/XO ratio through modulation of cellular NADH/NAD ratio with aspartate. From these data, it is suggested that aspartate can be an antidote to ethanol toxicity by its effect on metabolic facilitation of ethanol oxidation and metabolic regulation of radical generation through the correction of cellular NADH/NAD ratio deranged by ethanol oxidation.

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Biochemical mechanisms of myocardial protection against ischemia/reperfusion injury by amino acids

Exogenous glutamate (Glu) and aspartate (Asp) and products of their catabolism, 2-oxoacids, are able to maintain cardiac function during ischemia and reperfusion at a higher level. This implies the influence of these naturally-occuring compounds on myocardial metabolism. The aim of this work was to assess an association of Glu and Asp contents in mitochondria (mito) and myocardial tissue with energy state of ischemic heart and to study effects of exogenous amino acids (AA) on intermediary and energy metabolism of ischemic and reperfused heart. Isolated working rat hearts perfused with Krebs-Henseleit buffer supplied with glucose were used. Isolation of mito, polarographic and metabolite determinations were carried out by standard methods. During ischemia, a reduction of Glu in mito positively correlated with a loss of state 3 respiration supported by Glu, a decrease in adenine nucleotide pool and an increase in $NADH/NAD^+$ ratio. A gradual decrease of myocardial Glu and Asp was related to depletion of tissue ATP and reduction of the energy charge. Thus, a decreased tissue/mito contents of AA indicate a limited energy-producing capacity of the heart. Treatment of ischemic heart with Glu or Asp plus 2-oxoglutarate augmented anaerobic energy formation in glycolysis and substrate phosphorylation at succinate level in mito. These metabolic events were accompanied by stimulation of ATP-dependent reactions of glutamine, asparagine and urea synthesis and reduction of myocardial ammonia level. Glu treatment of reperfused heart recovered mito/tissue contents of the malate-aspartate shuttle (MAS) reactants, increased the rate of [5-^{3}H]glucose oxidation, reduced cytosol $NADH/NAD^+$ ratio and lactate formation. The improved operation of MAS and tricarboxylic acid cycle was associated with a greater myocardial oxygen consumption and more effective repletion of ATP and phosphocreatine stores. We conclude that Glu and Asp are involved in anaerobic and oxidative metabolism to meet energy requirements of the heart. This dual mechanism of action substantiates clinical application of both AA for correction of metabolic disorders induced by myocardial ischemia and reperfusion.

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Genetic aspects of beta-alanine metabolism in the fruit fly and yeast

Beta-alanine is an unusual amino acid which is primarily derived from pyrimidine catabolism. However, it also represents a key intermediate in the metabolism of many other compounds. In higher organisms, beta-alanine is found to be a neurotransmitter and it is also involved in the formation of many neural dipeptides. From the applied point of view, beta-alanine and its derivatives represent degradation products of many currently employed anti-cancer drugs, such as 5-fluoro uracil. However, so far only very little is known about the regulation of beta-alanine. Recently, we have started to employ two model organisms, the fruit fly and yeast, to study the genes involved in the metabolism of beta-alanine.

In *Drosophila melanogaster* beta-alanine represents the third and final step in the catabolism of the pyrimidine bases. Apart from being an insect neurotransmitter, beta-alanine is involved in pigmentation of the cuticle, giving a characteristic yellowish colour. Alleles of the *black (b)* mutation are defficient in beta-alanine and as a consequence the flies carrying this mutation are black. Two suppressor mutations, which restore the normal levels of the beta-alanine and yellowish cuticle coloration, have been described. One of them, *Su(b)*, has been characterized as a defect in the endproduct inhibition of the first enzyme activity in *de novo* pyrimidine biosynthesis. The mutant flies have an increased amount of pyrimidines and consequently also an increased beta-alanine pool. The second mutation, *su(b)*, which also alleviates the phenotypic effect of *b*, has been characterized with dimished beta-alanine catabolism. All three mutations, *b, su(b)* and *Su(b)* are presently under further molecular characterization.

Saccharomyces cerevisiae can not utilize pyrimidines or beta-alanine as a sole nitrogen source. However, another yeast from the same genus, *S. kluyveri,* can grow on pyrimidines as well as beta-alanine. Recently we have developed a genetic system for this yeast to study the genes involved in pyrimidine and beta-alanine metabolism. Two classes of mutants characterized by a defect in pyrimidine catabolism, *pyc,* and beta-alanine degradation, *bac,* have been isolated and are currently being investigated.

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Antifibrotic and antihypertensive properties of a *cis*-4-hydroxy-L-proline polymer in liposomes

An iv injection of the proline analogue, *cis*-4-hydroxy-L-proline (cHyp), (200 mg/kg) in liposomes ameliorates hypoxic pulmonary hypertension (HPH) in rats for 5 days by inhibiting collagen (coll) accumulation in pulmonary arteries (PA). Poly(ethylene glycol) based copolymers of cHyp have been synthesized and have sustained antifibrotic activity in vitro. Localized delivery of a copolymer in liposomes to PA_S may reduce the effective encapsulated dose of cHyp and prolong the antifibrotic and antihypertensive effects. We studied whether hypoxic rats injected iv with a single dose of liposomes containing ~ 2 mg/kg cHyp in copolymer (HP) had lower right ventricular pressure (RVP) and PA coll content than hypoxic rats injected with saline (HS) after 3 and 7 da exposure to 10% O_2. Air controls received saline (CS) or encapsulated copolymer (CP).

Table 1

Group:	CS		CP		HS		HP	
Day:	3	7	3	7	3	7	3	7
RVP (mm hg)	9±1	9±1	9±1	9±1	14±1	20±2	10±1*	16±1*†
Coll (ug hyp/*ves*)	**94±4**	**95±4**	**95±9**	**95±7**	**121±9***	**129±4***	**106±5†**	**115±8**

Data are $\bar{x}$ ± sem. *p < 0.05 vs. da matched CS; †p < 0.05 vs. da matched HS. n = 5–6

Results (Table 1) showed that at 3 da RVP and coll were not increased in the HP group. At 7 da RVP and coll were reduced in the HP compared to HS group. We conclude that: (1) a single injection of cHyp copolymer in liposomes prevented HPH for 3 da. Compared to previous studies with encapsulated monomeric cHyp the effective dose was reduced ~100 fold by cHyp in encapsulated copolymer and (2) the antihypertensive effect was prolonged to 7 da. (Support HL24264, and the Medical Research Service of the VA).

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Protein kinase C specifically phosphorylates peptides corresponding to two syndecan cyctoplasmic domains *in vitro*

The transmembrane heparan sulfate proteoglycans of the syndecan family are implicated to participate in several cellular reactions which are dependent on protein kinase C. We have used an in vitro assay to assess whether any of the known syndecans may become phosphorylated directly by protein kinase C. Peptides corresponding to the complete cytoplasmic domains of rat syndecans 1 through 4 were used as substrates for the enzyme. The syndecan-2 (fibroglycan) and syndecan-3 (N-syndecan) peptides were both found to be phosphorylated by protein kinase C with Kms of 15 ± 3 μM and 85 ± 25 μM, respectively, while the syndecan-1 and -4 peptides were not phosphorylated under the conditions used. The sites of in vitro phosphorylation for syndecans-2 and -3 were localized to ser-197 and ser-339, respectively. Thus, among 13 available sites (serines and threonines) in the four peptides, two were selectively modified by the enzyme. The specificity and the kinetics of the reactions indicate that the cytoplasmic domains of syndecan-2 and -3 are likely to be physiological substrates for protein kinase C.

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The effect of tannic acid on free radicals formation in Maillard reaction

One of the most important reactions in the chemistry of foods and natural products is Maillard reaction. The investigations of the effects of some polyphenols on the Maillard reaction routes, first of all on the inhibition of the reaction of pyrazine cation radicals with creatinine which leads to the formation of cancerous and mutagenic compounds, the classes of imidazo azarenes, quinolines and quinoxalines, and also on the stabilization of these reactive particles into pyrazine derivatives, flavor constituents, would contribute to obtaining of the wholesome and acceptable product. The effect of tannic acid on the formation of free radicals in Maillard reaction model systems of D(+)-glucoxe and 2-, 3- and 4-aminobutanoic acid, respectively, was examined in this work by Electron spin resonance spectroscopy (ESR).

The model systems were obtained by mixing the equimolar concentrations of D(+)-glucose and 2-, 3-, and 4-aminobutanoic acid, respectively, with the final concentrations of 1,0 M in alkaline water solutions, pH = 9,00. Tannic acid in the concentration range from 10^{-3} to $5 \cdot 10^{-2}$ M was added into the model systems. ESR spectral determinations were recorded on a Bruker 300E ESR spectrometer in reaction mixtures heated at 371K for

20 minutes. g-Values was determined by using PPADS (potassium peroxylamine disulfonate; g = 2.0036 ± 0.0002) as the standard. The hiperfine splitting constants of ESR spectrum were determined with a Bruker Data system ES 1600.

Pyrazine cation radicals, which were formed in reaction mixtures of all investigated model systems at above mentioned reaction conditions, have been recorded by ESR. The decrease of ESR signal intensity or even its disappearance in the systems with added tannic acid was registered.

The results obtained suggest that tannic acid decreases the concentration of pyrazine cation radicals in all investigated model systems. It is supposed that decreased concentrations of pyrazine cation radicals were caused by the stabilization of pyrazine cation radicals with phenoxy anion radicals into pyrazine-phenoxy compounds.

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Nitric oxide participation in the cardiovascular control by ventrolateral medulla in cats

In acute experiments on cats changes in the background and reflector sympathetic activities in the renal and inferior cardiac nerves together with shifts of haemo- and cardio-dynamic parametres have been found following unilateral injections of Nitric Oxide (NO) containing drugs into the ventrolateral medulla. Injections of sodium nitroprusside and L-arginine into sympathoexciting neurons of the rostral (RVLM) ventrolateral medulla resulted in remarkable attenuation in the sympathetic activities in both nerves and decrease in arterial pressure level (AP), peripheral vascular resistance, heart rate and cardiac contractile activity. Injections of L-N^G-monomethyl-L-arginin (L-NMMA) into the RVLM induced inhibition of the synthetic activity of L-arginin in the RVLM neurons and resulted in enhancement of the sympathetic activities in the peripheral nerves and increase in AP level.

Unilateral injections of NO into the sympathoinhibitory neurons in the caudal ventrolateral medulla (CVLM) resulted in both enhancement of the sympathetic activities and increase of haemo- and cardiodynamic parameters due to disinhibition of the neurons in the RVLM which are the origin of the descending sympathoexciting influences to the vessels and heart.

NO effects the RVLM and CVLM neurons depend on the cellular gyanylate cyclase and thus, of the cGMP level.

The data obtained evidence that NO plays an important role in the mechanisms of the central cardiovascular control by the ventrolateral medulla, perhaps as a central vasodilator.

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Synthesis and analysis of contaminants in EMS-related tryptophan

Several trace contaminants in tryptophan (Trp), among them 1,1'ethylidenebis-tryptophan (1,1'-EBT) and 3-anilinoalanine (AAL) were statistically associated with the occurrence of the eosinophilia-myalgia syndrome (EMS) [1]. The in vitro formation conditions of 1,1'-EBT and AAL were compared with those to be found during the manufacturing process of the EMS implicated Trp lots. 1,1'-EBT is formed preferentially in slightly acidic, Trp saturated, acetaldehyde containing solutions at low temperatures. AAL is formed at slightly acidic or slightly basic conditions from the precursors aniline and dehydroalanine by Michael-type addition. In consequence both compounds are likely to be formed during the ion exchange purification process. Both contaminants were synthesized in good yields (> 70%) and at high purity (> 90%, HPLC/UV) and could serve as reference substances.

Most efforts in contaminant search in Trp was done by RP-HPLC. RP-HPLC separation of a solution of EMS related Trp that was first digested by Tryptophanase (EC 4.1.99.1), indicated that several unidentified peaks coelute with the large Trp peak.

Occurrence of EMS sets new demands for an analytical quality control of Trp raw material. Therefore an RP-HPLC separation of 19 Trp related substances and simultaneous UV and fluorescence detection is applied to biotechnically manufactured Trp. The presence of Trp-metabolites and non-physiological Trp oxidation and carbonyl condensation compounds serves as marker for the quality of the purification process after fermentation. Selectivity and sensitivity achieved by the presented method allow the detection of 1 ppm, 1,1'-EBT or 1 ppm AAL in Trp raw-material. Two different pharmaceutical grade Trp raw materials (one using glucose and ammonia; the other, serine and indole as substrates for fermentation) have been examined. The number and the amount of trace contaminants were significantly lower than in EMS-implicated Trp. Both materials contained less than 1 ppm AAl and 1,1'-EBT. In 8 Trp containing infusions 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCC) (0.04–0.66 mg/l) and 1-methyl-THCC (MeTHCC) (0.03–0.20 mg/l cis-MeTHCC and 0.01–0.04 mg/l tr-MeTHCC) could be determined.

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Involvement of amino acids in adaptation to different environmental stresses in wheat

Environmental stresses represent important limiting factors to agricultural productivity. Plant biotechniques give tools for constructing species with improved physiological properties, such as enhanced tolerance to stresses, resistance to pathogens or increased quality of products.

The aim of the study was to obtain more knowledge to better understand the adaptation of wheat to different environmental stresses (e.g. drought, cold, salt) of wheat at the molecular level. Involvement of amino acids in this process is well known, however, only the role of proline has been emphasized.

In our work stress induced free amino acid accumulation has been compared in tissue cultures and in plants of Chinese Spring, Cappelle Desprez and Cheyenne wheat cultivars and in disomic chromosome substitution lines of Cappelle Desprez into Chinese Spring and Cheyenne into Chinese Spring.

The profile of free amino acid acumulations caused by stress conditions was different depending on the stress tolerance of the varieties and the nature of the treatment. Results also suggested that there is a link between drougt and frost tolerance of wheat.

In vitro techniques using tissue cultures offer powerful tools for biochemical and genetical investigations in environmental stress studies.

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Formation of amino acids in wheat during grain ripening

Wheat has long been studied extensively in search of relationship of its proteins to flour baking quality.

Plant cultivation technology (fertilizer system, preparation of soil) is one of the major factors which influencing formation of amino acids and protein synthesis in grain. Protein content of grain during ripening is also important to determine harvest time.

Growth stages of Hungarian wheat variety "Martonvásár 21" was studied in the period of three years.

The aim of this research was to investigate the changes of nitrogen containing compounds during the development of grain.

The content of free and peptide bound amino acids and protein in different organs (stem, leaf, grain) of plant were determined from 5th day to 42nd day after anthesis.

Formation of free amino acids showed characteristic trends during the period of seed development. Proline is present of higher quantity in seeds up to the waxy-ripe period. Hydrophobic amino acids (Ala, Val, Ile, Leu, Phe) can be found predominantly in leaves. There were no significant differences in basic and acidic amino acid concentrations in stem, leaf and grain. Interesting changes in ornithine content was observed during grain development which could merite particular attention. This will be further discussed in the presentation.

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Comparative studies the influence of externally applied L-arginine and phosphatidylcholine liposomes on endothelium-dependent relaxant responses in thoracic aorta of spontaneously hypertensive rats

The present study was designed to investigate the effects of L-arginine and phosphatidylcholine liposomes (PCL) on endothelium-dependent vascular responses in spontaneously hypertensive rats (SHR) compared with those in Wistar-Kyoto (WKY) normotensive rats. It was shown that isolated thoracic aorta rings from SHR preconstricted with noradrenaline had significantly decreased relaxant responses to acetylcholine (Ach). When added to the bath solution, L-arginine (10^{-4}M) caused an increase in the amplitude of the Ach-included relaxation from 33.5 ± 4.2 to 57.2 ± 9.3% ($p < 0.05$). However, using PCL (10^{-4}M), which can restore lipid content of plasma membrane, we observed that they not only promoted more-pronounced relaxation to Ach (from 33.5 ± 4.2 to 69.0 ± 4.7, $p < 0.01$) but transformed abnormal constrictor responses in SHR to dilation responses. The dilator responses to Ach were not different in WKY rat aortae with or without L-arginine and PCL suggesting that L-arginine and PCL can modify the endothelium-dependent responses in damaged aortae only.

The results are consistent with the hypothesis that the loss of endothelium-dependent vascular responses in SHR may be due to, at least, two reasons: 1) a lack of nitric oxide precursor in vascular wall; 2) endothelial cell plasma membrane damage.

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Detection of multiple 4'-phosphopantetheine cofactors as the thioester binding sites for the substrate amino acids of gramicidin S synthetase – the Multiple Carrier Model of nonribosomal peptide biosynthesis

Microbial organisms produce a great variety of low molecular bioactive peptides, depsipeptides and peptidolactones. In general, the biosynthesis of such compounds is accomplished nonribosomally by large multienzyme systems activating their amino acid substrates in a two step process at specific thiotemplates. The biosynthesis of the cyclic decapeptide gramicidinS from *B. brevis*, for example, is catalysed by two multifunctional proteins, gramicidinS synthetase 1 and 2 (GS1 and GS2) showing a modular architecture of amino acid activating modules. GS1 contains one, GS2 four of them. Every of such building blocks is equipped with a highly conserved thioester binding motif LGGH/DSL/I. Both multifunctional proteins were specifically labeled at their thiotemplates either directly with the ^{14}C-substrate amino acids or indirectly with radioactive thiol inhibitors. After enzymatic digestion the enzyme-substrate/inhibitor complexes were isolated in pure form by multistep separation methodology and identified as the thiolation site peptides by pulse liquid phase sequencing. By FAB and electrospray mass spectrometry in combination with amino acid analysis it was demonstrated that in every module a 4'-phosphopantetheine (Pan) cofactor was attached to the active serine of its thiolation motif. The sulfhydryl groups of these Pan carriers form the thioester binding sites of the amino acid substrates and function as the mobile structural elements for the assembly of the peptide product in a series of transpeptidation steps. A Multiple Carrier Model of nonribosomal peptide biosynthesis is discussed.

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Cyclophosphamide induced pulmonary fibrosis in the rat: Inhibition by taurine

The antiinflammatory, antioxidant and antifibrotic activity of taurine against cyclophosphamide induced lung fibrosis in rats was investigated. A single intraperitoneal injection (20 mg/100 g body weight) of cyclophosphamide markedly altered the levels of several biomarkers in lung lavage fluid. Total protein, albumin, angiotensin converting enzyme, lactate dehydrogenase, lactate, N-acetyl-β-D-glucosaminidase, alkaline phosphatase, acid phosphatase and lipid peroxidation products were elevated. In contrast, decreased levels of total reduced glutathione, ascorbic acid and antioxidant enzymes were observed. Cyclophosphamide significantly increased malondialdehyde levels in serum and lung tissue. Significant increases in lung content of lipid hydroperoxides were seen that paralleled the decreased levels of total reduced glutathione and total sulfhydryl groups. Pretreatment of rats with daily intraperitoneal injection of taurine 7 days prior to and 2 days after cyclophosphamide insult significantly inhibited the development of lung injury, prevented the alterations in lavage fluid biomarkers associated with inflammatory reactions, with less lipid peroxi-

dation and restoration of antioxidants. Cyclophosphamide significantly increased lung hydroxyproline, elastin, hexosamine uronic acid, histamine and 5-hydroxytryptmine. Taurine pretreatment and daily injections of the same resulted in significant reduction in lung collagen content, connective tissue components and lung lipid peroxidation 28 days after intraperitoneal administration compared with cyclophosphamide alone. In addition, taurine treatment significantly inhibited lung protein and DNA increases after cyclophosphamide treatment compared with cyclophosphamide treatment alone. These data indicate that taurine treatment prevents early inflammatory processes and late phase collagen accumulation in cyclophosphamide induced lung fibrosis.

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In vivo effect of cystathionine on ischemia-reperfusion injury in rats

Cystathionine is a metabolical precursor of cysteine. In vivo effects of cystathionine, especially as a role of radical scavenger, have not been examined. Therefore, we studied protective effect of cystathionine on acute gastric mucosal injury induced by ischemia-reperfusion in rats. Under the pentobarbital anesthesia, the celiac artery was clamped with a small clip for 30 min and reperfused by removal of the clip to make the ischemia-reperfusion state. Sixty minutes after the reperfusion, total area of erosions and thiobarbituric acid reactive substances (TBA-RS) in tissue, as an index of lipid peroxidation, were measured and compared between control and cystathionine treated groups. In this model, superoxide radical generated from xanthine-xanthine oxidase (X-XOD) system is the trigger of lipid peroxidation on gastric mucosal injury. Intraperitoneal administration of cystathionine (1 mg/kg – 20 mg/kg) 10 min before the ischemia significantly reduced the total area of erosions and the level of TBA-RS. Oral administration of cystathionine (10 mg/kg) also significantly reduced the total area of erosions and the level of TBA-RS. A good correlation between total area of erosions and the level of TBA-RS was seen. No effect of cystathionine on blood flow during ischemia-reperfusion was observed. On the other hand, cystathionine caused the scavenging effects of superoxide derived from X-XOD system in vitro. These results indicate that the protective effect of cystathionine on acute gastric mucosal injury induced by ischemia-reperfusion may be due to the scavenge of superoxide derived from X-XOD system in vivo.

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L-Arginine supplementation and blood pressure circadian rhythm in renal allograft recipients

Nocturnal hypertension is associated with a greater risk for end-organ damage and often complicates renal transplantation (Tx) even if on antihypertensive treatment. It is recently suggested that L-arginine (L-arg) supplementation in animals might have beneficial effects on hypertension, progression of renal damage and cyclosporine nephrotoxicity. The effects of L-arg on BP pattern and renal hemodynamics in renal Tx patients have not yet been studied.

In a double-blind study, 11 patients (6 M, 5 F) aged 11–22 yrs, post-Tx follow-up time 2–7 yrs, Creatinine Clearance 30–70 ml/min/1.73 m2, proteinuria 0.5–1.8 g/day, mean SBP (expressed as standard deviation score for age and sex, SDS) 1.8 ± 1.1 SDS, mean DBP 0.9 ± 1.1 SDS, treated with various antihypertensive drugs (excluding angiotensin converting enzyme inhibitors), were randomized in a cross-over fashion to receive a 6-week treatment with Placebo (Pla) or L-arg followed by a 6-week treatment with the other substance. L-arg was administered as L-arginine hydrochloride p. o. at the dose of 0.2 g/kg/day (max. 8 g/day). At the end of each treatment period, GFR (Inutest clearance) and RPF (PAH clearance) were determined. 24-hour BP monitoring was performed using a Spacelab device. BP load (percentage of BPs exceeding the upper limits of normal for age and sex) during waking and sleeping was calculated.

L-arg treatment did not modify GFR (37.3 ± 12 vs. 36.5 ± 17 ml/min/1.73 m2) and RPF (181 ± 70 vs. 193 ± 103 ml/min/1.73 m2). Also BP did not change significantly (mean systolic 1.45 ± 0.5 vs. 1.49 ± 0.6 SDS; mean diastolic 1.24 ± 0.5 vs. 1.18 ± 0.8 SDS). BP load values are shown in the Table 1. Data are presented as: mean ± sd.

Table 1

	Systolic BP load (%)		Diastolic BP load (%)	
	waking	sleeping	waking	sleeping
Pla	18.6 ± 18.7	19.9 ± 31.0	21.2 ± 12.1	15.2 ± 14.2
L-arg	26.5 ± 34.0	11.3 ± 15.0	27.5 ± 26.0	9.0 ± 13.0*

* $p < 0.05$ vs. waking

L-arg supplementation did not change renal hemodynamics and mean BP but selectively reduced BP during sleep. BP circadian rhythm seems therefore to be restored by L-arg in renal allograft recipients.

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Lack of renal hemodynamic effect of L-arginine infusion in renal allograft recipients

It has been recently suggested that L-arginine, a precursor of nitric oxide (NO), might ameliate cyclosporine nephrotoxicity and have beneficial effect on hypertension and renal function. We have assessed effects of L-arginine infusion on systemic and renal hemodynamics in cyclosporine A-treated renal allograft recipients with mild-to-moderate renal insufficiency.

11 patients (6 M, 5 F), range of age 11–21 yrs, CrCl (30–73 ml/min/1.73 m2), post-transplantation time 2–7 yrs, underwent an infusion of L-arginine at the rate of 290 mg/min/1.73 m2 bsa for 70 minutes. Before and during the infusion mean arterial blood pressure (MAP), GFR (Inutest Clearance), RPF (PAH Clearance) and cGMP urinary excretion have been determined. Data are presented as mean ± sd. Statistical analysis: t-test for paired data (Table 1).

Table 1

	Before	During	p
MAP (mmHg)	115 ± 15	112 ± 12	NS
GFR (ml/min/1.73m2)	37 ± 12	40 ± 13	NS
RPF (ml/min/1.73m2)	181 ± 70	200 ± 69	NS
cGMP (nmol/100mlGF)	2.0 ± 0.9	1.8 ± 0.9	NS

In renal allograft recipients, L-arginine infusion did not affect both systemic and renal hemodynamics. The fact that urinary cGMP excretion during L-arginine infusion did not increase is consistent with the hypothesis that NO synthesis is not enhanced by L-arginine, and this might account for the observed lack of hemodynamic effect.

Polyamines

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Ornithine decarboxylase as an oncogene

Recent studies from our laboratory and elsewhere indicated that ornithine decarboxylase (ODC), is a marker of proliferation, is expressed early during the cell cycle and is deregulated in transformed cells. In the present study we used NIH 3T3 fibroblasts, transfected with a construct in which the Ha-*ras* oncogene is driven by a corticosteroid-inducible MMTV-LTR promoter. In the presence of dexamethasone (DEX), *ras* was expressed and cells were morphologically transformed. Concomitantly, the activity of ODC increased and polyamines accumulated in the transformed cells. The increase in cellular polyamines had far reaching consequences; they enhanced the transcription of nuclear oncogenes such as *myc* and *fos*. The activation of ODC and the accumulation of polyamines appeared to be pre-requisite for the transformation process. α-difluoromethylornithine (DFMO), a specific inhibitor of ODC, prevented the expression of *myc* and *fos* and also the transformation of DEX-treated cells. This inhibition was reversed by the addition of exogenous polyamines. MMTV-ras transfected cells grew on soft agar in the presence of DEX. Similar anchorage-independent growth on soft agar was also observed when cells were treated with polyamines in the absence of the inducer DEX. These findings suggest that polyamines are essential for transformation and that ODC, which catalyzes the formation of these polycations, could be regarded as an oncogene.

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Polyamine biosynthesis in bacteria

Polyamines are essential constituents of most bacteria. Various aliphatic di-, tri-, tetra-, penta-, hexa, and heptaamines have been detected and only in halophilic bacteria the content of polyamines is rather low or not detectable. Recently, it has been demonstrated that the distribution and variation of polyamines are useful for differentiation and classification of methanogenic *Archaea* [1] and *Proteobacteria* [2]. To introduce polyamine patterns as a tool for classification to other groups of bacteria members of the familiy *Pasteurellaceae* as well as coryneform bacteria were analyzed for their polyamine contents. Within the family *Pasteurellaceae* 1,3-diaminopropane, putrescine, cadaverine, *sym*-norspermidine, spermidine, and spermine were detected and the presence of high amounts of one or more of these polyamines resulted in a characteristic polyamine pattern, which allowed a clustering of organisms and differentiation from others. In most of the coryneform bacteria analysed the overall polyamine content was lower than in members of the *Pasteurellaceae* and other Gram-negative bacteria. The polyamines detected in this group were 1,3-diaminopropane, putrescine, cadaverine, spermidine, *sym*-homospermidine, spermine and a substance not identified so far. The coryneform bacteria also displayed different polyamine patterns useful for differentiation and confirming recently proposed taxonomic rearrangements.

The correlation of the clustering according to the different polyamine patterns of the two groups to published phylogenetic data will be discussed.

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Putrescine as a biochemical marker of excitotoxicty in the rat brain

In previous work we have demonstrated that the systemic injection of some convulsants (kainic acid or lindane) to the rat produce an important increase in brain putrescine (PUT) concentration. However, this effect was not observed after convulsions induced by other agents such as picrotoxinin and pentylenetetrazol. In this communication it is shown that the convulsions produced by different agonists of the ionotropic and metabotropic glutamatergic receptors induced an increase of PUT in the brain areas studied (frontal cortex and hippocampus ipsi- and contra-lateral to the injection). The agonists assayed were: NMDA (80 nmol), kainic acid (2.3 nmol), AMPA (15 nmol), ACPD (2 μmol) and DHPG (1.5 μmol), and were injected into the lateral ventricle of male Wistar rats using a permanent canula implanted under halothane anaesthesia 24 hrs. before. Polyamines were determined by HPLC with fluorometric detection. Increases in brain PUT (up 400–1000%) were observed 24 hrs. after each treatment. The effect was more pronounced in the hippocampus than in the frontal cortex. Spermidine and spermine modifications were not statistically significant. The increase of PUT concentration was paralleled by brain damage. Histological examination of the hippocampus, also performed 24 hrs. after injection, revealed the presence of neuronal damage after all the treatments studied. These results support that brain PUT is a biochemical marker of the brain damage mediated by ionotropic and metabotropic glutamate receptor activation. (Work supported by the FIS 93-0350 and SAF 92-0913 grants.)

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Transglutaminase activation by tetanus toxin in secreting synaptosomes

The recent evidence about the ability of tetanus toxin (Tetx) to activate both a tissue liver and a synaptic-vesicle associated transglutaminase (Tgase) [1–4], has been further investigated by studying such activation in living synaptosomes (isolated nerve terminals). It is well known that Tetx is able to bind, enter and poison brain synaptosomes, thus specifically interfering with a

crucial step of neurosecretion [5]. The current model of Tetx action involves the proteolytic activity of the toxin on VAMP/synaptobrevin [6], although the proteolytic cleavage of other intracellular protein or the involvement of more than one enzymatic activity in Tetx action cannot be completely excluded [7, 8].

Rat brain synaptosomes, in which one of the two VAMP isoforms is not cleavable by Tetx, have been used to examine the ability of Tetx to activate synaptosomal Tgase and to inhibit the secretion of neurotransmitters. A Tetx dose-dependent activation of synaptic-Tgase, as measured by labeled spermidine incorporation into dimethylcasein *in vitro* was indeed found. This effect of Tetx appears to correlate with the level of glutamate release inhibition. Moreover, measures of the amount of glutamyl-lysine and mono- and bis-spermidine derivatives in lysates from intoxicated synaptosomes indicated a strong Tetx-dependent increase of Tgase activity in intact secreting synaptosomes. These results support our hypothesis that Tgase activation might play a role in the action of Tetx. SDS PAGE analysis of intoxicated synaptosomes indicated the presence of a 80 Kda protein (presumably synapsin I) covalently modified by Tgase, supporting a possible role of this protein in the Tetx-mediated activation of synaptic Tgase [4].

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Decrease in cell viability due to the accumulation of spermidine in spermidine acetyltransferase-deficient mutant of *Escherichia coli*

Physiological functions of spermidine acetyltransferase (SAT) in *Escherichia coli* have been studied using the SAT (speG) gene-deficient mutant CAG2242 and the cloned *speG* gene. The growth of *E. coli* CAG2242 in the defined M9 medium was normal in the presence and absence of 0.5 mM spermidine. However, cell viability of *E. coli* CAG2242 at 48 hrs. after the onset of growth decreased greatly by the addition of 0.5 mM spermidine. The amount of spermidine accumulated in the cells was approximately 3-fold that in the cells grown in the absence of spermidine. Transformation of the cloned *speG* gene to *E. coli* CAG2242 recovered the cell viability. Decrease in cell viability of *E. coli* CAG2242 was observed even when 0.5 mM spermidine was added at 24 hrs. after the onset of growth. The results indicate that accumulated spermidine functions at the late stationary phase of growth.

The accumulation of spermidine caused a decrease in protein synthesis, but not in DNA and RNA synthesis at 28 hrs. after the onset of growth. The synthesis of several kinds of proteins was particularly inhibited. They included ribosome modulation factor and ompC protein. Since the ribosome modulation factor is essential for cell viability at the stationary phase of growth (Yamagishi, M., Matsushima, H., Wada, A., Sakagami, M., Fujita, N., and Ishihama, A. [1993] EMBO J. 12, 625–630), the decrease in the protein was thought to be one of the reasons for the decrease in cell viability. The decrease in the ribosome modulation factor occurred at the translational level.

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Functions of potA and -D proteins in spermidine-preferential uptake system

Spermidine uptake system in *Escherichia coli* consisted of potA, -B, -C, and -D proteins. PotA protein is a membrane-associated protein having the nucleotide binding site. PotB and -C proteins are transmembrane proteins probably forming channel for spermidine and putrescine. PotD protein is periplasmic substrate-binding protein. In this study, we examined the functions of potA and -D proteins.

PotA protein was purified to homogenity and some of its properties were examined. PotA protein showed Mg^{2+}- and SH-dependent ATPase activity. The specific activity was approximately 400 nmol/min/mg protein and the K_m value for ATP was 385 μM. The binding site of ATP was explored by identification of the amino acid residue photoaffinity-labeled with 8-azido-ATP. It was found that 8-azido-ATP was attached to cysteine 26. 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 (50 to 57 and 168 to 173), was replaced by methionine. This mutated potA protein could be labeled with 8-azido-ATP, but did not show any ATPase activity. To identify which cysteine is involved in the function of potA protein, cysteins 26, 54 and 276 were replaced by alanine, threonine and alanine, respectively. The mutated potA protein C54T lost both spermidine uptake and ATPase activities. Thus, it was shown that cysteine 54 is also involved in the function of potA protein. The results taken together indicate that adenine portion of ATP interacts with a domain close to the NH_2 terminal end of potA protein and the active center of ATP hydrolysis is located between two consensus amino acid sequences for nucleotide binding.

Spermidine binding sites on potD protein were analyzed by X-ray crystal analysis of potD protein, and site-directed mutagenesis of *potD* gene. The results indicate that Trp34, Glu36, Tyr85, Asp168, Glu171, Trp255 and Asp257 were involved in the interaction with spermidine.

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Effects of alanine on polyamine metabolism and galactosamine-induced injury of rat hepatocytes in primary culture

We investigated the effects of alanine on hepatocellular injury induced by galactosamine (GAL). Rat hepatocytes were isolated by collagenase perfusion method and incubated in Willium's E medium at low cell density (1.25×10^6 cells) with GAL in the presence or absence of alanine. Then, ornithine decarboxylase (ODC) activity and the extent of cellular injury were assayed. The extent of the cellular injury was represented as a percentage of extracellular lactic dehydrogenase activity to the sum of extracellular and intracellular lactic dehydrogenase activity after 48 hrs. incubation. Alanine increased ODC activity about 5 fold in GAL-treated cells at 6 hrs. and the enzyme activity was remained at high level until 12 hrs. GAL caused hepatocellular injury (51 ± 9%), compared to GAL-untreated cells (13 ± 2%). The addition of alanine and putrescine attenuated the extent of the cellular injury to 34 ± 6% and 36 ± 8%, respectively. However, the combined addition of alanine and putrescine augmented GAL-induced cellular injury (81 ± 2%). The addition of alanine or putrescine increased intracellular putrescine content of cells treated with GAL. The combination of alanine and putrescine caused a synergistic increase in the putrescine content.

These results suggest that alanine protects hepatocytes from GAL-induced cellular injury due to the increase in cellular putrescine content, resulting from the induction of ODC activity, but the excess of intracellular putrescine cannot protect cells from the cellular injury, but rather augment it.

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IRF-1 regulates the expression of ODC gene during IFNγ macrophage activation

The enzyme ornithine decarboxylase (ODC) is the key regulator of the synthesis of polyamines that are essential for cell growth and proliferation. Moreover, the role of polyamines does no seem to be restricted only to proliferation, in fact it has been found that cell differentiation and function of terminally differentiated cells also involve activation of ODC. We have observed an induction of ODC gene expressen in human and mouse macrophages activated by Lypopolysaccharide (LPS), Tumor Necrosis Factor (TNF) and Interferon γ (IFNγ). Furthermore in our last report we hypothesized that the transcription factor Interferon Regulatory Factor 1 (IRF-1), an IFNγ-inducible nuclear protein that controls the expression of interferon (α and β) genes and some IFN-inducible genes, regulates ODC gene expression during human macrophage IFNγ activation. In fact, we demonstrated that: 1) both IRF-1 and ODC genes, are expressed in human macrophages activated with IFNγ, 2) IRF-1 binds to the first intron of the ODC gene (+2711 –2722), 3) a hamster fibroblast cell line, ts 13 tk-, constitutively transfected with IRF-1, overexpressed the ODC gene. To better establish the role of IRF-1 in the regulation of ODC gene during IFNγ macrophage activation, we transfected a human promonocytic cell line, U937, with the cDNA of IRF-1 cloned in antisense under the control of an SV40 constitutive promoter. After the selection of a mix population which integrated IRF-1 antisense cDNA, the parental and the transfected cells were stimulated only with IFNγ for 2 h, the time in which there is the highest expression of IRF-1, or with phorbol myristate acetate (PMA) for 72 hrs. to differentiate the cells into monocytes/macrophages and than with IFNγ. Total RNA was extracted from these cells and a Northern blot was performed. This filter was hybridized with the human ODC probe. Only the RNA extracted from the transfected cells stimulated with IFNγ or with PMA and then IFNγ, did not hybridize. These preliminary results confirm our hypothesis that IRF-1 regulates the expression of ODC gene in human macrophages activated with IFNγ, in fact in this system the block of IRF-1 protein causes the inhibition of the ODC gene. (This work was supported by grants from MURST [40%], AIRC [Associazione Italiana per la Ricerca sul Cancro], CNR [Consiglio Nazionale delle Ricerche].)

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A requirement of zinc for the induction of ornithine decarboxylase in cultured rat hepatocytes

Zinc is necessary for growth in animals. In cell culture, zinc has been shown to be required for DNA synthesis and proliferation of several cell types. The biochemical basis for the growth failure associated with zinc deficiency is not clear.

The polyamines putrescine, spermidine and spermine are present in many cells, and the evidence is that polyamines play an essential role in cell growth and differentiation. Eukaryotic cell proliferation proceeds by an increase in the activity of ornithine decarboxylase (ODC) and by polyamine biosynthesis. Here, the effect of a restricted Zn^{2+} availability on ODC activity was studied in primary cultured adult rat hepatocytes.

Hepaotcytes were isolated from the rats by the collagen perfusion method. The cells were plated in 20 mm plastic dishes at a density of 2.5×10^5/ml in William medium E supplemented with 10% FCS, 1×10^{-7} mol/L insulin and 1×10^{-5} mol/L dexmethazone. The cells were cultured at 37 °C for 24 hrs in a humidified atmosphere of 5% CO_2 and 95% air. After the medium was replaced with fresh medium, cells were cultured in the absence of hormones for another 24 hrs. This medium was exchanged for medium with added inducer (10^{-7} mol/L insulin, 10 ng/ml EGF) and supplemented with diethylenetriaminopentaacetic acid (DTPA) at a concentration of 0.6 mM. After 1 hr., $ZnSO_4$ was supplemented to a final concentration of 0.6 mM.

ODC activity increased and peaked 12 hrs. after the addition of insulin and EGF, thereafter it decreased, and it returned to the control level by 24 hrs. The addition of the chelating agent diethylenetriamine penta-acetic acid (DTPA) caused an inhibition of the induction of ODC activity by up to 50%, and Zn^{+2} supplementation was effective in reversing this effect. The level of ODC messenger RNA did not decrease with addition of DTPA. The half-time of ODC activity was shorted about twofold by DTPA treatment. These results suggest that Zn^{2+} is involved in ODC induction at the level of posttranslation.

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A polyamine-derived amino acid, hypusine: Its post-translational formation in eIF-5A and its role in cell proliferation

The unusual amino acid hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] is a unique component of one cellular protein, eukaryotic translation initiation factor 5A (eIF-5A, old terminology, eIF-4D). It is formed posttranslationally and exclusively in this protein in two consecutive enzymatic reactions, (i) modification of a single lysine residue of the eIF-5A precursor protein by the transfer of the 4-aminobutyl moiety of the polyamine spermidine to its ε-amino group to form an intermediate, deoxyhypusine [N^{ε}-(4-aminobutyl)lysine] and (ii) subsequent hydroxylation of this intermediate to form hypusine. The amino acid sequences surrounding the hypusine residue are strictly conserved in all eukaryotic species examined, suggesting the fundamental importance of this amino acid throughout evolution. Hypusine is required for the activity of eIF-5A in vitro. There is strong evidence that hypusine and eIF-5A are vital for eukrayotic cell proliferation. Inactivation of both of the eIF-5A genes is lethal in yeast and the hypusine modification appears to be a requirement for yeast survival (Schnier, J., Schwelberger, H., Smit-McBride, Z., Kang, H. A., and Hershey, J. W. B. [1991] Mol. Cell. Biol., 11, 3105–3114; Wöhl, T., Klier, H., Ammer, H., Lottspeich, F. and Magdolen, V. [1993] Mol. Gen. Genet. 241, 305–311). Furthermore, inhibitors of either of the hypusine biosynthetic enzymes, deoxyhypusine synthase or deoxyhypusine hydroxylase, exert strong antiproliferative effects in mammalian cells, including many human cancer cell lines. These inhibitors hold potential as a new class of anticancer agents, targeting one specific eukaryotic cellular reaction, hypusine biosynthesis.

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Spermidine regulation of protein synthesis at the level of initiation complex formation of Met-tRNA$_i$, mRNA and ribosomes

Spermidine regulation of protein synthesis (stimulation at low concentrations and inhibition at high concentrations) was studied using mRNAs with GC- and AU-rich 5'-untranslated regions (5'-UTR) in a rabbit reticulocyte cell-free system. When the 5'-UTR contained a continuous GC stem region, the synthesis of ornithine decarboxylase, S-adenosylmethionine decarboxylase and β-actin was strongly stimulated by low spermidine concentrations and greatly inhibited by high spermidine concentrations. When the 5'-UTR contained an AU-rich sequence, both the degree of stimulation by low spermidine concentrations and that of inhibition by high spermidine concentrations were less than those with the 5'-UTR having the continuous GC stem. The results showed that the contiouos GC stem region in the 5'-UTR plays an important role in the spermidine regulation of protein synthesis. Spermidine regulation of protein synthesis reflected the initiation complex formation of Met-tRNA$_i$, mRNA and ribosomes. However, the binding of mRNA to ribosomes in the presence and absence of Met-tRNA$_i$ or the binding of Met-tRNA$_i$ to ribosomes in the absence of mRNA$_i$ was not significantly influenced by spermidine. The results suggest that the binding sites of mRNA and Met-tRNA to ribosomes partially overlap, and spermidine regulates the simultaneous binding of mRNA and Met-tRNA$_i$ to ribosomes through the configurational change of RNA by spermidine. The spermidine regulation of initiation complex formation of Met-tRNA$_i$, mRNA and ribosomes occurred before 40S ribosomal subunits reached the initiation codon AUG, and the inhibition by high spermidine concentrations was strengthened by spermidine inhibition of initiation factor-dependent RNA helicase activity.

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Synthetic polyamine analogs as antiproliferative agents

Nine novel chemically modified polyamine analogs were evaluated for their ability to inhibit the polyamine biosynthesis and to promote the polyamine degradation in cell-free test-system from rat regenerating liver. Oxy- and aminoanalogs of polyamines, modified by adenosine, and polyamines, modified by two uracils were investigated. All tested compounds were differed with the number of methylen groups in carbon chain. The final concentration of analogs and α-difluoromethylornithine (DFMO) were 0.1 mM in the reaction mixture. Ornithine decarboxylase (ODC) activity, polyamine levels and both diamine oxidase (DAO) and polyamine oxydase (PAO) activities were determined.

Compounds, modified by adenosine, could act as activators of ODC and as inhibitors of both DAO and PAO. In contrast, agents, modified by two uracils, as well as DFMO were able to inhibit ODC and to increase the PAO and DAO activities.

The data obtained indicate that such modified polyamine analogs as a new effective inhibitors of cell proliferation may be useful to regulate cellular polyamine levels and could serve as basis for the control of polyamine metabolism and RNA and DNA synthesis.

Synthesis

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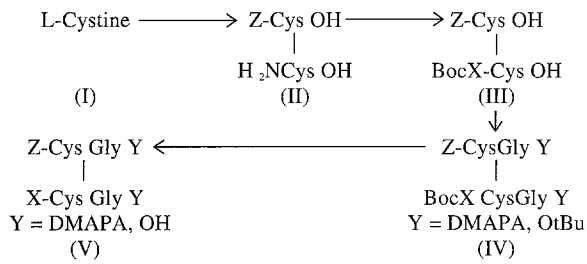
Synthesis of mixed disulphide potential alternative Substrates for trypanothione and glutathione reductases

Trypanothione reductase, a key metabolic enzyme unique to trypanosomitidae, is a potential target for trypanosomal drugs. The goal of this study was to design inhibitors that would incorporate key interactions once these had been mapped out by the use of mixed disulphide substrates. A key feature of the approach (Scheme) was the need to develop a route to asymmetrical open-chain cysteine derivatives. These could be studied as potential substrate analogues of trypanothione and glutathione disulphide with trypanothione and glutathione reductases respectively [1].

The synthesis was based on the elaboration of Zervas' product [2], mono-N-carbobenzoxy-L-cystine (II) formed by the interaction of excess of L-cystine with carbobenzoxychloride (Z-Cl) (20% aq. NaOH, RT, 24 hrs) under Schlotten-Baumann conditions, after which the pH of the reaction mixture has to be adjusted to 6, the highly insoluble excess cystine removed by filtration and the pH of the filtrate readjusted to 3.2 which precipitated (II) as hydrochloride salt.



Derivative (II) was then mixed with BocXoSu (X = Phe, Glut(OtBu), Trp) in a mixture of dioxane/water, in the presence of triethylamine, at room temperature (16 hrs). The aqueous layer was basified to pH 9, extracted with ethyl acetate, and acidified to pH 3.2 to deposit (in the cold) the disubstituted L-cystine (III) (80–90% yield). (III) was then coupled with 2 eq. of either H-GlyDMAPA [3], or H-GlyOtBu (DCC, N-hydroxybenzotriazole, 5 hrs, RT). The fully protected *bis* tripeptides (IV) were obtained highly pure after flash chromatography (70–90% yield).

Removal of the blocking groups and tBu ester in (IV) occurred smoothly in a mixture of triethylsilane/TFA/CH_2Cl_2 (45 min., RT) [4] to give (V) in quantitative yields.

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Stereoselective synthesis of β-hydroxy α-amino acids

There is considerable interest in the development of synthetic methods for the preparation of nonproteinaceous amino acids. These are often important constituents of various biologically active substances. We recently reported a general approach for the synthesis of α-amino acids based on addition reactions to a chirally stable serine aldehyde and to a threonine ketone equivalent where the carboxylic acid is protected as an ortho ester. This presentation will describe important improvements which allow for very large scale preparation of these unique synthons. Various addition reactions to these synthons will be discussed in terms of their stereoselectivity. Several syntheses of more complex α-amino acids such as MeBmt and analogs will illustrate the utility and versatility of this approach.

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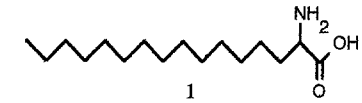
Conversion of lipidic amino acids into lipidic 1,3-diamines and taurines

Synthetic routes to diamines are of special interest because these compounds are useful as chelating agents (cis-platinum chelates) and as precursors in the synthesis of various interesting medicinal compounds. On the other hand long chain alkyl amines and polyamines exibit various biological acitivities [1]. We have recently reported methods for the conversion of amino and peptide alcohols into chiral 1,2-diamines and for the preparation of lipidic 1,2-diamines from lipidic amino acids [2]. We present here a facile method for the conversion of racemic lipidic α-amino acids (**1**) into lipidic 1,3-diamines (**2**) and taurines (**3**).

Boc-protected lipidic 1,2-aminoalcohols were easily derived from lipidic α-amino acids. The hydroxy group was activated as the mesylate and converted into nitrile by treatment with sodium cyanide. Reduction of the cyano group by $NaBH_4$ in the presence of $NiCl_2$ afforded the monoprotected 1,3-diamine. Replacement of the methanesulfonyloxy group by a sulfo group afforded lipidic taurines (long chain 2-substituted taurines) (**3**).

The biological activities of lipidic 1,3-diamines and taurines are under investigation.



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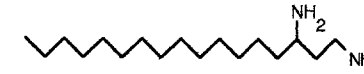

2

3

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Stereospecific synthesis of α-deuterated Boc-L-amino acids

Alkylation of *(2R)-N-{Bis(methylsulfanyl)methylene-2,2-d_2-glycyl}bornane-10,2-sultam* enolate with alkyl- and aralkyl-iodides in the presence of HMPA afforded α-deuterated L-amino acid derivatives in good yields and very high enatiomeric purity (ee% > 99%). Deprotection of the amino groups from these derivatives, cleavage from the sultam auxiliary and subsequent bocylation, afforded Boc-α-deuterated-L-Amino acids in good yields, such as *Boc-L-Ala-2-d, Boc-L-Leu-2-d.H2O, Boc-L-Phe-2-d_2 and Boc-(O-benzyl)-L-Tyr-2-d* along with *Boc-Gly-2,2-d_2* synthesized from a precursor in an earlier step.

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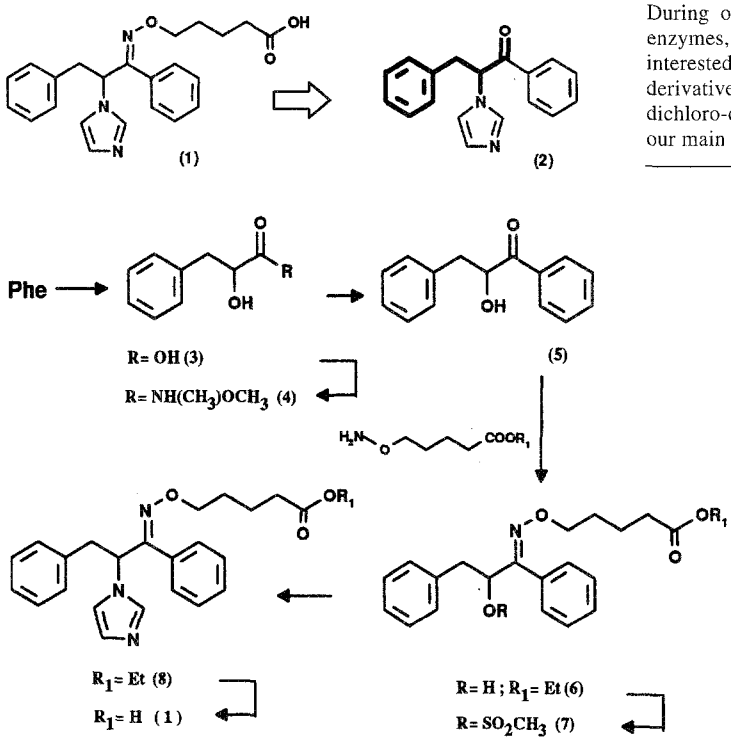
Use of amino acids as chiral building blocks in organic synthesis: Enantiospecific synthesis of 2-(imidazol-1-yl)-1,3-diphenylpropanone O-alkyloximes

During the course of our research for thromboxane A_2 synthase inhibitors endowed with thromboxane A_2 receptor antagonism [1] we identified compound **1** as a molecule endowed with both the above pharmacological properties. The need to analyse the relationship between the enantiomers and the corresponding biological activities, along with the identification of the corresponding absolute configuration, led us to carry out an enantioselective synthesis of compound **1**. Homochiral natural products such as sugars, terpenes and amino acids, are largely used as sources of carbon frameworks bearing suitable asymmetric centres. A retrosynthetic analysis for compound **1** showed the phenylalanine framework (highlighted) embedded in the synthon **2**.

Due to configuration integrity problems during imidazole ring synthesis, we preferred to introduce this moiety by a nucleophilic substitution on a proper intermediate, so giving rise to an inversion of configuration. Thus R- or S-phenylalanine afforded respectively to S- or R-1 according to the scheme reported below.

Phenylalanine was stereospecifically diazotized to the hydroxy acid **3**, which was in turn, converted into the hydroxy ketone **5** by Weinreb ketone synthesis. Configurational instability of **2** adviced us to introduce the imidazolyl moiety into the configurationally more stable hydroxy-alkyloxyme **6**. Nucleophilic substitution of imidazole onto the mesylate **7** afforded compound **8**, which was hydrolysed to the desired enantiomers of **1** with good optical purity, as confirmed by chiral HPLC analysis. The above nucleophilic substitution on 2-hydroxy-O-alkyloximes has been never reported so far.



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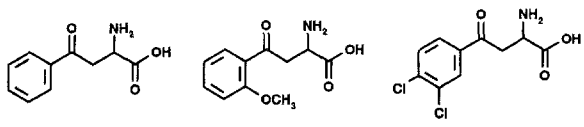
Use of amino acids as chiral building blocks in organic synthesis: Enantiospecific synthesis of benzoylalanine derivatives from aspartic acid

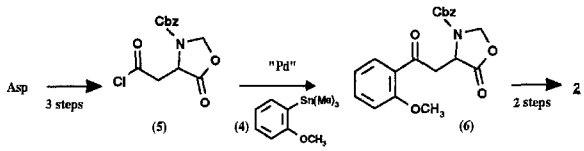
Chiral non racemic natural products have been widely utilised as building blocks in organic synthesis: in particular L- and D-amino acids represent an easily available source of chirality. During our research for inhibitors of tryptophane catabolism enzymes, kynureninase and kynurenine-3-hydroxylase, we were interested in the enantiospecific synthesis of benzoylalanine **1** derivatives. Particularly the 2-methoxy-derivative **2** and the 3,4-dichloro-derivative **3**, in addition to benzoylalanine itself, were our main targets.



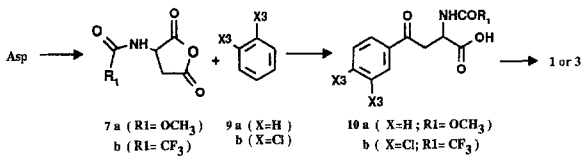
1 2 3

An inspection of the benzoylalanine carbon framework clearly pointed out aspartic acid as the chiral starting material of choice. Owing to different aromatic substitution patterns present in **2** and **3**, two different synthetic routes, both using aspartic acid as chiral source, were choosen for the enantiospecific synthesis of these compounds.

The Stille ketone synthesis, involving palladium catalysed addition of organostannane to acyl chlorides, was followed to prepare **2**. The palladium catalysed reaction of the organostannane **4** with the aspartate chiral synthon **5** gave the derivative **6**, that was deprotected to yield the desired enantiomer(s) of **2**.



The recently reported use of optically active N-carbomethoxy or N-trifluoroacetyl aspartic anhydride derivatives in enantiospecific Friedel-Crafts acylations prompted us to take advantage of this route for the preparation of **1** and **3**.



Thus $AlCl_3$ catalysed reaction on anhydride **7 a, b** with **9 a, b** gave high yield of **10 a, b**. Removal of the protecting group led to the enantiomers of **1** or **3**.

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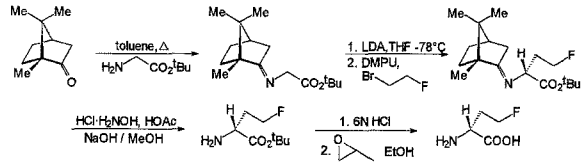
Synthesis of γ-fluoro-α-amino acids

Fluorinated amino acid and derived peptides claim an extraordinary interest in chemistry and biochemistry as well as in medicinal research because of their enormous variety of biological activity. Moreover, using ^{19}F-NMR-spectroscopy, the determination of conformations of these compounds becomes possible.

To now only few racemic γ-fluoro-α-amino acids were synthesized almost without exceptions by nucleophilic substitution of α-bromo-γ-fluorocarboxylic acids [1].

We present methods for the synthesis of several racemic and optically active title compounds. The key step is the alkylation with 1-bromo-2-fluoroalkanes of glycine ester-derived imines at low temperature. In this way the shown four step procedure yields (R)-4-fluoro-2-aminobutyric acid in 26% overall yield and 32% ee using (1R)-(+)-campher as the auxiliary and 1-bromo-2-fluoroethane as the alkylation agent. Analogously the other enantiomer can be obtained with > 95% ee using (2R,3R,5R)-(+)-2-hydroxypinan-3-one. Other required vicinal bromofluoro-alkanes are easily available by bromofluorination of the corresponding terminal alkanes with the system N-bromosuccinimide/triethylamine trishydrofluoride [2].



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Synthesis of orthogonally protected diaminopimelic acid derivatives

The biological importance of L,L- and meso-diaminopimelic acid (DAP) has stimulated the synthesis of these naturally occurring diamino dicarboxylic acids. On the one hand meso-DAP serves as substrate for the enzyme meso-DAP decarboxylase and is transformed into L-lysine which is an essential amino acid in mammals. On the other hand DAP functions as a crosslinking constituent of virtually all Gram-negative and some Gram-positive bacteria. Since mammals lack meso-DAP decarboxylase and require L-lysine in their diet, inhibitors of the diaminopimelic pathway represent possible anti microbial agents that should display selective toxicity.

Synthesis of different protected diaminopimelic acid derivatives was successful using the mixed Kolbe-electrolysis of glutamic acid and aspartic 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 diaminopimelic acid derivatives were transformed into very useful starting materials for the synthesis of peptides of pharmaceutical interest.

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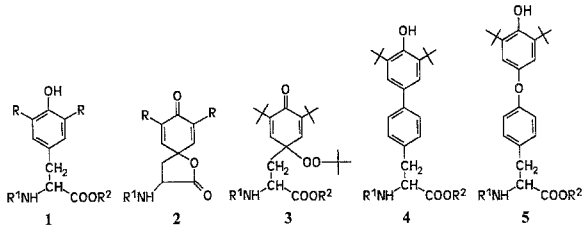
Non-natural phenolic amino acids. Synthesis and application in peptide syntheses

Tyrosine derivatives as **1** have been synthesized. They are redox active compounds which should be oxidizable to phenoxy radicals or cations reacting with other radicals or nucleophiles to give synthons useful in peptide synthesis. The oxidation to the phenoxenium ion [1] has been performed anodically (carbon anode/acetonitrile/$NaClO_4$ or NBu_4BF_4), with N-bromosuccinimide (20% acetonitrile/80% acetate buffer) [2] or with iodosobenzene diacetates (abolute methanol or acetonitrile) [3].

For R^2 = H intermediate phenoxenium ions yield spirolactones **2** by intramolecular cation/nucleophile addition in yields of 17–100%. These react as active esters with amino acid esters to give dipeptides in 7–77% yields.



For R = tBu, R^1 = CHO and R^2 = Me the amino acid can be oxidized catalytically to **3** by tBuOOH and Co^{II}(salen) as catalyst [4]. This stable quinolperoxide can be transformed to dopaquinone derivatives by acid treatment.

The non-natural phenolic amino acids **4** and **5** have also been synthesized and oxidized to give stable phenoxy radicals. The investigation of these radicals by ESR-spectroscopy demonstrated that they can be used as potential spin labels in peptide chemistry.

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Methyltrypsin catalyzed peptide synthesis by use of inverse substrate

Protease catalyzed peptide synthesis has many advantages. The reaction is stereoselective, racemization-free, and require minimal side-chain protection. A drawback of the method, however, is the loss of the product due to hydrolysis by the protease. In addition, amino acid and peptide derivatives used for the coupling reaction are limited to those meet the specificity of the protease. Previously we reported that "inverse substrate" was useful for trypsin catalyzed peptide synthesis and was able to overcome the disadvantage intrinsic to the enzymatic method. Recently, chemically modified proteases, which were lacking the amidase activity, were used for peptide synthesis eliminating the secondary hydrolysis. Methyltrypsin is the enzyme in which His-57 residue in the active site is methylated. The enzyme is lacking its amidase activity though it retains binding activity toward the specific substrates. It is of interest to study the interaction between modified trypsin such as methyltrypsin and inverse substrate with a view to applying to the enzymatic peptide synthesis.

Methyltrypsin catalyzed peptide coupling raction was performed as follows. The reaction mixture of acyl donor (Boc-amino acid p-guanidinophenyl esters as inverse substrate, 1 mM) and acyl acceptor (amino acid p-nitroanilides, 20 mM) was incubated with methyltrypsin (10 μM) in aqueous DMSO at 25 °C. The progress of the coupling reaction was monitored by HPLC. The coupling products were obtained in 60~90% yield within 4 hours using L-amino acid as acyl donor and acyl acceptor. When using D-amino acid as acyl donor, coupling products were also obtained in 60~90% yield within 25 hours. In these case, secondary hydrolysis of the coupling peptide was not observed. However when using the D-amino acid as acyl acceptor, the coupling products were not obtained. Further requirement of acyl donor and acyl acceptor on the coupling reaction will be discussed.

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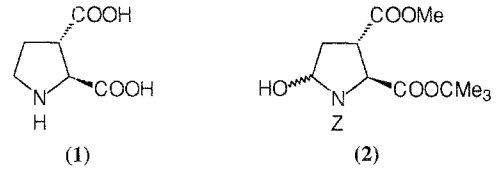
The synthesis of conformationally constrained amino acids and their incorporation into biologically active peptides

The incorporation of conformationally constrained amino acids into biologically active peptides is a well established method for probing the biologically active conformation of a peptide. In this poster, our recent synthesis of *trans*-3-carboxy-(**S**)-proline (**1**) and a range of its protected derivatives will be reported. Compound (**1**) can be considered either as a conformationally constrained aspartic acid derivative, or as a proline residue with modified acidity. As such it combines the properties of two amino acids into a single structure. The incorporation of compound (**1**) into a conformationally constrained RGD analogue will also be described, as will the preparation of six membered ring analogues of compound (**1**).

Compound (**1**) was prepared utilising our previously reported methodology for the generation of the aspartic acid β-anion and its trapping with allyl bromide. Ozonolysis of this adduct lead to the key intermediate (**2**), hydrogenation of which followed by deprotection gave compound (**1**). Alternatively, treatment of (**2**) with a Lewis acid and an organosilicon derivative resulted in substitution of the OH group, allowing a range of 5-substituted analogues to be prepared.



(1) (2)

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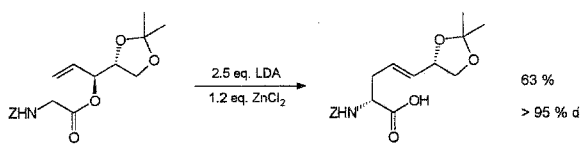
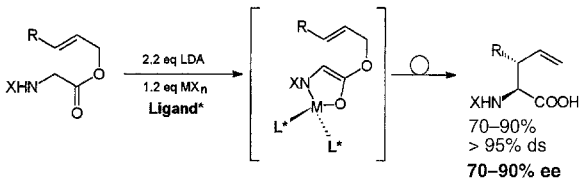
Asymmetric synthesis of γ,δ-unsaturated amino acids via ester enolat Claisen rearrangement of chelated allylic esters

The first synthesis of γ,δ-unsaturated acids by Claisen rearrangement was described in 1975 by Steglich et al. [1] The reaction proceeds via an oxazole intermediate and is especially suitable for the synthesis of α-alkylated allylic amino acids. In 1982 the Ireland-Claisen rearrangement of glycine allylic esters was studied by Bartlett and coworkers [2]. Recently we developed another new variation of the ester enolate Claisen rearrangement, proceeding via chelated allyl ester enolates [3].

This methodology is suitable for acyclic as well as cyclic allylic esters [4], and can also be applied to peptides [5]. If the reaction is carried out with allylic esters of other amino acids α-alkylated amino acids can be obtained by this procedure. Rearrangement of chiral allylic esters proceeds in a highly diastereoselective fashion with a nearly complete transfer of chirality.



Another possibility for the synthesis of chiral amino acids is the rearrangement in the presence of bidentate chiral ligands (L*). The optically active amino acids are obtained in very good yields and excellent enantio- and diastereoselectivities.



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Structural feature of peptides for the biosynthesis of N^G-methylarginine

N^G-Methylarginines in protein molecule are enzymatically synthesized posttranslationally by protein methylase I (S-adenosylmethionine: protein-arginine N-methyltransferase; EC.2.1.1.23) on the specific arginyl residue in polypeptides. To understand structural features which are essential for serving as the methyl acceptor for protein methylase I, we have investigated substrate capacities of several synthetic oligopeptides whose sequences are homologous and/or analogous to the methyl acceptor region of the naturally occurring arginine-methylated proteins. These studies have led to the following conclusions. 1) The preferred amino acid sequence of methyl-accepting peptides was shown to be an arginine-containing peptide with glycine in both the N- and C-flanking positions. While a tetrapeptide with such a sequence (residues 106–109 of bovine myelin basic protein) exhibited almost negligible substrate activity, an overlapping hexapeptide was a moderate substrate. 2) Substitution of the N- or C-flanking glycine in GKG**R**GL (residues 104–109 of myelin basic protein) with histidine, phenylalanine, lysine, aspartic acid and leucine abolished the ability of these hexapeptides to serve as substrates. 3) A heptapeptide with a repeated glycine-arginine motif (G**R**G**R**G**R**G) was an excellent substrate for the enzyme, indicating the presence of three potential arginine methylation sites in the peptides. 4) A cyclic octapeptide (CGKG**R**GLC), which was formed by cyclization of GKG**R**GL by introduction of disulfide bridge to cross-link N- and C-terminus of the hexapeptide, was an even better substrate than the hexapeptide, indicating that not only the primary sequence but also the secondary structure of the peptide play an important role in the enzymatic methylation. 5) Upon HPLC amino acid analysis, all enzymatically [*methyl*-^{14}C]-labeled oligopeptides yielded predominantly N^G-monomethylarginine with a minor fraction of N^G,N^G-dimethylarginine in certain peptide samples. However, no N^G,N'^G-dimethyl-arginine formation was detectable.

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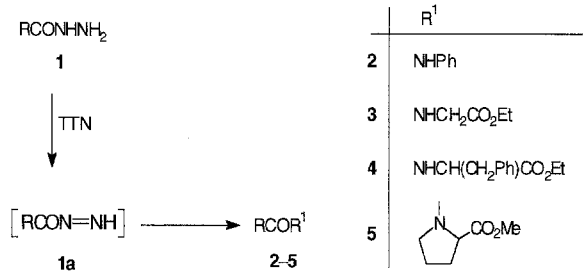
N-Protection of α-amino acids using hydrazides

Hydrazides **1** are easily oxidized with thallium(III) nitrate (TTN) in the presence of the appropriate nucelophiles. The reactions probably proceed via acyl diimide **1a,** which then reacts with the nucleophile. Thus, oxidative cleavage in the presence of aniline results in the formation of N-phenyl amide **2.** On the other side, reaction with esters of α-amino acids give N-protected esters **3-5.** Racemization was not observed during the acylation. Those transformations were found to display a certain level of chemoselectivity.



Some other applications of hydrazide/TTN system will also be discussed.

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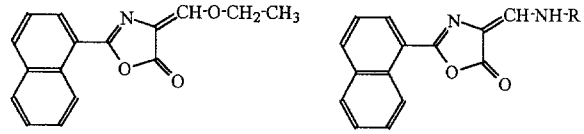
Synthesis and application of naphtyl-oxazolone derivatives of amino acids



(I) **(II)**

4-(ethoxymethylene)-2-[1]-naphtyl-5(4H)-oxazolone **(I)** was synthesized in ethylacetate solution with the reaction of 1-naphtoyl-glycine triethyl-orto-formiate and acetic anhydride. This compound was characterized by RP-HPLC, NMR, and mass spectroscopic (MS) methods. When reacting with primary amines **(I)** undergoes displacement of the ethoxy group by the amine with elimination of ethyl alcohol and forms type **(II)** derivatives. Some amino acid-naphtyl oxazolone derivatives were synthesized with direct reaction of **(I)** and amino acids in water or methanol. During the synthesis of the ε-lysine derivative the temporary protection of the α-amino group was realized by using the copper **(II)** complex of free lysine. The structure of the amino acid derivatives were verified by NMR, FAB-MS, UV spectrophotometry. In contrast to the respective phenyl derivatives – commonly used as synthetic hapten in basic immunology – naphtyl-oxazolone-amino acids possess fluorescence properties. Stability studies in aqueous solution were carried out using RP-HPLC. It was found that **(I)** can be applied for fluorescent labeling of the free amino groups of peptides and proteins. (This work was supported by the Hungarian Research Fund OTKA No. T014964.)

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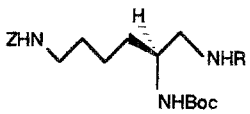
[2]Chemical Laboratories, Agricultural University of Athens, Athens, Greece

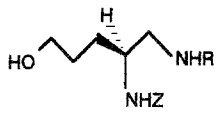
Synthesis of chiral triamines and diamines from amino acids

Triamines and vicinal diamines are important intermediates in the synthesis of ligands used for radiolabelling and imaging, of heteromacrocycles and in chelation chemistry. Although there are many approaches for the preparation of racemic compounds, only a few for the enantiomerically pure forms exist [1]. Natural α-amino acids have proven to be versatile substrates for the synthesis of optically active molecules. We have reported the conversion of α-amino acids into chiral 1,2-diamines [2]. We present here a method for the synthesis of selectively protected chiral triamines and vicinal diamines starting from L-lysine and L-glutamic acid.

Boc-Lys(Z)-OH was reduced to the corresponding alcohol. Replacement of the hydroxy group by the azido group, followed by chemoselective reduction afforded compound **1a** isolated as the Fmoc derivative. Each protecting group of the selectively protected 1,2,6-triaminohexane (**1b**) can easily and preferentially be removed. The carboxyl group of Z-Glu(OMe)-OH was reduced and, following the same procedure as described for lysine, derivatives of 4,5-diaminovaleric acid and 4,5-diaminopentane-1-ol (**2**) bearing selectively protected amino groups were prepared.



1a : R = H
1b : R = Fmoc



2a : R = H
2b : R = Boc

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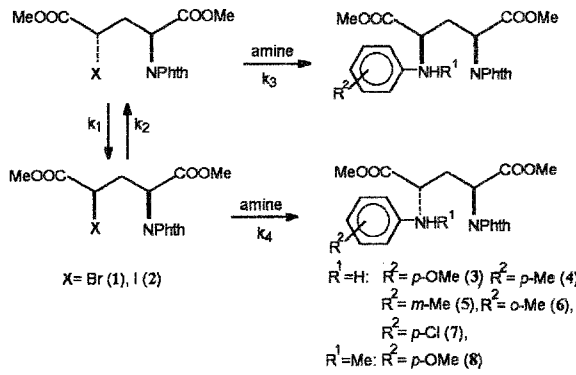
Substitution reaction of 4-haloglutamates with arylamines

Reaction of dimethyl *(2S,4RS)*-N-phthaloyl-4-bromoglutamate (**1**) with arylamines proceeds diastereoselectively and the yield of *threo* isomer is 3–4 times that of *erythro* one. The mechanism of substitution was found to agree closely with classical S_N2 displacement. Kinetic model of this reaction was suggested.

To determine the nucleophile structure effect of relative reactivity of epimers of compounds **1** and **2** ($S = k_3/k_4$) we studied the kinetics of the reaction with substituted anilines using the HPLC method.



X= Br (1), I (2)

R^1=H: R^2= *p*-OMe (3) R^2= *p*-Me (4), R^2= *m*-Me (5), R^2= *o*-Me (6), R^2= *p*-Cl (7),
R^1=Me: R^2= *p*-OMe (8)

The reaction was carried out in acetonitrile at 68 °C, arylamine was in 12-fold excess. k_3, k_4 are observed pseudo-first-order rate constants.

When +M-effect of *para* substituent (R^1 = Me and Cl) decreased k_3 and k_4 values became an order lower. Parameters S for the formation of products **3, 4, 7** from substrate **2** being 3.5, 4.2, 3.9, respectively, provide evidence for the weak influence of amine basicity on diastereoselectivity. S values for forming products **5, 6, 8** were 5.0, 3.1, 4.7.

Maximum value S = 5.0 was observed when substrate 2 reacted with *meta*-toluidine. It is likely that steric interaction of bulky functional groups at C^2-atom with *meta* substituent in benzene ring of arylamine to a great extent causes different reactivity of haloglutamate epimers. A transition state model is suggested.

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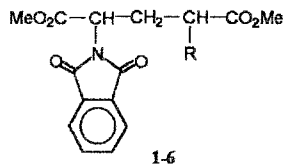
Racemization of 4-arylamino-2-phthalimidoglutarates

Earlier we established that reaction of dimethyl 4-bromo-2-phthalimidoglutarate with substituted anilines proceeded diastereoselectively. As a result the yield of *threo* diastereomer was 3–4 times that of *erythro* one. To determine whether this reaction is kinetically or thermodynamically controlled we studied the racemization of 4-arylamino-2-phthalimidoglutarates (**2-5**) under the action of potassium phthalimide in dimethylformamide at 68 °C.



1-6

R= Br (1), *p*-MeO-C_6H_4-NH- (2), *m*-Me-C_6H_4-NH- (3), *p*-MeO-C_6H_4-N(Me)- (4), *o*-Me-C_6H_4-NH- (5), PhthN- (6)

Equilibrium ratio *threo/erythro* (P) was determined by HPLC method (Silasorb C18, column 64 × 3 mm, detection at 230 nm, eluent hexane-isopropanol 40 : 1 for substrate **2**; hexane-THF 7 : 1 for **3-5**) and for compounds **2-5** was 1.06, 1.14, 1.17, 1.58, respectively.

Maximum P value was observed in the case of compound **5** whose *ortho* CH_3-substituent most intensively interacted with neighbouring groups.

Mechanism of racemization is likely to consist in proton elimination followed by its additon at C^2-atom. Symmetric displacement of phthalimido group does not take place since only the mixture of stereomers **6** was formed from *threo* stereomer of diphthalimido derivative **6** under the action of potassium-2-nitrophthalimide.

Thus equilibrium ratio of stereoisomers of 4-arylamino derivatives **2–5** was significantly lower than that observed in their synthesis from **1,** which provides evidence for kinetic control of the substitution process.

Possible synthetic application of the results obtained is discussed.

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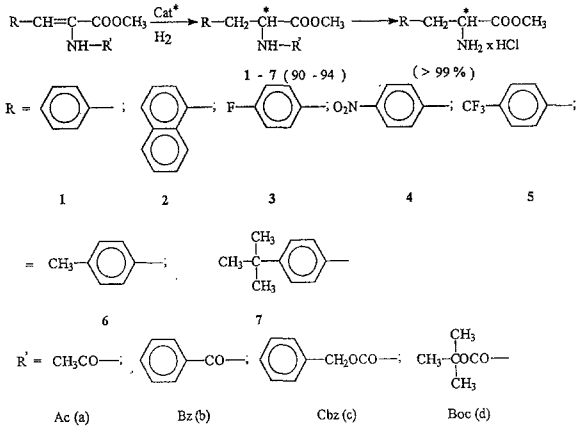
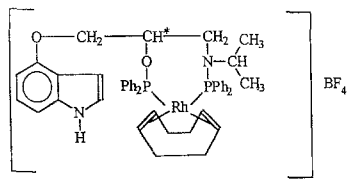
Synthesis of non-proteinogenic (D)- or (L)-amino acids using the new hydrogenation catalyst pindophos-Rh

Pindophos, the aminophosphine phosphinite derivative of the commercial β-blocker Pindolol, has been prepared and used as chiral ligand in the rhodium catalyzed asymmetric hydrogenation of amino acid precursors. The (R)- or (S)-configured rhodium complexes are highly active catalysts leading to L- or D-amino acids. Optical yields between 90 and 95% ee could be realized. After deprotection the hydrochlorides are practically pure.





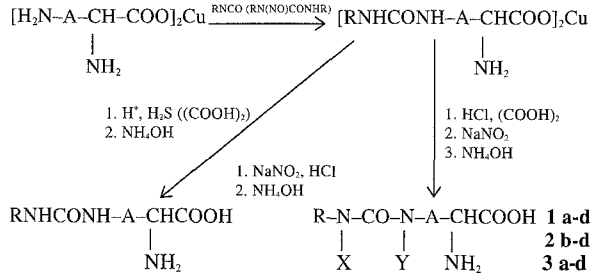
II

G. L. Levit, L. B. Radina, V. F. Gopko, N. M. Peretolchina, and V. P. Krasnov

Institute of Organic Synthesis, Ekaterinburg, Russia

Synthesis of N_ω-alkylnitrosocarbamoyl derivatives of α,ω-diamino acids possessing anticancer activity

The nitrosoureas are among the most active anticancer drugs whose activity is often paralleled with various degrees of toxicity. Linking cytotoxic fragments to carrier molecules is a rational approach to improve anticancer chemotherapy. Besides other compounds a series of amino acids and oligopeptides have been used as such carriers. But most of these compounds were characterized by integration of the α-amino group of the amino acid to the alkylnitrosocarbamoyl (ANC) moiety. We think that binding of ANC moiety to the terminal ω-amino group of α,ω-diamino acids and thus leaving intact amino acid backbone which is significant for the active transport through cell membranes will result in active anticancer agents possessing low toxicity.



a: A = $(CH_2)_2$; **b:** A = $(CH_2)_3$; **c:** A = $(CH_2)_2SCH_2$; **d:** A = $(CH_2)_4$. **1:** R = CH_3, X = NO, Y = H. **2:** R = C_6H_{11}, X = H, Y = NO. **3:** R = $ClCH_2CH_2$; X = H, Y = NO and X = NO, Y = H (mixture of isomers).

We synthesized a series of N_ω-ANC derivatives starting from copper complexes of α,ω-diamino acids (L-α,γ-diaminobutyric acid, L-ornithine, L-4-thialysine and L-lysine) which were carbamoylated with respective alkylisocyanates RNCO (R = CH_3, C_6H_{11}, $ClCH_2CH_2$) or 1,3-dialkyl-1-nitrosoureas followed by decomposition of copper complexes and nitrosation with $NaNO_2$ in dilute HCl. Nitrosation of N-methyl (R = CH_3) or N-cyclohexyl (R = C_6H_{11}) ureas in such conditions yielded appropriate individual nitrosoureas **1** or **2.** However, nitrosation of N-(2-chloroethyl)ureas resulted in mixtures **3** of two nitrosoureas, the one having the nitroso group at the N_ω-position of the starting amino acid (X = H, Y = NO) accounted for the larger amount (~ 75–80% by HPLC and ^{1}H NMR).

The nitrosoureas **1b–d, 2c, d** and mixtures of isomers **3a–d** were examined for their anticancer activity against murine L1210 leukemia and several solid tumours. The compounds were tested at the highest tolerated doses. In general the range of the activity and spectrum of action depended on the structure of alkylating fragments. Thus N-methyl and N-(2-chloroethyl) nitrosoureas showed from moderate to high activity and N-cyclohexyl nitrosoureas from moderate to low one. The structure of amino acid moiety affected the toxicity of tested compounds. For example, the mixtures of isomers derived from L-ornithine (**3b:** A = $(CH_2)_3$) and L-4-thialysine (**3c:** A = $(CH_2)_2SCH_2$) when compared with that from L-lysine (**3d:** A = $(CH_2)_4$) were more toxic without enhancing their activity in the case of **3b** and with activity decrease in the case of **3c,** the isometric composition of the mixtures being equal.

The mixture of isomers **3d** derived from L-lysine has demonstrated the best therapeutic properties at doses of 175–200 mg/kg among examined compounds.

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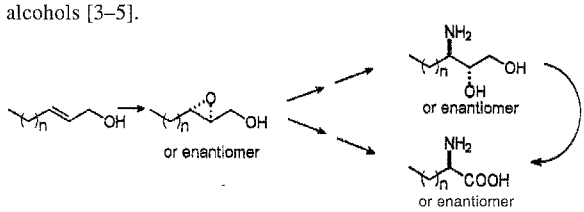
An approach to the enantiomeric synthesis of lipidic α-amino acids

The lipidic amino acids (LAAs), non-natural α-amino acids with long alkyl side chains, and their homo-oligomers, the lipidic peptides, represent a class of compounds which combine structural features of amino acids and peptides with those of lipids. Of particular interest is their use as a drug delivery system [1], as an adjuvant/carrier system and as starting material for the synthesis of biologically interesting compounds [sphingosin and ceramide analogs and lipidic 1,2-diamines [2], inhibitors of phospholipase A_2]. Racemic LAAs are prepared by reacting 1-bromoalkanes with dialkyl acetamidomalonate, followed by hydrolysis and partial decarboxylation of the intermediate and can be resolved enzymatically or by chemical methods.

We present in this communication an approach to the enantiomeric synthesis of lipidic α-amino acids and 3-amino-1,2-diols based on the regioselective opening of chiral 2,3-epoxy alcohols [3–5].



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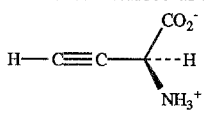
New developments in the synthesis of optically active ethynylglycine derivatives

Ethynylglycine **1** (FR 900130) is a naturally occuring unusual α-amino acid isolated from fungus *Streptomyces Catenulae* in 1980 [1]. This notoriously labile compound is obtained as an hygroscopic 70% pure powder in a very low yield, and has been characterized as its N-acetyl derivative.

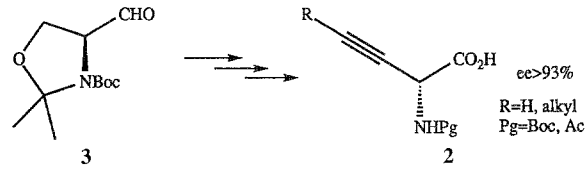


1

It displays antimicrobial activity against gram positive bacteria which could be explained by its inhibitory activity on L-alanine racemase [1–3].

Much remains to be known about the biological properties of β,γ-acetylenic α-amino acids. Since more stable protected derivatives of **1** have only been described in racemic form [4], a general synthetic route to optically active β,γ-acetylenic α-amino acids **2** had to be found. We have achieved this challenge from protected L-serinal **3** as a configurationally stable optically pure starting material.

Our latest results will be presented [5].



3 **2**

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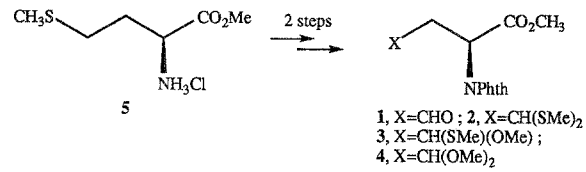
Synthetic uses of methionine, serine and threonine in the synthesis of unusual α-amino acids and amino alcohols

The synthetic utiliy of aminoacids for the generation of chiral reagents, intermediates and final products including amino acids and amino alcohols is now well documented.

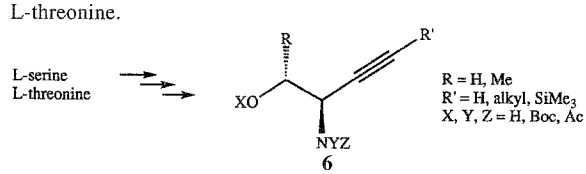
Derivatives of (S)-2-amino-4-oxobutyric acid (aspartic acid β-semialdehyde) are interesting chiral intermediates for the synthesis of biologically relevant molecules of wide interest like nicotianamine and analogues, which are iron chelating agents [1], or naturally occuring unusual α-amino acids [2] among others. We have achieved a straightforward synthesis of aldehyde **1** and derivatives **2, 3, 4,** from methionine methyl ester **5**.



5

1, X=CHO ; **2**, X=CH(SMe)$_2$
3, X=CH(SMe)(OMe) ;
4, X=CH(OMe)$_2$

Substituted optically active propargylic amines are also useful synthetic intermediates [3] in the synthesis of biologically relevant molecules like oxotremorine [4] or GABA [5] analogues. Some new protected optically active α-amino β,γ-acetylenic alcohols **6** have been synthesized from L-serine and L-threonine.



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Biphenyl analogues of AP-7, a new class of competitive N-methyl-D-aspartate(NMDA)-receptor antagonists

By restricting the conformational flexibility of (2R)-2-amino-7-phosphonoheptanoic acid (D-AP-7), the "prototype" of a competitive NMDA antagonist, a number of more active and selective representatives was found, e.g. (R)-4-(3-phosphonopropyl)-piperidazine-2-carboxylic acid (D-CPP), (R,E)-4-(3-phosphonoprop-2-enyl) piperidazine-2-carboxylic acid (D-CPP-ene). Insertion of a phenyl ring in the middle of the chain of the AP-7 molecule does not reduce the NMDA antagonistic activity but inverses enantioselectivity from R to S, whereas the addition of a second phenyl ring further increases both potency and enantioselectivity [W. Müller et al., Helv. Chim. Acta 75, 855 (1992)].

A series of substituted biphenyl analogues of AP-7 were synthesized and their NMDA antagonistic activity determined by measuring inhibition of NMDA-induced depolarization in a rat cortical wedge preparation (CWP) and affinities in a [3H]CGP-39653 binding assay. Structure-activity relationships (SAR) show that attachment of a hydroxyl group at position 4 in the inserted phenyl ring as well as introduction of a chloro substituent in the ortho position of the additional phenyl ring brings an increase of activity by a factor of 6 to 8 independent of other substituents at the biphenyl skeleton. Expansion of the angle between the planes of the phenyls caused by orthosubstituents seems to be an important parameter for NMDA antagonism *in vitro* and *in vivo*. In fact some representatives with this substitution pattern show good bio-availability and activity in pharmacological *in vivo* tests (e.g. maximal electroshock test) suggesting a central mechanism of action.

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Synthesis and properties of p-guanidinophenylesters derived from amino acid and peptides

Previously we reported that p-guanidinophenyl esters behave as specific substrate for trypsin and trypsin-like enzymes. In these esters the site specific group for the enzyme (cationic group) is included in the leaving portion instead of being in the acyl moiety-"inverse substrate". In an extension of the applicability of "inverse substrates", we found that p-guanidinophenyl esters derived from N-blocked amino acid and N-blocked peptide were useful substances for the enzymatic peptide coupling method. However, difficulty arose in the preparation of these esters. Coupling reaction of N-blocked guanidinophenol and N-blocked amino acid (or peptide) derivatives was not successful because of the diminished nucleophilicity of guanidinophenol hydroxy group.

Alternative route for the preparation of the p-guanidinophenyl esters has been investigated and the following procedure has been shown to be useful for the purpose. At the first stage of the reaction route, Boc-amino acid p-aminophenyl ester was prepared. Boc-amino acid p-aminphenyl ester was easily prepared by catalytic hydrogenation of the corresponding p-nitrophenyl ester. Boc-amino acid p-aminophenyl ester was treated by amidination agent 1-(N,N'-bis-Z-amidino) pyrazole to give p-N,N'-bis-Z-guanidinophenyl ester. Deblocking of Z group at the guanido group was carried out by catalytic hydrogenation and desired compound was obtained as p-toluenesulfonate. Preparation of Boc-peptide p-guanidinophenyl esters was successfully carried out in a same manner.

Kinetic parameters of p-guanidinophenyl esters for trypsin catalyzed reaction were determined. The esters were shown to behave as specific substrate for the enzyme and the reaction followed Michaelis-Menten equation. Applicability of the esters for the peptide synthesis will be discussed.

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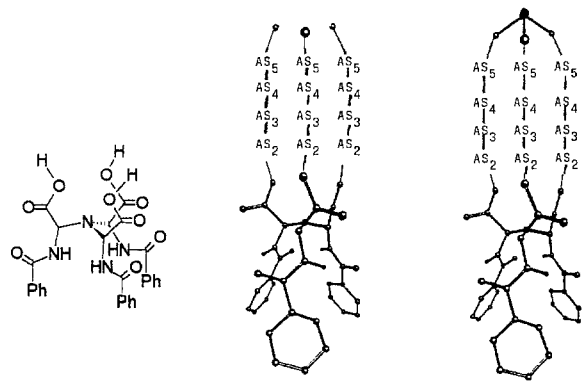
Synthetic amino acids and peptides with defined metal-binding sites

We developed a new template [1] for the synthesis of peptides with well-defined structures [2, 3]. The synthesis of this template is straightforward [4]. Reaction of methyl N-benzoyl-2-bromoglycinate with ammonia yields a molecule with three glycine-residues connected by a nitrogen atom. The stereochemistry follows from the X-ray structure analysis. The conformation is stabilized by three intramolecular hydrogen-bonds. The corresponding triacid can be connected with various peptides. The structures of these peptides are determined by the conformation of the template. The peptide chains adopt a C_3-symmetrical helical structure stabilized by intramolecular hydrogen bonds. Preorganization of these compounds can be used for the rational design of synthetic peptides with defined metal binding sites [5].



template | preorganized peptide-bundle | metal-complex

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Taurine

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Effects of ammonia on taurine release from glial cells in culture

Pulse treatment with ammonium ions at concentrations reached in brain in acute hyperammonemic conditions (0.05–5 mM) stimulates the release of newly loaded radiolabelled taurine (TAU) from cultured cortical astrocytes (Albrecht et al., Brain Res. 660, 288, 1994) and Müller cells (Faff-Michalak et al., Glia 10, 114, 1994). The ammonia-induced release, comparable in magnitude to that accompanying high (65 mM) K^+ or hypotonicity-induced cell swelling is thought to counteract ammonia-induced neuronal disinhibition, in line with the postulated neuromodulatory, gliotransmitter role of TAU. Here we show that ammonia releases TAU by a mechanism that differs from the release elicited by K^+ in that it is largely Cl^--independent (insensitive to furosemide), unaffected by conditions modulating the intracellular level and/or transport of Ca^{2+}, and insensitive to changes in medium tonicity. However, ammonia-induced TAU release shows a good correlation with the increase of cAMP levels (both are dose-dependent in the 0.5–5 mM range). The release is attenuated by compounds that reduce generation of cAMP by ammonia and by an inhibitor of cAMP-dependent protein kinase. The results suggest that ammonia-induced TAU release is mediated by cAMP, in which it resembles TAU release following β-adrenergic stimulation of different types of glia (cf. Martin, Glia 5, 81, 1992). The release of TAU may be causally related to cAMP-dependent phosphorylation of either/or: a) some as yet unidentified cytoskeletal proteins, b) a TAU carrier c) an ion channel permeable to TAU. (Supported by SCSR grant no 6P 207 05905.)

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Role of taurine in the regulation of luteinizing hormone secretion. Studies in immature and adult female rats

Taurine (2-aminoethanesulphonic acid, Tau) acts as a putative inhibitory amino acid neurotransmitter (or, at least, neuromodulator) in the brain of different vertebrate species, exerting a hyperpolarizing effect probably caused by changes in Cl^- conductance. Also in coincidence with γ-aminobutyric acid (GABA), Tau has been shown to affect hypothalamic/pituitary hormone secretion in rodents, e.g.: 1) somatostatin release by median eminence tissue is increased by Tau; 2) Tau inhibits stimulated luteinizing hormone (LH) release; 3) a stimulatory effect of this amino acid on prolactin secretion has been described.

In an attempt to further characterize the effects exerted by Tau on LH release in female adult rats, we performed, initially, in vitro studies. A 5 h incubation with Tau (10^{-3} – 10^{-8} M) did not affect basal of LHRH-stimulated LH release by cultured anterior pituitary cells. Basal LHRH release from superfused mediobasal hypothalamic fragments was not affected by Tau (10^{-3} M). However, this substance clearly diminished LHRH release after stimulation with KCl (50 mM) or N-methyl-D-aspartate (10^{-4} M).

As regards to the role played by Tau in sexual development, we demonstrated that, in female rats, the hypothalamic concentrations and release of Tau diminish from postnatal day 20–25 onwards. Given the inhibitory effect of Tau on LH secretion, this decrease might contribute to sexual development (taking place around day 30–35). Hence, investigations were designed to assess the effect of preventing the fall in hypothalamic Tau levels on LH secretion in peripubertal rats. Tau supplementation was achieved with daily intracerebroventricular injections of this amino acid (0.15 μmoles), administered during postnatal days 23–29. Treatment with Tau lowered serum LH (Tau: 0.20 ± 0.04; Controls: 1.04 ± 0.21 ng/ml; mean ± SEM; $p < 0.05$) as well as hypothalamic LHRH levels (Tau: 82.6 ± 9.5; Controls: 128.7 ± 14.1 pg/mg wet tissue, $p < 0.05$). Tau treatment doubled hypothalamic GABA levels (Tau: 31.3 ± 2.9; Controls: $15.6 \pm 1,2$ nmoles/mg wet tissue, $p < 0.001$). In a second group of similarly treated animals, Tau delayed vaginal opening by more than two days ($p < 0.05$ vs. Controls).

It is concluded that Tau exerts an inhibitory effect on LH secretion acting at the hypothalamic level, by diminishing stimulated LHRH release. As regards to the possible role of Tau in sexual development, hypothalamic Tau levels decrease significantly prior to sexual maturation, and the supplementation with Tau during the fourth postnatal week reduces LHRH/LH secretion and postpones vaginal opening, perhaps by increasing the activity of the hypothalamic GABAergic system.

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Dietary protein influences taurine requirement in cats

Determining dietary taurine requirement of cats is complicated for two reasons: 1) taurine requirement varies with diet composition, and 2) the nature of ingredients that affect taurine requirement are unknown. We have recently found evidence indicating that the concentration and quality of protein in diets given to cats affects taurine requirement. Plasma taurine concentration was decreased by 70% in cats (n = 4) given for 6 wks a purified diet containing 0.1% taurine and 50% soybean protein isolate. In cats (n = 4) given for 6 wks the same diet but with 25% protein, taurine concentrations were unchanged. In other experiments with purified diets, taurine depletion was observed when soybean protein isolate, gelatin, or casein cooked with glucose were substituted for a more digestible protein, casein, at

concentrations ranging between 43 and 60%. Apparent nitrogen digestibilities of the soybean protein diets (≈ 92%) were less than those found when the taurine-sustaining, casein, diets (≈ 95%) were fed. Experiments with commercial canned-type diets containing 0.15% taurine and 51 to 56% crude protein showed that the thermal processing of canning decreased nitrogen digestibilities from ≈ 92 to ≈ 84% and exacerbated taurine depletion. These studies indicated that taurine requirement is positively correlated with dietary protein content and negatively correlated with protein digestibility. We suggest that taurine requirement is largely determined by peptides which escape digestion and stimulate microbial deconjugation of taurocholate and degradation of taurine.

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Amino acid transport in renal cells secreting taurine: Characteristics and regulation

Taurine can be reabsorbed and/or secreted by the renal tubules of vertebrate kidneys. Mammalian kidneys physiologically exhibit only net reabsorption of taurine, whereas the kidneys of marine teleost fishes exhibit only net secretion of this amino acid. Ophidian reptiles demonstrate both net reabsorption and net secretion of taurine in vivo. In reptilian kidneys where taurine can undergo bidirectional renal transport, concurrent studies using purified membrane vesicles from both renal brush-border and basolateral membranes have been carried out. There are high-affinity secondary active cotransport systems for taurine on both luminal and basolateral membranes that are Na^+ und Cl^- dependent but otherwise characteristically distinct. The luminal taurine uptake system is electrogenic with a stoichiometry of 3 Na^+: 1 to 2 Cl^-:1 taurine and has high affinity for both Na^+ and Cl^-. The basolateral system is non-electrogenic, exhibiting a stoichiometry of a 1 Na^+ :1 Cl^-:1 taurine with much lower affinity for Na^+ and Cl^-. Only the luminal taurine transport system is stimulated by a Cl^- gradient. In addition, the luminal taurine transporter has slightly higher affinity for taurine but half the capacity of the basolateral taurine transporter. Taurine efflux across the basolateral membrane, occuring via a mediated process, is higher than that across the luminal membrane. Net transepithelial reabsorption of taurine is accomplished through the high-affinity luminal taurine uptake system coupled with passive, mediated basolateral efflux. The basolateral taurine cotransporter could also facilitate basolateral taurine efflux when intracellular taurine concentration is high. The membrane mechanisms for transepithelial taurine secretion are not yet well characterized. These mechanisms have been partially elucidated in the kidneys of teleost fishes that can secrete taurine, using primary monolayer cultures of the winter flounder renal proximal tubule cells. The cultured epithelium displays net taurine secretion when short-circuited in Ussing chambers. Taurine secretion (cell to lumen) appears to be mediated, partly, through a luminal transporter that is sensitive to bromocresol green and probenecid but distinct from the luminal Na^+-taurin cotransporter. In nonmammalian vertebrates that secrete taurine, renal taurine excretion is modulated by changes in plasma taurine concentration associated with a change in extracellular osmolality. A decrease in extracellular osmolality and the resultant increase in plasma taurine concentration (because of cellular volume regulation) are associated with increased net taurine secretion in fishes in vivo. Acute exposure (2 h) of the flounder proximal tubule cell cultures to hypoosmotic media also increases taurine secretory flux with no effect on the reabsorptive flux. The enhanced taurine secretory flux in cultured renal monolayers is associated with a decrease in basolateral membrane apparent permeability to taurine as well as an increase in the luminal membrane apparent permeability. The response of the taurine transport systems to extracellular osmolality in nonmammalian proximal renal cells is quite different from that observed in mammalian cells, where only the basolateral taurine transport system of the distal nephron exhibits the response. (Supported by National Science Foundation.)

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Taurine modulation of calcium and sodium transport in heart and vascular endothelial and smooth muscle cells

Taurine has been reported to exhibit beneficial effects in heart failure as well as calcium overload by affecting Ca^{2+} influx as well as Ca^{2+} release. However, little is known concerning the action of taurine on vascular endothelial and smooth muscle (VSM) cell function. In this work, we studied the effect of taurine on different ionic channels of heart and VSM cells using the whole cell voltage clamp techniques as well as cytosolic and nuclear Ca^{2+} and Na^+ concentrations using microfluorometry and confocal laser microscopic techniques. In heart cells of both human and chick heart cells, taurine increased R- and T-type Ca^{2+} channels and decreased K^+ current without affecting the L-type Ca^{2+} channel and the TTX-sensitive fast Na^+ channel. Short exposure to taurine increased cytosolic and nuclear $[CA^{2+}]_i$ without affecting $[Na]_i$. However long term exposure to taurine had no significant effect on cytosolic $[Ca^{2+}]_i$ but decreased nuclear $[Ca^{2+}]$. In aortic endothelial vascular smooth muscle cells of human and rabbit, taurine stimulated the R-type Ca^{2+} channel and decreased I_K and increased tension of rabbit aortic VSM. These results highly suggest that the action of taurine on heart muscle is not only mediated by affecting sarcolemic Ca^{2+} transport, but also affects nuclear Ca^{2+} transport in both heart and vascular endothelial and smooth muscle cells. (Supported by MRCC grant PG-12350.)

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The influence of taurine on various parameters of hearing development

The presence of high levels of taurine in the cochleas of animals [1] suggest a biological function in hearing. Its distribution within the organ of Corti remains uncertain [2, 5] as does its function. It is most unlikely to be a neurotransmitter but a role as a membrane stabiliser, a modulator of calcium channels

or an osmotically active agent controlling the elasticity of the basilar membrane are all possibilities.

Tyson et al. [4] have suggested that dietary taurine levels are influential in the development of hearing in pre-term babies but the taurine supplementation of baby food remains a contentious issue [3].

We have used various audiological parameters to assess the influence of diets containing various taurine supplements on the hearing development of normal babies. Auditory brain stem evoked potentials (ABRs) and transient evoked oto-acoustic emissions have been used to measure the integrity of different parts of the auditory system including the two different types of hair cells in the organ of Corti.

Three groups of babies have been studied over a period of three months, one group received a normal casein/whey diet supplemented with 3 mg percent of taurine, one received the same diet supplemented with 35 mg percent of taurine and the third group were breast fed, assumed to be variable and containing an intermediate level of taurine.

Data will be presented on the peak latencies and inter-peak intervals of the ABRs and the frequency and intensity characteristics of the oto-acoustic emissions.

(We gratefully acknowledge the financial support of Action Research and Cow and Gate Ltd.)

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Urinary taurine excretion after partial hepatectomy (PH)

Background: Administration of various hepatotoxic agents to rats increased the urinary excretion of taurine (TAU) significantly (Waterfield et al. 1993, Toxicology 77:1–5). We investigated the TAU excretion after PH.

Methods: Male wistar rats (250–350 g) were kept in metabolic cages. Under ether anesthesia, the median and left lateral lobe were surgically removed. Control groups consisted of pairfed SHAM-operated and of untreated (NORM) rats. Twenty-four and 48 h after surgery, the TAU concentration was determined in urine and in normal and regenerating liver tissue using a HPLC technique. Creatinine was measured with a Boehringer kit.

Discussion: During the first 24 h after PH the total amount of TAU excreted in urine increased +155%. This was accompanied by a 50% reduction in the TAU concentration in the remaining liver tissue. No significant changes in urinary TAU excretion were observed in the pairfed SHAM-operation animals, which seems to exclude ether anesthesia, laparotomy or (decreased) food intake as possible causes. After 48 h, the TAU concentration in the liver of PH animals had returned to NORM values. This suggest that after PH hepatic TAU synthesis and/or uptake is increased.

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Changes in rat tissue taurine levels following administration of clenbuterol

The β-amino acid taurine is believed to be important in the functioning of many mammalian tissues, especially the myocardium, where it is found in especially high concentrations. Although the physiological role of taurine in the heart remains unclear, it is thought to play a protective role [1]. However, recently we have found, exposure to β-agonist drugs, may be important in affecting tissue taurine levels [2, 3]. Previous studies with a related drug, isoprenaline (isoproterenol), a less selective β-agonist than clenbuterol, have shown a marked dose related decrease in cardiac taurine levels after a single dose of this drug were given to rats [4]. Therefore, the objective of the current study was to investigate the effect of clenbuterol on taurine levels in the heart and other tissues in relation to dose and time.

Female Random-Hooded (RH) rats (150 to 200 g) housed in communal cages were given a single subcutaneous (sc.) dose of clenbuterol (250 μg/kg). Control animals received saline. After designated time periods (1–24 hr), animals were killed and heart, lung, liver, serum and muscle were removed, weighed and taken for (taurine and biochemical) analysis. During the course of the experiment, no food was given to the animals; water was given *ad libitum*. Taurine was measured by HPLC. At 3 hr post clenbuterol administration, the taurine content of the heart decreased significantly and remained lower until 12 h after clenbuterol treatment, returning to control values by 24 hr. The content of taurine in the liver increased at 3 hr after clenbuterol administration but was lower than the control value at 24 hr post administration. Lung taurine levels were significantly lower than control at 12 hr post dose and remained depressed at the 24 hr. Clenbuterol did not seem to have a significant effect on taurine levels in muscle, or serum.

Results:	NORM	PH-24	PH-48	SHAM-24	SHAM-48
Urine:	(n =8)	(n = 12)	(n = 10)	(n = 12)	(n = 10)
volume (ml/24h)	21 ± 3	11 ± 2*	15 ± 1	6 ± 1*	9 ± 1*
conc TAU (mM)	4.8 ± 1.4	25.6 ± 7.4	4.8 ± 1.3	12.5 ± 3.7	11.4 ± 3.4
total TAU (μmol/24h)	86 ± 19	219 ± 32*	55 ± 7	63 ± 12	79 ± 15
total creat (μmol/24h)	1293 ± 207	855 ± 86	1129 ± 62	891 ± 72	963 ± 53
ratio TAU/creat	.072 ± .017	.292 ± .103*	.048 ± .008	.080 ± .012	.074 ± .011
Liver:					
weight (g)	9.1 ± 0.2	4.7 ± 0.2*	5.6 ± 0.2*	8.9 ± 0.3	7.9 ± 0.5
conc TAU (μmol/g)	5.5 ± 0.9	2.7 ± 0.5*	5.4 ± 0.7	5.1 ± 0.9	7.4 ± 1.1

Values are mean ± S.E.M. Significant from NORM $p < 0.05$: *

Clenbuterol was administered as a single sc. dose of 63, 125, 250, 375 or 500 μg/kg to female RH rats (150 to 250 g), housed in communal cages. Control animals received saline. At 12 hr post dose animals were killed and the heart, lung, liver, serum and muscle were removed and weighed and taken for analysis as above. Taurine levels in the heart were significantly lower than controls at all doses studied except at the lowest dose. The reduction in taurine however was not dose dependent. Liver taurine levels increased at all doses used and were significant at the highest dose of 500 μg/kg. Lung taurine levels were significantly reduced at all doses. There was no significant effect on taurine levels in muscle.

The significant decrease in the level of taurine in the heart tissue could be important in view of the clinical use of this drug and especially its abuse by athletes.

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A long-lasting increase of synaptic potentials induced by taurine

On the bases of electrophysiological studies, taurine has been envisaged as an inhibitory substance acting on $GABA_A$ or glycine receptors (see Huxtable, 1989, for review). There are, however, very few reports studying if taurine could modulate synaptic potentials. Our aim has been to assess the possible modulatory actions of taurine on synaptic potentials in the CA1 area, using rat hippocampal slices.

Field extracellular excitatory postsynaptic potentials (EPSP) were evoked by electrical stimulation of Schaffer collateral fibers. Bath application of taurine (10 mM, 30 min) induced two opposite actions on these synaptic responses: a decrease of fEPSP slope prevented by $GABA_A$ antagonists, and a long-lasting potentiation of the fEPSP, independent on $GABA_A$ or NMDA receptors activation. Two long-lasting processes accounted for this taurine-induced potentiation: 1) an increase in synaptic efficacy that is not accompanied by modifications in the postsynaptic membrane electrical properties, nor by those prosynaptic changes involved in fEPSP paired-pulse facilitation, and 2) an increase in the fiber volley amplitude explained by a reduction in the threshold for antidromic action potential generation. Furthermore, this taurine induced fEPSP potentiation seems to be dependent on the previous taurine uptake into intracellular compartments, as judged by saturation experiments, and also by its dependence on temperature and extracellular sodium.

These data describe a new action of taurine on synaptic transmission, and indicate that intracellular taurine levels couls modulate, perhaps interacting with voltage-dependent Na^+ channels, synaptic efficacy and axon excitability.

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Procollagen gene expression is down-regulated by taurine and niacin at the transcriptional level in the bleomycin-hamster model of lung fibrosis

Taurine and niacin have previously been found to block the accumulation of collagen in lung in the multidose bleomycin (BL) hamster model of pulmonary fibrosis (PF). This study was designed to evaluate if taurine and niacin would block the increases in procollagen I and III mRNA levels in the same model of PF. Hamsters were intratracheally (IT) instilled with three consecutive doses of saline or BL at weekly intervals (2.5, 2.0, 1.5 units/5ml/kg). Animals were fed diet containing either 2.5% taurine and 2.5% niacin or the same diet without the drugs throughout the experiment. The four groups were saline-instilled with the control diet (SCD), saline-instilled with taurine-niacin in diet (STN), BL-instilled with control diet (BCD), and BL-instilled with taurine-niacin in diet (BTN). Steady-state transcript levels in the total RNA prepared from lungs of all 4 groups were determined at 0, 3, 7, 14 and 21 days after the BL-instillation by slot blot and Northern blot analyses. Results indicated that the procollagen I mRNA level was elevated as compared to saline control by 1.5, 2.25, 1.75, 1.6, and 1.5 fold at 0, 3, 7, 14, and 21 days after the last IT instillation, respectively. Treatment with taurine and niacin decreased the steady state level of BL-induced increase of procollagen I mRNA gradually from day 0 through 21. However, it showed maximal inhibition at day 21. We observed a similar pattern of procollagen III inhibition by combined treatment with taurine and niacin which decreased the abundance of this mRNA from day 7 through day 21. Nuclear run-off analysis was performed to determine if the inhibitory effects of taurine and niacin on BL-induced procollagen I and III mRNA accumulation and protein production were mediated by alterations in procollagen gene transcription. Transcription of procollagen I and III genes was readily detected in nuclei prepared from BL-treated lung samples. In contrast, transcription of procollagen I and III genes was barely detectable in nuclei prepared from BL+taurine and niacin treated lungs. Control groups SCD and STN also exhibited the levels of gene transcription similar to the BTN group. There was no alteration in the rate of transcription of the gene encoding for 18S rRNA in the lung nuclei of hamsters treated either with BL or BL+taurine and niacin. Taurine and niacin treatment also caused a significant reduction in the BL-induced increase in the hydroxyproline content from 1734 μg/lung to 1052 μg/lung at 14 days and from 2204 μg/lung to 1285 μ/lung at 21 days. Our results suggest that procollagen I and III gene expression in BL-induced lung fibrosis of hamsters are transcriptionally down-regulated by combined treatment with taurine and niacin. (Supported by NHLBL#RO1 HL-27354)

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Cytoprotective mechanisms of L- or D-cysteine and hypotaurine against xenobiotic toxicity

The role of cellular glutathione in protecting macromolecules from toxins is well recognized. Depleting cellular GSH levels markedly increases hepatocyte susceptibility to toxins which on metabolism form alkylating species or reductively

activate oxygen to reactive oxygen species including H_2O_2. The much smaller pool of L-cysteine needed for GSH biosynthesis plays another role by supplying inorganic sulfate for cunjugating and detoxifying phenolic toxins. Sulfate is believed to be formed from cysteine via cysteine sulfinate. However using hepatocytes we have discovered the following: 1) Sulfate is formed much more effectively from L-cysteine or D-cysteine than cysteine sulfinate. Furthermore transaminase inhibitors prevented sulfate formation from L-cysteine or L-cystine but not D-cysteine whereas D-amino acid oxidase inhibitors prevented sulfate formation from D-cysteine. Thus sulfane sulfur formation from cysteine or cystine is the preferred pathway for inorganic sulfate formation. 2) L- and D-cysteine or L-cystine but not cysteine sulfinate also prevented cyanide and nitrile induced cytotoxicity and markedly increased thiocyanate formation. Transaminase inhibitors prevented thiocyanate formation from L-cysteine whereas D-amino acid oxidase inhibitors prevented thiocyanate formation from D-cysteine. Thus, sulfane sulfur formation from cysteine via cystine also represents an important pathway for the detoxification of cyanide. 3) Cysteine sulfinate markedly prevented H_2O_2 or CCl_4 toxicity but not in the presence of decarboxylase inhibitors. Hypotaurine but not taurine was also markedly cytoprotective. It can be concluded that hypotaurine formation from cysteine via cysteine sulfinate represents an important novel intracellular antioxidant pathway.

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Taurine metabolism in experimental ischaemia/reperfusion (I/R) injury of skeletal muscle and its clinical relevance

Taurine concentrations (μmol/dm^3) in plasma (TCP) and in muscle cells (TCM) were investigated in New Zealand white rabbits. 1 h (group A) or 2.5 h (group B) of bilateral hindlimb ischaemia followed by 2 h of reperfusion were compared to sham operated animals. Venous blood and hindlimb muscle tissue samples were deproteinized, separated by HPLC and determined with OPA.

No significant changes of TCP were observed in animals of group A and B at the end of ischaemia, as well in group A during reperfusion. The elevated TCP were expressed in animals of group B one hour (133 ± 59.3 vs 65 ± 23.7 in control, $p < 0.05$) and 2 h (161 ± 70.8 vs 72 ± 35.9 in control, $p < 0.01$) after reperfusion. TCM showed no significant changes in groups A and B, as compared to control value (700 ± 130).

Elevation of TCP could be a result of leakage of taurine from the muscle cells which seems to be dependent on the duration of the preceding ischaemia. We did not find a significant depletion of cell taurine content possibly due to high concentration gradient of taurine.

TCP was investigated in the patients with intraoperative temporary clamping of magistral arteries. Five from six patients with peripheral arterial occlusive diseases of lower limbs (Fontaine 2b stage) or popliteal aneurysm showed an evident increase of TCP at the end of ischaemia or after the reestablishment of blood circulation.

Monitoring plasma taurine concentration, as a sensitive marker of cell membrane leakage during ischaemia/reperfusion injury, may provide useful diagnostic or prognostic information.

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Comparison of depolarization-evoked release of taurine from synaptosomes of olfactory bulb and cerebral cortex in rat

Taurine (Tau) has been postulated to play an important role as a neurotransmitter or modulator in the central nervous system. Therefore, the possibility of depolarization-evoked release of taurine from nerve terminals were examined using a superfusion of synaptosomes prepared from rat olfactory bulb. In addition, the effects of taurine on release of endogenous glutamate (Glu), aspartate (Asp), and γ-aminobutyrate (GABA) were investigated and compared with those in cerebral cortex. Contents of released amino acids were determined by HPLC after a precolumn derivatization. Tau content and basal releasing rate from synaptosomes were the highest in the olfactory bulb (83.3 nmol and 110 pmol/min per mg protein, respectively). However, the 2-min stimulation with high KCl evoked only 1.2-fold increase in release of Tau (130 pmol/min). On the other hand, the depolarization cuased 1.5- to 2.6-fold increases in release of Glu, Asp and GABA from the synaptosomes. The addition of Tau (10 μM9 significantly reduced the depolarization-evoked increase in GABA release by 63%, but not those in Glu and Asp release, differing from the results in cerebral cortex. The reduction in GABA release was attenuated by phaclofen (10 μM, a GABA-B antagonist) but not by bicuculline (10 μM, a GABA-A antagonist). These data suggest that Tau may interact upon GABA-B autoreceptors located at the nerve terminals of GABAergic neurons to regulate the release of the inhibitory amino acid transmitter.

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The effect of taurine on the contractile response of the isolated rat trachea

Taurine (2-aminoethane sulphonic acid) is a stable sulphur containing amino acid whose possible role(s) in mammals has received much attention, but whose precise role(s) remains to be fully elucidated. Although being present in relatively large quantities in the lung, little attention has been directed towards its function in this organ. One possibility, is that it may modulate the contractility of respiratory smooth muscle. Certainly, it is able to modulate the contractile responses of both gastrointestinal and vascular smooth muscle. With this in mind we decided to investigate the effect of taurine on acetylcholine (ACh) induced contractions of isolated rat trachea.

Experiments were performed on male adult rats (400–500 g) killed by stunning and exsanguination. The trachea was rapidly removed and placed in a petri dish containing Krebs-Henseleit solution (KHS) aerated with 95% O_2/5% CO_2. The trachea was cleaned, mounted between two glass cannulas and placed in a 250 ml organ bath containing KHS maintained at 37 °C. The trachea and glass cannulas were filled with KHS, one cannula was closed to the atmosphere via a three way tap whilst the other was connected to a pressure transducer. Contractile responses were recorded as increase in intra-luminal pressure. Cumulative dose response curves to ACh were obtained in the absence and presence of taurine (1, 2, 5 and 10 mM). In a further series of

experiments, the effects of 5 mM taurine on the dose response curve to ACh were investigated in a calcium free KHS.

The Table 1 shows the effect of taurine on EC_{50} values for ACh. Results are reported as the mean +/– SEM of six experiments.

In a further series of four experiments, the effect of taurine (5 mM) in normal and calcium free KHS were investigated. The Table 2 shows the percentage maximal responses (mean +/– SEM) evoked by ACh at a final bath concentration of 512×10^{-8} M. All percentages are calculated against the maximal responses occurring in normal KHS with taurine absent.

In conclusion, we have demonstrated that taurine inhibits the contraction of respiratory smooth muscle by a mechanism that appears to be calcium dependent. The precise mechanism by which this occurs remains to be elucidated.

Table 1

Taurine (mM)	0	1	2	5	10
EC_{50} (10^{-7}M)	6.78 +/– 1.02	10.06 +/– 1.22	12.91 +/– 1.16	20.99 +/– 3.87	21.94 +/– 4.13

An analysis of variance showed that the EC_{50} values obtained in the presence of 2, 5 and 10 mM taurine were significantly different from the control EC_{50} values ($p < 0.05$).

Table 2

	Normal KHS		Ca^{++} Free KHS	
	Control	Taurine (5 mM)	Control	Taurine (5 mM)
% Max. Response	100	69.5 +/– 4.3	29.0 +/– 6.2	23.5 +/– 6.8

An Analysis of variance indicated that in normal KHS, taurine (5 mM), significantly reduced the mean maximal response ($p < 0.5$). However, in calcium free KHS, the presence of taurine (5 mM), did not significantly reduce the mean maximal response ($p > 0.05$).

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The effects of taurine on perfused heart muscle malondialdehyde levels

1. Taurine is found as a free amino acid in plasma and many tissues such as heart, muscle, brain and blood. Its exact role is not fully defined but it appears to have an important effect on the function of the heart.

2. The purpose of this study was to investigate the effects of taurine (10^{-3} M and 10^{-2} M) on malondialdehyde levels in perfused heart muscle.

3. In this study we found that taurine administered before anoxia and reperfusion increased the malondialdehyde levels but taurine administered after anoxia decreased malondialdehyde levels in perfused heart muscle compared to the taurine administered group before anoxia.

4. Anoxia did not change the perfused heart muscle malondialdehyde levels.

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Relationship of dietary taurine to reproductive efficiency in the queen

Taurine-deficient reproductive failure in cats is manifested by fetal resorptions, abortions, stillbirths and low birthweight kittens. The dietary taurine requirement for feline reproduction is not defined. The dietary requirement to maintain adequate plasma or whole blood taurine is dependent upon protein source and method of diet processing. This study evaluated the taurine requirement for reproduction in queens using a dry-expanded diet. Taurine status and reproductive performance were evaluated in 53 queens fed 0.01%, 0.04%, 0.06%, and 0.09% taurine. Blood and tissue samples were collected to monitor taurine status. Conceptions, in utero resorptions, and abortions were monitored using ultrasound. Plasma taurine concentration at breeding was significantly greater ($P < 0.05$) in the 0.09% group (62.1 μmol/L) than all other groups (38.0 μmol/L, 33.4 μmol/L, and 16.1 μmol/L for 0.06%, 0.04%, and 0.01% groups, respectively). Whole blood taurine concentration at breeding was significantly different ($P < .001$) in all groups (455, 332, 161, and 105 μmol/L; 0.09%, 0.06%, 0.04%, and 0.01% groups, respectively). Conception rate was highest in the 0.09% group (92.3%), intermediate in the 0.06 and 0.04% groups (84.6% and 83.3%), and lowest (76.9%) in the 0.01% group. The % live-births was approximately 3 fold greater in the 0.09% and 0.06% group compared to queens fed 0.04% and 0.01% taurine. Fetal loss occurred predominately between days 25–28 of gestation.

Mean kitten birth weights were significantly different ($P < 0.01$) among all groups; 106.6 g, 87.0 g, 73.2 g, and 58.1 g bw from 0.09%, 0.06%, 0.04%, and 0.01% groups, respectively. In conclusion, reproductive performance was highest in the 0.09% taurine group, intermediate at 0.06%, and poor in queens receiving 0.04% and 0.01% dietary taurine. Therefore, the minimum taurine requirement for adequate reproductive efficiency in queens fed a expanded-dry diets is about 0.09% dietary taurine or 450 μmol/L in whole blood.

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Taurine uptake under starvation conditions by the protozoan

Tetrahymena pyriformis

Tetrahymena cells, suspended in a non-nutrient medium, take up taurine primarily by diffusion. However, prolonged exposure to micromolar concentrations of taurine induces a capacity for concentrative taurine uptake. This induction requires de novo protein synthesis. Growth overnight in the presence of 1 mM taurine prior to transfer of the cells to the non-nutrient medium enhances the capacity for taurine diffusion as well as for concentrative taurine uptake. Under these conditions the unidirectional taurine influx consists of a saturable component with $K_m = 257$ μM, $V_{max} = 21$ nmoles · g dry wt^{-1} · min^{-1}, and a diffusion component with a diffusion constant of 0.20 ml · g dry wt^{-1} · min^{-1}. About 20% of the unidirectional influx in cells grown in the absence as well as in the presence of taurine is Na^+-dependent when measured ½ hr after transfer to starvation conditions, i.e. non-nutrient medium. However, even though the Na^+-dependent fraction increases to 40% after 4 hrs of starvation in cells grown in the absence of taurine, concentrative taurine uptake also occurs in the nominal absence of extracellular Na^+. Furthermore, taurine influx is Cl^--*independent* and *not* inhibitable with structural analogues to taurine (hypotaurine, β-alanine, GABA). Thus, taurine influx in Tetrahymena does not in any way resemble taurine influx via the Na^+-and Cl^--dependent β-system seen in vertebrate cells. About 40% of the total taurine influx is sensitive to DIDS, indicating that taurine diffusion could be mediated by a channel similar to the DIDS-sensitive taurine channel seen in mammalian cells.

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Urea and volume-regulatory taurine efflux in cerebral cortical cells

Variations in the rate of efflux of amino acids, notably taurine, play a key role in stabilizing brain cell volumes during anisosmotic stress [1, 2]. Such stress occurs during uraemia, a condition accompanied by neurological symptoms whose pathophysiological basis at cellular level is not well understood [3]. A variety of "uraemic toxins" and "middle molecules" have been speculatively implicated. The present study concerns the effects of urea itself on volume regulation and taurine efflux in cells in cerebral cortical slices pre-loaded with taurine (1 mM) (for basic methodology see [2]).

The main findings were as follows – (1) In phosphate-bicarbonate Ringer made hyperosmotic by addition of 50 mM urea, rate constants for efflux of taurine were significantly reduced ($0.58 \pm 0.04(12)$ (urea) vs $0.73 \pm 0.03(12)$ (control) $\times 10^{-2} \times min^{-1}$, $P < 0.01$) with increased slice non-inulin spaces (cell volumes) ($3.06 \pm 0.13(10)$ (urea) vs $2.33 \pm 0.09(17)$ μl/mg dry weight, $P < 0.001$) (values mean ± s.e.m.); (2) Urea had no effects on either variable in the absence of taurine; (3) Both effects of urea were almost totally abolished by trimethylamine (TMA, 1 mM). TMA alone did not influence taurine efflux or cell volumes.

It is well recognized that osmotic perturbations are commonly manifest as disturbances of CNS function [4]. The present findings are consistent with the suggestion that during uraemia urea itself may induce reduction of taurine transport and that this is causally linked to cytotoxic cerebral oedema. Aliphatic amines are present during uraemia [5] and may afford protection against the deleterious effects of urea.

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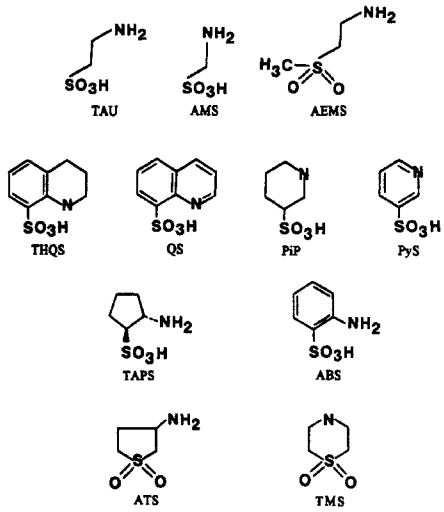
Combination effects of taurine and taurine analogues on the phosphorylation of a ~ 20 kDa protein present in the mitochondrial fraction of the rat retina

While it is well documented that all mammalian tissues contain high concentrations of taurine and also that taurine is involved in a myriad of physiological actions in excitable tissues such as the retina, brain and heart, the mechanisms of action for this amino sulfonic acid remain unknown. It has also been established that taurine is a necessary component of the visual system in a variety of species, including the rat, cat, monkey, and man. However, while pathologies due to taurine deficiency have been recorded, again the exact mechanism as to how taurine functions is elusive. The objective of the present study was to investigate the conformational requirements of cyclic analogues of taurine essential to their function as inhibitors of the phosphorylation of a specific ~ 20 kDA molecular weight protein present in a mitochondrial fraction of the rat retina. We previously reported (Lombardini, Biochem. Pharmacol. 46: 1445, 1993) that a taurine concentration of 34.2 ± 2.1 mM is required to inhibit the phosphorylation of this specific protein by 50% (IC_{50}). However, since the structure of taurine has no conformational restrictions, the preferred conformation that taurine assumes when it exerts its inhibitory effect is unknown. Because of the lack of rigidity in the taurine molecule various analogues of taurine and especially cyclic analogues of taurine have been tested for inhibitory activity.

Median-effect plot parameters and combination index values (Lombardini et al., Mol. Pharmacol. 36: 256, 1989) which determine whether the combinations of taurine plus taurine analogue are additive, synergistic, or antagonistic were determined by analyses of the dose-effect relationships. The compounds tested in this study are shown below.



The only combination of taurine plus taurine analogue that was demonstrated to be additive by the quantitative analysis of the dose-effect relationships was taurine plus THQS (ratio of 8:1). The combination of taurine plus TAPS (ratio of 10:1) was shown to be additive at 30% saturation but at higher saturation levels (50 and 70% saturation) the combination index values are 1.68 and 2.75 and thus indicate antagonism. All other combinations such as taurine plus PiP (ratio of 1:1), taurine plus PyS (ratio of 1:1), taurine plus QS (ratio of 2:1), taurine plus ATS (ratio of 1:1), and taurine plus ABS (ratio of 1:1) had combination index values greater than 1 and clearly demonstrated an antagonistic relationship. These analogues of taurine which have a wide variety of steric, electronic, and conformational features will provide insight and precise information concerning the structural requirements for inhibition of the phosphorylation of the ~ 20 kDA protein. (Supported in part by a grant to J. B. L. from the RGK Foundation of Austin, Texas).

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The phosphorylation of a ~ 20 kDA protein present in the mitochondrial fraction of the rat retina is increased after *in vivo* taurine depletion with the taurine transport inhibitor guanidinoethanesulfonic acid

Taurine (2-aminoethanesulfonic acid), an ubiquitous amino β-sulfonic acid found primarily in the animal kingdom, is a constituent of all mammalian tissues. While some of the highest tissue contents of taurine are reported to be in the retina, attaining levels of 50 μmoles/g wet tissue in the rat, there is at present no well-defined function or mechanism of action at the molecular level for taurine in this tissue. However, there is considerable evidence that taurine has a modulatory effect on protein phosphorylation in various subcellular fractions of the retina (Lombardini, Adv. Exp. Med. Biol. 359/9, 1994). Evidence for a vitamin-like role for taurine in species such as the rat has been delayed because of the ability of the rat to synthesize a portion of its body stores of taurine and not be solely dependent on dietary taurine. However, the rat can be partially depleted of its tissue stores of taurine by the addition of guanidinoethansulfonate (GES) to the drinking water. GES, a structural analogue of taurine, depletes the taurine content of the various rat tissues 70–80% by inhibiting taurine transport.

In the present study GES was added to the drinking water (1.5%) of rats with the objective being to investigate the effect of retinal taurine depletion on the phosphorylation of an ~ 20 kDa protein present in the rat retina. Because previous studies have demonstrated that exogenous taurine has a significant inhibitory effect on the phosphorylation of this protein, it was hypothesized that one function of taurine may be to regulate retinal protein phosphorylation.

Taurine levels decreased by 50% after 1 week of GES treatment and by 80% at 16 weeks. Replacing GES with taurine (1.5%) from week 16 to 22 returned the taurine content to the control value. The amplitude of the electroretinogram (ERG) b-wave was also measured in these animals. The ERG decreased by 60% after GES-treatment for 16 weeks and returned to normal after taurine replaced GES after an additional 6 weeks. At 6 weeks of GES treatment when the retinal taurine content was reduced by 70% and the amplitude of the b-wave was reduced by 50%, phosphorylation of a specific protein with an approximate molecular weight of 20 kDa was increased by 94%. The data support the theory that taurine might have a regulatory effect on retinal protein phosphorylation. (Supported in part by a grant to J. B. L. from the RGK Foundation of Austin, Texas, and a grant from Research to Prevent Blindness, Inc. to The Department of Ophtalmology and Visual Sciences)

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Molecular modeling of taurine-phospholipid interaction

Taurine exhibits several functions which are related to modulation of membrane activity, including alterations in cation transport, regulation of protein phosphorylation, changes in enzyme activity and membrane stabilization. These multiple effects account for many of the neuromodulatory, cardiomodulatory and cytoprotective actions of taurine, some of its metabolic effects and most of its pharmacological actions. It has been proposed that all of taurine's membrane-linked functions are caused by its interaction with membrane phospholipids. We have previously shown that taurine interacts weakly with biological membranes and that the binding of one molecule of taurine modifies the binding of another taurine molecule to an adjacent site. Our data also reveals that taurine probably binds close to a cation (calcium or sodium) binding site. In order to provide a clearer understanding of the binding process on a molecular level we have employed the finite-difference Poisson-Boltzmann method to calculate the free energy of binding of taurine to a phospholipid membrane. Initially we modeled this system by placing taurine near the head group of a single phosphatidylethanolamine, such that the taurine solfonic acid group was close to the lipid amino group and the taurine amino end was close to the lipid phosphate group. The free energy of binding for this arrangement is – 8 kcal/mol at 0 ionic strength and the free energy of binding is – 8 kcal/mol when the ionic strength is near physiological conditions of 150 mM. The characteristics of this interaction change in a phospholipid bilayer. More detailed calculations in which taurine is placed on a model membrane will be discussed.

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The effects of taurine administration on taurine and other amino acid levels in rat plasma, aorta, vena cava, and heart

Taurine levels and their effects on other amino acids and related amino compounds in plasma and some tissues were examined by HPLC-fluorometric analysis 30 min after injection of 1 g/kg of taurine into rats. In the plasma, taurine values (mean ± SEM; nmol/ml or g) were 3084 ± 166 (control 328 ± 28). Taurine levels increased in the heart from 31360 ± 1886 to 50013 ± 4936, in the aorta from 2129 ± 195 to 6907 ± 283 and in the vena cava from 6249 ± 310 to 12033 ± 1345. In addition, significant changes in other compounds were noted in the plasma (threonine, serine, asparagine, glutamine, valine, leucine, ethanolamine, tyrosine, histidine, arginine), heart (reduced glutathione, serine, alanine, citrulline, methionine, isoleucine, tryptophan, lysine), vena cava (aspartic acid, threonine, serine, glutamic acid, glycine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ornithine, lysine, histidine, arginine) and aorta (aspartic acid, reduced glutathione, serine, glycine, isoleucine). These changes were tissue specific and not always related to plasma changes. After taurine injection, positive correlations were found between taurine and aspartic acid, leucine and isoleucine in the vena cava, glutamic acid and threonine in the heart and none in the aorta. Thus, taurine administration raised taurine levels and affected a variety of other amino compounds significantly.

Pharmacological effects seen after taurine administration could result from taurine per se and/or via changes in specific amino acids or related compounds. (This study was supported in part by the Anesthesia Research Foundation of Wilmington, Delaware, U. S. A.)

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Effect of taurine administration on amino acid and 3-methyl-istidine concentrations in man

This work was performed to study the possible effect of taurine on amino acid and protein metabolism in man. For this purpose we evaluated plasma (at 8.00 a. m.) and urine (24 h) amino acid concentrations and urine (24 h) 3-methyl-istidine concentration in two volunteers groups (30 to 35 years old) for a period of 10 days. During this period the volunteers were fed on a standard diet in calories, protein, carbohydrates and lipids. Taurine was administrated per os (1.5 gr/die in three administrations of 500 mg each) from the 6th day of the experiment. Blood for amino acid analysis was drawn in heparinized syringes after a fast of at least 12 hours. Amino acid and 3-methyl-istidine analysis was performed by ion-exchange chromatography using a Beckman System 6300 High Performance Amino Acid Analyzer. In the treated group compared to the control group the results showed a sharp increase in plasma (control = 3369 ± 120 micromoles/l; treated = 6585 ± 180 micromoles/l; $p < 0.001$) and urine (control = 6580 ± 221 micromoles/l; treated = 12566 ± 380 micromoles/l; $p < 0.001$) amino acid concentrations from the 6th day of the experiment. Furthermore a decrease in urine (24 h) 3-methyl-istidine concentration was noted after taurine administration compared to the first 5 days of the experiment (control = 389 ± 25 micromoles/l; treated = 315 ± 19 micromoles/l; $p < 0.001$). Finally the urine (24 h) urea concentration did not show significant differences in the two phases of the experiment. Seeing these results we think that taurine may play an important physiological role in amino acid and protein metabolism in man.

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Intracellular modulation between taurine response and adrenoceptors in rat substantia nigra neurons

Taurine is highly concentrated in substantia nigra (SN) and SN neurons receive afferents accumulating taurine from the striatum, suggesting that taurine might play a functional role in the SN. Receptors of catecholamines such as dopamine and noradrenaline (NA) are highly observed in the SN neurons and are linked to the mobility of intracellular second messenger system. Taurine is an agonist of glycine receptor, which is regulated by intracellular substances (Song and Huang, 1990, Inomata et al., 1993). In the present study, we examined the modulatory effect of NA on the taurine response in acutely dissociated SN neurons of the rats using a nystatin-perforated patch clamp recording.

An activation of α2 adrenoceptors by the application of NA with prazosin (10^{-5} M) and propranolol (10^{-5} M) gradually enhanced taurine-induced chloride current ($I_{taurine}$) in a dose dependent manner (NA: 10^{-7} M to 10^{-4}). This enhancement by α2 was blocked by the treatment with pertussis toxin. Either forskolin or IBMX lessened the effect of α2 on the $I_{taurine}$. Membrane permeable cyclic AMP, dibutyl cyclic AMP (10^{-4} M), applied in the extracellular solution also decreased the $I_{taurine}$. In the presence of dibutyl cyclic AMP, α2 adrenoceptor activation failed to potentiate the taurine response. Intracellular perfusion with cyclic AMP (2 mM) in a conventional patch clamp configuration gradually decreased the $I_{taurine}$, but this suppression was disinhibited by an application of H-89 (10^{-6} M), protein kinase A inhibitor. These findings suggest that an activation of α2 adrenoceptor coupled with G_i protein inhibits adenyl cyclase activity and decreases an intracellular cyclic AMP. Decrease of PKA activity results in the enhancement of taurine response in SN neurons.

On the other hand, an activation of α1 adrenoceptor also increased the $I_{taurine}$. H-7 (10^{-6} M) and staurosporine (10^{-7} M) blocked the α1 effect. Phorbol diacetate could mimic the α1 adrenoceptor activation. Neither modulators for IP_3 nor for cyclic AMP affected the enhancement of $I_{taurine}$ by α1, suggesting that α1 adrenoceptor increased the $I_{taurine}$ via PKC.

In conclusion, adrenoceptors regulate the glycine receptor-mediated taurine responses in, at least, two different intracellular signaling pathways in SN neurons.

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Hypo-osmotic stress induces a neuronal-glial exchange of taurine *in vivo*

Taurine is thought to act as an osmoregulator in several tissues. As for the CNS, most studies of how osmotic stress affects

the cellular contents of taurine have been based on in vitro preparations in which the normal spatial relationship between neurons and glia is lost. We set out to examine how the intact brain handles taurine under hypo-osmotic stress, by combining biochemical analyses with quantitative light- and electronmicroscopic immunocytochemistry. Specifically we asked, using the rat cerebellum as a model, whether hypo-osmotic conditions induce intercellular fluxes of taurine, as opposed to a diffuse loss from all cell compartments. In other words, does taurine contribute to the maintenance of glial volume, neuronal volume, or both?

Reductions in plasma osmolality by 15–20% were induced by intraperitoneal injections of distilled water. The rats were allowed to survive for 4 h. The biochemically recorded level of taurine in the cerebellum was decreased by 12%. Semithin and ultrathin plastic sections incubated with antibodies specifically recognizing glutaraldehyde-fixed taurine revealed a loss of taurine from Purkinje cells. This loss amounted to about 50–60%, as judged from quantitative immunogold analyses supported by a calibration system based on known taurine concentrations. In contrast, the glial cells in the immediate vicinity of the Purkinje cells showed a 70–80% *increase* in the contents of fixed taurine. The changes in both cell compartments were reversed in animals whose plasma osmolality had been normalized by injections of hypertonic saline.

Our data from the rat cerebellum suggest that hypo-osmotic stress induces a flux of taurine from a neuronal to a glial compartment and that this flux is reversed when the plasma osmolality is normalized. This pattern of change was specific for taurine and suggests that the exchange of this amino acid gives priority to the regulation of neuronal rather than glial volume.

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Urinary concentration defect and taurine

Taurine (TAU) as well as sorbitol, myo-inositol, betaine, and glycerophosphorylcholine has been demonstrated to accumulate in renal papillary cells during antidiuresis. These cells apparently use these organic compounds to adjust their intracellular osmolality, because, unlike perturbing solutes such as NaCl, KCl, and urea, the organic osmolytes inhibit enzymes and other cellular processes. Although these compounds play an important role in urinary concentration (UC), the relation between organic osmolytes and UC defect has not been well defined. To test whether the reduction in medullary osmolytes cause the impairment of UC, we measured the medullary content of TAU as well as other organic osmolytes in a model of UC defect, protein deprived (PD) rats, when these animals were dehydrated or infused with hypertonic NaCl infusion. In the protocol without hyperosmolality, TAU content was comparable to control (C). In the protocol of water deprivation, urine osmolality as well as medullary TAU content was significantly lower in PD rats. Hypertonic NaCl solution failed to increase TAU content to the level of C rats, when urine osmolality and Na concentration were as much as C. Other organic osmolytes behaved similar pattern. In conclusion, UC defect due to PD was associated with decreased accumulation of TAU as well as other organic osmolytes. PD inhibited the accumulation of organic osmolytes even in the hypertonic medullary tissues, which might precede UC defect.

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Taurine uptake by brain slices in varying ionic environments

Taurine hyperpolarizes neurons by increasing the membrane chloride conductance inhibiting neuronal activity and participates in cell volume regulation as osmolyte. We have now studied the uptake of taurine in cerebral cortical slices from adult (3-month-old) and developing (3-day-old) mice when the ionic composition of medium is varied. In a number of experiments the incubation medium was rendered hyperosmotic or hypoosmotic, in both cases also supplemented with depolarizing concentrations of potassium ions. In other experiments chloride ions in medium were partially or totally replaced by other anions which could or could not permeate across cell membranes. In this way dramatic changes were induced in the intra- and extracellular spaces of the slices. The results were subjected to computer-assisted analyses and fitted by nonlinear equations containing nonsaturable diffusional terms and saturable terms of the Michaelis-Menten type. In standard HEPES-buffered Krebs-Ringer-glucose medium taurine uptake consisted two saturable components, high- and low-affinity, and nonsaturable penetration. At very low concentrations of taurine the major part was mediated by the high-affinity uptake whereas at high concentrations nonsaturable penetration was predominant. Depolarizing concentrations of potassium ions completely abolished the saturable high-affinity component and markedly subdued the low-affinity one in both developing and adult mice. The high-affinity uptake likewise disappeared in hypoosmotic media but hyperosmolality tended to enhance the low-affinity uptake. Omission of chloride ions caused the high-affinity component of uptake to disappear and the low-affinity component to be markedly reduced, irrespective of the nature of anion used as the replacer of chloride. The slices from developing mice were more sensitive to chloride omission than the slices from adults. The conditions which are known to induce taurine release concomitantly block the uptake, particularly at relatively low extracellular concentrations of taurine. We may infer from the present *in vitro* experiments that by virtue of this propensity taurine is also more efficiently enriched in extracellular spaces *in vivo* when the excitation state is elevated or interstitial fluid tends to be hypoosmotic in the brain.

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Glycation and associated alterations of collagen in diabetic rats: Effects of taurine

Nonenzymatic glycation and its late stage products, Advanced Glycosylation End products have been implicated in the pathogenesis of various complications of diabetes and aging. Recently, studies on the role of oxidative stress in glycation and associated alterations of proteins have received much attention. To understand the involvement of increased oxidative stress in glycation, in the present work the effect of an antioxidant, taurine, on glycation and associated alterations of collagen in diabetic rats was investigated. From the second day of diabetes induction animals were given taurine 65 mg/100 g body wt intraperitoneally for 10 weeks. Rat tail tendon collagen was extracted and extent of glycation, solubility in acetic acid and pepsin, susceptibility to denaturing agents, SDS-PAGE of CNBr peptides, relative fluorescence and absorbance spectra were

carried out and the results compared with control collagen samples. Taurine treatment significantly reduced the solubility characteristics and crosslinking pattern of collagen in diabetes. The results clearly demonstrated that taurine prevents glycation associated alterations of collagen, suggesting the role of oxidative stress in glycation of collagen.

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Taurine and hypoxic neural cell damage

Taurine has been shown to protect neural cells from harmful effects of excitotoxicity and hypoxic cell damage. The mechanism of this neuroprotection is not known but may be related to neuromodulatory and osmoregulatory functions of taurine. We studied now the uptake and release processes of taurine in hypoxic experimental conditions. The uptake of [^{3}H]taurine by synaptosomal preparations from the cerebral cortex of adult mice was not markedly affected by short-term incubation in glucose-free media under nitrogen, whereas the uptake was significantly reduced in developing mice. Excitotoxic agents such as N-methyl-D-aspartate (NMDA), 2-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate had no marked effects on taurine uptake in normal or hypoxic conditions. The basal release of preloaded [^{3}H]taurine from superfused hippocampal slices from both adult and developing mice was markedly increased by hypoxic conditions, whereas the K^+-stimulated (50 mM) release was decreased. However, there was still considerable calcium-dependent release left. NMDA, kainate and AMPA enhanced taurine release in hypoxia. The effect of NMDA was antagonized by dizocilpine, a non-competitive antagonist of the function of NMDA receptors. Moreover, the actions of AMPA and kainate were also reduced by the respective antagonists of these glutamate receptor subclasses. The results indicate that in conditions provoking neural cell damage the release of taurine is increased, apparently contributing to its neuroprotective effects.

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Regulation by taurine of ionic channel currents in cardiomyocytes

It has been shown that taurine produces many functions on the ion fluxes and the contractile force of myocardial cells. Under ischemia and cardiac failure, taurine causes a negative inotropic effect. In contrast, at low $[Ca]_o$ taurine causes a positive inotropic effect. Aim for this study was to examine the effects on ionic channel currents in guinea pig ventricular myocytes. Whole-cell and cell-attached patch voltage-clamp and current-clamp experiments were performed (36 °C).

Taurine at pCa 6 inhibited the I_{Ca} current, but at pCa 8 taurine enhanced I_{Ca}, suggesting that taurine may exert a regulation of the $[Ca]_i$ level by modulating I_{Ca}. The inactivation time course was increased by 10 and 20 mM taurine at pCa 6, and by 20 mM at pCa 8. In cell-attached experiments (using 100 mM Ba^{2+} in the pipette solution), the open probability of Ba^{2+} channel current was enhanced by about 120% at 0.9 mM $[Ca]_o$, and inhibited by about 38% at 5.4 mM $[Ca]_o$. Time constants of histograms for the open and closed times were increased and decreased at low $[Ca]_o$, respectively, and vice versa at high $[Ca]_o$. The conductance was not affected by taurine.

The effects of taurine were modulated by the $[Ca]_i$ or/and $[Ca]_o$ levels, consistent with previous reports. Taurine acted on the open probability of L-type Ca^{2+} channel, but not on the channel conductance. These dual action is an antagonism against the Ca^{2+}-induced cardiac functions (Ca^{2+} homeostasis), and taurine would protect the cell demage. These results indicate that taurine exerts potent cardioprotective actions under the conditions at low Ca^{2+} level as well as of calcium overload. Taurine actions are complex, there being a number of actions on the cardiac muscle cells, which may show the possible therapeutic use of this sulfur amino acid.

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Modulation of Na^+-Ca^{2+} exchange by taurine

The Na^+-Ca^{2+} exchanger is an electrogenic antiporter, which transports 3 Na^+ in exchange for 1 Ca^{2+}. In the heart, influx of Ca^{2+} via the calcium channel is usually balanced by extrusion of Ca^{2+} via the Na^+-Ca^{2+} exchanger. However, recent studies suggest that the exchanger may also promote Ca^{2+} influx during the initial phases of the action potential. In this mode, it has been implicated in the release of Ca^{2+} from the sarcoplasmic reticulum. We have recently demonstrated that the Na^+-Ca^{2+} exchanger is regulated by signal transduction pathways initiated by several key effectors. For example, incubation of isolated sarcolemma with 9.5 U/l insulin, 5 nM angiotensin II, 5 nM endothelin 1 or 100 μM phenylephrine leads to a 40%, 115%, 210%, and 110% increase in Na^+-Ca^{2+} exchanger activity, respectively. However, preloading the membrane vesicles with 25 mM taurine virtually eliminates the stimulation of the Na^+-Ca^{2+} exchanger by these agents. A dose-response of the taurine effect yields an ED_{50} of approximately 0.5 mM. Since all of these agents activate protein kinase C, an attractive scenario is that taurine functions by altering membrane phosphorylation. In addition, taurine modulates Na^+-Ca^{2+} exchanger activity by affecting the phospholipid composition of the membrane. An elevation in membrane phosphatidylcholine content mediated by the conversion of phosphatidylethanolamine to phosphatidylcholine results in a 75% decrease in Na^+-Ca^{2+} exchanger activity. Taurine prevents the loss in transporter activity by inhibiting the formation of phosphatidylcholine. It also reverses the effects of insulin on the methyltransferase reaction. Thus, taurine serves as an important regulator of the Na^+-Ca^{2+} exchanger by altering the actions of several key effectors, as well as indirectly modulating Na^+-Ca^{2+} exchanger activity through changes in the phospholipid content of the membrane.

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Net taurine transport and its physiological role

The net-transport of γ-aminobutyric acid (GABA), glutamic acid and taurine was recorded in synaptosomes isolated from rat brain. The isolated synaptosomes were in separate experiments incubated in increasing external concentrations of radio labeled amino acids. Following the incubation, the entry of radio labeled

amino acid was assessed by liquid scintillation and compared to the net-transport, derived from changes in the external concentration of the incubation medium measured by HPLC.

The experimental set-up used, promptly, illustrates a complex relationship between the intraterminal concentration, the net-transport and the external concentration of the amino acid. In fact, various ways to manipulate the external concentration of GABA during the incubation was previously used to show a product inhibition of GABA synthesis in the nerve-terminal.

Net-transport of GABA and glutamic acid became active at external concentrations $\leq 10\ \mu M$, whereas net-transport of taurine became active at an external concentration of ~ 370 μM, i.e. below these concentrations net-release of the amino acids to the incubation medium was observed.

Using the suggested taurine antagonist 6-aminoethyl-3-methyl-4H, 1,2,4-benzothiadiazine-1,1-dioxide (TAG) and $^{86}Rb^{+}$ in additional studies of the taurine transport. TAG was found to competitively inhibit the uptake of [^{3}H]taurine. This inhibition of uptake was paralleled by a reduced accumulation of $^{86}Rb^{+}$ into the synaptosomes, which suggests a reduced hyper polarisation.

Our results illustrate that the transmembranal gradient maintained of taurine is substantially lower than that for GABA and glutamic acid. The electrogenic taurine transporter, accordingly, seems to have its equilibrium for intracellular-to-extracellular taurine concentrations in a range where a rapid transport of large number of molecules may be achieved into or out from the intracellular compartment. The physiological significance of this transport will be discussed.

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Differential regulation of taurine transport in neuronal and glial cells by PMA and okadaic acid

Recent evidence indicates that taurine transporter is regulated by protein kinase C (PKC). Kulanthaivel et al. [1] reported selective impairment of taurine transporter in JAR placental chorionica cell line. These observations were extent to human colon carcinoma cell lines [2] and LLC-PK1 renal cell line [3]. We reported here that taurine is differently regulated by both phorbol 12-myristate 13-acetate (PMA), a PKC activator and okadaic acid in rat glial and neuronal cells. Uptake of taurine in rat astrocytes was inhibited in a time dependent manner by PMA and okadaic acid. Maximal inhibition was obtained at 1 μM after 1 hour of treatment and 0.1 μM after 30 minutes of treatment respectively for PMA and okadaic acid. The effect of PMA was prevented by pretreatment of the cells with chelerythrine, a potent and selective inhibitor of PKC [4]. This effect was mimicked by oleoyl acetyl glycerol (OAG), an endogenous stimulator of PKC and by R59949, an inhibitor of diacyl glycerol kinase. In contrast the taurine transport in rat neurons was not affected by PMA, OAG, R59949 or okadaic acid. This was not only the feature of rat cells since the same differences were observed in human glioma GL15 cells and human neuronal cells IMR 32. Although PKC activity was not measured, these differential effects of PMA and okadaic acid in neuronal and glial cells are difficult to explain since many studies reported that this activity is higher in both astrocytes and neurons [5, 6]. Difference between neuronal and glial cells may be related to the different isotypes of PKC present in cells [7] or to different PKC-taurine transporter coupled signal transduction in the two cell types. As reported for glial and neuronal insulin receptors, we can speculate that neuronal and glial taurine transporters are structurally different or differently glycosylated.

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Radioautographic and immunohistochemical studies on localization of taurine in vascular cells of several organs of the mouse

It is generally accepted that taurine has hypotensive or vasodilator effect. However, its ultrastructural localization in the vascular cells has not been elucidated. We studied both the incorporation of ^{3}H-taurine into the vascular cells by radioautography and the intracellular localization of the endogenous taurine by immunohistochemistry.

Small pieces of skeletal muscle tissues of mice were incubated in a culture medium, Eagle's MEM, containing 3.7 MBq(ml of ^{3}H-taurine for 5 to 60 min. Tissues were fixed, embedded, sectioned and prepared for light and electron microscopic radioautogram. For immunohistochemical study, blood vessels of the brains, hearts, livers and kidneys of mice were stained with an immunoperoxidase method using taurine antibodies.

By light microscopic radioautography, silver grains were observed on the blood capillary walls in the 5 minute incubation tissues. The number of grains increased with the prolongation of incubation time. By electron microscopy, silver grains appeared in skeletal muscle cells; endothelial cells, smooth muscle cells of the blood vessels; adipose cells; fibroblasts; erythrocytes; and myelin and perineural sheaths of the peripheral nerves. The silver grains were observed on cytoplasm and nuclei of the endothelial cells and pericytes of capillaries. The silver grains were observed in nuclei and cytoplasm of endothelial cells or smooth muscle cells. In the nuclei, silver grains were observed attaching to chromatin, in the cytoplasm ultrastructural localization of silver grains were unclear. The results obtained by immunohistochemical observation revealed intracellular localization of endogenous taurine.

From the results obtained, it was demonstrated that taurine was localized and incorporated in vascular cells, which means that taurine should influence vascular cells directly.

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Possible role for taurine in regulating pituitary hormone release in fish

In our initial HPLC analysis of the amino acid neurotransmitters present in the goldfish pituitary we discovered high

concentrations of taurine (5-15 ng/μg protein). This prompted us to investigate the effects of taurine on *in vivo* gonadotropin (GTH-II) and growth hormone (GH) release from the pituitary. Intraperitoneal or brain injection of taurine rapidly stimulated the release of GTH-II. The precursor hypotaurine also stimulated GTH-II release, and the taurine metabolite isetheonic acid was without effect. Taurine-stimulated GTH-II release was enhanced by pretreatment with the gonadal steroids testosterone and estradiol, and by the catecholamine synthesis inhibitor α-methyl-p-tyrosine. Studies on GH secretion demonstrated that during most of the reproductive cycle, taurine was inactive. However, during the period of seasonal gonadal redevelopment, taurine exerted a slight stimulatory effect on GH release that was enhanced by estradiol. The cellular site of taurine synthesis in the hypothalamic-pituitary complex of goldfish is unknown. The *in vivo* GTH-II and GH release data together with the demonstration of a substantial content of taurine in the neuroendocrine system suggests that it is involved in the regulation of GTH-II and GH secretion in the goldfish.

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Taurine uptake in retinal pigment epithelium of diabetic rats

The blood retinal barrier consists of two components, an inner barrier composed of the retinal endothelium and the outer barrier formed by the tight junctions of the retinal pigment epithelium cells (RPE). Changes in RPE permeability might alter the intraretinal environment that can lead to alterations founded in diabetic retinopathy. Taurine has been found to be important in maintaining retinal function. High and low affinity transport systems for taurine has been characterized in RPE. Therefore we studied the uptake of taurine in the RPE of diabetic rats and compared it with that of normal rats.

Diabetes was induced in adult long Evans rats by stroptozotocin administration (65 mg/kg) and used after 20 and 45 days of treatment. Rats were considered diabetic if serum glucose exceeded 300 mg/dl. The isolated RPF was incubated at 37 °C in a Krebs bicarbonate buffer containing the radiolabeled taurine. Radioactivity accumulated was estimated by liquid scintillation counting.

Taurine uptake was increased at progressively longer time intervals following the onset of diabetes (40%). Under in vitro hyperglycemic conditions, only one transport system of low affinity (KM 2.5 mM) and high capacity (312 nmol per g/min) was observed, indicating changes in the affinity compared to the control rats. The results suggest that diabetes induced changes in the RPE permeability. Whether the changes observed on taurine uptake are due to lack of insulin and/or hyperglycemic conditions remains to be studied.

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Measurement of whole blood and plasma taurine in adult cats with reference to age and sexual status

The WALTHAM Centre For Pet Nutrition currently measures whole blood taurine as an indicator of taurine status in the cat. Before 1990 the measurement of plasma taurine was widely used to assess taurine status. However, Trautwein and Hayes [1] suggested that plasma taurine in humans fluctuated widely and artificially elevated values may occur due to the release of taurine from blood cells, particularly platelets, as a result of sampling techniques. In addition, plasma taurine fluctuates widely with food intake. For the above reasons, Trautwein and Hayes [1] recommend the measurement of whole blood taurine as an indicator of taurine status since it is more stable and reflects cellular taurine levels more reliably. This is now the required measurement for the American Association of Feed Control Officials (AAFCO). This study was conducted to obtain normal ranges for whole blood and plasma taurine in the cat and to establish any relationships between the two taurine pools. The influences of age and sex were also considered.

80 blood samples were collected from 40 neutered males and 40 entire females ranging in age from 12 months to 12 years. The cats were then allocated into four equal three-year groups of 20 cats (10 entire females, 10 neutered males). All cats were fed nutritionally complete commercially prepared petfood for at least 2 weeks and were fasted for 12 hours prior to the bleed. After blood samples were analysed for plasma and whole blood taurine the data were subjected to multi-analysis of variance with age-group, sexual status and bodyweight as the main effects.

Plasma taurine values were all within the normal range for the cat (> 60 nM/g). However, some of the whole blood taurine results were higher than the range identified by Trautwein and Hayes [2], possibly due to sex status since both whole blood and plasma taurine pools varied significantly with sex status ($p \leq 0.05$), neutered males having higher values for both taurine pools. All the males sampled were neutered and this may affect their requirement for taurine. There was no significant effect on taurine values due to bodyweight although neutered males were significantly heavier than entire females ($p \leq 0.05$). Whole blood taurine was also found to increase with age only in the neutered males, from 495.7 ± 33.2 nM/g at the $0 \leq 3$ year age group to 668.7 ± 37.6 nM/g for the $9.1 \leq 12$ year group suggesting either a reduced requirement, an accumulation in the whole blood pool or reduced excretion. No similar relationship was seen in entire female cats. Finally, weak relationship between whole blood and plasma taurine was established in neutered males ($r = 0.48$, $n = 38$), but not entire females.

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Urinary taurine as a biomarker for protein synthesis status: Studies with cycloheximide and clenbuterol

We have shown that rats given hepatotoxic compounds and known to inhibit protein synthesis (carbon tetrachloride, hydrazine, ethionine, thioacetamide) excrete increased amounts of urinary taurine [1] and compounds which increase protein synthesis (phenobarbital, salbutamol) decrease urinary taurine [2, 3]. To demonstrate whether this was due to changes in protein syntheses, hepatotoxicity or both, we have examined the effect of a non-hepatotoxic protein synthesis inhibitor, cycloheximide

and a protein synthesis stimulator, the β-agonist drug, clenbuterol, on tissue and urinary taurine levels.

Rats were acclimatised in metabolism cages for 48 hours and predose urine (24 h) collected. Animals were given a single dose of cycloheximide (0.1, 0.5, 1.0, 1.5, 2.0 $mg.kg^{-1}$, i.p.) or clenbuterol in drinking water (0.02, 0.2, 2.0 $mg.kg^{-1}day^{-1}$for 4 days), urine was collected for 24 h or 4 days respectively. [^{3}H]-leucine was given in the drinking water 24 h before necropsy. Tissues were removed for analysis of taurine, glutathione and [^{3}H] incorporation into protein. Blood was taken for serum analysis and measurement of taurine, total protein, albumin and markers of liver injury. Taurine was measured by HPLC [4].

Cycloheximide resulted in a dose dependent increase in urinary taurine which was maximal 8–12 h after dosing. Liver and serum taurine were raised 24 h after dosing but not in a dose dependent manner. Total urinary protein was significantly decreased by cycloheximide.

After exposure to clenbuterol, liver, muscle, urinary and serum taurine were decreased in a dose dependent manner; the decreases in liver and urinary taurine after the highest dose were statistically significant. There was a corresponding increase in incorporation of [^{3}H]-leucine into muscle protein. Reduced urinary taurine correlated significantly with the cumulative dose of clenbuterol.

The data show that taurine levels *in vivo* are influenced by changes in protein synthesis resulting from xenobiotic administration in the animal in the absence of liver damage and that urinary taurine is a non-invasive biomarker for these changes.

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Effect of *N*-acetyl-L-cysteine on urinary taurine in rats *in vivo* and cellular taurine in rat hepatocytes *in vitro*

Taurine is known to have protective properties both *in vivo* (as an endogenous compound) in tissues which generate free radicals eg. the retina and neutrophils [1] and *in vitro* in lymphoblastoid cells and hepatocytes [2, 3]. *N*-Acetyl-L-cysteine (NAC) is used routinely to treat paracetamol poisoning and is reported to limit the toxic effects of compounds such as halothane, nitrogen oxides, zinc and amanita phalloides toxin. It replenishes depleted glutathione via metabolism to cysteine, maintains protein thiols and can bind directly to compounds via -SH. However, some of the protective properties of NAC remain unaccounted for [4].

NAC is an effective precursor for cysteine and its metabolism to taurine has been demonstrated in isolated hepatocytes [5]. We propose that this may account for some of the protection afforded by NAC after toxic insult, possibly at a latèr stage in the toxicity. We have therefore investigated the synthesis of taurine from NAC in rats and in isolated rat hepatocytes.

NAC was administered (4 $mM.kg^{-1}$, ip) to male Sprague Dawley rats housed in individual metabolism cages. Taurine was measured in 24 h pre- and postdose urine collections and liver and serum 24 h after dosing. Urinary taurine was elevated 3 times above predose levels, representing a conversion of 17% of the NAC administered, on a molar basis. Liver glutathione and taurine were raised but serum taurine levels were the same as the control values.

Rat hepatocytes were isolated by two stage collagenase perfusion. Cells were incubated in rotating flasks (1.5×10^6.ml, $95\%O_2{:}5\%CO_2$), with NAC (0, 0.5, 1.0, 2.0 or 5 mM), cysteine sulphinate (2 mM) or hypotaurine (2 mM) for 3 h and samples removed each hour to measure taurine, glutathione and lactate dehydrogenase leakage. Glutathione synthesis was maximal after 1 h incubation and was independent of NAC concentration. Neither cysteine sulphinate nor hypotaurine altered GSH synthesis. After an initial lag phase taurine synthesis increased in a time dependent manner. Unlike GSH synthesis, taurine synthesis was dependent on NAC concentration. During the incubation taurine concentration in the buffer also increased. Taurine synthesis from cysteine sulphinate continued for 3 hour but was greater than that from NAC. Synthesis of taurine from hypotaurine was greater than from cysteine sulphinate and was maximal at 1 h.

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Addendum

Basic Chemistry

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Dielectric study on molecular dynamics in aqueous solutions of protein and polymer

Synthetic homo polymer has usually a simple structure and will give us a fundamental information on the structure of biopolymers. To make clear the interaction between water and biopolymers, dielectric measurements on several kinds of aqueous solutions of synthetic polymers were carried out in a concentration range of 0–50 wt% at 25 °C using a time domain reflectometry (TDR) method at frequency between 100 MHz and 10 GHz. Relaxation peak of water shifts to low frequency and the distribution of relaxation times becomes broad with increasing the polymer concentration for all solutions. The shift of the relaxation peak and broadness of the distribution vary with the polymer species. If the parameter describing the distribution of relaxation time was plotted against the relaxation time, the plot can be classified into two categories. One consists of non-electrolyte polymers and the other consists of electrolyte polymers and PVA. This suggests that the non-electrolyte polymer except for PVA makes the motion of water slow but keeps water structure uniform, if compared to the electrolyte polymers. In order to compare the relaxation of water in the mixture of homo polymers and that of biopolymers, dielectric relaxation measurements an aqueous solutions of biopolymers such as globule protein, collagen, denatured albumin and gelatin, were performed. As the results, the relaxation behaviour of water in biopolymer-water systems except for collagen is just the same as that in water mixture of electrolyte polymers and PVA.

Metabolism and Transport

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Regulation of hepatic arginase by Mn(II) ions and pH

Human liver arginase was purified by a new and mild procedure. EDTA caused a time-, temperature- and pH-dependent loss of activity over 1 h or more, fastest at pH 5.5 and very slow at pH 9. Mn^{2+} restored activity within 0.5 min at 37 °C to severalfold the original value. Reactivation by Mn^{2+} (100%) was also given by Ni^{2+} (59%), Cd^{2+} (54%) and Co^{2+} (27%) but not by other ions. Manipulation of Mn^{2+} status allowed repeated cycling between active and inactive states. Reactivation with a Mn-triphosphate buffer giving very low [free Mn^{2+}] had slower and hyperbolic (K_d ca. 36 nM) kinetics. At 6 nM Mn^{2+} reactivation was strongly pH-dependent, a Hill coefficient for [OH^-] of 2 indicating $2H^+$ displaced/Mn^{2+} bound. Catalytic activity did not require free Mn^{2+}, and was routinely monitored at pH 9 in presence of EDTA. Both V_{max} and K_m for arginine were markedly pH dependent.

These studies imply active and inactive conformational forms of arginase. At about 10^{-8} M free Mn^{2+}, and at physiological pH and [arginine], overall arginase activity is predicted to rise ca. 450-fold per 1 unit rise in pH. These conditions afford a mechanism for pH control of hepatic [arginine] and the ornithine cycle.

Polyamines

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The incorporation of polyamines into proteins: a potential mechanism of modulation of cellular transglutaminases

Cross-Linking of proteins, an event which occurs post-translationally is one of the vital physiological processes involved in stabilization of tissue and cellular matrices. Among the various cross-links identified, ε-(γ-glutamyl) lysine and N,N bis(γ-glutamyl)polyamine are the most abundant that are formed by enzymatic catalysis. The formation of these two types of cross-links is catalyzed by Ca^{2+}-dependent acyltransferases (glutaminyl-peptide γ-glutamyltransferase E.C. 2.3.2.13.), known as transglutaminases (TGases). In the reaction catalyzed by TGases, the γ-carboxamide groups of peptide-bound glutamine residues are the acyl donors while primary amino groups in a variety of compounds may function as acyl acceptors with the subsequent formation of mono-substituted γ-amide of peptide-bound glutamic acid. Based on their distinct catalytic characteristic and distribution, several forms of TGase have been identified to date and they have been found to exhibit differences in specificity. These differences are expresseds in terms of variations in susceptibility of glutamine residues to catalytic modification and appear to be dependent, at least in part, upon amino acid residues surrounding a given glutamine. In contrast to their limited glutamine substrate specificity, TGases possess an exceptionally wide specificity for polyamine substrates. Although the catalytic action of the TGases and their limited specificity are known, much remains to be learned concerning tissue specificity, regulation, and structural relationships. Polyamines, putrescine, spermidine and spermine may act as a substrate competitor to the peptide-bound lysine moiety, resulting in a "non-lysyl" isopeptide linkage. This remarkable feature provides a basis for the modulation of intracellular TGases activity by polyamines.

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Behavioral effects induced by systemic NMDA in the rat. Changes in brain putrescine concentration

The effect of 150 mg/kg of NMDA i.p. on the behavior of male Wistar rats was evaluated by both direct observation and image dynamic analysis (Videotrack) during 1 h. NMDA-induced behavioral response was classified as convulsant and non-convulsant. In a previous work, we observed that at this dose, the convulsant episode begins very fast after injection and animals only survive during a short time (few minutes). For this reason, in this study convulsant rats were killed immediately after convulsion. Non-convulsant animals exhibited abnormal behavior consisting in stereotypic movements and were killed at 8 or 24 h after NMDA administration. The concentration of polyamines in blood plasma and brain (frontal cortex and hippocampus) was determined by HPLC. In convulsant rats, the plasma putrescine concentration was increased 55% over the saline controls, but there were no modifications of polyamines content in brain. In non-convulsant animals, the increase of putrescine was found in the brain: In frontal cortex, was of 170% at 8 h and of 91% at 24 h and in hippocampus the increase was of 40% at 24 h. Moreover, there is a correlation between the concentration of putrescine in brain and the developed motor activity evaluated with Videotrack at 8 ($r = 0.8194$, $p < 0.01$ for frontal cortex; $r = 0.8563$, $p < 0.001$ for hippocampus) and 24 h ($r = 0.7397$, $p < 0.01$; for frontal cortex; $r = 0.8073$, $p < 0.01$ for hippocampus). These results are discussed in relationship to the behavioral response. (Work supported by the FIS 93-0350 and SAF 02-0913 grants.)

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Methylxanthines increase polyamine incorporation into proteins and affect the metastatic activity of B16-F10 melanoma cells

Tumor invasion and metastasis require complex changes in normal cell-cell and cell-matrix interactions. Transglutaminases [Tgase, E.C. 2.3.2.13]-catalyzed cross-link formation may be related to the adherence of tumor cells to the extracellular matrix (ECM), since fibronectin, laminin and other ECM-proteins are good substrates for the enzyme. The activation of Tgases seems to be inversely correlated with the invasive potential of metastatic cells, suggesting that post-translational modification of cellular protein may alter tumor cell malignant behaviour (Int. J. Cancer, 53: 792, 1993). We tested methylxanthines as Tgases activators for their effects on the metastatic potential of B16-F10 melanoma cells. Among methylxanthines, theophylline was the most effective inhibitor of tumor cell adhesion to basal membrane proteins, invasion through Matrigel-coated filters and pulmonary colonization after i.v. injections in mice. We provided evidence for the synthesis of polyamine derivatives as a product of the catalytic action of Tgases. Theophylline-treated cells contained higher levels of N^1,N^8-bis (γ-glutamyl) spermidine with respect to control cells, thus indicating that the induction of protein cross-links catalyzed by Tgases resulted in an impairment of tumor cell metastatic activity. Our observations highlight the role of Tgases and polyamines in the intricate processes connected with melanoma cell adhesion, migration and colonization. Moreover, fluctuations in Tgase activity and spermidine-derived protein cross-link levels could result in perturbations of membrane structure and function. The impairment of cellular adhesion and other cell surface characteristics, associated with the metastatic potential of tumor cells, would aid in contrasting their dissemination from the primary tumor to distant sites. (This work was supported by a grant from CNR [target project 9200339])

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Polyamines prevent transglutaminase-induced opacity in rabbit eye lens

It is unquestionably assumed that post-translational modifications of protein must have important biological roles in the regulation of enzyme activities and proteins interactions turnover. Although these functions appear plausible in many instances, a final proof is often difficult to provide, and indeed it cannot be excluded that certain modifications are functionally unimportant and may occur as innoxious by-product of the intracellular metabolism. Cultured mammalian eye lens represents an experimental model proved to be useful in studying this type of modifications. It has been reported that transglutaminase (TG. E.C. 2.3.2.13)-mediated ε-(γ-glutamyl)lysine crosslinking of lens proteins may occur during *in vivo* cataract formation (Lorand et al., P. N. A. S. 1981). Since all bonds produced by TG catalysis are in the form of amides of the γ-carboxyl group of protein-bound glutamic acid, polyamines, may act as acyl-acceptor substrates (Folk et al., J. B. C., 1980). Naturally occurring polyamines could exert a controlling influence on protein crosslinking by competition against lysine residues of β- and γ-crystallins. We well report some recent findings on the working hypothesis that rabbit eye lens transparency preservation may derive from a precise equilibrium between the levels of Ca^{2+} and polyamines coming from the surrounding ocular fluids. (Supported by a grant from CNR [target project 9200339].)

Physiology and Pharmacology

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Mechanism of ATP hydrolysis by polymeric actin

In view of the importance of ATP hydrolysis for the dynamics of actin assembly and in view of the different models of hydrolysis proposed we present a study in which we analyzed the lag of ATP hydrolysis behind actin polymerization in terms of the mechanism of ATP hydrolysis by polymeric actin. ATP that is incorporated into filaments by polymerization of ATP-actin-monomeres onto the ends of actin filaments, has been found to be hydrolyzed some time after polymerization. Under the experimental conditions (100 mM Kcl and 1 mM $MgCl_2$, or 0 Kcl,

1 mM $MgCl_2$ and 0.4 mM EGTA, 25 °C) ATP hydrolysis lagged behind polymerization by about 100s independently of the concentration of polymerizing filament ends and of the actin monomer concentration. Three models of ATP hydrolysis were compared to experimental data: (i) Random ATP hydrolysis, ATP is assumed to be hydrolyzed at a rate that is independent of the type of nucleotide bound to adjacent filament subunits. (ii) Cooperative hydrolysis, the rate of ATP hydrolysis is thought to depend on the type of nucleotide bound to adjacent subunits. (iii) Sequential hydrolysis, ATP is assumed to be hydrolyzed only at the interface between ATP-subunits and ADP-subunits. The model of sequential ATP hydrolysis could be excluded. The results were in agreement with random or cooperative ATP hydrolysis. The differences of the rates of ATP hydrolysis by a random or cooperative mechanism are so small that based on the experimental results no distinctions between these two mechanisms could be made. All available evidence points towards a mechanism of ATP hydrolysis in which several or perhaps many interfaces between ATP- and ADP-subunits are formed within a filament (Ohm & Wegner, 1994, Biochim Biophys Acta).

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