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Abstracts

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Amino Acids Racemization

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Hydrolysis of proteins performed at high temperatures and for short times with reduced racemization, in order to determine the enantiomers of D- and L-amino acids

Racemization of free amino acids is considerably lower than that of amino acids bound in peptide. In the same experimental conditions, the rate of racemization of free amino acids is only 20–80% of that of peptide bound amino acids. When using to traditional protein hydrolysis, racemization was 1.2–1.6 times as high as that obtained at high temperatures (160–180 °C), under conditions ensuring total hydrolysis of the protein. This lower degree of racemization may be explained by the fact that, at high temperatures, the protein hydrolyses more rapidly into free amino acids and the racemization of free amino acids is considerably slower that of amino acids bound in polypeptides. When hydrolysis is conducted at lower temperatures for longer times, the amino acids bound in the peptide chain are exposed for a longer time to the effects actually causing racemization. As a result, we may say that any factor which speeds up hydrolysis, will lower the degree of racemization.

Racemization was higher for proteins in milk powder than for pure proteins. This may be explained by catalysis of racemization by the heavy metals present. After 48 hours at 110 °C and in presence of 4M barium hydroxide, all amino acids (whether free or bound in peptide) totally racemized. Therefore the racemization of tryptophan cannot be determined using barium hydroxide promoted protein hydrolysis. High temperature hydrolysis (at 160 °C for 45 to 60 minutes, at 170 °C for 30–45 minutes and 180 °C for 30 minutes) are recommended for those who would like to hydrolyse the protein for short times and to determine the degree of racemization occurring in the polypeptide chain, but do not wish to use enzyme hydrolysis.

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Optical resolution of amino acids via enantioselective hydrolysis of their esters catalyzed by microbial proteases

Homochiral non-protein amino acids are useful as building blocks for the synthesis of analogs of biologically active peptides and as chiral starting materials or chiral catalysts for other syntheses. For their supply in required amounts, chemical synthesis followed by optical resolution is still a method of choice. In this connection we have reported some novel enzymatic resolution procedures for amino acids using lipases and proteases. For example, a number of nonprotein amino acids were obtained with a high degree of chiral integrity via the microbial protease-catalyzed hydrolysis of their *N*–protected methyl esters [1]. In some cases, however, the hydrolysis rates were rather slow. When the methyl esters with a free α-amino group were employed as substrates for the microbial proteases (e. g., ex *Aspergillus oryzae*), hydrolyses generally proceeded more smoothly but with low enantioselectivities. Accordingly, the influence of ester groups on enantioselectivity was examined next [2]. It was increased progressively with the length of the ester alkyl chain, while it deteriorated strikingly with the C_8 ester. Thus, by employing esters with longer alkyl chains such as the isobutyl ester, sufficiently high enantioselectivities were achieved with several halogenated phenylalanines. The hydrolysis of the isobutyl esters of aliphatic amino acids also proceeded with rather high enantioselectivities, which, however, were lower than those observed with the aromatic amino acid derivatives, the highest enantioselectivity being obtained with norleucine carrying a C_4 side chain. We found that conducting the hydrolysis at low temperature was quite effective in improving the enantioselectivity.

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Species and age related excretion of D-serine in mammalian urine

Urinary D-serine (D-Ser) levels in human, rat and dog with different ages were investigated by a highly sensitive HPLC method previously developed [1, 2]. High D-Ser level was consistently excreted in human urine throughout life. No age-dependent changes were observed in urinary D-Ser/Total-Ser ratios from premature infants to old. D-Ser/creatinine ratios in adult human urine were found relatively constant in individuals. The constant excretion of D-Ser in human urine was confirmed by the consecutive 24 h analyses in three volunteers. Hith levels of D-Ser and D-Ala were excreted in adult dogs. Urinary D-Ser level was high in young rats at unweaned and

weaned periods and then declined with the age increased. In contrast, urinary D-Ala level was much lower in suckling rats, and increased rapidly after weaned state and then declined with the age increased. The origin of D-Ser in mammalian urine was discussed.

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Analysis

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Analysis of primary amino acids in urine by an automated HPLC method: comparison with ion exchange chromatography

Many reversed phase HPLC methods for the analysis of amino acids in urine have been described in the literature. However, no satisfactory comparisons between HPLC and classical ion exchange procedures have been made. Therefore, we developed a relatively rapid, simple and precise method for the quantitation of urinary amino acids and compared this HPLC method with the ion exchange method. Our method is based on reversed phase HPLC and automated pre-column derivatization with *o*–phthaldialdehyde. Total run time was 49 min. The ion exchange method made use of the Biochrom 20 chromatography system and post column detection with ninhydrin; total run time was 161 min. Forty urinary samples were analyzed by both methods and the correlation coefficient for the common amino acids was greater than 0.90. For the rare amino acids this value ranged between 0.304 and 0.891. Disadvantages of the HPLC method are the interference of ammonia with the determination of valine and a slight overestimation of glutamic acid because of the presence of an unknown interfering compound. Disadvantages of the ion exchange method are coelution of methionine and homocitrulline, susceptibility for interference by mixed disulphides, drugs and metabolites, overestimation of aspartic acid, and sometimes baseline fluctuation. The consequence of the latter was that low amino acid levels could not be determined accurately. Therefore, the more sensitive HPLC method is superior to the ion exchange method in case of low amino acid levels. Although this HPLC method was not able to detect secondary amino acids and quantify cystine or homocystine, the accuracy, speed and relative unsensitivity for exogenous factors make this method an excellent alternative to the classical ion exchange method.

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Total free and free D-amino acid content of cheeses produced by different technologies

The concentration of total free amino acids (AA) and free D-AA were determined by ion exchange column chromatography and by high performance liquid chromatography in the outer layer and inner part of Ardrahan (Irish smear-ripened cheese) and Camembert cheeses, Danish blue, Emmental, Gouda, Mozzarella, Parmesan and five Cheddar cheeses produced by different technologies. It was established that the total free AA concentration was highest in Parmesan and Gouda cheeses (39000–24000 µmol/100 g), and lowest in Mozzarella and Cheddar cheese produced by different technologies (2400–7400 µmol/100 g), while the other cheeses examined contained 1300–1900µmol/100 g free AA.

The average concentrations of free D-AA in the different cheeses were the following: D-Asp 58 µmol/100 g (30.3%), D-Glu 117 µmol/100 g (15.8%), D-Ala 276 µmol/100 g (37.2). The values in brackets are the free D-AA concentration as a percentage of total free (D + L) AA. The amount of free D-AA showed differences between cheeses. D-AA as a percentage of total free AA was changed 13.9–46.3% for D-As, 12.9–26.6% for D-Glu and 16.1–48.1% for D-Ala. Except for these three D-AA, the other D-AA were present in the cheeses at very low concentrations, at the limit of the identification and determination. In the case of Cheddar cheese concentrations of the D-AA were a little higher, when lactobacilli were added during cheese making.

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Comparison between classical amino acid analysis and HPLC-methods

In classical amino acid analysis the amino acids are determined by separating the free amino acids on an ion-exchange column, derivatising them with a post-column reagent and detecting them with a suitable detector. As most of the amino acids have no absorbance in a suitable wavelength-range, detection without derivatisation is not possible. In the classical method, first introduced by Stein and Moore, this derivatisation is done by reacting the amino acids with ninhydrin, another reagent sometimes used is OPA. When derivatised with ninhydrin the reaction products can be detected either at 440 nm (secondary amino acids) or at 570 nm (primary amino acids). The fluorescent OPA reaction products are only formed with primary amino acids. Up to now many attempts have been made to find other derivatisation methods with the aim to be able to use standard HPLC-equipment for the detection of the amino acids, and to take advantage of the higher speed and sensitivity of HPLC methods. In HPLC-methods for amino acids it is common to perform a pre-column derivatisation reaction followed by separation of the reaction products on a reversed-phase (RP) column. The reagents used for this purpose are mainly the same as they are known in peptide or protein chemistry for end-group determination or as protecting groups during peptide synthesis.

In the present lecture the advantages and disadvantages of the classical amino acid analysis method and of the "modern" HPLC-methods are to be compared. It will be shown that even nowadays the classical method is still the method of choice for the majority of the routine applications, and this expecially if a large number of different amino acids have to be separated in a single run, whereas HPLC-methods can be of advantage in cases where a high sensitivity is necessary and only a limited number of amino acids must be seperated in one chromatographic run.

The differences in the applicational usability of the different methods, based on three main reasons (instrumentation, stationary phases, and chemistry necessary for separation and detection) will be shown.

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Technology and possibilities of routine amino acid analysers

Routine amino acid analysers have been commercially available since the 1960's. They are also called "classical amino acid analysers" because this type of analyser still refers to the method according to Stein & Moore who published as early as 1951. The method for classical amino acid analysis is done by separation of the amino acids by cation-exchange chromatography followed by post-column derivatisation with Ninhydrin and detection at a wavelength of 440 and 570 nm.

During the last 25 years there have been quite a few world wide operating companies who supplied routine amino acid analysers. However, since the introduction of HPLC-pre-column derivatisation methods, most of the companies have left the field of routine amino acid analysers. Since the last three to five years there seems to be an increasing interest for the classical analysers again. Especially where the analysis of physiological fluids is involved.

The current state of technology for a routine amino acid analyser is presented. The analytical possibilities for physiological fluid analysis are shown. Routine analysis programs as well as specific separation programs for individual amino acids are explained.

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Evaluation of cocoa flavour produced by amino acids and starch interact at roasting conditions

The reaction of leucine, threonine and leucine/threonine (3 + 2 by wt.) with corn starch (1 + 10 by wt.) produced a complex mixure of compounds with an overall flavour resembling that of roasted cocoa. The reaction is carried out at roasting conditions (180–200°C) for 8 hours in a closed reflux system. The proper time and temperature that provided the highest intensity and stability of the desired aroma were adjusted by using a simple designed Sniffing Port. The aroma components were isolated by means of the simultaneous steam distillation/solvent extraction method according to Lirsens-Nickerson. The extraction was carried out using n-pentane/diethyl ether (1 + 1 by vol.). The extract was concentrated at room temperature with a rotary evaporator and then by Vigreux distillation column (25 × 1 cm). The flavour concentrate was separated into acidic/neutral and basic fractions and subsequently analysed by GC/MS. The identified components include furans, furanones, pyridines, pyrroles, pyrazines and their alkyl derivatives in addition to the Aldol condensation product, 2-isopropyl-5-methyl-hex-2-enol. which is supposed to be formed via the Strecker degradation product of leucine, 3-methyl butanal. Aroma panel evaluation test is carried out on ten experienced panialists.

In most cases, identification was confirmed by authentic samples or by organic synthesis. The results are represented by tables and discussed in details.

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Volatiles of roasted coffee generated in model systems involving starch and different amino acids

Coffee is one of the most popular beverage all over the world. Coffee aroma was prepared by roasting different model mixtures of starch, and threonine, serine, and cysteine. Each model mixture was examined for its aroma description in relation to coffee aroma. The aroma concentrates was fractionated and subjected to GC-MS analysis. The results showed that the mixture containing cysteine has the best sensory results, it was described as excellent roasted coffee. This may be confirmed by the presence of the most contributor components for coffee aroma such as 2-furfuryl mercaptan (2.22%) which has a roasted coffee note, in addition to 2-methyl-3-furanthiol (2.25%), and 2-ethyl-3,5-dimethyl pyrazine (0.63%), and 2,5-dimethyl-4-hydroxy-5[2H] furanone "furaneol" (1.76%). The latter component is considered as degradation product of carbohydrate and have marked caramel-like smelling and tasting, and it is important odourant of roasted coffee.

The reaction of starch with threonine only generate a good roasted coffee odour. The aroma of this mixture contained high concentration of 2-ethyl-3,5-dimethyl pyrazine (14.35%) which has earthy and roasty note and had an influence on the coffee aroma. Whereas the pyridines which are important ingredients of coffee aroma were present with total concentration of 22.62%.

The poor sensory results of the volatile system containing starch and serine may be attributed to the absence of furaneol and the small concentration of some of the most important components for coffee aroma. The volatile components generated by reacting starch with either serine or threonine were represented in the aroma of reacting starch with their mixture. However, the high concentration of furanones (10.80%) and cyclotene (8.26%) in the aroma of this mixture might be responsible for its sweet note.

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Large-scale identification of proteins by amino acid analysis

Two-dimensional protein maps of microorganisms are useful tools for the elucidation and detection of target proteins, a process essential in the development of new pharmaceutical products. We applied amino acid composition analysis, following separation by two-dimensional gel electrophoresis, for large-scale identification of proteins of *Haemophilus influenzae. H. influenzae* is a bacterium of pharmaceutical interest whose entire genome, comprising approximately 1700 open reading frames, has been sequenced and published. For amino acid analysis, we used both pre-column derivatization of amino acids followed by reversed-phase chromatography of the derivatized residues and post-column derivatization of the residues after separation on an ion exchanger. The composition analyses derived from both methods allowed the efficient identification of 110 protein spots. The proteins were identified using the AACompldent software on the ExPASy server accessible via the WorldWideWeb with a success of identification of approximately 90%. The introduction of the analysis data of 12 residues was sufficient for a correct identification. In addition, proteins which contained an unusually high percentage of one residue could unambiguously be identified. Amino acid composition analysis proved to be an error robust, efficient method for protein identification. The method can be practically established in every biochemical laboratory and as complementary to mass spectrometry represents an important analytical tool for the mapping of the proteomes of organisms of interest.

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Effect of the hydrolysis method on the determination of the amino acid composition of proteins

Fast and reproducible separation and determination of amino acids serves the economical and reliable characterization and quantification of peptides and proteins and the large-scale identification of proteins by amino acid composition analysis. A prerequisite of a successful compositional analysis is a complete hydrolysis of the peptides and proteins and a quantitative recovery of the residues in the hydrolyzate. We investigated the effect of the different acid-hydrolysis methods on the compositional analysis of known proteins in solution and after blotting onto polyvinylidene difluoride membranes and worked out the conditions for the processing of large numbers of samples daily. The reliability of each method was studied by introducing the analysis data into the AACompIdent software and deducing the protein identification scores. All acid hydrolysis methods delivered reliable analysis data. The most accurate data were obtained by conventional, thermal hydrolysis of proteins in solution in the presence of methanesulfonic acid, closely followed by hydrolysis with hydrochloric acid and microwave radiation-dependent hydrolysis in the presence of hydrochloric and methanesulfonic acids. For blotted proteins, conventional hydrolysis delivered more accurate analysis data in comparison with the microwave radiation-induced hydrolysis. The extraction of the hydrolyzate residues from the membranes was a critical step for the unambiguous protein identification. Microwave radiation-induced hydrolysis was responsible for a higher degree of racemization of the residues.

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Application of amino acid analysis in critical ill patients – methodology, indications and interpretations of amino acid analysis with low pressure liquid chromatography in medicine

The development of methods to determine free amino acids in biology fluids has already a long history. The complicated and time consuming analytical procedure with the sensitive preanalytical phase, the time consuming separation by ion exchange chromatography with low or high pressure application, the data acquisition and the very complicated interpretation by transforming the original chromatografic areas into clinically usable data made it very difficult and sometimes frustrating to integrate these methods into the daily clinical routine procedures. There are defined fields of interest where the analysis of free amino acids in biological fluids should play a role in the clinical diagnostic strategies. Besides the field of clinical nutrition with controlling the indication and the follow up of clinical nutrition mostly in intravenous form has rationalized the application of specific amino acid solutions like solutions containing branched chain amino acids. The field of inborn metabolic errors and the field of detection of imbalances by specific pathological changes mainly in concern with intensive care medicine of critical ill mostly septic patients plays an important role. The problem was increased by the lack of methods to interpretate on the basis of relevant reference ranges the individual result of a total free amino acid analysis.

In this presentation a precise description of the preanalytical, analytical and postanalytical conditions is given especially of the principally different methodological approaches of low pressure liquid chromatography with ion exchange and postcolumn ninhydrine-detection and high pressure liquid chromatography with pre- and postcolumn derivatisation of orthophthalaldehyd or fluorometric detection with FMOC.

Main focus in this paper is given to the data documentation *and* interpretation on the basis of percentage-patterns of amino acids. The result are given as concentration profiles in body fluids like plasma, liquor and urin. The amino acid pattern as a rational basis of interpretation is done with the percentage of each individual amino acid of the total content because the relationship of different amino acids determines the efficacy of the transmembrane transport-process, where amino acid groups are competing like the aromatic and the branched chain amino acids.

In the last part the history of a child is presented where many years epileptic states were treated neurologically till the differentiated analysis of blood amino acid with analysis of urea- and ammoniapriduction showed an insufficiency of the final urea production especially in fasting states where the imbalance of amino acids can be detected only in the relative pattern with an high increase of glutamine and alanin as compensating biochemical reaction for the overproduction of ammonia.

Arginine

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Identification of N^G–methylarginine residues in heterogenous nuclear RNP protein A1

Heterogenous nuclear (hn) RNP protein A1 is the major core protein in hnRNP, the precursor for pre-mRNA, and known to be one of the most highly arginine methylated proteins in nature. The formation of N^G–methylated arginines are post-translationally catalyzed by protein methylase I (S-adenosylmethionine:protein-arginine N-methyl-transferase; EC. 2.1.1.23). We have identified four sites of N^G,N^G–demethyl-(asymmetric)arginines in protein A1 isolated from HeLa cell, which are located at residues 193, 205, 217 and 224, respectively. When the recombinant unmethylated protein A! was methylated *in vitro* with S-adenosyl-L-methionine and purified protein methylase I, residues 109, 205 and 224 arginines were methylated yielding a mixture of N^G–monomethyl- and N^G,N^G–dimethyl(asymmetric)-arginines. All of the four sties fall within a span of sequence between residues 190–233 that contains multiple Arg-Gly-(Gly) sequences interspersed with phenylalanine residues. These RGG boxes have been postulated to represent an RNA binding motif. Examination of sequences surrounding the sties of methylation in protein A1 along with a compilation from the literature of sites that have been identified in other nuclear RNA binding proteins suggests the protein methylase preferred recognition sequence of Gly-Gly-**Arg**–Gly-Gly, with the COOH-terminal flanking glycine being obligatory. Taken together with data in the literature, identification of the sites of A1

arginine-methylation strongly suggests a role for this modification in modulating the interaction of hnRNP protein A1 with nucleic acids.

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Effect of the amino acid L-arginine and its analog nitro-L-arginine methyl ester HCl (L-NAME) on central dopamine (DA) receptor reactivity

Nitric oxide (NO), a novel intracellular messenger of mammalian brain, affects a variety of physiological and pathological functions. Previously we showed that NO modulates central DA D_3 receptor reactivity in rats. n the current study DA D_1 (SKF 38393 HCl, 10.0 mg/kg) and D_2 (quinpirole HCl, 0.2 mg/kg) receptor agonists or DA D_1 (SCH 23390 HCl, 0.5 mg/kg) and D_2 (haloperidol, 0.5 mg/kg) receptor antagonists were administered IP to male and female adult Wistar rats, 10 min after administering the NO donor L-arginine (300 mg/kg IP) of NO synthase inhibitor L-NAME (25 mg/kg IP). Controls received saline. The following behaviors were recorded: locomotor activity time, grooming time, catalepsy time and numbers of rearings. We observed that L-NAME attenuated SKF 38393-induced locomotor activity, grooming time and rearing in male and female rats, although the effect of L-NAME was more prominent in females. L-NAME also enhanced SCH 23390-induced catalepsy, while L-arginine was without effect. Quinpirole-induced locomotor activity was enhanced by both L-NAME and L-arginine. Only L-arginine increased quinpirole-induced rearing activity; only L-NAME increased haloperidol-induced catalepsy. In summary, L-NAME and L-arginine each enhanced behavioral expression of DA D_2 receptor mediated actions, while L-NAME suppressed DA D_1 receptor mediated actions.

These findings on DA D_1 and D_2 receptor reactivity extend our previous report that NO has a modulatory role on central DA receptors.

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Regulation of nitric oxide synthesis by arginine-metabolizing enzymes

Nitric oxide (NO) is a major messenger molecule regulating blood vessel dilatation and immune function and functions as a neurotransmitter in the brain and peripheral nervous system. NO is synthesized from arginine by nitric oxide synthase (NOS), and citrulline which is generated can be recycled to arginine by argininosuccinate synthetase (AS) and argininosuccinate lyase (AL), forming a cycle termed "citrulline-NO cycle". When rats were injected with LPS, mRNAs for AS and AL as well as that for inducible isoform of NOS (iNOS) were induced in the spleen and lung. In immunoblot analysis, increase of iNOS and AS proteins was evident in these tissues. In immunohistochemical analysis, macrophages in the spleen were strongly positive for both iNOS and AS after the LPS treatment. These results indicate that citrulline-arginine recycling is important in NO synthesis in activated macrophages.

On the other hand, arginase may be involved in down-regulation of NO synthesis by decreasing availability of arginine for the NOS reaction. We found that mRNAs and proteins for iNOS and liver-type arginase (arginase I) were coinduced by LPS in cultured rat peritoneal macrophages and in the lung and spleen *in vivo*. mRNA for C/EBPβ, a transactivator of the arginase I gene, was induced more rapidly than that for arginase I. In addition to arginase I, an isoform (arginase II) that is expressed in some non-hepatic tissues, is present. cDNA for human arginase II was isolated. The predicted amino acid sequence was 59% identical with that of arginase I. Arginase I is located in the cytosol, whereas arginase II is located in the mitochondria. Arginase II mRNA was coinduced with iNOS mRNA in murine macrophage-lke RAW 264.7 cells by LPS. All these results suggest that there is a complex regulation of arginine-synthesizing and degrading enzymes that together control NO production in cells.

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New fundamental and biotechnological aspects in bacterial arginine biosynthesis and regulation

The cyclic pathway of arginine biosynthesis from glutamate to ornithine via acetylated intermediates has not been studied extensively. This more economical pathway, as compared with the linear one in *Escherichia coli*, functions in some thermophilic and mesophilic bacteria in different ways. In *Bacillus stearothermophilus*, the key *argJ* gene responsible for recycling of the acetyl group from *N*–acetylornithine to glutamate encodes a bifunctional enzyme which also provides the first catalytical step, the acetylation of glutamate using acetyl-CoA (Sakanyan et al., 1993a). In *Corynebacterium glutamicum, argJ* encodes a monofunctional enzyme (Sakanyan et al., 1996). The overexpressed ArgJ is deleterious for *E. coli* host cells. The possible mechanisms responsible for the features of the acetylation steps in different bacteria will be discussed. The advantage of the cyclic pahtway allowed us to overcome the limiting steps and to construct *E. coli* recombinant strains overproducing L-arginine.

Specific regulation of gene expression in thermophiles remains today another relatively little-explored area of investigations. In *B. stearothermophilus* arginine biosynthesis genes are organized in the *argCJBD* operon. A very strong promoter was found to mediate the operon transcription. This promoter was successfully used for overexpression of several reporter genes in *E. coli* and for construction of shuttle expression vectors. In the native *B. stearothermophilus* host the *argCJBD* operon transcription is governed by the extremely thermostable repressor, ArgR (the first repressor cloned from thermophiles; Dion et al., 1997). A new helix-turn-helix motif is involved into DNA recognition/binding and several distinct amino acids participate in oligomerization and/or stabilization of the ArgR/DNA complex. ArgR binds to the operator, that largely overlaps the P*arg* promoter of the *argCJBD* operon (Savchenko et al., 1996). The control region of the operon displays some distinct particularities and, therefore, it was used as a target for studying the regulatory network in thermophiles in this laboratory.

Finally, the *E. coli argE* mutant strains were used for searching heterologous genes which encode biocatalytically important enzymes (Sakanyan et al., 1993b). Two genes encoding L-stereospecific aminoacylase and carbomylase were cloned from thermophiles (Batisse et al., 1997). Structural and

functional similarities suppose evolutional relationship between the amino acid amydohydrolases of different metabolic pathways in distant bacteria.

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Peroxisomal aging is influenced by arginine and taurine

Peroxisomes are present in all mammalian cells except mature red blood cells. Several anabolic and catabolic pathways are exclusively localized in peroxisomes. Peroxisomes are involved in the production and detoxification of reactive oxygen species, which makes them, at least in part, responsible for metabolic changes during the process of aging.

To understand the metabolic importance of peroxisomes in the process of aging we studied the time course of peroxisomal enzyme activities in NMRI-mice at the age of 6 weeks, 6 months and 24 months. Since arginine and taurine are considered to influence the process of aging based on their known ability to reduce oxidative stress, animals were fed these two substances.

The peroxisomal enzyme activities catalase, acyl-CoA oxidase and Crotonase and the activity of mitochondrial glycerol-3-phosphate dehydrogenase were determined. Significant ($p < 0.05$) age dependent changes of activities in liver samples from control mice were seen only for the peroxisomal marker enzymes catalase and acyl-CoA oxidase. Both activities decreased in parallel until the age of 24 months. After 24 months addition of arginine to the food, increased acyl-CoA oxidase activity by about 100%. Taurine more than doubles the specific activity of the rate limiting step of the peroxisomal oxidation of fatty acids. Interestingly the activity of catalase is unchanged in both arginine and taurine treated animals which might give rise to enhanced intraperoxisomal levels of hydrogen peroxide followed by a diffusion of hydrogen peroxide into the cytosolic compartment.

Long term treatment (24 months ?) with taurine enhances the activity of acyl-CoA oxidase, the rate limiting enzyme of the peroxisomal ί-oxidation of fatty acids. This rise of activity is not accompanied by a rise in catalase activity for the detoxification of hydrogen peroxide, being produced by this enzyme. This is contrary to the observations in control mice where the activities of hydrogen peroxide producing acyl-CoA oxidase and catalase, the enzyme responsible for the detoxification of hydrogen peroxide, are both decreasing with age. On the other hand, based on the antioxidant ability of taurine, the enhanced level of hydrogen peroxide which can occur if the diet contains a high amount of fatty acids (long and expecially very long fatty acids), which are mainly or solely degraded via the peroxisomal ί-oxidation which yields hydrogen peroxide in the first (acyl-CoA oxidase) oxidative step. In case of arginine treatment, which also reduces lipid peroxidation in case of oxidative stress, the same assumption might be true.

Basic Chemistry and Modelling

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Intra- and extracellular amino acids in relation to protein metabolism in Belgian Blue double muscled bulls on 2 different growth patterns

The assessment of protein nutritional status and the adequacy of amino acid (AA) supply is a major challenge in both human and animal nutrition. In this communication plasma AA (AAp) and intracellular AA (AAi) were measured in 16 Belgian Blue double muscled bulls (BBDM) on 2 different growth rates. Rour groups of 4 bulls were given either a control fattening diet over the entire experimental period (from 300 to 600 kg weight) control group, CG), or a low growth diet (0.5 kg/d; LG) during respectively 4 (group 2, G2), 8 (G3) or 14 months (G4) followed by a period of compensatory rapid growth (RG). In both periods muscle biopsy and blood plasma samples were taken and N-balance was measured.

Total [AAi] were substantially higher than [AAp]. Among individual AA β-Alanine was particularly high. Upon changing between LG and RG [AAp] followed no particular systematic pattern, while [AAi] increased in CG and G2 and remained almost constant in G3 and G4. Total essential AAi, [EAAi] rose in G2 and G3 but not in G4. To verify to what extent AA supply meets demands for muscle protein synthesis (MPS) the ratio free to bound (F/B) EAAi was computed. Total F/B EAAi rose substantially from LG to RG in all 3 restricted groups, e. g. from 0.36, 0.44 and 0.46% in LG to 0.48, 0.92 and 0.64% in RG, respectively in G2, G3 and G4, and did not change in CG, indicating a better AA supply in RG. Leucine had the lowest F/B ratio in LG, making it a candidate for limiting AA. It was low in CG and G2 during both periods while in G3 and G4 an increase was observed. Total and individual [AAi] and [AAp] could also be related to overall AA and protein metabolism. N-shuttle ALA decreased both in plasma and in intracellular compartments between LG and RG. This could indicate a lowering of degradation in favor of synthesis. However, urinary 3-methylhistidine excretion, a marker of myofibrillar protein degradation (MPD), and urinary urea excretion, which could be considered as a measure of total protein degradation, rose both. The latter could be ascribed to a higher degradation related to a higher N intake. So, during RG, degradation of both dietary protein and MP increased. N balance, as an extimate of protein deposition rose significantly from LG to RG: from 20–25 to about 60 g/d. This rise resulted from increases in both MPS and MPD, the former being more important. The magnitude of these responses was inversely proportional to the length of the restriction period.

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Fluorescence studies of model peptides

Biopolymers often oscilate within an ensemble of structures and this fact presents frequently an enormous challenge in the studies establishing the bioactive conformation. Among the various modern techniques the fluorescence spectroscopy is one of the most useful in studies of the mutual interactions in

the biomolecules. The technique requires a negligible amount of a studied compound but it is often necessary to incorporate additional chromophores into a molecule in order to determine an energy transfer between acceptor and donor and the donor-acceptor distance. Analysis of a distance distribution between chromophores allows to establish the degree of the conformational flexibility of the studied molecule.

In this communication we present synthesis of model helical peptides containing two chromophores: dansyl (Dns) at N-terminus and naphtylalanine (Nal) at C-terminus

Dns-AAAAKAAAAKAAAAKA-Nal-NH_2

Dns-AKAAKAKAAKAKAAKA-Nal-NH_2

The peptides were subjected to conformational studies by means of fluorescence and circular dichroism. The conformational parameters obtained (the distance between the chromophores, the distance distribution and the molar elipticity at 222 nm) will be presented in connection with a helix content in the studied molecules. Influence of pH, temperature and a type of a solvent used in the measurements on conformational parameters will be also discussed.

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Photoreactive amino acids in conformational studies of peptides

One approach to map directly the ligand-receptor bimolecular interface is to identify "contact points" by covalently cross-linking hormone to the receptor when the two are in close proximity [1]. Photoaffinity labelling is a method which has been successfully used for the identification and localization of macromolecular receptors [2] and allows light-induced covalent linking of a ligand to the binding site of its receptor. Subsequent purification and proteolysis of the photolabeled receptor allows localization and sequencing of the binding site.

Arylketone derivatives constitute an important class of photoaffinity labels. These photophores, often with benzophenone structural units, have several very desirable features: (i) they are chemically more stable than diazoesters, aryl azides and diazirines, (ii) they can be manipulated in ambient light, (iii) they can be activated at 360 nm, avoiding protein damaging shorter wavelenghts, (iv) they are activated to triplet biradicals capable to react with high specificity and efficiency with many types of alkyl carbon atoms, even in the presence of solvent water and bulk nucleophiles and they do not require occuring particular reactive functional groups in the binding site [3]. Moreover this type of photoprobes have chemical stability to the synthetic conditions including solid-phase peptide synthesis.

Benzophenone (BP) substituted ligands are ideal for this purpose because of their chemical stability and remarkably selective photochemistry.

Application of such BP moiety (Bpa;(p-benzoyl)-phenylalanine; Lys(ϵ-pBz_2;ϵ-([p-benzoyl]benzoyl)lysine) in peptides and its influence on conformation of these peptides using CD and fluorescence spectroscopy will be presented.

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Preliminary studies on structure-function analysis of a major proline transporter (PRNB) of the filamentous fungus *Aspergillus nidulans*

The filamentous fungus *A. nidulans* possesses a major proline transporter (PRNB) encoded by the *prnB* gene whose expression has been studied in detail. The PRNB transporter belongs to a well established family of amino acid transporters, conserved in both prokaryotes and eukaryotes, with a predicted structure of 12 α-helial transmembrane domains.

The long-term objective of our work is to study amino acid transport across biological membranes addressing a basic biological phenomenon (transport of solutes) having medical potentialities. Apart from their importance in mammalian metabolism amino acids act as neurotransmitters (proline, glutamate) and amino acid transport proteins act as receptors for certain leukaemia retroviruses.

In the present work, we studied structure-function relationships in the PRNB protein by molecular and biochemical approaches. A number of specific *prnB* mutants was studies: cryosensitive mutants *prnB115* (a frame-shift mutant resulting in the addition of seven amino acids at the carboxyl terminus of PRNB) and *prnB117* (duplication of 3 amino acids, NRT, at position +376 of PRNB) show dramatically reduced mRNA levels at the non-permissive temperature. At the permissive temperature where their *prnB* MRNA levels are similar to the WT, mutant *prnB117* shows reduced proline uptake rates while mutant *prnB115* does not mediate significant proline accumulation. This might suggest that in these mutants the PRNB protein is not fully expressed or it is unstable. Mutants *prnB119* (a F → V change at position +278) show similar mRNA levels to the WT. These mutants have increased K_M and unchanged Vmax for L-proline uptake. Since both mutations should not affect protein structure (phenylalanine, valine and leucine have similar atomic structures and hydrophobic characteristics) they might affect affinity for proline which in turn suggests that the mutated amino acid residues might be involved in proline binding.

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Kinetic analysis of tryptophanase-catalysed D-tryptophan degradation

The stereospecificity of enzyme is very rigid. This property is important in early metabolism to make asymmetric biological world. However, it is not at all clear why only L type of D and L-amino acids is dominant in contemporary biological world. The investigation of the mechanism on stereoselectivity is considered to give the clue to solve the problem. The enzymological first step to elucidate it is to study the difference between the reaction with L type and D type on the same enzyme. Tryptophanase (TPase) which has the very strict stereospecificity to L-tryptophan is one of the most extensively studied PLP-dependent enzymes. Nevertheless, the reaction with D type of tryptophans have not been investigated. We have so far studied whether TPase can react with D-tryptophan or not. TPase becomes active to D-tryptophan in highly concentrated diammoniumhydrogen phosphate solution (DAP). The reaction is not responsible for denaturation because the removal of DAP reversibly returns TPase to the original one. DAP makes the comparison of the reaction between L type and D type possible. The reaction process of D-tryptophan degradation is studied in terms of kinetics. DAP acts on TPase as activator below 3.1 M, and

as noncompetitive inhibitor over 3.1 M. Additionally, a pathway of the reaction and the equation of its initial velocity is provided on the basis of kinetic parameters. Kinetic results give the significance of TPase·DAP·D-Trp complex, which is essential to gain access of D-Trp to the catalytic site through some steric structural change of TPase. This reaction will contribute to clarify the selection mechanism for optical isomers in early metabolism.

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Amino acid composition of silks

Silk is the proteinaceous fiber extruded by various animal species in the classes *Insecta* and *Arachnida* and of particular commercial importance is the domesticated *Bombyx mori*. Readily available materials are transformed into insoluble filaments surrounded by a second glue-like protein under ambient conditions by the caterpillars in approximately one month. Various breeding projects have been undertaken to produce a fibers with specific characteristics, such as fine or thick filaments, or disease resistant silkworms, but the effect on the structure and composition of the silk itself has not investigated.

This study compares the amino acid compositions of several strains of *B. mori* silk to the compositions of the wild-silks. Standard acid hydrolysis, TN HCl at 110°C für 22 hr, was used to hydrolyze silk cocoon and fiber samples. Amino acid analysis was carried out using a Hitachi L-8500 automatic amino acid analyzer with ninhydrin detection. While the silkworm breeding program was improved some characteristics relating to disease-resistance and fiber properties, the amino acid compositions of the various strains of *B. mori* are essentially the same. The amino acid compositions of the wildsilks are different only between families and not between genus.

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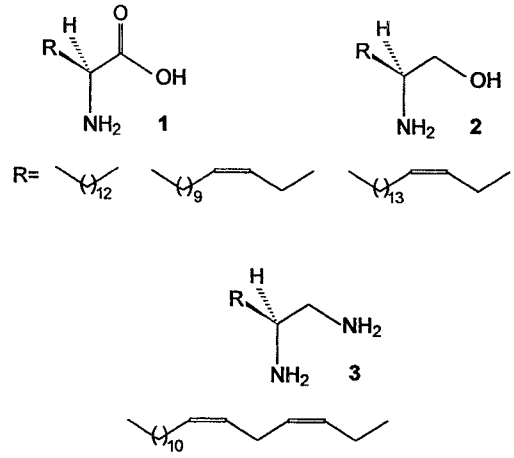
Conversion of chiral unsaturated lipidic amino acids into sphingosine analogs and diamines

Sphingosine is a metabolic intermediate of sphingolipids that modulates the activity of several enzymes involved in signal transduction and cell growth. On the other hand, long chain fatty amines exhibit a number of interesting properties: antimicrobial activity, antihyperlipidemic activity, growth inhibition of ras-transformed cells. Thus, new sphingosine analogs and lipidic diamines are expected to present interesting biological properties.

The lipidic α-amino acids (LAAs) are non-natural α-amino acids with long aliphatic side chanis and their poteintial use is wide [1]. The synthesis of chiral saturated LAAs is based on the oxidative cleavage of 3-amino-1,2-diols obtained by the regioselective opening of enantiomerically enriched long chain 2,3-epoxy alcohols [2]. Unsaturated LAAs may be prepared in their enantiomeric forms from glutamic acid semialdehydes using Wittig type reactions. We present here the synthesis of chiral sphingosine analogs **2** and lipidic diamines **3** starting from chiral LAAs **1**.

Boc-protected chiral LAAs were converted into alcohols by chemoselective reduction of their corresponding fluorides with sodium borohydride and dropwise addition of methanol. Replacement of the hydroxy group by an azido group followed by selective reduction ($NaBH_4$ in MeOH, 10% Pd/C) led to chiral long chain 1,2-diamines. The procedure was free of racemization as proved by ^1H NMR analysis of the suitable Mosher amides.



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Equilibrium of ion exchange of amino acid cations on a liquid sulphonic ion exchange extractant

Ion exchange extraction method for preparation of pure amino acids is based on interaction of their protonised forms with the carrier of sulfonic groups of a liquid ion exchanger (in our case dinonyl naphtalene sulfonic acid in a non-polar solvent). In difference with polymer ion exchangers, physical chemical regularities of these processes are almost completely unknown.

At the Institute of Physical Organic Chemistry their systematic studies have been done which allowed to develop ion exchange-extraction technology for their extraction from cultural liquids and recovery as pure substances. A part of these research is a subject of the communication.

In the paper are given results of experimental investigation of ion exchange of cations of the main aliphatic amino acids (Gly, Ala, Val, Leu) with hydrogen ion in acid media. It has been established that the selectivity of sorption of these ions by the liquid ion exchanger is substantially higher than on the sulphostyrene ion exchangers, as well as the difference in their selectivities. This is a favorable pre-conditions for selective removal and separation of amino acids. The selectivity coefficients have a wide region of weak dependence on the degree of loading of the liquid ion exchanger with the amino acid cations ($x = 0–0.5$). At $x > 0.5$ a sharp decrease in the selectivity occur. Complete saturation of the liquid ion exchanger with these cations appeared practically impossible and the saturation degree can reach values only 70–80% depending on the amino acid. Such a phenomenon is usually interpreted for resinous ion exchanger as an evidence of the sieve effect caused by steric

hindrances to permeability of bulky ions to the exchange sites. In our case the sieve effect is impossible and the phenomenon was explained by formation in the organic phase strong complexes including (apart of the amino acid cations and the extractant anion) one hydrogen ion per 3–6 amino acid cations. This suggestion has been supported by IR spectra measurements.

The selectivity of extraction as a function of degree of loading of the liquid ion exchanger has been described in terms of mathematical model developed earlier. It appeared that adequate description of the real processes is achieved in the assupmtion that there are at least four physically distinguishable state of the cations in the reverse micelles of the extractant. The micelles themselves, as it has been shown by vapour pressure osmometry, are undergone substantial change in size upon their conversion from H^+ into the A^+ forms, also exhibiting existence of at least four types.

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Liquid ion exchange extraction of amino acids from aqueous media

The main principles, advantages and drawbacks of amino acids extraction from aqueous media with abtaining the pure substances based on application of ion exchange resins and ion exchange extractants are discussed in the paper. The first method is well described in the literature and is applied in practice. Technologies of production of many amino acids from products of microbial syntheses are based on this process. Their physical chemical regularities have been extensively investigated and well described in a numerous publications and it is accounted that most of the principle questions connected with the mechanism of amino acid sorption by ion exchangers are clear. There are some problems predetermined by the nature of polymeric ion exchangers which do not allow to make this technology intensive and cheap. The main of these problems are: slow sorption-desoprtion process; large amount of waste waters; intensive side processes, fowling the ion exchanger and polluting the final product.

At the Institute of Physical Organic Chemistry an alternative technology of extraction of amino acids have been developed. It is based on application of liquid analogue of sulphostyrene ion exchanger, extractant Sulphex. The latter is a solution of branched polyalkyl naphtalene sulfonic acids in non-polar solvents. Similar to the resinous ion exchanger, the main process in the method is interaction between the sulphonic group and a protonised molecule of amino acid. Since liquid ion exchangers do not have a polymeric matrixes, there is no diffusion obstacles for sorption of amino acids of any molecular mass. Therefore the rate of sorption processes in this case can exceed those for polymeric ion exchangers by orders of magnitude. By applying traditional extraction technology, it appeared possible to develop a technologies for extraction of amino acids from cultural liquids with their following purification, with small amounts of waste water (ten times less than in the traditional technologies). The process is fast and requires compact inexpensive equipment.

In spite of similarity of the main processes in the ion exchange and extraction technologies, there is large difference between the whole processes due to completely different molecular and overmolecular structures of polymeric and liquid ion exchangers. In the paper are discussed the main scientific problems connected with development of technology for industrial ion exchange extraction of amino acids.

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Modeling properties of biochemical compounds with connectivity terms

The descriptive and utility power of linear combinations of connectivity terms (LCCT) derived by a trial-and-error procedure from a medium-sized set of 8 connectivity indices: $\{\chi\} = \{D, D^v, {}^0\chi, {}^0\chi^v, {}^1\chi, {}^1\chi^v, \chi_t, \chi_t^v\}$ has been tested on properties of heterogeneous classes of biochemical and inorganic compounds centered on the homogeneous class of natural L-amino acids. Two techniques have been tested to choose the appropriate combination of indices: the forward selection and the complete combinatorial technique. While the latter searches the entire combinatorial space, the former searches only a subspace of it. The forward selection technique has nevertheless many advantages among which to be a good tool for an elementary and direct test for newly defined indices. The modeling has been achieved centering the attention both on the predictive power of the proposed linear equations and on their utility. Thus, the modeling of the solubility of the entire heterogeneous class of n = 43 amino acids, purines and pyrimidines could be satisfactorily accomplished with a set of connectivity terms based on the χ_t^v index. The unfrozen water content of a mixed class of inorganic salts and natural amino acids has also been satisfactorily described with composite terms. In both cases the modeling shows a remarkable utility.

Further, the δ cardinal number is used as a basis for the definition of a supravalence delta index used to encode the genetic code. In fact, the newly defined delta index can be applied to the triplet code words to generate the different families and subfamilies of the genetic code. Besides, five experimental propterties of DNA/RNA bases have been modeled by the aid of linear combinations of connectivity indices and terms (LCCI and LCCT): singlet excitation energies δE_1 and δE_2, oscillator strenghts f_1 and f_2, and molar absorption coefficient ϵ_{260}.

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Structural characterization of HSA adsorbed on an anion exchanger chromatographic supports by FT-IR analysis

Adsorption of proteins onto surfaces has long been a subject of interest in the biomaterial world. Access to the fine three-dimensional structure of adsorbed proteins should be a key to the evaluation of the energy variation associated with conformational changes. The most efficient techniques, such as X-ray diffraction, NMR spectroscopy, circular dichroism, used for protein structure determination, are not adapted to adsorbed molecules. Infrared and Fourier transform infrared spectroscopy (FT-IR) may be applied yet to turbid samples in contrast to the above-mentioned techniques.

In order to better understand the adsorption behavior of human serum albumin (HSA) on chromatographic supports, we compared the structural infrared characteristics of this protein when adsorbed on chromatographic supports of small pore size. Two different materials were used: C6 alkyl chains grafted on silica for the reversed phase support and polyvinylimidazole cross-linked on silica for the ion-exchanger. On both supports, the column capacities are very similar. With 20% acetonitrile in the eluent, the column capacity is 1.5 times higher than that with the eluent buffer at pH 7.4.

When HSA is adsorbed with 0–30% acetonitrile in the solvent on a C6 reversed phase support, one-tenth of HSA cack-

bone is unfolded. In the adsorbed state, the protein is more hydrated and self-associated than in the corresponding solutions. These conformational changes result from the competition of the solvation of acetonitrile and of the grafted alkyl chains for the interaction with the hydrophobic domains of the protein. This adsorption behavior is compared to that observed when HSA is adsorbed on an anion exchange stationary phase, the polyvinylimidazole cross-linked on silica. The protein remains in its native state, without any considerable conformational changes. This is important in cases that we are interested to reserve the three-dimensional structure of the protein and maintain the catalytic activity.

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A method of side-chain modeling using information of conserved side-chain torsion angles within homologous family of proteins

When numerous structures of homologous proteins for the target protein were known, homology modeling is effective because it is sometimes able to obtain a reasonably accurate model to explain their structure, properties, and functions. And in homology modeling, side-chain modeling is an important process.

In this work, we introduce a method of side-chain modeling using information from conserved side-chain torsion angles within a homologous family of proteins, and these were calculated for a pair of residues at topologically equivalent positions as a result of structural alignment. These probabilities were obtained from many homologous families in the Brookhaven Protein Data Bank. Side-chain modeling using these probabilities was performed for 11 known proteins from their native and modeled backbones, respectively. As a result, the percentage of the $\chi 1$ angle correct within 30 degree was found to be 67.4% and 81.7% for all and core residues. Moreover, to obtain an index of accuracy for a prediction method of side-chain conformations, we investigated the probability of conserved side-chain torsion angles for the highly similar proteins having > 90% sequence identity and < 2.5 Å X-ray resolution. As a result, 83.4% of the side-chain conformations were conserved for the $\chi 1$ angle in the highly similar proteins.

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Structure of glycylhistamine dihydrochloride by X-ray and NMR spectroscopy

Glycylhistamine has been found in bee venom and it can serve as radioprotective agent. For this study glycylhistamine was obtained from BOC-glycine and histamine dihydrochloride in the presence of BOP, which was used as coupling reagent.

White crystals of glycylhistamine dihydrochloride from solution in methanol crystallize in the triclinic P-1 space group. The cell parameters are a = 7.401 Å, b = 8.327 Å, c = 10.422 Å, α = 102.95°, β = 97.15° and γ = 112.78°. Two molecules per asymmetric unit cell have been found. This is confirmed by solid state NMR, where we observed single peaks for different nuclei, because of a symmetric center, (x, y, z) yielding (–x, –y, –z). The bond lengths and bond angles of the peptide group in glycylhistamine are in good agreement with the generally observed values for the oligopeptides containing protonated imidazole ring. The two groups of atoms, constituted by the peptide group and imidazole group are coplanar. The distances of the atoms from a mean plane of the imidazole cycle are not significant, for the peptide group they are rather small. The torsion angle around the C-N bond is –176.9°. The crystal cohesion is characterized by a very dense lattice of hydrogen bonds, in fact all protons are engaged at least in one bond. This explains the high density of the crystal (1.425).

Any labile proton (amino, amido, imidazolium protons) is bound by chlorine atoms except H-N(3). The chlorine (Cl1) is bound to three molecules *via* the N(1)-H, N(4)-H and N(5)-H, whereas the chlorine (Cl2) is bound to two molecules *via* the H-N(5). On the contrary, the H-N(8) proton is bound strongly by hydrogen bond to the carbonyl, the distances being 2.696 Å and 1.70 Å for O(1)-N(3) and O(1)-H-N(3), respectively. This bound induces a small modification of the imidazole cycle distances, probably due to a change of population of the different isomeric forms of the peptide group and the imidazole group.

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NMR study of copper(II) complexes with histamine and glycylhistamine

The carbon-13 and proton relaxation times (T_1 and T_2) and the isotropic contact shifts ($\delta\omega$) were measured, at 100.62 MHz and at pD = 10.6, on histamine (0.33 mol.L^{-1}) in aqueous solutions, containing 0 to 10^{-3} mol.L^{-1} of copper(II) perchlorate over a temperature range of 5–90 °C. Under these conditions, the Cu^{2+} cation is bound to two histamine molecules. The longitudinal relaxation of carbons and protons of histamine in this complex is shown to be purely dipolar and is controlled by the rotational reorientation of the complex, $\tau_R = 9.0 \times 10^{-11}$ s at 36 °C. The metal to carbons distances are 2.92, 3.71, 3.12, 3.92 and 3.16 Å for the C(2), C(5), C(4), CH_2(gly), C(NH_2) terminal respectively. The metal to proton distances are 3.74 and 5.01 Å for the C(2)-H and C(5)-H respectively. This is a proof of the coordination by the N(3) nitrogen atom of the imidazole ring and the N-amino terminal atom. The transverse relaxation of bound histamine molecules is mainly scalar, the ratio T_{1M}/T_{2M} being much higher than 7/6. The transverse relaxation is controlled by the electronic relaxation. The study of the chemical shift as a function of the temperature shows a relatively fast exchange between bound and free ligands. The measurements of T_2 and $\delta\omega$ allowed us to determine the following values: the activation energy of the reorientation of the complex: E_R ='17.9 kJ.mol^{-1}, the mean time of residence of a bound molecule $\tau_M = 1.1 \times 10^{-8}$ s, the hyperfin constants A(H5) = 0.92 MHz and A(H6) = 0.76 MHz and the electronic relaxation time $T_{1e} = 4.25 \times 10^{-9}$ s at 36°C.

The same measurements were made on glycylhistamine (0.2 mol.L^{-1}) as ligand in presence of copper (0 to 10^{-3} mol.L^{-1}). The variation of the chemical shift in function of the temperature is characteristic of a fast exchange leading to $\tau_M \sim 1.3 \times 10^{-8}$ s. The following values: $\tau_R = 1.2 \times 10^{-10}$ s (reorientation correlation time); E_R = 18.6 kJ.mol^{-1}, $T_{1e} = 6 \times 10^{-8}$ s, and A(H5) = 0.45 MHz were determined at 36°C. The paramagnetic ^{1}H relaxation rates are equal to 1049 and 907 s^{-1} for C(2) and C(5)H respectively. The ratio of these values is 1.15 for glycylhistamine against 2.80 for histamine. This shows another mode of coordination for glycylhistamine, comparatively to histamine; the complex CuL_2H_{-1} is consti-

tuted by one molecule of glycylhistamine coordinated through the amino, amido and N(3)-imidazole nitrogen atoms and one molecule through the N1-imidazole nitrogen atom only. This is confirmed by the value of the deprotonation constant (pK = 7.60) in the complex, found by potentiometry, which corresponds to the deprotonation of the free NH_3^+ group of the second ligand. The values of the RPE parameters (g_o = 2.103 and A_0 = 82.0 Gauss) and of λ_{max} (568 nm) in UV-vis spectrum are characteristic of a coordination by four nitrogen atoms.

Biochemistry and Medicine

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The role of glutamine synthetase in differentiation in *Dictyostelium discoideum*

Dictyostelium discoideum is a lower eukaryote which has been widely used as a model system for the study of differentiation. This organism has a number of attractions; it is easy to grow and manipulate, it undergoes true differentiation, there is a substantial body of data on its genetics and mutants can easily be obtained either by conventional means or using molecular techniques, it has a limited number of cell types and there is clear separation of its growth and differentiation phases.

There is widespread acceptance of the hypothesis that gradients of morphogens play a major role in the determination of cell fates. However experimental evidence for this hypothesis is relatively sparse, mainly for technical reasons. The *Dictyostelium* system has provided some of the best information on the nature of morphogens. Here four low molecular weight compounds have been identified, these being cyclic AMP, DIF (differentiation inducing factor – a chlorinated phenyl alkanone), adenosine and ammonia. In very general terms cyclic AMP and ammonia favour the spore pathway while adenosine and ammonia favour the stalk pathway. Our interests lie in the mechanisms by which ammonia the concentration of ammonia is controlled.

We have been studying the properties and control of two key enzymes involved in ammonia metabolism, glutamate dehydrogenase(s) and glutamine synthetase (GS). The major glutamate dehydrogenase is the NAD-dependent form and the evidence suggests that it functions primarily in a catabolic role (deamination of L-glutamate). Our more recent work has been on GS, which is likely to play a major role in the removal and detoxification of ammonia during differentiation. This enzyme increases approximately 3 fold during differentiation and is localised in prespore cells, which are predicted to be the site of ammonia removal. The enzyme has been purified to homogeneity and its properties examined. It has a native molecular weight of 376 kDa and a subunit molecular weight of 43.5 kDa, suggesting it is an octamer. It appears to be a typical eukaryotic GS-II enzyme.

Using degenerate oligonucleotide primers based upon conserved eukaryotic GS gene sequences we have amplified part of the *D. discoideum* gene. Sequence analysis of the PCR product shows over 90% homology to yeast GS and 75% towards the enzyme from mammalian and plant sources. Southern and Northern blot analysis indicates that a single copy of the gene is present in *Dictyostelium discoideum* and that a single mRNA is transcribed from that gene. This mRNA increases during differentiation and it is preferentially localised in pre-spore cells, in line with the findings for the protein.

Methionine sulfoximine (MSX) inhibits the enzyme in a specific and irreversible manner. Cells grown in the presence of 5 mM MSX have no detectable GS activity initially but eventually do recover activity during differentiation. They do not proceed through culmination until this recovery of activity occurs. Cells grown in the presence of higher MSX (50 mM) do not recover activity and do not culminate. We conclude that GS plays an important role in the removal of ammonia during differentiation and that this removal is essential for culmination.

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The effect of 1,2-benzo-8-(alanyl)-3-phenoxazone (BLP) on proliferation of prostatic carcinoma epithelial (PCE) cells

By reacting tyrosine with 1-nitroso-2-naphthol in the presence of nitric acid 1,2-benzo-8-(alanyl)-3-phenoxazone (BLP) an analog of actinomycin D is produced. The structural similarity of BLP to actinomycin D prompted the National Cancer Institute (NCI) to investigate its antitumor activities. NCI investigations revealed that BLP exhibits growth inhibitory effect on various cancer cells and as a result BLP has received the U. S. Patent by the U. S. Patent Office. The purpose of this investigation was to study the effect of BLP on PCE cell proliferation.

For this study, cultured PCE cells were exposed to different concentrations of BLP ranging from 10^{-12} to 10^{-5} M for 24, 48 and 72 hr. At 24 hr interval, cells were analyzed for BLP's effect on cell proliferation, cell viability and DNA synthesis. The cells were enumerated using a hemocytometer. The cell viability effect was assessed by vital staining and DNA synthesis was measured by the incorporation of ^{3}H-thymidine.

The results of the study indicate that none of the concentrations of BLP had any significant effect on PCE cell number up to 24 hr of exposure. However, increase in concentrations of BLP and time incubation period did inhibit cell proliferation. The exposure of PCE cells for 72 hr at concentrations greater than 10^{-10} M BLP caused drastic decrease in cell viability. At 10^{-5} M BLP, the cell viability was only 30% indicating that BLP between the concentrations of $10^{-10} - 10^{-5}$ M are both cytostatic and cytotoxic. Similar parallel studies using human fibroblasts revealed that BLP is not cytotoxic to human fibroblasts. These preliminary observations suggest that BLP may exhibit selective antiproliferative effects. The focus of future investigations is to elucidate the possible mechanism(s) of BLP induced cell death; whether it is due to apoptosis or cell necrosis.

(This study was supported by grant from Research Centers In Minority Institutions (RCMI) Grant No. 2G12RR03045–06.)

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Effect of fentanyl citrate on protein and glucose metabolism after TNF-α injection in rat brain

The present study was to determine whether metabolic response induced by intracerebroventricular (ICV) administration of TNF-α would be reduced by prophylactic administra-

tion of fentanyl citrate. SD.rats (n = 60, BW: 225–250 g) were catheterized into the jugular vein for TPN and simultaneously a catheter was inserted to ICV under pentobarbital anesthesia, on day 0. On day 5, TPN was begun with a half strength diet. On day 6, a full strength diet (250 kcal/kg/day, 1.5 gN/kg/day) was given and the animals were randomly divided into 5 groups; 1) Control (C, saline ICV), 2) TNF (T, TNF-α ICV, 0.6 μg/rat), 3) Fenta. (F, fentanyl IV, 50 μg/kg), 4) Fenta.+TNF (FT, fentanyl IV prior to the injection of TNF-α ICV), 5) Systemic TNF (ST, TNF-α IV, 0.6 μg/rat). After the treatment, TPN solution containing 6-^{3}H-glucose (7 μCi primed plus 2 μCi/hr constant) and 1-^{14}C-leucine (2 μCi/hr constant infusion) were given for 4 hours to determine glucose production (Glu.Pro, mg/kg/min), whole body protein breakdown rate (WPBR, μmole LEU/kg/hr) and fractional synthesis rate (FSR) of the muscle and mixed plasma protein (%/day). Data are mean (SEM), stat. by ANOVA and different superscripts indicate significant difference; *p < 0.05;

	Cont.	TNF	TNF+Fenta.
n	14	14	10
Mus. FSR	13.0 (1.4)	*7.7 (0.6)**	11.5 (1.5)
Plas. FSR	40.4 (2.8)	*52,1 (2.3)**	37.2 (3.6)
WPBR	45.6 (22.3)	*93.4 (24.4)**	48.4 (25.5)
Glu. Pro.	3.7 (0.8)	*7.5 (0.7)**	4.4 (0,8)

	Fenta.	Systemic TNF
n	10	12
Mus. FSR	13.2 (1.6)	11.6 (0.9)
Plas. FSR	39.9 (2.5)	31.5 (1.5)
WPBR	51.6 (20.5)	61.3 (9.4)
Glu. Pro.	3.8 (0.4)	4.6 (1.2)

We concluded that prophylactic administration of fentanyl citrate attenuated an enhancement of proteolysis and gluconeogenesis induced by ICV administration of TNF-α.

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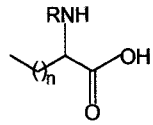
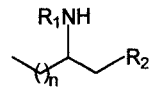
Anti-inflammatory activity of lipid mimetics derived from lipidic amino acids

Currently available nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of inflammatory conditions including rheumatoid arthritis. However, since NSAIDs and steroids cause side effects, the discovery of novel anti-inflammatory agents as an alternative is in demand.

The lipidic amino acids **1a** are non-natural α-amino acids with saturated or unsaturated long side chains exhibiting interesting properties [1]. A series of lipid mimetics, molecules which resemble lipids either structurally or functionally, have been prepared and tested for their anti-inflammatory activity. *N*–Protected lipidic α-amino acids **1b,c** were rapidly converted into alcohols by *in situ* reduction of their corresponding fluorides with sodium borohydride in high yields. The synthesis of lipidic 1,2-diamines **2c,d** has been reported [2]. Lipidic 1,3-diamines **2e,f** were prepared by replacement of the hydroxy group of **2a** with the cyano group followed by reduction. The rat carrageenan-induced paw edema assay was employed as a model for acute inflammation and indomethacin was used as a reference drug. Compounds **2b** and **2d** (n = 13) caused 85.6% and 93.5% inhibition of paw edema at 0.15 mmol/kg, while compounds **2c** and **2e** (n = 13) inhibited the edema by 90.8% and 87.6% respectively at 0.08 mmol/kg.



1	R
a	H
b	Boc
c	Ac



2	R_1	R_2
a	Boc	OH
b	H	OH
c	Boc	NH_2
d	H	NH_2
e	Boc	CH_2NH_2
f	H	CH_2NH_2

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Effect of metabotropic glutamate receptor agonists and antagonists on the synthesis of kynurenic acid in cortical rat brain slices

Kynurenic acid (KYNA) is an endogenous antagonist of all ionotropic excitatory amino acid (EAA) receptors. In the brain KYNA synthesis occurs mainly in glial cells due to irreversible enzymatic transamination of L-kynurenine, the metabolite of amino acid tryptophan. It has been found that KYNA production may be affected not only by alterations in KYNA biosynthetic enzyme activity, but also by depolarizing agents which stimulate the presynaptic release of neurotransmitters. Since neither selective agonists nor antagonists of ionotropic EAA receptors influence KYNA production, we decided to study the effect of agents interacting with metabotropic glutamate receptors (mGLUr) on KYNA synthesis.

KYNA production was examined in rat brain cortical slices according to the method of Turski et al., (J. Neurochem. 1989, 52: 1629–1636). KYNA was detected fluorimetrically according to the method of Shibata (J. Chromat. 1988, 430: 376–380).

The synthesis of KYNA was concentration-dependently inhibited by selective mGLUr agonists: (S)-3,5-dihydroxyphenylglycine, (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPD), (2S, 1'S,2'S)-2-carboxycyclopropylglycine (L-CCG-I) and L(+)-2-amino-4-phosphonobutyric acid (L-AP4). Also quisqualate which acts on metabotropic receptors and ionotropic AMPA receptor, L-glutamate and L-aspartate, endogenous nonselective agonists of all metabotropic and ionotropic EAA receptors, effectively decreased KYNA synthesis. On the other hand, all mGLUr antagonists studied: (S)-4-carboxyphenylglycine, (2S)-α-ethylglutamic acid, and (RS)-α-methylserine-O-phosphate monophenyl ester (MSOPPE) influenced neither control nor glutamate-inhibited synthesis of KYNA.

Our results suggest that KYNA production within the brain can be inhibited by agonists of mGLUr. However, the specificity of this action needs to be further investigated.

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Influence of stereoisomers of 4-fluoroglutamate on glutamate decarboxylase *in vitro*

Glutamic acid, beside its participation in general amino acid metabolism, acts as an excitatory neurotransmitter in the CNS and the substrate for synthesis of γ-aminobutyric acid (GABA). By *in vivo* inhibition of the GABA-synthesizing enzyme glutamate decarboxylase (EC 4.1.1.15, GAD), experimental epilepsy can be induced [1], Among the inhibitors of amino acid metabolism, fluorinated amino acids represent an important group. The present authors prepared stereoisomers of 4-fluoroglutamic acid from a DL-*erythro*, *threo* mexture [2, 3]. Radiometry of the $^{14}CO_2$ liberated from 1-^{14}C-glutamate was used for the GAD assay with the rat brain enzyme. The inhibition of glutamate decarboxylation by a mixture of all stereoisomers at concentrations of 0.1, 1.0, and 10 mM was 6.9 ± 1.8%, 13.0 ± 11.2%, and 46.5 ± 9.9%, resp. The inhibition type was competitive, the apparent inhibition constant K_i being 1.2 x 10^{-2} M. No effect on GAD was found with D- or L-2-fluoro-GABA as the products of fluoroglutamate decarboxylation. The decreasing inhibition effect of 4-fluoroglutamate on GAD decarboxylase during its preincubation with enzyme (0–12 h) suggests that at least one enantiomer is an alternative substrate for mammalian GAD, similarly to GAD from *E. coli* [4]. Inhibition effects of individual stereoisomers at 2 x 10^{-2} M concentration are: *erythro*–D,L- 56.8 ± 0.7%, *erythro*–D- 69.8 ± 4.6%, *erythro*–L- 35.8 ± 4.6%, *threo*–D,L- 42.0 ± 3.6, *threo*–D- 30.6 ± 2.1%. The structure-activity relationship for individual stereoisomers reflects their space-arrangements: The amino group probably binds to the pyridoxal part of the enzyme molecule and the affinity of binding is affected by electronegativity, lipophilicity, and position of fluorine. D-isomers seem to be inhibitors of GAD. As brain L-glutamate concentration is ranging about 10^{-5} M/g tissue, 4-fluoroglutamate might influence GAD activity *in vivo*.

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Effect of glutamine supplement on immune and gut barrier function in advanced esophageal cancer patients with radio-chemotherapy

The aim of this study was to determine whether glutamine supplement enhanced lymphocyte and gut barrier function in patients receiving radio-chemotherapy for advanced esophageal cancer. 12 exophageal cancer patients in stage IV were randomized into two groups, control (C) and glutamine (G). The patients nutritional status prior to the radio-chemotherapy was equivalent between the two groups. All the patients received 2.4 Gy/day of radiotherapy from day 1 through day 21 and chemotherapy composed of CDDP (9.6 mg/m²/day) and 5-fluorouracil (500 mg/body/day) from day 1 through day 5 and from day 8 through day 12. Glutamine (30 g/day) was given by oral intake from day 1 through day 28. Lymphocytes in the peripheral blood were counted. Lymphocytes blastogenation was stimulated by either phytohemagglutinin (PHA) or concanavaline A (Con A), and ^{3}H-thymidine uptake was counted to determine mitogenic activity. Secretory IgA (sIgA) levels were measured in salvia and plasma by ELISA. The gut permeability was evaluated by an oral administration of phenolsulfonphtalein (PSP, 30 mg) and collecting urine for the following 6 hours. Glutamine levels in the plasma were measured by HPLC.

Results: The radio-chemotherapy reduced glutamine levels in the plasma with C, but glutamine supplement prevented this reduction on day 7. Lymphocytes count was significantly higher with G group than with C group on day 7. sIgA levels in salvia were significantly higher with G than with C on days 7 and 14. Data are mean (SEM), and statistical analyses by Student t-test, *:$p < 0.05$ as follows:

		pre. (day 0)	7	28
PHA	C (cpm)	30941 (3106)	14505 (766)	18918 (3121)
	G	31108 (4285)	29756 (2053)*	29072 (2820)*
Con A	C (cpm)	25439 (3746)	11636 (3036)	13524 (3546)
	G	28223 (5980)	27287 (3053)*	27099 (3257)*
PSP	C (%)	11.2 (1.4)	13.2 (3.4)	13.8 (4.6)
	G	10.0 (1.6)	6.8 (1.2)*	9.4 (4.2)

We concluded that impairment of lymphocytes and gut barrier function caused by radio-chemotherapy was prevented by glutamine supplementation in esophageal cancer patients.

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Effect of combination therapy of methionine-mitomycin C conjugate and methionine deficient diet on tumor growth

We examined whether methionine-mitomycin C conjugate (M-M conj.), provided in conjunction with methionine free nutrition, reduced the survival of exophageal cancer cells in vitro and depressed tumor growth in vivo compared to mitomycin C (MMC) administration. Methionine was conjugated with MMC, using water-soluble carbodiimide. The human esophageal cancer cell line, KE-3 (1 x 10^5/200 µl), which was incubated in either standard or methionine free media, were treated with phosphate buffered saline (PBS), M-M conj. in PBS, or MMC in PBS. At the end of incubation, cell survival was determined by fluorescence staining. The tumor bearing mice were maintained on either standard or methionine free diet (MFD) and treated with PBS, MMC (120 µg/kg/day), or M-M conj. (120 µg/kg/day). Although neither concentration of 0.05 nor 0.1 µg/ml of M-M conj. plus standard media reduced survival rate compared with the MMC added media, the lowest tumor cell survival rate was found with M-M conj. plus methionine free media at every dose tested ($p < 0.05$ vs the other groups). In an in vivo, M-M conj. plus standard diet did not inhibit turmo growth compared with MMC treated mice. Tumor weight was, however, significantly lower with M-M conj. plus MFD than any other groups ($p < 0.003$ vs the other groups). In conclusion, combined administration of methionine free diet and M-M conj. suppresses tumor growth rather than either MMC or M-M conj. plus standard diet, suggesting methionine targets MMC to tumor during administration of MFD.

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The effects of iminodipeptides in serum of a patient with prolidase deficiency on superoxide generation in human neutrophils

Iminodipeptides containing C-terminal proline or hydroxyproline in serum from patients with prolidase deficiency were determined using liquid chromatography-mass spectrometry. Several iminodipeptides, Gly-pro, Pro-pro, Ser-pro, Leu-pro, Ile-pro and Gly-hyp were found in the serum of a patient with prolidase deficiency.

Preincubation of human peripheral blood polymorphonuclear leukocytes with an iminodipeptides containing proline at the terminus, Pro-pro, Gly-pro, Ala-pro, significantly enhanced N-formyl-methionyl-leucy-phenylalanine-induced superoxide generation in a concentration-dependent manner. These iminodipeptides also enhanced the superoxide generation induced by opsonized zymosan but not that induced by arachidonic acid or phorbol myristate acetate. The iminodipeptides containing hydroxyproline at the C-terminus, Pro-hyp, Gly-hyp, did not show stimulative action on the superoxide generation induced by fMLP. The rates of enhancement by these iminodipeptides follow in the order; Pro-pro>Gly-pro>Ala-pro>>Ser-pro.

The superoxide generation induced by Pro-pro was inhibited by genistein, an inhibitor of tyrosine kinase, and was enhanced by 1-(5-isoquinoline-sulfonyl)-2-methyl-piperazine, an inhibitor of protein kinase C.

Tyrosyl phosphorylation of the 45-kDa protein occurred in parallel with the iminodipeptides dependent enhancement of superoxide generation in neutrophils. Herbimycin A and genistein, inhibitors of protein tyrosine kinase also decreased tyrosyl phosphorylation of 45-kDa protein after priming by Pro-pro. On the other hand, 1-(5-isoquinoline-sulfonyl)-2-methyl-piperazine and staurosporine, inhibitors of protein kinase C, did not decreased, or rather enhanced the superoxide generation in a low concentration range. These results suggest that the priming effect of iminodipeptides on superoxide generation in neutrophils is coupled with phosphorylation of 45-kDa protein by protein tyrosine kinase.

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Effects of a novel tumoricidal protein, trichotumoricin, on cell cycle of SV-40 transformed mouse fibroblast

A novel tumoricidal protein, Trichotumoricin (TTM) was purified from an edible mushroom, *Trichoroma matsutake* with its LD_{50} value to SV-40 transformed mouse fibroblast cells (SV-T2) of as low as 5 ng/mL. The cytotoxic effect of TTM extended over tumor cell lines such as HeLa and HL60 cells. TTM induced blebbing of plasma membrane, cell shrinkage and nuclear condensation typical of apoptosis in SV-T2 cells. In this study we investigated how TTM would affect the progression of cell cycle using SV-T2 cells.

The addition of TTM to SV-T2 interfered with the ongoing of the cell cycle from G0/G1 to S phase up to 12 hours which is the doubling time of SV-T2. The effect of TTM on cell cycle progression was examined with staurosporin (STS)-arrested SV-T2 cells, since SV-T2 cells were reversibly arrested at G0/G1 phase in the presence of STS. As much as 95% of SV-T2 cells were arrested at G0/G1 phase by STS with the concomitant suppression of the cytotoxic effect of TTM. After removal of STS from STS-arrested SV-T2 cells, the addition of TTM to the cells at 7 hours later when the cells had not entered into S phase, effectively prevented the onset of S phase, leading to accumulation of the cells into G0/G1 phase. The results imply that TTM may induce apoptosis into the transformed cells by disturbing a step promoting the transition from G0/G1 to S phase. We are currently investigating what molecular events in cell cycle TTM is involved in.

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Where does indolylacrylic acid come from?

Tryptophan belongs among the amino acids with the highest number of alternative degradation pathways. In addition to the main catabolic routes there exist minor and less thoroughly investigated pathways; one of these leads to indolylacrylic acid (IAcrA). IAcrA is a plant growth hormone, whereas its biological role in animals is still obscure, as is the way and site where it is formed in the organism. A two-stage production is likely: Intestinal micro-organisms catabolize tryptophan to indole derivatives which are then absorbed and converted to IAcrA and its glycine conjugate, indolylacryloyl-glycine (IAcrGly). Our finding of IAcrGly in the urine of proved germ-free piglets points to the possibility that tryptophan can be converted to IAcrA without the intervention of intestinal micro-organisms. The excretion of IAcrGly, obviously a physiological urinary constituent in man (monkey, rat), was described on several occasions: There have been reported some seasonal and age variations, the influence of light and some connection with polymorphous light eruption. Besides other pathological conditions (liver cirrhosis, some types of leukaemia and bladder cancer) the differences in IAcrGly excretion relative to normal controls were expecially pronounced in some myopathies, namely in boys with Duchenne muscular dystrophy. Some alternative explanations of these results are put forward.

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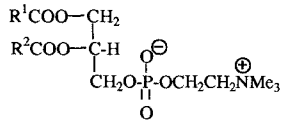
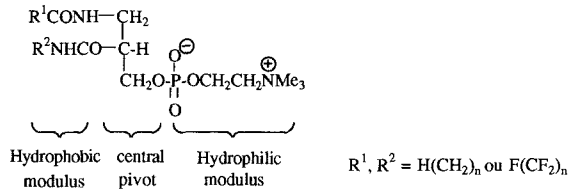
New mimics of lecithines derivated from α-substituted β-alanine

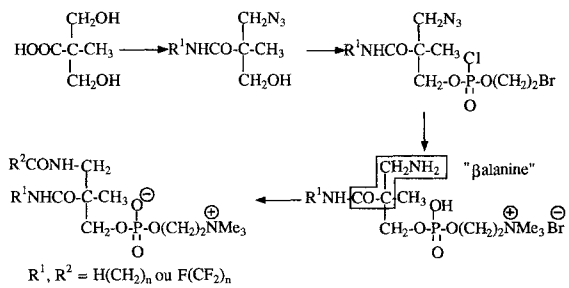
Phospholipids are one of the most important classes of natural surfactants. Among them, phosphatidylcholine is one of the most frequent structure.



These compounds are remarkable because of their ability to form organised molecular systems. For example, lecithines are used for emulsions or encapsulation by liposomes in food, cosmetics, pharmacy . . . For these reasons, we are interested in the conception and the synthesis of new mimics of lecithines or ceramide derivatives. The analogues, that we propose here, have the structure shown below:



The glycerol, as usual central pivot, is replaced here by the 3-hydroxy-2-hydroxymethyl-2-methylpropionic acid. The strategy is based on the method of modular synthesis. This method consists of synthetizing independently the polar and the apolar modulus and connecting them by a good linkage function. The synthesis is described below: the global yield is about 35%.



The evaluation of the physicochemical properties shows that these compounds have classical surfactant properties. Moreover, we can underline their great capacity to associate molecules of water. This property can be used for application in the domain of microencapsulation.

The test of their toxicity on cellular cultures shows that these compounds cause apoptosis but in vivo evaluation is necessary to draw further.

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Possible involvement of rat brain glutathione S-transferase in the protection of nigrostriatal neurons

Conjugation with glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is considered to be an important detoxifying reaction for electrophilic compounds, both endogenous and exogenous. This reaction is the first one in the mercapturic acid pathway and it is catalyzed by glutathione S-transferase (GST), a multigene family of isoenzymes widely distributed in animal tissues. 6-Hydroxydopamine (6-OHDA) lesion in the nigrostriatal pathway is a widely used animal model of Parkinson's disease. The molecular events linked to the neurotoxicity involve oxygen active species, as well as cytotoxic quinones non-enzymatically generated. The stereotaxic procedure is well standardized, but not all the rats become totally lesioned animals (TLA) according with the behavioral and biochemical markers. The group of animals, suffering a lesser extent of damage, could be provided with a more capable molecular defensive machinery. In order to evaluate the role of GST in the protection of nigrostriatal neurons against 6-OHDA lesion we measured GST activity in the striata from TLA, partially lesioned animals (PLA) and untreated animals. Moreover, the kinetic parameters (Maximum reaction velocity and Michaelis constants for glutathione, chlorodinitrobencene and cumene hydroperoxide) as well as the molecular profile by high performance liquid chromatography and isoelectric focusing of GST purified from the brains of other three similar groups were evaluated. Striatal GST activity showed a significant bilateral increase in PLA while TLA exhibited only an ipsilateral augment. GST purified from PL brains differed from those purified from the other groups because of the presence in the former of a selenium independent glutathione peroxidase activity and the presence of a component with a basic isoelectric point. These results suggest the induction of a GST isoenzyme in PL brains that protects these animals against the neurotoxin-induced oxidative damage.

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Metabolism of glycine in freshwater fish's organism

Experiments with carp's white muscles and liver homogenates showed that glycine practically does not take part in transamination reactions. This amino acid loses its amino group by way of oxidizing desamination turning by this into glyoxilate acid. This process occurs with participation of glicineoxidase proper, which is flavoproteid and of desamination NAD-dependent dehydrogenases. Glycine oxidizing desamination in muscles is more intensive than in liver. So, in case of muscles glycineoxidase is 5 times more active (0.98 ± 0.03 against 0.19 ± 0.05 mg of protein during 30 minutes).

Studied enzymes activity depends on fish acclimation temperature. Glycine oxidizing desamination by glycineoxidase proper and by desaminating NAD- and NADP-dependent dehydrogenases is almost 3 times higher in muscles of fish acclimated to +2+3 °C temperature than in awes acclimated to +7+8 °C.

Glyoxilate in carp's muscles and liver formed in the result of oxidizing desamination very quickly furns further with TPP-dependent glyoxilatecarboligase through tartron acid semialdegyde to glycerate. Glyoxilate is also used in condensation reaction with acetyl-CoA and malatesynthetase participation. By this malate is formed malatesynthetase presence and found earlier isocytratase are indicative of glyoxilate cycle functioning in carp tissue. Glyoxilate transformation in glyoxilatecarboligase and malatesynthetase reactions ensure glucose synthesis in carp fish organism, using glyoxilate parallel with fat acids (fats), and in reality, using the simplest amino acid – glycine. It is namely fats and glycine that are accumulated in carp muscle tissue before wintering period. Glucose is necessary for carp's nervous system and muscles optimum activities during winter starvation period.

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D-amino acid utilization by halophilic bacteria

Using an extremely halophile, *Halobacterium syrinalium* VO107, the utilization of D-leucine was studied comparing with that of L-leucine. D-leucine transported into cells was immediately isomerized to L-leucine and concomitantly metabolized to keep chirality in cells, whose activity was inductively increased when growing cells in a medium containing D-leucine, -valine or -allo-isoleucine. L-leucine thus isomerized was found in *de novo* proteins. D-leucine was also transported into vesicles by forming an ion gradient across the membrane, which was facilitated with illumination, suggesting that D-leucine was actively transported by an energy-coupled process, possibly due to electrochemical gradient of Na^+, similar to that for L-leucine. On the other hand, a halophilic mutant, *Haloferax volvanii* VO109, which required leucine for growth, showed that D-leucine could be substituted for L-leucine for growth. Therefore, only *Halobacteria* but also *Haloferax* might preserve the D-amino acid utilizing system, suggesting that the utilization of D-amino acids in *Halobacteria* does not compensate for that of amino acids requirement, but provides other object. Also, the D-amino acid utilization system might be conserved in all the halophilic *archaebacteria*.

Since the utilization of D-amino acids has been suggested in high acido-thermophilic bacteria, such evidence would contribute to a new frontier research in achaebacterial world. If the utilization system of D-amino acids in *achaebacteria* was made clear in the future, its evolution mechanism can be understood in comparison with those in eubacteria and eukaryotes.

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Tyrosine hydroxylase activity in human adrenal gland

Background. Tyrosine hydroxylase (TH) is the first and rate-limiting step in the catecholamine biosynthesis. The enzyme catalyzes the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA). Intracerebral grafts of dopamine-producing cellular systems constitute an alternating treatment for Parkinson's disease. The chromaffin cells of the adrenal gland are an abundant and accessible catecholamines source and the fetal tissue has high survival and functional plasticity. In spite of that, the human fetal adrenal gland (HFAG) has been poorly studied. In the present study we measured TH activity in HFAG and in the human adult adrenal gland (HAG).

Methods. TH activity was determined based on measurement of L-DOPA formed from L-tyrosine by HPLC with electrochemical detection [1]. HFAGs were obtained from fetuses of therapeutic abortions and HAGs were obtained from patients dying of nonneurological disorders.

Results and interpretation. The enzyme activity increased with fetal age and it is higher in the adult tissue than in the fetal one (Table 1). The results suggest that TH activity may be influenced by adrenal cortex maturation and the consequential glucocorticoids release.

Table 1. TH specific activity in the fetal and adult adrenal gland

	Age	N	pmol L-DOPA/min/mg protein
Fetal	9–10 weeks	4	2.61 ± 0.37 °
Fetal	11 weeks	3	9.95 ± 1.76 * °
Adult	54–79 years	3	37.65 ± 4.70

Values are mean ± S. E. M., * $P < 0.01$ as compared with values at 9–10 weeks; ° $P < 0.01$ as compared with adult values in the Mann-Whitney U test.

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The effects of induced kynurenin pathway on TNF-α secretion in rats fed an excessive L-tryptophan diet

Abnormalities in metabolism of tryptophan have been reported to be associated with tissue fibrosis and inflammation in connective tissue diseases. It is thus possible that L-tryptophan intake is involved in stimulation of the immune system. The present study investigated which of the tryptophan metabolites is involved in the induction of cell proliferation and activation of inflammatory reactions in skeletal muscle, lung and spleen of rats. 3-month-old female CD-1 rats were fed for 3 weeks on a diet containing 20% protein derived from casein and supplemented with 1%, 2% and 5% L-tryptophan. On the 3rd week of feeding half of the animals fed on a control diet and half of the animals fed on a diet supplemented with L-tryptophan, were injected with 2 injections of para-chlorophenyl alanine (p-CPA), (300 mg/kg body weight i.p) followed by 3 injections of 100 mg/kg body weight on every alternate day. Results indicated that treatment with p-CPA, an inhibitor of tryptophan hydroxylase, resulted in a reduction of serotonin levels in the blood. Increased amounts of connective tissue and induced cell proliferation were observed in lungs and in gastrocnemius muscles of rats treated with the higher level of tryptophan alone or in combination with p-CPA. Indirect immunohistochemistry revealed cells (macrophages) that reacted positively for TNF-α. The results were supported by increased secretion of TNF-α from cultures of isolated spleen macrophages. In addition, large numbers of eosinophils were observed in the connective tissue adjacent to the blood vessels in lung and muscle of the animals treated with p-CPA. It is concluded that excessive L-tryptophan consumption induced in muscle and in lung inflammatory processes and tissue fibrosis. These effects were further augmented through the kynurenin pathway.

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Nuclear magnetic resonance spectroscopy of biological fluids. A new technique for identification and quantitation of accumulating amino acids in cystinuria

Proton nuclear magnetic resonance (P-NMR) spectroscopy of urine (as well as of other biological fluids) is a very powerful technique enabling multi-component analyses for both diagnosis and follow-up of a wide range of inherited metabolic diseases. Among these pathologies, cystinuria is characterized by accumulation in urine of four dibasic amino acids, namely lysine, arginine, ornithine and cystine; the last one, being only slightly water soluble, generates urolithiasis. The mentioned amino acids can be detected in the urine NMR spectrum of cystinuric patients, the most abundant being the lysine (5 mM and over are often detected), whose typical signals become very high; arginine and ornithine are also usually detectable in cystinuric patients, although in lower concentrations (usually below 2 mM). Cystine is also detectable in the urine of the patients, but only in sub-millimolar concentration. Its clear identification and quantitation may need in some cases special NMR techniques such as decoupling procedures, and/or identification in the urine precipitate.

NMR spectra of urine allow, in few minutes, a more safe diagnosis when compared to the Brandt test (often questionable), while providing amino acid quantitation comparable with that obtained by other well established techniques such as amino acid analyzers or HPLCs. The proposed NMR technique is also suitable in the follow-up of therapy with α-mercaptopropionylglycine, because it simultaneously provides quantitation of cystine, citrates (also used during therapy to enhance urine pH and hence cystine solubility), and creatinine, thus allowing the therapist to monitor cystine excretion in order to keep its level below the 300 mg/g of creatinine threshold, in order to permit its solubilization. It is also worth noting that in urine samples of heterozigores, enhanced levels of lysine (though always below 1 mM, thus in concentrations unambiguously distinct from the urinary levels in homozigotes) can be clearly detected, providing an easy identification of etherozigotes; the other dibasic amino acids seem to be instead barely detectable.

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Clonidine action in spontaneously hypertensive rats (SHR) depends on the GABAergic system function

γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system participates in the regulation of the cardiovascular system function. Abnormalities in GABAergic inhibition has been found in several regions of the brain and may contribute to the development and maintenance of spontaneous hypertension. Current evidence suggests that GABAergic transmission plays an important role in the central mechanism of some antihypertensive drug action.

The aim of the present work was to investigate the influence of GABAergic system activity on clonidine hypotension. The alterations in the GABA turnover after acute drug treatment were also studied. Initially the effect of clonidine and $GABA_A$ receptor antagonists (picrotoxin and bicuculline) on blood pressure in SHR and normotensive WKY rats were determined. Systolic blood pressure (SBP) was measured in conscious animals by the indirect tail-cuff method in 45 min after clonidine and in 15 min after antagonists. Co-administration of clonidine at a dose 10 μg/kg or 20 μg/kg with these agents at doses 0.5–2.0 mg/kg i.p. on blood pressure were

assessed. In both strains the GABA turnover after aminooxyacetic acid at a dose 50 mg/kg i.p. in hypothalamus and pons-medulla was examined.

Picrotoxin and bicuculline administered alone had no influence on SBP in both strains. The single administration of clonidine induced significant and dose-dependent decrease of SBP in rats but this effect was more pronounced in SHR. Co-dosage of clonidine with these agents reduced its hypotensive effect in dose dependent manner and the effectiveness of both antagonists was higher in SHR rats. It was found that the colonidine elevated the GABA turnover in the studied areas. However the effect of clonidine was weaker in SHR than in WKY rats. The present results indicate that the more pronounced inhibitory action of $GABA_A$ receptor antagonists in SHR is connected with the central GABAergic system dysfunction in hypertension. These data are corroborated by experimental findings showing weaker the GABA turnover in SHR than in WKY rats after clonidine administration. Based on the results of our studies mentioned above we suggest that the stronger action of clonidine on blood pressure in SHR may be explained by other neurotransmitter systems involving in its hypotensive effect.

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Polyamine-oxidizing enzyme activities during the postnatal development of mice. Effects of testosterone

Diamine oxidase (DAO) and polyamine oxidase (PAO) are important enzymes in the catabolism of polyamines, a group of aliphatic cations associated with cellular proliferation. DAO and PAO activities were investigated in kidney of 20-, 50- and 70-day-old male and female mice. The influence of testosterone (T) (10 μg/100 g body weight) on renal DAO and PAO activities was also studied. The renal DAO activity in 20-day-old (immature) mice was 2-fold higher in males than in females. In both sexes, DAO activity increased gradually in the course of postnatal development, reaching the highest level in 70-day-old (mature) mice. A close pattern of changes in renal PAO activity was observed during the femal mouse postnatal development. In male mice, no significant changes in renal PAO activity as a function of age were determined. T (24 h after the treatment) caused a marked increase in DAO activity in 20-, 50- and 70-day-old female and in 70-day-old male animals. PAO activity was slightly elevated in 20- and 50-day-old males and females in response to T-administration. T-treatment provoked a decrease in PAO activity in 70-day-old male and female animals. The results obtained show a sexual dimorphism in renal polyamine-oxidizing enzyme activities during the postnatal development of mice. In both sexes, renal DAO and PAO are T-sensitive enzymes, but the two enzymes exhibit different responses (more significant in females) to T-treatment in the course of postnatal development of mice.

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Bis(uracilyl)-polyamine analogs as potent antitumor agents

Several bis(uracilyl) polyamine analogs, N,N'-bis(uracilyl-5-methylene)-tetramethylenediamine (BUTD), and N,N'-bis(uracilyl-5-methylene)-hexamethylenediamine (BUHD), were tested in different cell and cell-free systems as new agents with poteintial antitumor activity, particularly their ability to inhibit growth of cultured carcinoma ovary (CaOv) cells, polyamine biosynthesis and enzymes activities (ornithine decarboxylase [ODC] and polyamine oxidase [PAO]).

Both investigated compounds exhibited no significant cytotoxicity against CaOv cells during the first day of incubation (CE_{50} >> 3 mM for BUTD and BUHD). At the same time these substances in low concentration (0.1 mM) effectively depressed the proliferation of CaOv cells on the 3rd day of incubation.

The data of polyamine content in cancer cells during incubation time in the presence of BUTD and BUHD showed the induction of polyamine synthesis in cells in the first 2 days and reduction of this synthesis on the 3rd day of incubation, according of cell cycle.

In contrast of this both bis(uracilyl) polyamine analogs could significantly inhibit the ODC activity and elevate the PAO activity in cell-free systems of regenerating rat liver and rat hepatoma G-27.

It was suggested that bis(uracilyl) polyamine analogs strongly inhibit the de novo synthesis of polyamines directly through enzymes or, probably, due to lethal DNA synthesis.

Genetics

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On multiple alignment of genome sequences with three dimensional structure of protein

The alignment of amino acid or base sequences is important to compare these sequences and one can align them automatically by means of a development of algorithm with a computer. There are two kinds of alignments, one of which is the pairwise alignment for two sequences and another is the multiple alignment for many (more than three) sequences. The pairwise alignment have been studied by several authors [3, 5, 6]. The multiple alignment using the simulated annealing was studied in [4].

Recently, three dimensional structure of protein was studied by X-ray analysis and NMR (Nuclear Magnetic Resonance). Protein is a polymer by the peptide bonds of many amino acids. Backbone is folded and it makes specified three dimensional structure.

In order to obtain the results with more higher validity, we try to introduce the algorithm of the multiple alignment by taking account of three dimensional structure. In this paper, we proper two algorithms with three dimensional structure of protein and discuss the performance of these algorithms.

(1) We previously match a particular part of amino acid sequences obtained from the analysis of three dimensional configuration, and the rest of the sequences is matched by the multiple alignment.

(2) Based on the probability distributions of the bond angles of the backbone of amino acids, we make a distance matrix among several amino acid sequences, by which we take the multiple alignment.

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On code structure of HIV

Organisms carry genetic information in their base sequences. The mutation is caused by the variation of the amino acid or base sequence. This information is realized as the order of four bases in a sequence, so that each sequence has its own code structure. An index measuring the difference between the code carried by a group of species or organisms and the artificial code was introduced in [1]. In this paper, we analyze the code structures of the genome sequences of HIV based on the data reported in [2, 3, 4] by a help of the entropy evolution rate.

We obtained the following results:

(1) The code structure for the primary data of HIV is close to that of the cyclic code.

(2) The variation of the code structure of HIV is related to the appearance of symptoms of AIDS.

(3) We used the data of six patients, there are several patterns depending on the codes of each patient.

(4) The CD4 count of a patient is related to the code structure of his sequence.

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Analysis of HIV by entropy evolution rate

We analyze the variation of HIV after infection by means of an information measure called the entropy evolution rate which was introduced [1] on the basis of information theory. In our analysis, we use a part of external glycoprotein gp120 including the V3 region obtained from six patients.

The main purpose of this study is to find a new criterion grasping the processes of the change of CD4 count and the immunity of the patients from gene level after HIV infection.

First, we explain the entropy evolution rate (EER for short) which is a fundamental tool for our analysis, and we mention the data of HIV gene of the patients [2, 3, 4] used in this paper. Then we analyze the data with the EER.

We can make clear the following two aspects;

(1) the relation between the variation of the entropy evolution rate and the appearance of symptoms of disease (AIDS),

(2) the relation between the variation of the entropy evolution rate and that of the CD4 count of patients.

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Information theoretic approach to genome sequences

After Shannon, there exists a useful theory, called information theory, which tells us how to proceed and how to communicate the messages. Genome sequences is considered to carry information, and the information is stored in genome or amino acid sequences so that it originates the life itself. Here we present how information theory is used to investigate the "information" stored in DNA. In particular, we shall discuss the uses of several informations (entropies) and the artificial codes.

Genome sequence carries information as an order of four bases, and the information is transmitted to m-RNA, which makes a protein as a sequence of amino acids by a help of t-RNA. In information theory, the concept of information has two aspects, one of which expresses the amount of complexity of a whole system like a sequence itself and another does the structure of the system (or message) such as the rule stored in the order of sequence [1]. From Shannon's philosophy, as a system has the larger complexity, the system carries the larger information, from which the information of a whole system has been expressed by the entropy. The structure of the system is studied in the field named "coding theory", that is, how to code the messages is essential in communication of information.

Pioneering works for applications of the information theory to genome sequence were done by Smith and Gatlin, since then few works have been appeared along this line. In 1989 [2], I introduced a measure representing the difference of two genome or amino acid sequences, which is called the entropy evolution rate and has been used to make phylogenetic trees [3] and to study the appearance of symptoms of disease. The coding theory is applied to the study of genome sequences in order to examine the coding structure of several species [4] and HIV virus. In this paper, we shall explain fundamentals of the information and coding theory and show how we can use them for the study of base and amino acid sequences.

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Cloning and localization of GABA and taurine transporters in mouse retina

Neurotransmitter transporters are involved in such important functions as transmitter inactivation and amino acid homeostasis. During the course of screening a mouse retina cDNA library for neurotransmitter transporters, we have isolated cDNA clones encoding GABA and taurine transporters. RNA transcribed *in vitro* from the cDNA clones induces Na^+ and Cl^-–dependent, ^{3}H-GABA or ^{3}H-taurine uptake in microinjected *Xenopus* oocytes. In each case, the K_m values for uptake suggested that the transporters were of the high affinity type. GABA uptake was inhibited by the GABA analogs, DABA, nipecotic acid and guvacine but not by β-alanine. Taurine uptake was blocked 50% or more by 100 µM β-alanine, hypotaurine, guanidionoethanesulfonic acid and guanidinopropionic acid. Homotaurine and glycine, however, did not block uptake. Furthermore, taurine uptake was inhibited by the phorbol ester, PMA but not by its inactive analog, 4-α-PDD suggesting a role for phosphorylation in taurine transport. Finally, *in situ* hybridization studies showed that the retinal GABA transporter was expressed by amacrine cells in the inner nuclear layer and the ganglion cell layer. However, taurine transporter was found only at a low level in retina but was highly expressed in the ciliary body. These results suggest that GABA and taurine transporters are localized in different cells in the eye, and may serve distinct ocular functions.

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Bacillus stearothermophilus thermostable aminoacylase and carbamoylase: genetic analysis and biocatalytical application

Amino acid amidohydrolases represent an important class of enzymes capable of mediating stereospecific resolution of amino acids. A DNA region of the moderate thermophile *B. stearothermophilus* was cloned in *Escherichia coli* by complementation of the *argE* mutation (Sakanyan et al, 1993). The cloned DNA harbours two genes, *amaB* and *amaA* encoding *N*–carbomoyl-L-amino acid and *N*–acetyl-L-amino acid amidohydrolases, respectively (Batisse et al., 1997). The highest activity for both enzymes was detected at the early stationary growth of *B. stearothermophilus* cells in a minimal medium. Almost no activities were detected during the cellular growth in rich medium. These results were verified by Northern-blot analysis of total RNA extracted at different growth stages. The abundant and unique *ama*–specific transcript was detected along the early stationary growth in minimal medium, whereas no *ama*–specific mRNAs were detected in cells grown in rich medium. These data prove that both amidohydrolases are involved into the secondary metabolism of *B. stearothermophilus*. Furthermore, the *amaB* and *amaA* genes were cotranscribed as a bicistronic mRNA from σ^{70}–specific promoter in *E. coli* cells, as judged by primer-extension experiments. However, in *B. stearothermophilus* the *ama* genes appear to be mainly cotranscribed from another promoter detected a few nucleotides upstream from the first one. These results suppose transcriptional regulation of the two *ama* genes operon in the native thermophile.

The amino acids sequences of AmaB and AmaA contain several conserved regions. Moreover, L-carbamoylase hydrolyzes some *N*–acetyl-L-amino acids considered as substrates for L-aminoacylase. Thus, a common ancestor could be supposed for both amidohydrolases.

The distinct properties of the thermostable enzymes are useful for biocatalytical synthesis of proteinogenic and non-proteinogenic amino acids in non-organic or organic solvents: starting from acetylated racemates (for aminoacylase, see Dion et al., 1995) and from hydantoines (for carbamoylase in combination with thermostable hydantoinases). Moreover, the *amaA* gene was expressed under the control of a strong promoter from a thermophilic origin and tested in different bacterial hosts, as a prerequisite for the construction of new vectors. Taking into consideration the facility of the aminoacylase detection (by complementation or by measurement its activity) the *amaA* gene might be convenient reporter-gene for mesophilic and thermophilic microbial hosts.

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Expression-cloning and biochemical characterisation of kynurenine 3-hydroxilase

Quinolinic acid (QUIN) and kynurenic acid (KYNA) are two endogenous neuroactive metabolites of the kynurenine pathway. Most of the current interest in this pathway arises from the observations that KYNA and QUIN seem to play a role in neurological diseases, the former acting as a neuroprotectant and the latter as a neurotoxic agent. Several lines of evidence suggest that an enhancement of KYNA formation and a reduction of QUIN production by pharmacological means may offer new opportunities to develop neuroprotective molecules. Kynurenine 3-hydroxylase (kyn 3-OHase), catalysing the conversion of kynurenine to 3-hydroxykynurenine has been proposed as the major target for the pharmacological manipulation of the kynurenine pathway. Since attempts for the purification of kyn 3-OHase have been so far unsuccessful, an expression cloning strategy was designed. We have isolated a cDNA clone (K3OH) by screening a rat kidney library for expression of kyn 3-OHase activity in *Xenopus laevis* oocytes. The deduced amino acid sequence of K3OH contains 478 amino acids. Sequence analysis revealed a putative mitochondrial targeting signal and putative FAD and NADPH binding sites. Northern blot analysis showed two mRNA species of 2 kb and 4.3 kb highly expressed in rat liver, kidney and testis. In vitro translation of K3OH cDNA gave rise to a product having the expected molecular weight (~ 50,000 kDa).

Identification of K3OH cDNA as coding for kyn-3-OHase was based on substrate specificity and detailed kinetic analysis of the expressed activity in comparison with that of the native rat kidney enzyme. The K_m value of the expressed activity was 16 ± 1.1 µM, in the same order of magnitude of that obtained for the native enzyme. Furthermore, PNU156561, a well characterised kyn 3-OHase inhibitor (Speciale et al., 1996) showed identical Ki value (0.06 ± 0.01 µM) for the expressed and native rat liver enzymes.

Geochemistry

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The first stable nitrogen isotope values for extraterrestrial amino acid enantiomers: Implications for their origin

The ability to determine the stable carbon and nitrogen isotope compositions of individual amino acid enantiomers in carbonaceous meteorites can provide insights with respect to their origin(s) and mechanisms of formation. The development of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) permits such measurements to be made at nanomole to subnanomole levels. This is because the CO2 and N2 effluents resulting from the combustion (and, in the case of nitrogen, reduction) of individual compounds eluting from the gas chromatographic column are introduced directly into the source of the isotope ratio mass spectrometer. Our research to date has focused on determining the stable carbon and nitrogen isotope compositions of amino acid enantiomers in unhydrolyzed and hydrolyzed water extracts of the Murchison meteorite (type CM). Our results indicate moderate (delta 13C) to substantial (delta 15N) enrichments of the individual compounds relative to terrestrial, biological materials. In general, the amino acids are not racemic (L-enantiomer excess). Amino acid distributions and the similarity of delta 13C and delta 15N values for the D- and L-enantiomers of individual amino acids supports an extraterrestrial origin for this observed optical activity.

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Stable nitrogen isotope analysis of amino acid enantiomers at natural abundance levels

The analysis of the stable nitrogen isotope compositions of individual amino acid stereoisomers through the use of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) is presented. Nitrogen isotopic compositions of single amino acids or of their enantiomers is possible without the labor-intensive and time-consuming preparative-scale chromatographic procedures required for conventional stable isotope analysis. Following hydrolysis and derivatization, single component isotope analysis is accomplished on nanomole quantities of each of the stereoisomers of an amino acid, utilizing the effluent stream of gas chromatographic separation. Nitrogen isotope fractionation is minimal during acylation of the amino acid, with no additional nitrogen being added stoichiometrically to the derivative. Thus, the isotopic composition of the nitrogen in the derivative is that of the original compound. Replicate stable nitrogen isotope analysis of 11 amino acids, and their trifluoroacetyl (TFA)/isopropyl (IP) ester derivatives, determined by both conventional isotope ratio mass spectrometry (IRMS) and GC/C/IRMS, indicate that the GC procedure is highly reproducible (standard deviations typically 0.3 to 0.4 per mil) and that isotopic differences between the amino acid and its TFA/IP derivative are, in general, less than 0.5 per mil.

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Stable isotope studies of ancient hair

Fundamental to the understanding of human history is the ability to make interpretations based on artifacts and other remains in order to gather information about an ancient population. Sequestered in the organic matrix of these remains can be information, for example, concerning incidence of disease, genetic defects and diet. Stable isotopic compositions, expecially those made on isolates of collagen from bones, have been utilized to help suggest principal dietary components. A significant problem in the use of collagen is its long term stability, and the possibility of isotopic alteration during early diagenesis, or through contaminating condensation reactions. In this paper, we suggest that a commonly overlooked material, human hair may represent an ideal material to be used in addressing human diet of ancient civilizations.

Through analysis of the amino acid composition of modern hair, as well as samples that received intense radiation (thus simulation of aging of the hair) and hair from humans that is up to 5000 years old, we have observed little in the way of change. The principal amino acid signatures observed in all of these samples are essentially identical in relative abundances and content. Dominating the compositions are serine, glutamic acid, threonine, glycine and leucine, respectively accounting for approximately 20%, 17%, 9%, 8.5% and 8.5%. This constancy clearly indicates little has altered the amino acid composition of the hair. Further it would indicate that hair is well preserved and is amenable to isotopic analysis as a tool for distinguishing sources of nutrition. Slight decreases in amino acid content are seen with increasing age of the sample.

Based on this observation we have isotopially characterized individuals for whom the diet has been documented. Both stable nitrogen and carbon were assessed, and together should give an indication ot trophic status, and principal type (C3 oder C4) vegetation consumed. True vegans have nitrogen isotope compositions of about 6% whereas humans consuming larger amounts of meat, eggs, or mild are more enriched in the heavy nitrogen isotope. We have also analyzed large cross sections of other modern humans to yield an indication of the variability seen in a population (the supermarket diet). There is a wide diversity in both carbon and nitrogen based at least partially on the levels of seafood, corn-fed beef, and grains in the diets. Following analysis of the ancient hair, we have observed similar trends in certain populations, with some diets apparently of similar diversity as seen in the modern group, whereas in other ancient populations we have observed a much mor tightly constrained diet. It appears that the often overlooked hair in archeological sites may represent a significant new approach for understanding ancient human communities.

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Genetic analysis of CAT-mediated arginine transport: Does it control nitric oxide production?

Four transporter proteins, CAT1, CAT2, CAT2A and CAT3 are believed to encode transport system y^+, a major carrier of arginine in mammalian cell membranes. Their three genes, *Cat1, Cat2* and *Cat3* are differentially expressed in tissues and organs. *Cat1* expression is nearly ubiquitous, *Cat2* is more tissue specific and *Cat3* is reported to be brain specific. Because *external* arginine is an absolute requirement for NO synthesis via inducible nitric oxide synthase (iNOS), and arginine transport is considered rate limiting, it is of considerable interest to identify the protein(s) responsible for meeting the specific arg transport requirements of iNOS expressing cells. A common feature of many cells that transcriptionally induce iNOS is the coordinate induction of CAT2 and the stable expression of CAT1. Since arg transport mediated by CAT1 & CAT2 is highly similar, and the genes are co-expressed in most iNOS expressing cells, a genetic approach might permit discrimination of these carriers. Hence we investigated arg transport in CAT1 knockout embryonic fibroblast cell lines (EF-$CAT^{-/-}$) and compared them to wild type EF cells (EF-wt). EF-$CAT^{-/-}$ cells express both CAT2 and CAT2a (the low affinity transporter isoform). $CAT^{-/-}$ cells transport arg 2–3 times slower than EF-wt cells. The apparent K_m for arg uptake is 81.49 ± 11.5 μM (attributed to CAT2) and 2.27 ± 0.4 mM (attributed to CAT2a). Data from these EF-$CAT^{-/-}$ mammalian cells contradict data obtained by assessing the function of the CAT2/2a transporters in *Xenopus* oocytes. EF-$CAT^{-/-}$ cells are trans-stimulated 6–8 fold following arg preloading, whereas EF-wt cells expressing abundant CAT1 and low levels of CAT2 are poorly trans-stimulated by arg. In contrast, in *Xenopus* oocytes expressing CAT1 or CAT2, trans-stimulation of arg uptake is > 5 fold higher when CAT1 is mediating transport.

The literature documents numerous, contradictory effects of NO in tumor growth, metastasis and angiogenesis. A genetic approach was designed to conclusively determine whether iNOS induced NO and/or dietary arginine modifies these tumor properties. We introduced a polyoma middle T oncogene (MMTV-PyV) into knockout mice lacking functional iNOS gene ($iNOS^{-/-}$). 100% of females bearing the transgene develop multifocal, highly angiogenic mammary tumors by 2 months of age which regularly (80%) metastasize to the lung. Females with and without functional iNOS and bearing the PyV transgene will reveal whether iNOS and/or dietary arginine modify tumor growth and spread. The results of our analysis will be discussed.

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Expression of the surface antigen 4F2hc (CD 98) affects system L-like neutral amino acid transport in mammalian cells

Neutral amino acids pass the blood-brain barrier and the astroglial cell membrane by means of the system L transporter. By expression cloning we recently isolated a clone responsible for sodium independent neutral amino acid transport in rat brain glioma cells [Bröer et al. (1995) Biochem J 312: 863–870]. The clone was identified as the rat surface antigen 4F2hc (CD 98). Injection of 4F2hc CRNA into *Xenopus laevis* oocytes strongly induced sodium independent neutral amino acid transport activity. Large neutral amino acids were the preferred substrates for the transport process. Comparison with transport experiments in cell culture revealed that sodium independent transport of large neutral amino acids was solely due to system L. No system y^+L-like or system $b^{0,+}$–like activity could be detected in rat brain glioma cells. This was in accordance with antisense experiments performed in *Xenopus* oocytes. Injection of mRNA from rat glioma cells led to expression of system L-like transport activity. This activity was completely suppressed by 4F2hc antisense cRNA or corresponding antisense oligonucleotides. The 4F2hc mRNA could be detected in a variety of cell types known to have system L-like transport activity. In lymphocytes induction of system L activity by phorbolesters was accompanied by a parallel increase of 4F2hc mRNA. Overexpression of 4F2hc in CHO cells resulted in a small increase of system L-like activity, whereas incubation with antisense oligonucleotides directed against 4F2hc resulted in a decrease of system L-like activity [Boöer et al. (1997) Biochem J 323 (in press)]. Arginine transport was unaffected in these experiments.

Immunocytochemical analysis of brain slices revealed strong expression of the 4F2hc surface antigen in all brain areas. Expecially high expression was found in the endothelial cells of the blood-brain carrier.

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Glutamate and glutamine transport in rat cerebral mitochondria:
Modulation by amino acids and their derivatives

Seventeen different L-amino acids (aa) were tested for their effects on the uptake of [14C]glutamate (GLU) or [3H]glutamine (GLN) to rat cerebral mitochondria. GLU uptake was abolished by an equimolar concentration (e.c.) of the GLU carrier cosubstrate L-aspartate (ASP), and was inhibited to 15% of control by 10-fold excess of L-artinine (L-ARG). GLU uptake was not significantly affected by any other aa added in a 10–25-fold excess of GLU. Of the L-ARG metabolites added in 25-fold excess, ornithine, putrescine or ammonia had no effect, whereas creatine and the NO generator SNP increased the uptake by ~ 50%. A 25-fold excess of D-ARG was 3 times less effective in inhibiting GLU uptake than a 10-fold excess of L-ARG. The results suggest that L-ARG may function as a specific endogenous modulator of GLU transport in cerebral mitochondria. Similarly to GLU, GLN uptake was inhibited by L-ARG but not by either of the two other basic aa ornithine or lysine. However, GLN uptake was stimulated by pathophysiologically relevant (3 mM) concentration of ammonia. In vivo, the ammonia-induced GLN uptake may promote accumulation of water in mitochondria, and may be responsible for mitochondrial swelling accompanying hyperammonemic conditions. In contrast to GLU uptake, GLN uptake was inhibited by a range of neutral or acidic amino acids exept proline, taurine and cysteine. Both GLU and GLN uptake were moderately inhibited by dicarboxylate carrier ligands α-ketoglutarate and phenylsuccinate, suggesting some degree of functional coupling of the carriers.

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Regulation of amino acid transport in early embryos: Developmental implications and possible importance of the discovery of novel systems

Numerous novel transport processes have been discovered in preimplantation mouse conceptuses, and as many as eight of these novel processes receive amino acids as substrates. Extensive "ABC" texting and analog inhibition analysis have been used to demonstrate that these amino acid transport systems have novel substrate selectivities and other characteristics. While more recent data are consistent with the theory that systems similar to the embryonal systems $B^{o,+}$ and $b^{o,+}$, are also present in adult endothelia and epithelia, the substrate selectivities of the systems in adult tissues await verification utilizing quantitative "ABC" testing. If these systems are not unique to early embryos, then they are major routes of amino acid absorption, reabsorption and interorgan flows. If some of the systems are unique to early embryos, however, then they are potential targets for relatively innocuous birth control devices. Among the novel systems in oocytes and early embryos, those that transport cationic amino acids are the most numerous. Moreover, the systems that receive selectively only cationic amino acids even in the presence of Na^+ are the most likely actually to be unique to oocytes and preimplantation embryos. Of these systems, b^2_2 is highly selective for arginine which is essential for protein synthesis during growth and for synthesis of nitric oxide for chemical signaling. Either or both protein synthesis and nitric oxide signaling may be needed for implantation of early embryos in the uterus. Hence, agents that interact selectively with the novel system b^+_2 to inhibit arginine transport might also selectively interfere with implantation but not other biological processes.

Developmental regulation of amino acid transport system activities appears also to regulate the levels of nonessential amino acids in preimplantation embryos *in vivo*. Morreover, these nonessential amino acids promote preimplantation development *in vitro*, and they increase the proportion of resultant blastocysts that implant in the uterus after transfer to surrogate mothers. A principle function of nonessential amino acid uptake may be to help to prevent the catabolic state that might otherwise occur in preimplantation embryos that are in a hypertonic environment *in vivo*. Similarly, we suggest that insulin-like growth factors I and II may influence embryos to swell *in vivo* in a manner analogous to insulin-induced hepatocyte swelling. Hence, *nonessential* amino acid uptake and growth factors may work together to produce an anabolic state in embryos during preimplantation development. Moreover, exposure of preimplantation embryos to *essential* amino acids *in vitro* has been shown to enhance the development of fetuses after transfer of the embryos to surrogate mothers. For this reason, we propose that an important anabolic process during the preimplantation period is the synthesis of proteins needed for subsequent development of the embryo.

Finally, developmental regulation of expression of amino acid transport system activities in early embryos appears to have been subject to reprogramming during evolution of different species. For example, system $B^{o,+}$ is absent from one-cell mouse conceptuses, and although it may be present at the two-cell stage, system $B^{o,+}$ increases dramatically in activity when mouse and rat embryos form blastocysts. In contrast, system $B^{o,+}$ is conspicuous in porcine oocytes, but it is absent at the blastocyst stage. Similarly, system $B^{o,+}$ activity decreases dramatically in *Xenopus* oocytes just before they are deposited in pond water. Finally, system $B^{o,+}$ activity is not detectable prior to fertilization of sea urchin eggs, but it appears dramatically following this event. We propose that such developmental reprogramming of the timing of the expression of transport system activities in early embryos is needed for them to develop in different environments. Most of the mechanisms for the beneficial effects of reprogramming on development in different environments are, however, still under investigation.

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Molecular sites of control of arginine transport in liver cells

The cationic amino acid transporter 1 (Cat-1) protein mediates type C ecotropic retrovirus infection of mouse and rat cells by acting as a receptor for the viral envelope glycoprotein. Given that retroviruses infect dividing cells, Cat-1 has also been linked to cell proliferation. Nutrients and hormones regulate the activity of Cat-1 by altering the expression of the cat-1 gene. Two promoter regions and one initiator sequence are involved in transcription of Cat-1. Regulation of cat-1 expression is critical in catabolic stages such as sepsis and trauma and when the supply of amino acids is limited. During amino acid starvation, cellular protein synthesis decreases to protect the energy sources of the cells. We demonstrate that mammalian cells respond to amino acid starvation by upregulation Cat-1 amino acid carrier. Expression of the high affinity cationic amino acid transporter (Cat-1) increased in amino acid starved cells and decreased when cells were shifted from starvation to amino acid rich media. Therefore, expression of the Cat-1 gene appears to be regulated by inducing its expression when the amino acid supply is limited and suppressing it when there is an excess of amino acids. This mechanism involves changes in Cat-1 mRNA stability, suggesting that the Cat-1 gene during amino acid starvation is regulated at the post-transcriptional level. The upregulation of Cat-1 in response to amino acid deprivation was dependent on transcription and protein synthesis.

We propose that a labile protein is synthesized in response to amino acid starvation in mammalian cells, that stabilizes the amino acid transporter-mRNAs. A potential site of post-transcriptional regulation of the cat-1 gene is the 3.6 kb of 3'-untranslated region (3'-UTR). mRNA from different tissues contains an unspliced intron within the 3'UTR which has a very stable stem-loop secondary structures. This intron is slpiced in a tissue specific manner, generating Cat-1 mRNAs with alternative 3'-UTRs. The importance of these structures in mRNA stability and efficiency of Cat-1 mRNA translation is under investigation.

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Cationic amino acid transport in peripheral blood mononuclear cells; pathways and their regulation by cell activation

Peripheral blood mononuclear cells (PBMs) are readily obtained from both experimental animals and humans; since they are nucleated they provide a ready source of cells in which regulation (both transcriptional and post-translational) of transporters can be studied under different physiological conditions in vitro (human, sheep) and in vivo (sheep). The ready availability of these cells means that they are of particular interest as a model in septic shock where the delivery of the cationic amino acid arginine to activated cytoplasmic nitric oxide synthase (NOS) is an important step (which may become rate limiting) necessary for the biosynthesis and subsequent release of NO and thus to pathogenesis of the syndrome.

We have shown previously (Boyd and Crawford 1992 Pflugers Archiv) that there are two transport systems for cat-

ionic amino acid (lysine, 2uM) influx. Kinetic and inhibitor studies show these to be systems y+ and y+L, with no functional evidence for additional influx via either system b0+ or system B0+. Following PBM activation (either in vitro with PHA, or in vivo with the endotoxin LPS) influx of lysine through system y+ is strongly stimulated over a time course of 6–8 hours. System y+L is activated to a smaller extent and with a slower time course. With the endotoxin stimulus (both in vivo and with whole blood in vitro) there is an initial (maximal at approximately 1 hour) inhibition of system y+; in vitro studies show this transient inhibition to be insensitive to pre-treatment with cycloheximide, thus it appears to be via a mechanism operating at the post-translational level. In contrast the subsequent (slower) stimulation of lysine influx (observed both in vivo and in vitro at 12 hours) is (in vitro) completely inhibited by cyclohexamide showing that it depends on de novo protein synthesis. Since this cyclohexamide-sensitive, endotoxin-dependent stimulation is aboloshed by corticosteroids (dexamethasone), by a lipoxygenase inhibitor (ibrufen) and by a tyrosine kinase inhibitor (genistein), it seems reasonable to suggest that transcription is regulated by a prostaglandin-coupled phosphotyrosine-dependent intracellular signalling pathway. However there is no evidence of control at the level of CAT2 mRNA.

In patients with septic shock (defined both by circulatory and chemical criteria) the activity of system y+ ind PBMs is increased dramatically; there is a much smaller increase in system y+L. The molecular basis of these observations is being explored.

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Two adjacent residues of the glutamate transporter GLT-1 are important for ion coupling and selectivity

Glutamate transporters prevent neurotoxicity by maintaining low synaptic concentrations of the transmitter and limit receptor activation. They achieve this by an electrogenic process where the transmitter is cotransported with three sodium ions [1], followed by countertransport of a potassium ion [2, 3]. It has been postulated that potassium binds at a site previously occupied by one of the sodium ions [4]. Despite the detailed mechanistic knowledge, little is known about the structural determinants of the translocation pathway. Recently we identified a residue of the cloned glutamate transporter GLT-1 important for potassium coupling (glutamate-404) [5]. Mutation of this residue to aspartate (404D) prevents both forward and reverse transport induced by K^+. Sodium-dependent transmitter exchange and a transporter-mediated chloride conductance are unaffected by the mutation, indicating that this residue selectively influences potassium flux coupling. The results support the above kinetic model in which Na and K are translocated in distinct steps [2, 3] and suggest that this highly conserved region of the transporter is intimately associated with the ion permeation pathway. Mutation of the neighbouring tyrosine-403 to other aromatic residues similarly abolishes the interaction of GLT-1 with potassium, but also increases the sodium affinity approximately 10-fold and causes a striking broadening of its ion-specificity. Other functional properties are not significantly affected by the mutation. Our results suggest that the residue at position 403 affects sodium binding directly and that – while not identical – the binding sites for sodium and potassium share common determinants.

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Glutamine transport, cell-volume and muscle metabolism

Physiologically-relevant changes in muscle cell-volume of the sort seen e.g. after exercise or insulin treatment have important effects on muscle metabolism, with swelling stimulation anabolic processes and shrinking having opposite effects. Important anabolic effects of the conditionally-essential amino acid glutamine on protein and glycogen turnover in skeletal muscle are also linked to cell-swelling which occurs as an osmotic consequence of glutamine accumulation in the cytosol. These results may help explain the observed positive correlation between muscle cell volume and nitrogen balance in man during critical illness. A potentially-important modulator of the overall effects of glutamine-induced anabolism is the rate of glutamine transport across the sarcolemma. System N^m (the major Na^+–dependent glutamine transporter in skeletal muscle) is stimulated acutely by increases in muscle cell volume and also chronically by insulin; these features make it a likely site for the initiation and modulation of glutamine's metabolic effects in skeletal muscle. Wortmannin (phosphatidylinositol 3-kinase inhibitor) and genistein (tyrosine kinase inhibitor), but not rapamycin (inhibitor of $p70^{S6k}$ activation), prevent both hypo-osmotic swelling-induced stimulation and hyper-osmotic shrinkage-induced inhibition of system N^m transport in primary culture of rat skeletal muscle. In contrast, insulin stimulation of system N^m is prevented by both wortmannin and rapamycin. G-protein inhibitors (pertussis, cholera toxins) abolished responses of glutamine transport to cell-volume change but these responses were sustained by G-protein activators (MAS-7, LPA). Swelling-induced activation of system N^m is the converse of that expected for a regulatory volume-decrease mechanism and may act as a component of a feedforward loop promoting anabolism in proportion to nutrient supply. We propose a mechanism in which phophatidylinositol 3-kinase and G-proteins interact with a sarcolemmal "volume-sensor" to initiate transduction of changes in cell volume/membrane stretch to metabolic signals. Modulation of system N^m activity will alter the rate of glutamine-induced change of cell volume, offering a potential target for novel therapies to ameliorate muscle wasting during severe illness or after injury.

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The human cationic amino acid transporters hCAT-1, hCAT-2A and hCat-2B: Three related carriers with distinct transport properties

In this study, we aimed at analyzing the human homologues of the murine cationic amino acid transporters mCAT-1, mCAT-2A and mCAT-2B. cDNAs encoding hCAT-1 had been previously reported ba two independent groups [Albritton et al.

(1993) Genomics 12: 430; Yoshimoto et al. (1991) Virology 185: 10]. We isolated cDNAs encoding hCAT-2A and hCAT-2B from a human liver cDNA library and from cDNA derived from the human hepatoma cell line HepG2, respectively. Analyses of the deduced amino acid sequences of both carriers demonstrated 90.0% identity with the respective murine proteins. In their functional domains (42 amino acids) both hCAT-2A and hCAT-2B differ only by one residue from the respective mouse proteins. Thus, CAT-2 proteins demonstrate a higher interspecies conservation than CAT-1 proteins that are overall 86.5% identical between mouse and human and differ by 7 residues in the functional domain. The high degree of sequence conservation was reflected by the functional similarity of the human carriers with their mouse homologues. When expressed in Xenopus oocytes hCAT-1 and hCAT-2B demonstrated transport properties consistent with y+. Unlike the mouse CAT-1 and CAT-2B whose transport properties could hardly be distinguished, the transport properties of the human CAT-1 and CAT-2B isoforms showed clear differences: hCAT-1 had a 3-fold higher substrate affinity and was more sensitive to transstimulation than hCAT-2B. In contrast to the y+-carriers, hCAT-2A exhibited a 10 to 30-fold lower substrate affinity, a greater maximal velocity and was much less sensitive to transstimulation at physiological substrate concentrations.

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Levels of cysteine and related compounds in Saccharomyces cerevisiae cultured under aerobic and anaerobic conditions

Introduction. Last year we reported effects of age on levels of cysteine and related compounds in livers of mice and rats. In this experiment we had to keep animals over 2 years. It was the time-wasting experiment. The same experiment was carried out using yeast. **Method.** Sacchromyces cerevisiae was grown under aerobic or anaerobic condition. The cells were disrupted by Vibrogen Cell Mill. **Results.** The growth rates of yeast cells were same under both conditions within 30 h. The levels of compounds and the enzyme activities in the cells cultured aerobically were changed as follows compared to those in the cells cultured under anaerobically. The numbers in parentheses indicate how many times are higher than the levels of anaerobiosis. GSH (x 2.4), Cys (x 25), Glu (x 2.8), Gly (x 0.4), Cystathionine (Cysta) (x 1.9), S-Ade-methionine (x 0.5), NAD (x 1.5), NADH (x 3.9), NANP (x 1.1), NADPH (x 3.5), ATP (x 0.8), Cysta-synthase (x 3), Cysta-synthase (x 0.5), Cysta-lyase (x 0.7), Cystalyase (x 0.5), O-Acetyl-homoserine sulfhydrase (x 0.8), O-Acetyl-serine sulfhydrase (x 0.9), Xanthine oxidase (x 3.6), Xanthine dehydrogenase (x 5), Total superoxide dismutase (x 3.2), Mn-superoxide dismutase (x 4.7), Catalase (x 4.5), Glucys synthase (x 0.9), GSH synthase (x 1.1), -Glutamyl transpeptidase (x 1.2), GSH reductase (x 0.8), GSH peroxidase (x 1.2), GSH S-transferase (x 1.2), HO generating rate (x 6.4), O generating rate (x 2.3), Thiobarbituric acid reactive substance (x 2). **Conclusion.** The Cysta overproduction led to the enormous increase of the Cys level, in turn Glu-Cys and finally GSH level. The activities of enzyme involved in destroying the oxygen species were extremely elevated.

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Cloning and functional characterization of a system B°-like amino acid transporter

We have isolated a cDNA from a human placental choriocarcinoma cell cDNA library which, when expressed in HeLa cells and in *Xenopus laevis* oocytes, induces a Na^+–dependent amino acid transport system with preference for neutral amino acids. Basic amino acids and N-methylated amino acids are excluded by the system. Acidic amino acids interact with the system at acidic pH. We have subsequently cloned and characterized this transporter from a human intestinal cell line, human kidney, rabbit intestine, and a rat liver cell line. ATB° is also expressed in the brain. The human transporter consists of 541 amino acids with 10 putative transmembrane domains. The gene for ATB° is located on human chromosome 19q13.3. Amino acid sequence homology predicts this transporter (ATB°) to be a member of a superfamily consisting of glutamate transporters (EAATs) and ASC transporters (ASCTs). Functional analysis in ATB°-expressing oocytes has revealed that the activity of ATB° is obligatorily dependent on Na^+, with no involvement of any anion. The Na^+/amino acid stoichiometry is 1:1. Interestingly, ATB° catalyzes hetero-exchange of amino acids during the transport cycle in a Na^+–dependent and substrate-specific manner. Another functional feature of ATB° is its ability to induce inward currents in ATB°-expressing oocytes and these currents are sensitive to membrane potential, reversing at about –30 to –40 mV. Interestingly, these currents are not related to the translocation of Na^+ and amino acid substrates, suggesting that ATB° may indeed possess channel-like properties as has been showon in the case of other members of the glutamate transporter gene family. The expression of ATB° in the intestine and the kidney and the preference of ATB° for a broad-range of neutral amino acids as substrates suggest that this transporter is a likely candidate for the amino acid transport defect known to occur in Hartnup disease.

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Binding specificity and cation dependence of the two transporters for cationic amino acids in human erythrocytes: Systems y^+ and y^+L

Two systems are involved in cationic amino acid transport across the human erythrocyte membrane. One transporter is highly selective for cationic amino acids (K_{mLYS} = 109 ± 15.6 μM) and is inactivated by N-ethylmaleimide. The kinetic properties are compatible with those of system y^+, as described by others. The second transporter has broad specificity and interacts with L-leucine and L-lysine with similar apparent affinities ($K_m \approx 10$ μM). Interestingly, its specificity is modulated by monovalent cations; thus, when Na^+ in the medium is replaced with K^+ (or choline), it becomes selective for cationic amino acids. This transporter was first identified in human erythrocytes and has been designated system y^+L.

In spite of the functional differences of the two transporters, experimental studies often fail to distinguish between them. The difficulty arises from the fact that system y^+ is not totally selective towards cationic amino acids but shows weak interaction with neutral amino acids. Moreover, as in the case of system y^+L, the interaction of system y^+ with neutral amino acids is reduced when external Na^+ is replaced with choline.

We have conducted a comparative analysis of the binding specificity and cation dependence of these two transporters with two aims: 1) to investigate the mechanisms underlying the stimulation of amino acid binding observed in the presence of some monovalent cations and 2) to establish criteria that may be used to functionally discriminate these activities. Our studies, which involve a large number of analogues, show that system y^+L and system y^+ not only differ in their relative affinities towards neutral amino acids (the apparent inhibition constants are 10^2 to 5 x 10^4 times larger in the case of system y^+), but also in the structural determinants of binding and in their cation dependence. Whereas with both transporters the

affinity for neutral amino acids increases with chain length and decreases in the presence of β substitution, the effects are more pronounced for system y^+L; that is, this system discriminates better among different ligands. System y^+L showed the highest affinity for L-leucine and system y^+ for L-homoserine. In Na^+ medium the apparent inhibition constants were K_{iLEU} = 10.7 ± 0.72 μM and $K_{iH\text{-}SER}$ = 14.2 ± 0.39 mM for systems y^+L and y^+, respectively. The specificity towards neutral amino acids was studied in the presence of various ions (Na^+, K^+, Li^+, Mg^{2+}, choline, guanidinium) and the results show different patterns of interactions for the two transporters. Possible mechanisms involved in the stimulation of binding by cations will be discussed.

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H^+/amino acid symport in intestinal epithelia

Although the H^+–electrochemical gradient is recognised as an importent driving force in amino acid transport in both plants and bacteria, in mammalian tissues the Na^+–electrochemical gradient predominates, H^+–gradient driven transport being limited to the intestinal and renal H^+/di-tripeptide symport mechanisms PepT1 and PepT2 [1]. However, recent studies using confluent monolayers of the human intestinal epithelial cell line Caco-2 provide five pieces of evidence that demonstrate H^+/neutral amino acid (e.g. proline, taurine, β-alanine, L-alanine, glycine, MeAIB, GABA, AIB, sarcosine and betaine) co-transport across the intestinal epithelial apical surface [2–4]. (i) In both Na^+–containing and Na^+–free conditions net absorptive transport and accumulation are saturable and dependent on the presence of a transepithelial pH gradient (apical pH 5.5–6.0, basolateral pH 7.4). (ii) Uptake across the apical surface is dependent on the maintenance of a transmembrane pH gradient, uptake being reduced after NH_4Cl (30 mM) pre-pulse. (iii) Superfusion of amino acid across the apical surface of Caco-2 cell monolayers loaded with the pH-sensitive dye BCECF is associated with an intracellular acidification (suggesting H^+/amino acid symport). (iv) pH-dependent amino acid transport is rheogenic in Na^+–free conditions; addition of amino acid to the apical membrane of voltage-clamped Caco-2 cell monolayers being associated with an increase in inward I_{SC}. (v) Uptake of amino acids with a range of pK_a values (1.50–4.23) show similar dependence on apical pH (over the pH range 5–8, half-maximal uptake is observed at pH 5.99–6.20). In conclusion, a Na^+–independent, pH-dependent, H^+–coupled amino acid transport mechanism is present at the apical membrane of human intestinal epithelial (Caco-2) cells that is involved in the absorption of a number of neutral amino acids.

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Regulated L-arginine transport in human endothelium and smooth muscle cells

Transport of L-arginine is mediated by the Na^+– and pH-independent cationic amino acid transporters (*m*CAT) in vascular endothelial and smooth muscle cells (SMC, see reviews, Closs, 1996; Sobrevia and Mann, 1997). Expression of the gene for the high-affinity cationic system y^+/*m*CAT-1 has been shown to be increased by insulin, L-arginine and a diet rich in carbohidrates (Wu et al., 1994; Contreras et al., 1997). L-arginine is the substrate for the synthesis of the potent vasodilator nitric oxide (NO, Moncada et al., 1991) and several studies have suggested that the activity of NO synthase may be dependent on the availability and/or transport of L-arginine (see Sobrevia and Mann, 1997). Therefore, the activity and/or expression of L-arginine transporter proteins may be associated with and play important roles in modulating the vascular tone. This paper summarises the effects of the vasoactive nucleoside adenosine, D-glucose and human insulin on the activity of L-arginine transport in vein endothelial cells and artery smooth muscle cells from human umbilical cords.

Endothelial and smooth muscle cells were cultured in medium 199 and MCDB131 (Clonetics), respectively. Initial rates of L-[^{3}H]arginine transport (0–1 mM) were determined over 30 s in Hepes-buffered Krebs (37°C). Cells were exposed to increasing concentrations of D-glucose (5–40 mM, 0–48 h), human insulin (0.01–10 nM, 8 h) or adenosine (0.01–100 μM, 2 min). The effects of insulin on L-arginine transport was also assayed in cells cultured in elevated D-glucose.

The maximal velocity of saturable L-arginine transport was increased by elevated D-glucose in smooth muscle cells (Sobrevia et al., 1996a) and endothelial cells (Sobrevia et al., 1995), whilst affinity values were not altered significantly. In preliminary experiments elevated D-glucose (25 mM, 24 h) and human insulin (1 nM, 8 h) increased the expression of the endothelial NO synthase. Incubation of cells with human insulin also resulted in an activation of L-arginine transport which was protein synthesis dependent (Sobrevia et al., 1996b) and blocked by inhibition of tyrosine kinase activity. Adenosine activated endothelial cell A_2–purinoceptors leading to a membrane hyperpolarization and stimulation of L-arginine transport (Sobrevia et al., 1997), and preliminary studies have shown that adenosine increased tyrosine kinase activity.

Our findings suggest that the activity of the human vascular endothelial and smooth muscle L-arginine transporter(s) are increased by high D-glucose, insulin and adenosine. These results complements our observations of an increased activity of system y^+/*m*CAT-1 and reduced transport and metabolism of adenosine in human umbilical vein endothelium from gestational diabetic pregnancies (Sobrevia et al., 1994, 1995).

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Amino acids and neurotransmitter transporters

The uptake of amino acids in mammalian cells is mediated by passive and energy-dependent transporters with overlapping substrate specificities. Early work have specified the various transporters according to their substrate and inhibitor specificities. The cloning of cDNA encoding amino acid transporters showed considerable deviation from the dogma. Several of the well defined transport systems were not cloned so far and the cDNAs encoding neurotransmitter transporters have bleared the borders between the former and the latter. A family of transporters with a common structure of presumably 12 transmembrane helices has been defined. Sequence analysis revealed that this family of transporters contains four subfamilies of monoamine, GABA, amino acid and "orphan" (NTT4) transporters. Genomic cloning of genes encoding neurotransmitter transporters and search for sequences without known function in the GenBank, revealed that insects and *C. elegans* contain genes encoding potential neurotransmitter transporters. Therefore it was assumed that this family of transporters evolved from a common ancestor about 1 billion years ago. Recently we identified and cloned a bacterial genes with relatively high homology to neurotransmitter transporters. Each bacterial genome contains a single gene homologous to the mammalian family of neurotransmitter transporters. These findings indicate that the family of neurotransmitter transporters is rooted to the onset of life and is present not only in Eukaryotes but also in Eubacteria and Archaea. Using immunocytochemistry with polyclonal antibodies raised against recombinant fusion proteins, as well as conjugated oligopeptides, we compared the distribution of the various neurotransmitter transporters. Their distribution will be discussed in light of their specific function.

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The genetic basis of cystinuria

Classic cystinuria is an autosomic recessive hyperaminoaciduria of cystine and dibasic amino acids, due to defective cystine and dibasic amino acids reabsorption in kidney and intestine; three types of cystinuria have been described. Expression cloning revealed that an integral plasma membrane protein from kidney (rBAT), with a low number of transmembrane domains (1 or 4 depending on the structural model) induces high-affinity sodium-independent transport for cystine, dibasic amino acids and some neutral amino acids (system bo,+ -like) in oocytes. The specific expression of rBAT in the apical plasma membranes of the renal straight proximal tubules and the small intestinal epithelium pointed *rBAT* as a candidate gene for cystinuria. Mutational analysis of the human *rBAT* gene resulted in the identification of ~ 30 cystinuria-specific mutations. For some of these mutations defective amino acid transport expression has been demonstrated. This stablished *rBAT* as a cystinuria gene, and demonstrated a role for rBAT/system bo,+ -like in the renal (at the nephron S3 segment) and intestinal reabsorption of cystine and dibasic amino acids.

This, raised several questions: i) What is the active transport mechanism of system bo,+ -like?: Studies in oocytes and in the renal cell line OK showed that rBAT / system bo,+ -like is an amino acid exchanger (i.e., tertiary active transport) that most probably reabsorbs cystine and dibasic amino acids by exchange with intracellular neutral amino acids. ii) What is the role of the rBAT protein in the transport function of system bo,+ -like?: rBAT / system bo,+ -like and 4F2hc / amino acid transport system y+L-like (4F2hc is an rBAT homologous protein) are heterodimeric, composed of the corresponding "heavy chain" (rBAT or 4F2hc) and unidentified "light chains", which are disulfide bound. Studies with the cystinuria-specific rBAT mutant Met467Thr and functional co-expression of system y+L -like transport activity with 4F2hc and purified rat mRNA strongly suggest that these heterodimers are the functional units of the corresponding transporters (Chillarón et al., 1997; Estévez et al., 1997). iii) Is rBAT responsible for all cases of cystinuria?: mutational and linkage analysis of the *rBAT* gene (chromosome 2p16.3-p21) demonstrated that mutations in *rBAT* cause only cystinuria type I. A wide genomic search sith cystinuria families transmitting types II or III demonstrated linkage with chromosome 19q13.1 (Bisceglia et al., 1997; Wartenfeld et al., 1997).

Purification of the rBAT complex, co-expression cloning, with 4F2hc, of its putative "light subunit" and positional cloning in the 19q cystinuria locus are currently under progress in order to identify the putative "light subunits" of rBAT and 4F2, and new cystinuria genes.

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Coupling factor glutathione: Introduction into the electrophysiology and thermodynamics of mitochondria

Th. Wieland and E. Bäuerlein formulated in 1968 a mixed anhydride of sulfenic and phosphoric acid, R-S-O-PO_3H^-, to explain the conversion of energy obtained by oxidation into the energy-rich binding between two phosphate groups, as for instance found in ATP. In 1970, Painter and Hunter showed high amounts of ATP-formation in a model system containing oxidized glutathione as essential catalyst. During my attempt to elucidate the mechanism of oxidative phosphorylation in mitochondria, I came to the conclusion that an (H^+) activated disulfide in the lipophilic membrane had to be involved [3]. I proposed a phosphate reaction with ATP on this activated disulfide in a synchronous reaction mechanism without the real build-up of a sulfenylphosphate intermediate. – The research performed during the last 20 years led inevitably

to the formulation of the mitochondrial F_0F_1–complex as coupled K^+/H_2O-pump (similar to the nAChR) with H^+/P_i^-–inducible ATPsynthase as well as to the respiratory chain substrate driven K^+/H^+–antiport system. These systems are linked together in anticyclic energy driven K^+/H_2O, H^+/P_i–movements and oscillations (swelling plus contraction of the mitochondrial matrix space by osmotically active K^+–ions), controlled by O_2 and the free Mg^{2+}– and Ca^{2+}–concentrations in the cytosol of the cells. The system is responsible for the thermoregulation of our body. – The cyclic hydrolysis/synthesis of ATP and the concomitantly cyclic release/binding of Mg^{2+} in the "steady state flow system" releases heat and the temperature (Δ T) is permanently raised. The released heat is constantly distributed throughout the entire body by the oscillating mitochondria, as well as the pumping heart, and is used up by the normal body functions. Disturbances of this system are normally compensated for by lower/higher respiration rates. For clarification, one should study the theory of Carnot. The essentially by iron and its state of oxidation dependent H^+/e^-–displacements, current (i), lead to high local voltages (Δ V) over the membrane with corresponding magnetic fields (H). The under physiological conditions operating mitochondriotic membrane acts thereby simultaneously as capacitor, transmitter and supraconductor (37 °C!). *The entire system is dependent on oxidized and reduced glutathione.* To gain more insight into this system, one should read an introduction to the theory and equations of Maxwell and Kirchhoff. Further studies based on Faraday's and Oerstedt's equations should lead to the complexity of mitochondrial electrophysiology, its influence on momory and thinking, and the basics of the chinese acupuncture.

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Adaptive responses in system y^+ and nitric oxide synthase in vascular disease

Over production of nitric oxide (NO) by vascular cells has been implicated as one of the major causes of hypotension associated with endotoxaemia [1], and recent evidence suggests that enhanced generation of NO may serve as a failing counter-regulatory mechanism in patients with chronic heart or renal failure [2, 3]. Under these clinical conditions, the increased synthesis of NO from its cationic precursor L-arginine is catalysed by the Ca^{2+}/calmodulin-insensitive NO synthase (iNOS). Once induced by endotoxin and/or proinflammatory cytokines, the activity of iNOS appears to be dependent on the availability of extracellular arginine [4, 5]. We have recently reported that plasma L-arginine concentrations are reduced significantly in patients with septic shock, uraemia and heart failure. Thus, it seems likely that transport of L-arginine via system y^+ and other cationic amino acid transporters may be rate-limiting for NO generation catalyzed by iNOS in vascular endothelial and smooth muscle cells.

Previous studies in cultured J774 macrophages and rat aortic smooth muscle cells demonstrated that bacterial endotoxin (0.1–30 µg/ml) and interferon- (50–100 U/ml) induced a dose- and time-dependent stimulation of L-arginine transport via system y^+ and NO production [5, 6]. In these cells L-arginine transport was Na^+–independent, unaffected by changes in extracellular pH and inhibited by L-lysine and L-ornithine but not by D-arginine or analogues selective for transport systems A (2-methylaminoisobutyric acid), L (phenylalanine) or N (6-diazo-5-oxo-L-norleucine). NO synthesis was critically dependent on extracellular L-arginine (K_i ~ 30 MM) and inhibited in the presence of either L-lysine or L-ornithine. Induction of L-arginine transport in both J774 macrophages and rat aortic smooth muscle cells in response to endotoxin and/or interferon- was abolished by cycloheximide, whereas dexamethasone (10 µM, 24 h) selectively inhibited NO production [6, 7].

The close coupling between substrate supply and NO production in J774 macrophages and vascular smooth muscle cells led us to investigate whether the L-arginine-NO signalling pathway could be induced in other cell types such as human red blood cells. Our recent studies have shown that activity of system y^+ (but not system y^+L, [8]) is stimulated in human red blood cells isolated from patients with chronic renal and heart failure [3, 9]. In addition to a 2-fold increase in the V_{max} for L-arginine transport, plasma and red blood cell levels of the arginine metabolite N^G–monomethyl-L-arginine (L-NMMA, an endogenous inhibitor of NO synthase and L-arginine transport) were elevated and would inhibit NO synthesis. Thus, this may help to explain why activation of the sympathetic and renin-angiotensin systems in uraemia, heart failure and septic shock cannot be fully compensated for by increased NO synthesis.

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Metabolism

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Homocysteine increase cyclin-dependent kinase in aortic rat tissue

Background. Hyperhomocyst(e)inemia is strongly associated with occlusive arterial disease. A direct effect of homocysteine on the proliferation of smooth muscle cells was proposed recently. This observation led us to examine the effect of homocysteine on cyclin-dependent kinase, the starter of mitosis and reflecting proliferation.

Methods and results. Seventy *Him*:OFA rats were divided into seven groups. For 12 weeks, 10 rats were fed homocysteine 25 mg/kg body weight per day, 10 were fed 50 mg/kg body weight per day, and 10 were fed 100 mg/kg body weight per day; 10 were given homocysteic acid 100 mg/kg body weight per day, 10 were administered cysteine 100 mg/kg

body weight per day, and 10 were given ascorbic acid 270 mg/kg body weight per day. Ten remained untreated and served as controls. Aortic cyclin-dependent kinase was determined at the transcriptional (mRNA) and protein levels. Phosphokinase C and aortic homocyst(e)ine also were evaluated in aortic tissue. Aortic cyclin-dependent kinase protein was significantly ($P = .0001$) elevated in the three homocysteine-treated groups, and mRNA cyclin-dependent kinase levels were significantly elevated in the rats given the 50 and 100 mg/kg body weight per day protocol. Endothelial damage was shown at higher homocysteine doses as reflected by circulating ACE and von Willebrand factor changes. Proliferation of cells of the aortic wall by bromodeoxyuridine incorporation could be shown in the high-dose homocysteine group only.

Conclusions. Our findings indicate that homocysteine specifically stimulates aortic cyclin-dependent kinase at the transcriptional level, with the possible consequence of proliferation of aortic cells as revealed by incorporation of bromodeoxyuridine in the aortic wall.

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Enzymatic and nonenzymatic glycosylation of hydroxylysine in the kidney. Effect oif various pathological states associated with glomerular basement membrane thickening: uremia with sucrose-rich diet, diabetes, hypertension

Glomerular basement membrane (GMB) thickening (T) is characteristic of diabetic microangiopathy. It is associated with type IV collagen accumulation. Capillary BMT has been reported in hypertension. We have observed that association of uremia (induced by subtotal nephrectomy), and sucrose-rich diet leads to very marked GBMT, whereas uremia alone does not induce any GBMT and the rucrose-rich diet alone induces a moderate GBMT. We have studied the relation between GBMT and Hyl glycosylation. Hyl is characteristic of collagens and type IV collagen is particularly rich in Hyl, Glycosylation on Hyl by UDP-Gal and UDP-Glc is catalysed successively by a galactosyltransferase (EC 2.4.1.50) and a glucosyltransferase (EC 2.4.1.66) resulting in Hyl-linked Glc-Gal disaccharide units characteristic of collagens. Their deglycosylation is catalysed by a specific α-glucosidase (EC 3.2.1.107) which has been described in kidney cortex by Sternberg & Spiro. The galactosidase activity on Gal-Hyl-peptides is very weak in rat kidney and its existence discussed. In GBM of diabetic patients, Garlick & Spiro have shown increased enzymatically glycosylated Hyl (Glc-Gal-O-Hyl and Gal-O-Hyl) concentration: 20.8 residues per 1000 aminoacids vs 15.9 in normoglycemic controls, whereas glycated Lys (Glc-N-Lys) and Hyl (Glc-N-Hyl, but also Glc-Gal-O-Hyl-N-Glc) levels were respectively 0.30 and 0.19 per 1000 (vs 0.18 and 0.08 in controls). The quantitative importance of enzymatic glycosylation is much greater than that of glycation. In experimental diabetes increased specific activity of Glc-transferase and Gal-transferase have been reported; the glucosidase activity is inhibited by clevated glucose levels but the enzyme concentration is increased; all these abnormalities are reversed by insulin. An aldose-reductase inhibitor, sorbinil, prevents cataract, hypoalbuminemia, GBM thickening, 3-Hyp accumulation and increase in glucosidase concentration, without modifying hyperglycemia. In spontaneously hypertensive rats, the glucosidase concentration, specific activity is decreased. This is not corrected by a preventive treatment by hydralazin which normalizes arterial blood pressure. Therefore this abnormality appears to be under genetic control. Uremia alone does not induce any change in glucosidase activity in the dialysed kidney 105000 × g supernatants. The sucrose-rich diet alone induces a moderate GBMT but no change in glucosidase activity. Association of uremia and sucrose-rich diet leads to very marked GBMT with significant increase in glucosidase activity. It appears that in uremia, as in diabetes, marked GBMT is associated with a rise in glucosidase level. This is consistent with an increase in glucosidase concentration secondary to GBMT.

Neurobiology

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Dopamine-glutamate interactions in the basal-ganglia

The present model of basal-ganglia (BG) circuitry posits that a cortical (glutamatergically mediated) signal reaching the striatum, is transmitted to the thalamus either via two GABAergic neurons (direct loop) or via three GABAergic neurons (indirect loop). Thus, it produces either disinhibition or inhibition of the thalamo-cortical signal respectively. Some evidence argues in favour of a predominantly inhibitory control of the BG over behaviour by way of the indirect loop. It is a matter of actual debate whether by virtue of this, the BG are involved in a process of evaluation that results in suppression of "unwanted" and in facilitation of "wanted" behaviour.

Dopamine: It is assumed that the release of dopamine in the striatum and in the nucleus accumbens does not influence what actually happens, but indicates how "good" or how "bad" the executed behaviour was. An increase in the activity of dopaminergic meso-accumbal neurons factilitates elicitation and strength of a given behaviour (sensitization). Evidence accumulates indicating that a similar mechanism in the nigrostriatal system may exist as well.

Glutamate: Besides its major role as a neurotransmitter in the cortico-striatal and in the subthalamic neurons, activation of glutamate-receptors is essential for the plasticity of the BG response: NMDA receptor activation in the midbrain seems to be prerequisite to initiation of behavioural sensitization and glutamatergic receptors may also facilitate expression of sensitization in the nucleus accumbens and possibly also in the striatum.

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The role of striatal glutamate receptors in models of Parkinson's disease

It is generally accepted that parkinsonian akinesia and rigidity stem from degeneration of the dopaminergic nigrostriatal pathway, or from blockade of striatal dopamine D2 receptors. Recently, it has been suggested that the primary

striatal dopaminergic hypofunction leads to a hyperactivity of glutamatergic neurotransmission. Therefore it is assumed that antagonists of glutamatergic receptors may be useful as antiparkinsonian drugs. Several brain structures which receive the glutamatergic innervation have been suggested as potential targets for the therapeutic effects of those compounds.

The aim of the present study was to examine the effect of drugs acting on the NMDA receptor complex on the muscle rigidity induced by haloperidol and reserpine. The muscle tone was estimated as resistance developed by the rat's hind foot during its passive flexion and extension in the ankle joint. The reflex electromyographic (EMG) activity in response to passive movements was additionally recorded in the gastrocnemius and tibialis anterior muscles. Reserpine (10 mg/kg ip) and haloperidol (0.5–10 mg/kg ip) induced parkinsonian-like muscle rigidity. Both drugs increased the muscle resistance of the hind foot to passive movements, as well as the reflex EMG activity. MK-801 (0.32–1.28 mg/kg sc), an uncompetitive antagonist of the NMDA receptor, inhibited the reserpine-increased muscle resistance and the EMG reflex activity. Similarly, L-701,324 (10–40 mg/kg ip), an antagonist of the glycine site of the NMDA receptor complex, reduced the muscle tone and the reflex EMG activity enhanced by haloperidol (5 mg/kg ip). Used in a lower dose of 5 mg/kg, L-801,324 inhibited the muscle rigidity induced by haloperidol (1 mg/kg ip). AP-5 (2 and 5 µg/0.5 µl), a competitive antagonist of the NMDA receptor, injected bilaterally into the rostral region of the striatum, inhibited the muscle rigidity induced by haloperidol (1 mg/kg ip). A similar inhibitory effect was observed after the glycine site antagonist 5,7 – dichlorokynurenic acid (1.0–4.5 µg/0.5 µl), injected bilaterally into the above mentioned striatal region, on the rigidity induced by haloperidol (2.5 mg/kg ip). In contrast, AP-5 (2 and 5 µg/0.5 µl), injected alone bilaterally into the intermediate-caudal region of the striatum, induced muscle rigidity. The present results seem to suggest that: (1) the inhibitory effect of different antagonists of the NMDA receptor complex on the muscle rigidity in animal models of parkinsonism depends, at least partly, on the blockade of NMDA receptors in the rostral region of the striatum; (2) the blockade of NMDA receptors in the intermediate-caudal region of the striatum induces an opposite effect (muscle rigidity) which, in turn, may reduce the beneficial impact of NMDA receptor antagonists after their systemic administration.

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Modulation of motor behaviour by NMDA- and cholecystokinin-antagonism

The neuropeptide cholecystokinin (CCK) is expressed at high levels in many regions of the brain involved in the initiation and execution of motor activity, e.g. in a corticostriatal projection where it may coexist with glutamate, and in the nigrostriatal dopamine neurones. Cloning and pharmacological characterization has to date revealed two receptors for CCK, CCK_A and CCK_B. These receptors have been localized with a partially overlapping distribution in the brain. The non-competitive NMDA antagonist phencyclidine (PCP) has been reported to induce a behavioural pattern of hyperlocomotion and stereotypies similar to schizophrenia. The anatomy of CCK innervation of the brain suggests the possibility that CCK may be involved in motor behaviour and related pathologies such as Parkinson's disease and schizophrenia. In this study, habituated male, adult rats were acutely injected with CCK_A- or $_B$-antagonists and placed in locomotor boxes for evaluation of motor activity. The antagonists had no effect when administered alone, but when administered together with PCP, the A-antagonist significantly reduced PCP-induced hypermotility and -locomotion, whereas the B-antagonists potentiated it. These data indicate that in the absence of a glutamatergic tone, CCK is endogenously released and will act on both CCK_A- and $_B$-receptors. The opposing effects of A- and B-receptor antagonism in this experimental model indicate that the relative balance between the receptors determines the effect of endogenous CCK release on motor behaviour. These results can be explained in the light of previously shown interactions between CCK and glutamate on signal transduction in the basal ganglia. In another experiment, CCK_B antagonism decreased exploratory activity in non-habituated rats, indicating a further example of endogenous CCK release influencing motor activity. Morphological studies on the neuroanatomical substrates for these results are in progress.

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Modulation of NMDA-receptor-channels in the striatum by adenosine A_{2A} receptor agonists

In order to investigate the modulatory effects of adenosine on excitatory amino acid projections onto striatal medium spiny neurons, whole-cell patch clamp experiments were carried out in rat brain slices. N-methyl-D-aspartate (NMDA; 1–1000 µM) caused a concentration-dependent inward current which was larger in the absence than in the presence of Mg^{2+} (1.3 mM). In a subset of striatal neurons, the current response to NMDA (10 µM) and the accompanying increase in conductance were both inhibited by the A_{2A} receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadensine (CGS 21680; 0.01–1 µM). The effect of CGS 21680 (0.1 µM) persisted in the presence of tetrodotoxin (0.5 µM) or in a Ca^{2+}-free medium, under conditions when synaptically mediated influences may be negligible. The A_3 receptor agonist N^6-2-(4-aminophenyl)ethyladenosine (APNEA; 0.1–10 µM) also diminished the effect of NMDA (10 µM), while the A_1 receptor agonists 2-chloro-N^6-cyclopentyladenosine (CCPA; 0.01–10 µM) and (2S)-N^6–[2-endo-norbornyl]adenosine [S(-)-ENBA; 10 µM] as well as the endogenous, non-selective P_1 purinoceptor agonist adenosine (100 µM) were inactive. The endogenous non-selective P_2 purinoceptor agonist ATP (1000 µM) also failed to alter the current response to NMDA (10 µM). Adenosine (100 µM), but not ATP (1000 µM) became inhibitory after blockade of nucleoside uptake by S(4-nitrobenzyl)-6-thioguanosine (NBTG; 30 µM). 8-(p-sulfophenyl)-theophylline (8-SPT; 100 µM), as well as the A_{2A} receptor antagonist 8-(3-chlorostyryl)caffeine (CSC; 1 µM) and the A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) at 0.03, but not 0.003 µM abolished the inhibitory action of CGS 21680 (0.1 µM). None of these compounds altered the effect of NMDA (10 µM) by itself. DPCPX (0.03 µM) prevented the inhibition by APNEA (10 µM). There was no effect of CGS 21680 (0.1 µM), when guanosine 5'-O-(3-thiodiphosphate (GDP-β-S; 200 µM) was included in the pipette solution in order to block G protein-mediated reactions. In conclusion, adenosine receptors, most likely of the A_{2A}-subtype, inhibit the conductance of NMDA receptor channels in a subset of medium spiny neurons of the rat striatum by a transduction mechanism which involves a G protein.

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Cross-talk between excitatory amino acids and histamine receptors

The aim of our work was to study the possible interaction between excitatory amino acids (EAA) and histamine receptors in neurons of the central nervous system. We used cultured cerebellar granule neurons for being glutamatergic and expressing histamine receptors. Exposure of cultures to either glutamic acid (50 mM) or domoic acid (10 mM) but not (S)-a-amino-3-hidroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 100 mM), induced both a rapid increase in intracellular calcium concentration as measured by confocal laser microscopy, and neurotoxicity. Glutamic acid and domoic acid effects are blocked by MK801 (1 mM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX 15 mM) respectively, indicating the selective activation of NMDA and NON-NMDA receptors.

Cultures exposure to the histamine H1 receptor antagonist, terfenadine (10 mM) but not to famotidine (500 mM), an histamine H2 receptor antagonist, slowly increased intracellular calcium concentration to levels comparable to those of excitatory amino acids, and did induce neurotoxicity. Terfenadine neurotoxicity was dependent upon concentration, time of exposure and neuronal age (days) in culture (DIC). Concentrations of terfenadine as low as 1 mM induced neurotoxicity in approximately 96 hours (h) while only 6–8 h were necessary for 10 mM. Moreover, terfenadine (10 mM) death time increased from 6–8 h, in neurons up to 24 DIC, to more than 13 h in neurons older than 24 DIC. Withdrawal of terfenadine from culture medium 3 h after beginning of the exposure to the drug, avoided cell death. In preliminary experiments, we have also observed that terfenadine may bind to neurons at higher amounts than expected for receptor binding.

Interestingly, terfenadine toxicity could not be prevented either by high concentrations of histamine (10 mM) or by the H1 receptor agonist (6-(2-(4-Imidazolyl) ethylamino)- N-(trifluoromethylphenyl) heptane carboxamide (HTMT) dimaleate, 100 mM). Terfenadine induced neurotoxicity was not significantly reduced by the NMDA receptor antagonist MK-801 (1 mM), while AMPA (100 mM) accelerated neurotoxicity and such effect was blocked by CNQX (15 mM). AMPA potentiation of terfenadine neurotoxicity occurred at any DIC and similar effects were observed for domoic acid. We suggest that terfenadine-mediated neurotoxic effects could occur via an histamine receptor-independent pathway. Calcium concentration could be an important clue in the mechanism of terfenadine neurotoxicity both alone or in association with EAA.

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Effect of glutamate on degeneration of dopamine neurons in several animal models of parkinsonism

Although controversial, studies with methamphetamine (METH) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) suggest a link between glutamate-mediated excitotoxicity and dopamine (DA) neurodegeneration. Both compounds are thought to create a metabolic stress in DA neurons. To further explore glutamate actions in DA degeneration, we investigated the effects of other metabolic inhibitors. In mesencephalic cultures, DA cell loss produced by 3-NPA (0.25 mM) or malonate (50 mM) was prevented by MK-801 (1 μM). Furthermore, DA toxicity was potentiated in the presence of malonate (10 mM) and NMDA (50, 100 μM). These findings implicate NMDA receptor involvement in DA degeneration produced by metabolic stress. *In vivo,* striatal DA loss produced by intranigral infusions of malonate (0.5 μmol/1 μl) was prevented by systemic MK-801 (2 ip injections, 2.5 mg/kg, 3h apart). Furthermore, intranigral NMDA (10 nmols/1 μl) with malonate (0.25 μmols/1μl) potentiated DA loss in striatum and substantia nigra. In contrast, systemic MK-801 (2.5 mg/kg) did not prevent DA loss produced by intrastriatal malonate (4 μmols).

Intrastriatal MK-801 (≥ 10 nmol) or CGS 19755 (3 nmol) did attenuate DA loss produced by METH (10,5 mg/kg; 2h apart), but was confounded by the findings that METH-induced hyperthermia, an important component in toxicity, was also attenuated.

Additionally, NMDA appears more potent when administered into the substantia nigra than into the striatum. Taken together, the data support the hypothesis of NMDA receptor involvement in DA degeneration. Furthermore, the data also suggest that this interaction is more likely to be at the level of the substantia nigra than the striatum.

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Caffeine indirectly enhances glutamate release in the hippocampus of awake, freely moving rats via muscarinic M_1 receptors

Caffeine is a popular drug which possesses a complex psychopharmacological profile. Although several mechanisms have been proposed to explain the physiological effects of caffeine, changes in various central neurotransmitter systems caused by antagonism of adenosine receptors are thought to be the most likely. We have used the technique of microdialysis in association with HPLC and fluorometric detection to study the ability of caffeine, in a dose range which corresponds to that ingested by humans, to influence the extracellular levels of glutamate in the hippocampus of awake, freely moving rats. Base-line extracellular levels of glutamate were increased when the microdialysis probe was perfused with a selective inhibitor of glutamate transport, L-*trans*–pyrrolidine-2,4-dicarboxylic acid, a Ca^{2+}-free Dulbecco's phophate buffered saline (DPBS) or the Na^+ channel blocker tetrodotoxin. Base-line extracellular levels of glutamate were not affected by perfusion of DPBS containing a low concentration of Ca^{2+} (0.1 mmol/L). Oral administration of caffeine dose-dependently (3–30 mg/kg) increased the extracellular levels of glutamate in the hippocampus of awake, freely moving animals. The levels were also enhanced when caffeine (1 mmol/L) was perfused directly through the microdialysis probe. Perfusion of the selective adenosine A_1 agonist N^6-cyclopentyladenosine (0.1–10 μmol/L) did not influence base-line release of glutamate, but did counteract the increase caused by the oral administration of caffeine (30 mg/kg). Interestingly, the levels of glutamate could also be enhanced by the local perfusion of the acetylcholinesterase inhibitor neostigmine (10 μmol/L) which enhances acetylcholine release. And this effect was counteracted by the local perfusion of the selective muscarinic M_1 antagonist pirenzepine at a concentration of 1 μmol/L which in itself had no effect on glutamate release. Moreover, the caffeine-induced increase in glutamate levels could be blocked by local perfusion of either N^6-cyclopentyladenosine or pirenzepine. In summary, the results of this study show that the interaction of caffeine with adenosine A_1 receptors causes increases in the extracellular levels of glutamate in the hippocampus of awake, freely moving rats. This effect may in part be due to an enhanced ACh release acting at muscarinic M_1 receptors.

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Effects of glutamate antagonists on dopamine synthesis

In a recent microdialysis study (Biggs et al., 1996), we showed that NMDA antagonists potentiated the conversion of L-DOPA to dopamine in the nigras of dopamine-depleted rats. This suggested to us that the converting enzyme, aromatic L-amino acid decarboxylase (AADC), is regulated by glutamate, and that AADC is maintained in an inhibited state by overactive glutamate pathways in the parkinsonian brain. We have therefore assayed AADC activity by the method of Hadjiconstantinou et al. (1995), in the striatum (CS) and substantia nigra (SN) of naive rats treated acutely with a variety of NMDA (1 h) and AMPA (30 min) antagonists. Drug doses (i.p.) were selected on the basis of their efficacy in behavioural experiments.

Basal AADC activity, expressed as nmol dopamine/mg protein/20 min, was found to be 19.7 ± 1.9 for SN. MK 801 (0.01 mg/kg) was ineffective, but 0.1 and 1 mg/kg increased enzyme activity ($p < 0.001$) in both CS (38.9 ± 2.6, 30.9 ± 0.9) and SN (72.7 ± 13.4, 47.0 ± 2.5) respectively. Other NMDA ion channel blockers were also highly effective in this regard, raising AADC activity in CS and SN as follows: 20 mg/kg memantine, 27.2 ± 4.2 and 31.6 ± 2.9; 40 mg/kg amantadine, 75.1 ± 7.3 and 198.2 ± 28.9; 40 mg/kg dextromethorphan, 66.5 ± 12.7 and 135.4 ± 34.3; 12.5 mg/kg budipine, 45.9 ± 3.6 and 93.5 ± 11.9. The NMDA glycine site antagonist (R)-HA 966 (5 mg/kg) elevated AADC activity in the CS (28.7 ± 2.5, $p < 0.001$) but not the SN (26.2 ± 3.7). By contrast, 10 mg/kg eliprodil, 5 mg/kg CGP 40116 and 10 mg/kg NBQX were without effect.

The existence of physiological regulatory mechanisms for AADC suggests the increase in activity of this enzyme by NMDA receptor-channel blockers may have clinical relevance. Our findings could help to explain the antiparkinsonian efficacy of enigmatic drugs such as the aminoadamantanes, and the newly introduced agent budipine, and pave the way for a new strategy of pharmacotherapy for Parkinson's disease.

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Direct and indirect control of dopamine release by excitatory amino acids

Dopamine (DA) release from nerve terminals of the nigrostriatal dopaminergic neurons not only depends on the activity of nigral cells, but also on presynaptic regulations. Glutamate presynaptically stimulates DA release through AMPA and NMDA receptors located on DA nerve terminals. Through its effects on striatal targets cells, glutamate also exerts indirect regulations of DA release involving transmitters such as GABA or acetylcholine or diffusible messengers such as nitric oxide (Hanbauer et al., 1992) or arachidonic acid (AA). Indeed, we have demonstrated that exogenous AA stimulates the release of DA from striatal synaptosomes of the rat (L'Hirondel et al., 1995). Acetylcholine which presynaptically facilitates the release of DA through muscarinic and nicotinic receptors allows the NMDA-evoked release of DA from striatal synaptosomes by removing the magnesium block of NMDA receptors (Chéramy et al., 1996). Experiments were thus performed on striatal microdiscs from the mouse to determine whether AA could be partly involved in this response since, as shown in our laboratory, the combined application of carbachol and NMDA markedly and synergistically stimulates the formation of AA from cultured striatal neurons (Tencé et al., 1995).

As expected, the co-application of NMDA and carbachol stimulated dramatically the release of preloaded [^{3}H]-DA from striatal microdiscs. In addition, this response was partially reduced (50%) in the presence of mepacrine (10^{-7} M), an inhibitor of PLA_2 which alone did not modify the K^+-evoked release of [^{3}H]-DA. These results indicate that endogenously released AA induced by the co-stimulation of NMDA and cholinergic receptors accounts, at least partly, in the evoked release of DA.

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Nitric oxide in thermoregulation

The rapidly expanding elucidation of the biology of the free radical gas nitric oxide (NO) has recently also focused on its modulator functions in thermoregulation, considering the widespread vascular and central nervous distribution of constitutive NO-synthases. Core temperature measurements and expecially vasodilatatory effects suggest hypothermic and, respectively, antipyretic actions of NO. Systemic applications of NO-donors and a NO-synthase inhibitor in conscious rabbits indicated co-ordinated activation of autonomic heat loss (skin vasodilatation, thermal panting) by NO. Since the blood-brain barrier is no obstacle for NO, it may act centrally when generated peripherally, but may also be released locally in the hypothalamus and in other central nervous thermosensory regions to modulate thermo-regulatory activities. NO-donor infusion into the 3rd cerebral ventricle of conscious rabbits showed that NO centrally facilitates heat loss mechanisms in a co-ordinated fashion. – The neuronal correlates of central nervous NO-actions specific for activation of heat loss have not been elucidated. In vivo recordings of hypothalamic neurons in rats showed excitatory and inhibitory actions of NO. Studies on slice preparations in vitro whowed mainly inhibition of warm sensitive hypothalamic neurons by NO, which is difficult to reconcile with its central facilitatory action on heat defense. However, in tissue slices from the spinal cord, a site of origin for temperature signals, warm sensitive neurons in the superficial dorsal horn presumably contributing to spinothalamic afferent transmission were mainly activated by NO, whereas warm sensitive neurons in deeper layers probably not contributing to the afferent thermosensory input were predominantly inhibited.

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Spinal nerve lesion induces upregulation of neuronal nitric oxide synthase in the spinal cord – An immunohistochemical investigation in the rat

The neurochemical basis of the pathophysiology of neuropathic pain and associated neurodegeneration is not well understood. Recently involvement of nitric oxide (NO) in many physiological and pathophysiological functions of the CNS is suggested [1, 2, 4, 5]. However, its role in neurodegeneration and cell injury is still unclear [1, 2]. The synthesis on NO in the nervous system is mediated by the enzyme nitric oxide synthase (NOS) which is normally present in some neurons of the CNS [2]. There are experimental evidences

which suggest that a focal trauma to the rat spinal cord is associated with an upregulation of NOS activity in neurons of the perifocal segments [3]. This upregulation of NOS is closely related with the pathological reaction of nerve cells indicating a putative role of NO in cell injury. The present investigation was undertaken to find out whether a chronic spinal nerve lesion is associated with alteration in NOS activity, and if so, whether this alteration in NOS expression is related with cell injury. Spinal nerve lesion at L-5 and L-6 level was produced according to the Chung model and rats were allowed to survive for 8 weeks, in one group of animals L-NAME was given intraperitoneally daily for 8 weeks. Sham operated rats, in which the spinal nerve was exposed but not ligated, served as controls. NOS upregulation was examined on Vibratome section (60 µm thick) obtained from L-5 segment of the lesioned control, L-NAME treated and sham operated rats according to the standard protocol [3, 4]. Selected tissue pieces of the cord from each group were embedded in epon for routine light and electron microscopy [5]. Ligation of spinal nerves in rats resulted in an upregulation of NOS which was most pronounced in the ipsilateral gray matter of the spinal cord compared to the contralateral side. In these rats, untrastructural investigations showed distorted neurons, membrane disruption, synaptic damage and myelin vesiculation. Sham operated rats did not show either NOS upregulation or such structural changes in the spinal cord. Pretreatment with L-NAME resulted in a significant reduction in NOS upregulation, and structural changes in the spinal cord were less pronounced. These observations strongly indicate a putative role of NOS in the pathophysiology of chronic nerve lesion. Further studies using other neuroactive drugs and growth factros in this model may provide a new strategy to treat chronic neuropathic pain or to minimise neurodegeneration in the patients suffering from such diseases of the nervous system.

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Spinal cord evoked potentials and edema in the pathophysiology of rat spinal cord injury

The possibility that spinal cord evoked potentials (SCEP) are a good indicator of pathological changes in the spinal cord was examined using epidural electrodes placed over the perifocal segments of the cord in a focal rat model of spinal cord trauma [1]. Experiments carried out in our laboratory in the past suggested an involvement of nitric oxide in the pathophysiology of spinal cord injury [2]. However, the contribution of nitric oxide (NO) in the early SCEP changes and the pathophysiology of edema and cell injury is not well understood.

The present investigation was undertaken to examine SCEP changes and expression of nitric oxide synthase (NOS, an enzyme responsible for conversion of NO from its precursor L-Arginine) in the traumatised spinal cord. In addition, the influence of various neuroprotective drugs or NOS antiserum on SCEP changes and NOS expression was also evaluated. The SCEP was analysed in rats pretreated with either NOS antiserum, L-NAME, a nitric oxide synthase inhibitor, p-CPA, a serotonin synthesis inhibitor, or diazepam, an antistress drug. In these treated or untreated rats NOS upregulation was examined using immunohistochemical techniques [2]. Spinal cord injury was produced in urethane anaesthetised rats by making an incision (about 2 mm deep and 5 mm long) into the right dorsal horn of T10–11 segments [2, 3]. Two min before injury, 20 µl of nitric oxide synthase (NOS) antiserum (1:20) was applied for 10 sec over the traumatised spinal cord segments [2]. In another group of rats, L-NAME, p-CPA or diazepam was given before injury according to the standard protocol [1].

A focal trauma to the rat spinal cord significantly attenuated the SCEP amplitude (about 60%) immediately after injury which persisted up to 1 h. However, a significant increase in SCEP latency was seen at the end of 5 h after trauma. The decrease in SCEP amplitude was not seen in rats treated with NOS antiserum, P-CPA or diazepam, whereas no significant changes in SCEP amplitude were seen after injury in rats treated with L-NAME. Upregulation of NOS, cell injury and edema were significantly prevented in rats received NOS antiserum, p-CPA or diazepam, whereas no significant difference in NOS expression or cell injury was observed in rats treated with L-NAME.

These observations suggest that SCEP appears to be a good indicator of cell injury and edema formation, and upregulation of NOS expression seems to be related with edema and cell injury.

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Nitric oxide in the pathophysiology of hyperthermic brain injury – A pharmacological study using immunohistochemistry in the rat

The possibility that nitric oxide (NO) is involved in the pathophysiology of brain injury [1, 2] caused by heat stress (HS) [3, 4] was examined by immunohistochemical techniques using an antiserum to constitutive isoform of neuronal nitric oxide synthase (NOS) in a rat model [3–5]. Furthermore, to find out a role of cellular and oxidative stress in inducing NOS activity in HS, effects of the antistress drug diazepam and a new antioxidant compound H-290/51 (Astra Hässle, Mölndal, Sweden) on HS induced expression of NOS immunoreactivity and brain pathology was examined.

Subjection of conscious young rat to a 4 h HS in a biological oxygen demand (BOD) incubator at 38 °C resulted in a marked upregulation of NOS in many brain regions compared to control rats kept at room temperature (21 ± 1 °C). This NOS immunoreactivity was found mainly in distorted neurons located in the edematous regions not normally showing NOS activity. Breakdown of the BBB permeability, increase in brain water and marked neuronal, glial and myelin reaction are a common findings in several brain regions showing upregulation of NOS

activity. Pretreatment with diazepam or H-290/51 significantly attenuated the upregulation of NOS in rats subjected to HS. In these animals breakdown of the BBB permeability, edema and cell changes were considerably reduced.

Our results suggest that hyperthermic brain injury is associated with a marked upregulation of NOS activity in the CNS. This upregulation of NOS and concomitant cell injury can be reduced by prior treatment with the antistress drug diazepam or and antioxidant compound H 290/51. This indicates that NOS upregulation, which probably causes an activation of NO production, is associated with cell injury. Our results further show that cellular and oxidative stress are important endogenous signals for NOS upregulation in thermal brain injury.

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Nitric oxide acts via cGMP to limit water-intake by its inhibitory action on subfornical organ neurons

The subfornical organ (SFO) is one of the circumventricular organs lacking a blood-brain-barrier (BBB). It has been amply documented that blood-borne angiotensin II (AngII) stimulates water-intake by activation of SFO neurons. We have recently shown that other blood-borne peptides which have so far not been implicated in water intake, like amylin, a pancreatic peptide co-released with insulin after food intake and calcitonin, a peptide released from the thyroid gland when the plasma calcium level is elevated, can also activate SFO neurons. The excitatory effects of amylin, calcitonin and AngII were observed on the majority (62–80%) of cells investigated, they were dose-dependent and had similar threshold concentrations (10^{-9} M). Like AngII, sc. injection of amylin and calcitonin caused a significant increase in water-intake. On the other hand, we could show that activators of the soluble or particulate guanylyl cyclase, like atrial natriuretic peptide (ANF) or nitric oxide (NO) have almost exclusively inhibitory effect on SFO neurons and reduce water intake. Incubation of SFO slices in vitro with ANF and NO results in an expected rise in cGMP immunoreactivity, but incubation with amylin, AngII and calcitonin caused also an (unexpected) rise in cGMP. The increase in cGMP by amylin, calcitonin and AngII was still observed in the presence of tetrodotoxin (10^{-6} M), but was completely prevented by L-NMMA. It is concluded that the water-intake stimulating peptides activate the majority of SFO neurons and cause a simultaneous release of NO from non-neuronal cells in the SFO. NO will then trigger the production of cGMP in nearby neurons. Thus NO might function as a servo-control mechanism in the SFO to prevent "overexcitability" of neurons to blood-borne peptides.

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Involvement of nitric oxide in the pathophysiology of spinal cord injury – An immunohistochemical study in the rat

The possibility that nitric oxide (NO) participates in the pathophysiology of spinal cord injury [1, 2, 4] was examined in a rat model topical application of nitric oxide synthase (NOS) antiserum of L-NAME (N^G-Nitro-L-arginine methyl ester hydrochloride), a potent inhibitor of NOS [1, 2]. Spinal cord injury was produced in urethane anaesthetised rats by making an incision (about 2 mm deep and 5 mm long) into the right dorsal horn of T10–11 segments [3]. Two min before injury, 20 µl NOS antiserum (1:20) was applied for 10 sec over the traumatised spinal cord segments using a microliter syringe [3]. In another group of rats, L-NAME was applied either topically (100 µg in 20 µl over 10 sec) or injected into the right femoral vein (2.5 mg/kg/min for 15 min) two min after injury [3]. Untreated injured animals received normal rabbit serum as controls. Five hour after injury NOS upregulation, water content, microvascular permeability to ^{125}I-labelled Met-Enk-Arg^6–Phe^7 (MEAP) and pathology of the spinal cord were examined in the perifocal (T9 and T12) spinal cord segments.

A focal trauma to the rat spinal cord induced a marked upregulation of NOS immunolabelling in the perifocal T9 and T12 segments. In these segments marked increase in the water content, microvascular permeability and profound cell changes were observed. Application of NOS antiserum significantly reduced the upregulation of NOS in the T9 and T12 segments of the injured spinal cord. This treatment also minimised the gross edematous expansion of the cord, microhaemorrhages and edema compared to the untreated group. Extravasation of MEAP, water content and cell changes were considerably reduced in the antiserum treated and traumatised rats. However, application of L-NAME, either topically or given intravenously, resulted in only a mild reduction of visual swelling, water content and the microvascular permeability. In these rats NOS upregulation was not reduced significantly.

These results suggest that upregulation of NOS, which probably represent an activation of NO, participates in the early pathophysiological reactions of the traumatised spinal cord. Further studies using microvascular flow alteration after application of NOS antiserum and a dose related response of L-NAME or other specific inhibitors of NOS are however needed to clarify this point.

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Neurotrophic factors attenuate upregulation of nitric oxide synthase and cell injury following trauma to the spinal cord – An immunohistochemical study in the rat

The possibility that neurotrophic factors induced neuroprotection is influenced by mechanisms involving nitric oxide [1, 2] was examined in a rat model of focal spinal cord injury. A focal trauma to the spinal cord results in marked upregulation of nitric oxide synthase (constitutive and neuronal type, nNOS) in the perifocal segments (T9 and T12) after 5 h injury [3]. The number of nNOS positive neurons correlates well with the edematous expansion of the spinal cord, microvascular permeability disturbances, edema and cell injury, indicating an involvement of NO in the early pathophysiological reactions of the spinal cord. Brain-derived neurotrophic factor (BDNF) or insulin like growth factor (IGF-I) are found to be neuroprotective in various other models of ischemic brain injury. However, the probable mechanisms of BDNF or IGF induced neuroprotection are not well known. This investigation was undertaken to examine whether pretreatment with BDNF and IGF is neuroprotective in our model of rat spinal cord injury and, if so, whether this neuroprotection is assiated with NOS upregulation. Spinal cord injury was inflicted on the T10-11 segments by making an incision of the right dorsal horn. In separate group of animals BDNF or IGF-I (0.1 µg/10 µl in phosphate buffer saline) was applied topically 30 min before injury on the exposed spinal cord followed by repeated doses of growth factors immediately before and 30 min after injury. Thereafter application of BDNF or IGF was carried out at every 1 h interval until sacrifice. Five hours after injury, the tissue pieces from the T9 segment were processed for nNOS immunostaining. Untreated injured rats showed a profound upregulation of nNOS which was most pronounced in the nerve cells of the ipsilateral side. These rats exhibited marked increase in the blood-spinal cord barrier (BSCB) permeability to ^{125}I-albumin and water content in these perifocal segments. Spinal cord samples of T9 and T12 segments showed marked cell injury and damage to the nerve cells, glial cells and myelin. Pretreatment with BDNF and IGF markedly reduced the upregulation of nNOS in the spinal cord. This effect of the growth factors was most pronounced in the contralateral side. These neurotrophic factors treated rats showed much less signs of BSCB damage, edema and cell injury. These results suggest that BDNF and IGF pretreatment is neuroprotective in spinal cord injury and that these neurotrophic factors has the capacity to down regulate nNOS expression following trauma to the spinal cord. Taken together, our results provide new experimental evidences which suggest that BDNF and IGF may exert their potential neuroprotective effects probably via regulation of NOS activity, a feature which require further investigation.

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Glutamate release inhibitors: a critical assessment of their action mechanism

Excessive activation of glutamate-mediated synaptic transmission is widely considered to contribute to the pathophysiology of neurological disorders as varied as epilepsy, stroke, amyotrophic lateral sclerosis (ALS) and traumatic brain injury (TBI). This concept has led to the development of drugs acting either post-synaptically on glutamate receptors (expecially antagonists of ionotropic NMDA- and AMPA/kainate-receptors), or pre-synaptically by inhibition of vesicular glutamate release (exocytosis). The so-called "glutamate release inhibitors" mainly include use-dependent blockers of voltage gated Na^+–channels (e.g. lamotrigine, its derivatives BW1003C87 and BW619C89, and riluzole) and κ-opioid receptor agonists (e.g. enadoline; CI-977).

With regard to brain ischaemia and TBI, it is often proposed that inhibition of glutamate exocytosis is the primary action mechanism underlying the cerebroprotective effects of "glutamate release inhibitors". However, this hypothesis conflicts with important data: (i) these compounds do not inhibit glutamate exocytosis selectively – lamotrigine inhibited veratridine-induced release of glutamate from cortical slices, but it was only 2-fold less potent in its inhibition of GABA release; (ii) lamotrigine derivatives and riluzole attenuated the efflux of glutamate provoked by ischaemia, but also that of aspartate which is not released by exocytosis because it is not concentrated in presynaptic vesicles; (iii) some of these compounds were protective even when administered after transient ischaemia, i.e. when extracellular glutamate levels had returned to baseline.

These drugs may actually enhance the resistance of nerve cells to insults, primarily by decreasing their energy demand, with reduced efflux of glutamate and other compounds being only a *consequence* of attenuated cellular stress. This action mechanism certainly applies to modulators of voltage-gated Na^+-channels (e.g. riluzole, lamotrigine and its analogues) because reduction of Na^+ influx results in energy preservation [Urenjak and Obrenovitch (1996) Pharmacol Rev 48: 21–67]. Cerebroprotection with enadoline may also be linked to reduced energy demand since this drug decreased cerebral glucose utilisation in rats [MacKay and McCulloch (1994) Brain Res 642: 160–168].

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Neuroprotection – rationale for pharmacological modulation of Na^+-channels

With anoxia, ischaemia and mitochondrial dysfunction, the primary factor detrimental to neurons is insufficient energy supply relative to their requirement. Accordingly, reduction of energy demand is a rational strategy for neuroprotection, which probably underlies the beneficial effects of hypothermia. As a large part of the energy consumed by nerve cells is used for the maintenance and replenishment of ionic gradients across their cellular membrane (expecially Na^+ gradient), down-modulation of voltage-gated Na^+-channels increases the resistance of brain cells during periods of deficient energy supply. Reduced density of Na^+-channels, and their inherent down-regulation during oxygen deprivation, contribute to the increased tolerance of the immature brain to hypoxia, and to the remarkable ability of some water turtles to survive anoxia. Preservation of the inward Na^+ gradient may be also beneficial because it is an essential driving force for ion exchange and transport mechanisms which

ensure vital functions (i.e. Ca^{2+} homeostasis, acid-base balance, cell volume regulation, and neurotransmitter uptake) [Urenjak and Obrenovitch (1996) Pharmacol Rev 48:21–67].

The capability of Na^+-channel modulation to increase the resistance of brain cells during an insult is clearly illustrated by drugs such as phenytoin, carbamazepine, or riluzole, which all prolong survival to hypoxia. Na^+-channel 'blockers' are also cerebroprotective when their administration is delayed (e.g. after transient ischaemia). This suggests that they help damaged or vulnerable brain regions to cope with secondary pathological processes (e.g. recurrent spreading depression, inflammation, delayed impairment of microvascular perfusion) or persistent abnormalities (e.g. upregulation of voltage-gated Na^+-channels, enhanced synaptic efficacy).

Finally, compounds such as lamotrigine and riluzole demonstrate that it is possible to interact with specific Na^+-channel states, or even with specific Na^+–channel types, to provide neuroprotection without unacceptable deficits of neuronal function, and without cardiotoxic effects.

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The importance of voltage-dependent sodium channels in cerebral ischaemia

Thromboembolic stroke causes neuronal death by depriving the brain of an adequate supply of oxygenated blood. Recently, clinical trials with the thrombolytic agent recombinant tissue plasminogen activator (rt-PA) have shown that this agent can increase the number of patients who survive a stroke without any permanent disability by up to 50%. There still may be scope, however, for improving therapy by administering neuroprotective agents. Results from a wide variety of different experiments have shown that subjecting brain tissue to ischaemia or hypoxia/hypoglycaemia causes massive depolarization, an increase in the intracellular concentration of calcium and an excessive release of excitatory amino acids such as glutamate and aspartate. These events act in concert to orchestrate cell death. Currently, the efficacy of several antagonists of one particular subtype of excitatory amino acid receptor, namely the N-methyl-D-aspartate (NMDA) receptor is being tested in clinical trials for stroke. Cerestat® is at most advanced stage of clinical development in this class. NMDA antagonists, however, possess drawbacks. They have a very narrow therapeutic window, do not prevent the initial depolarization of massive release of excitatory amino acids, and do not act neuroprotectively in the white matter of the brain. We know that human strokes include white matter as well as grey matter. Recently, interest has been focused on blockers of voltage-dependent Na^+ channels because they have been shown to prevent glutamate release and cell death in hypoxic/hypoglycaemic cell cultures as well as neuronal death in several different *in vivo* models of focal and global ischaemia. They also act neuroprotectively in models of white matter damage. Although there are several Na^+ blockers which are currently being tested in various phases of preclinical and clinical development, most of these agents are very weak and unselective. The selectivity with respect to voltage-dependent Ca^{2+} channels is particularly crucial because of the risk of cardiovascular side effects. Finally, blockers of voltage-dependent Na^+ channels should exhibit strong use dependency such that depolarized neurons in ischaemic areas are preferentially inhibited. It is therefore our goal in the pharmaceutical industry to design molecules which selectively block voltage-dependent Na^+ channels for the treatment of acute ischaemia stroke.

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Calpain inhibitors do not protect serum-free cultures of rat cortical neurons from the neurotoxic effects of glutamate or hypoxia/hypoglycaemia

Excessive synaptic release of excitatory amino acids such as glutamate causes the death of neurons which have been deprived of oxygen by increasing the intracellular concentration of calcium. Calpain is a protease enzyme which is activated by calcium and thought to contribute to neuronal death after ischaemia by breaking down cytoskeletal proteins. We have compared the neuroprotective effects of three calpain inhibitors, namely MDL 28170, calpeptin and calpain inhibitor I, with those of an antagonist of the NMDA receptor-channel complex, (+)MK-801, in pure, serum-free cultures of cortical neurons prepared from rat embryos. Neurotoxicity was induced by either the addition of glutamate or exposing the neurons to hypoxia/hypoglycaemia. The number of living cells was quantified with an assay based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and calpain activity was determined by measuring spectrin breakdown products with Western blots. Adding glutamate or subjecting the neuronal cultures to hypoxia/hypoglycaemia caused neurotoxicity and also increased the amount of spectrin breakdown products. The extent of neurotoxicity and amount of spectrin breakdown depended on the concentration of glutamate and the length of hypoxia/hypoglycaemia. (+)MK-801, an antagonist of the N-methyl-D-aspartate (NMDA) receptor-channel complex, concentration-dependently inhibited both glutamate- and hypoxia/hypoglycaemia-induced neuronal death and also prevented the accompanying spectrin breakdown. In contrast, three different calpain inhibitors, namely MDL 28170, calpeptin and calpain inhibitor I, failed to prevent neuronal death. They did, however, inhibit the accompanying spectrin breakdown. We therefore conclude that neuronal cell death induced by glutamate or hypoxia/hypoglycaemia is not caused by calpain-induced activation of spectrin breakdown in cultured rat cortical neurons.

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Glutamate plays a central role in mediating the neurotoxic effects of excitatory amino acids, Na^+ channel activation and hypoxia/hypoglycaemia in serum-free cultures of rat cortical neurons by opening the NMDA receptor channel

In this study, we have investigated the role of glutamate in mediating the toxic effects of excitatory amino acids, Na^+ channel activation and hypoxia/hypoglycaemia in serum-free cultures of rat cortical neurons. Neuronal cultures were prepared in medium conditioned by confluent monolayers of astrocytes. Neurotoxicity was induced by the addition of various excitatory amino acids, opening voltage-dependent Na^+ channels or exposing the neurons to hypoxia/hypoglycaemia. The number of living cells was quantified with an assay based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The concentration of glutamate in the medium was determined by HPLC and fluorometric detection. The addition of glutamate, N-methyl-D-aspartate (NMDA), kainate or the Na^+ channel activator veratridine caused a concentration-dependent neurotoxicity.

The neurotoxicity of all these agents was concentration-dependently inhibited by the NMDA channel blocker BIII 277 CL. Furthermore, tne neurotoxic effects of kainate could also be blocked by the AMPA/kainate antagonist NBQX and the effects of veratridine by the site 1 Na^+ channel blocker tetrodotoxin (TTX). Both kainate and veratridine caused a secondary release of glutamate into the culture medium which could be blocked by the addition of NBQX and tetrodotoxin, respectively. The time course for the neurotoxic effects of kainate and veratridine agreed with the time course for glutamate release. Subjecting the neurons to hypoxia/hypoglycaemia also caused neuronal cell death and glutamate release. The extent of neurotoxicity and the amount of glutamate released depended on the length of hypoxia/hypoglycaemia. Only the selective NMDA channel blocker BIII 277 CL prevented cell death at all time points. We therefore conclude that glutamate plays a central role in mediating the neurotoxic effects of excitatory amino acids, Na^+-channel activation and hypoxia/hypoglycaemia by opening the NMDA receptor channel.

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Destruction of catecholamine-containing neurons by 6-hydroxydopa, an endogenous amine oxidase cofactor

6-Hydroxydopa (6-OHDOPA), the amino acid that comprises the active site in aminooxidase, is thought to be neurotoxic owing to in vivo generation of reactive oxygen species (ROS) and/or decarboxylation to 6-hydroxydopamine (6-OHDA). Unlike 6-OHDA which destroys dopamine (DA) and norpinephrine (NE) nerves, 6-OHDOPA is relatively selective for NE neurons. When administered to rats in early postnatal ontogeny, 6-OHDOPA destroys NE-containing locus coeruleus perikarya and produces an axotomy of the dorsal bundle, with consequent permanent hypoinnervation of neocortex and hippocampus. This lesion, analogous to surgical transection of ther dorsal bundle, induces reactive proliferative sprouting of proximal branches of locus coeruleus neurons, with an eventual hyperinnervation of brainstem and cerebellum by NE neurons. The effects of 6-OHDOPA are partly modulated by opioids, as evidenced by morphine potentiation of 6-OHDOPA effects. L-Dihydroxyphenylalanine (L-DOPA) is known to produce ROS, as per 6-OHDOPA, but at a much slower rate. Findings with 6-OHDOPA may be illustrative of the neurotoxic potential of high dose L-DOPA used to treat Parkinsonism.

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Molecular mechanisms of cellular injury produced by neurotoxic amino acids that generate reactive oxygen species

The biochemical toxicology of semiquinones and quinones – the products of enzymatic or non-enzymatic degradation of catechol(amine)s-neurotransmitters was the object of very intense research during the past 20 years [1–4]. Generation of reactive oxygen species (ROS) is an unavoidable by-product of catechol(amine)s one – and two electron reactions. Damages resulting from brain trauma (ischemia), inflammation processes and/or certain neurological and psychotic disorders connected with neuronal lesioning (PD, AD), lead to excessive overproduction of ROS as a risk factor for neurodegenerative changes.

Recent discovery [5–7], that the basic precursors for the synthesis of catechol(amine)s-neurotransmitters, tyrosine and tryptophan can act as generators of ROS: endoperoxices (tyrosine-hydroperoxide, TyrOOH and tryptophan-hydroperoxide, TrypOOH), superoxide (O_2^-/HO_2) and peroxyradicals RO_2, raises the question about their neurotoxicity under pathological conditions. TyrOOH and TrypOOH can act as a metastable oxidants and therefor could diffuse far from the site of their formation [5–7]. Like other peroxides they should react with transition metals and promote lipid peroxidation and damage to DNA and proteins. However, cellular injury will depend on many factors which include the level of precursors (tyrosine and tryptophan), the oxygen tension and/or the redox status of the cell. Exposure of cells to organic hydroperoxide can cause a depletion of ATP (via changes in the brain level of GSH and piridine nucleotides), deficiency of mitochondrial activity and crucial changes in neuronal metabolism.

Although the generation of neurotoxic ROS by tyrosine and trypotphan is accepted to occur *in vitro*, doubts can exist as to the situation *in vivo*, which may be completely different. The relevance of the present finding with regard to a variety of neurological diseases will be discussed.

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Pharmacological approaches to counter the toxicity of Dopa

Dopa and related catecholamines and their degradation products have been demonstrated to have neurotoxic potential in a number of cellular and in vivo experiments. Several mechanisms have been hypothesized to be involved including generation of prooxidant products that subsequently oxidize membrane lipids and exposed macromolecules. We have utilized a neuronal culture of cerebellar granule cells to study the toxicity of Dipa and the ability of various neuroprotective and antiparkinsonian compounds to offer protection therefrom. We found that the neuroprotective antioxidant, PNU-101033 is more effective and potent than vitamin E and deprenyl in this regard. Similarly the D2/D3 agonist, pramipexole is also capable of blocking Dopa toxicity in this model and in a related model involving cultures of embryonic tyrosine hydroxylase expressing neurons. The activity in the former assay seems to be a function of the intrinsic antioxidant properties of pramipexole whereas the protection of the tyrosine hydroxylase phenotype apparently requires both that activity plus the D3 receptor agonist action of the compound. Potent antioxidants are effective blockers of Dopa toxicity. If the

mechanisms involved in this toxicity have relevance to the progression of Parkinson's pathology in dopa treated (or untreated) patients. these compounds have the potential to alter the course of the illness.

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Biological actions of endogenous excitatory amino acids and implications of their release under physiological and pharmacologically-induced conditions

Immunohistochemical studies have demonstrated the presence of glutamate (Glu) in the corticostriatal pathway of the rat, suggesting that this amino acid is released from nerve terminals and acts, via several receptor subtypes, as a major excitatory neurotransmitter. Aspartate (Asp) is also present in corticostriatal neurons, but its role as a neurotransmitter is questioned, since, in contrast to Glu, it does not appear to be taken up by presynaptic vesicles. Glu and Asp can be found, although without clear regional distribution, at subμM concentrations in most areas of the basal ganglia. When investigated with *in vivo* microdialysis, their concentrations in the extracellular space are largely regulated by transport mechanisms, although there is also a synaptotagmin-dependent exocytotic release. Whatever the origin of Glu and Asp, the concentrations reached are large enough to occupy junctional and extrajunctional receptors.

We have investigated whether Glu and Asp release can be selectively modulated by drugs acting on different neuronal systems. In the neostriatum, dopamine (DA) and cholecystokinin (CCK) exert a selective stimulatory modulation on Asp release, via D_1 and CCK_B receptor subtypes, respectively. Also κ-agonists increase Asp release. In contrast, local perfusion with opioid antagonists increases both Glu and Asp release. We propose that the selective modulation of Asp release by D_1-, CCK_B- and κ-agonists involves striatal neurons containing Asp, but not Glu. The effect of opioid antagonists on Glu and Asp is probably exerted on corticostriatal terminals, via μ-presynaptic inhibitory receptors. In the neocortex, CCK-8S exerts a modulation on both Glu and Asp release, that on Asp via CCK_B receptors, and that on Glu via CCK_A and CCK_B receptors. A similar effect has been observed in the substantia nigra. In this region, naltrexone and the μ-antagonist CTOP selectively increase Glu release. Thus, these results demonstrate that extracellular levels of Glu and Asp are modulated selectively by different neuronal systems, and suggest that there are different neuronal populations using Glu and/or Asp as messenger(s).

We have also investigated whether excessive release of Glu and Asp is related to the short- and long term effects of perinatal asphyxia. Glu and Asp appear to be involved in the effects elicited by mild, but not in those by severe asphyxia, suggesting that there are other mechanisms for the deleterious effects of persistent anoxia. In agreement, Glu receptor antagonism is only slightly protective against the effects of perinatal asphyxia. The hypothesis that Glu can be instead a protective substance during development is discussed, since it has been demonstrated that Glu metabotropic receptor agonists promote (and antagonists decrease) the density of TH-fibres into the striatum (Teng et al. 1996). The implication of these observations is discussed in terms of the potentials of treatments with drugs targeting excitatory amino acid systems.

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Modulation of NMDA receptors by glycine-introduction to some basic aspects and recent developments

Antagonism of NMDA receptors may potentially have a wide range of therapeutic applications. Although a number of uncompetitive and competitive NMDA receptor antagonists have been clinically characterised less is known about the therapeutic potential of full antagonists acting at the strychnine-insensitive, co-agonistic glycine site ($glycine_B$). Initial preclinical evidence for a more promising therapeutic profile of $glycine_B$ antagonists was obtained either with local i.c.v. administration of full $glycine_B$ antagonists with poor pharmacokinetic properties or systemic administration of partial agonists. In such studies $glycine_B$ antagonists have been reported to lack many of the side effects classically associated with NMDA receptor blockade such as neurodegenerative changes in the cingulate/retrosplenial cortex, psychotomimetic and learning impairing effects. Recently some full $glycine_B$ antagonists with somewhat improved pharmacokinetic properties have also been reported to have good therapeutic indices following systemic administration in models of hyperalgesia, as anxiolytics, as possible antipsychotomimetics and as neuroprotective agents in models of focal ischaemia and trauma. This improved therapeutic profile may be due to the ability of full $glycine_B$ antagonists to induce glycine-sensitive desensitization or via functional and regional NMDA receptor subtype selectivity. In this regard, attempts to improve systemic activity solely by increasing the *in vitro* potency of $glycine_B$ antagonists with poor pharmacodynamic properties may be the wrong approach as most very high affinity $glycine_B$ antagonists do not induce receptor desensitization.

Merz has recently developed a series of novel tricyclic pyrido-phtalazin diones which are moderately potent $glycine_B$ antagonists *in vitro* but show a much better *in vivo* systemic availability and/or penetration of the blood brain barrier. Provisional data indicate that these Merz $glycine_B$ antagonists may have improved potency and therapeutic indices in some indications and show a very different behavioural profile to competitive and uncompetitive NMDA receptor antagonists (Danysz et al., this meeting). These agents should prove to be useful tools to elucidate the true therapeutic potential of this class of NMDA receptor antagonist.

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Novel $glycine_B$ antagonists *in vivo*: Block of NMDA responses, and analgesic properties

A new series of pyridazine quinoline derivatives with selectivity for the $glycine_B$ site *in vitro* (Parsons, this meeting) has been tested by systemic administration *in vivo* for activity in three series of tests.

Selectivity for NMDA vs. AMPA was examined for several compounds in microiontophoresis experiments on spinal dorsal horn neurones in α-chloralose anaesthetised spinalised

Wistar rats. The most potent and selective NMDA antagonists were Mrz 2/502, Mrz 2/576 and Mrz 2/570 (ID_{50} ± s.e.m. 1.6 ± 0.3, 2.8 ± 0.7 and 4.5 ± 0.7 mg/kg i.v., n = 6–7, cf 1.3 ± 0.3 mg/kg i.v. for ketamine, n = 9). At the maximal doses tested, responses to NMDA were reduced by Mrz 2/502 (2–4, mean 3 mg/kg) to 17 ± 6% control (AMPA 76 ± 8%); by Mrz 2/576 (2–8, mean 4 mg/kg) to 30 ± 7% control (AMPA 88 ± 6%); by Mrz 2/570 (4–16, mean 9 mg/kg) to 29 ± 4% control (AMPA 100 ± 9%). On the same cells, ketamine (1–4, mean 3 mg/kg, n = 17) reduced NMDA to 15 ± 3% control (AMPA 91 ± 4%).

Mrz 2/502 and Mrz 2/576 were selected for electrophysiological tests of nociception. In α-chloralose anaesthetised spinalised Wistar rats, single motor unit reflex responses of hindlimb flexor muscles were evoked by noxious pinch stimuli applied to the hindpaw receptive field. These nociceptive responses had a strong NMDA receptor mediated component (ketamine ID_{50} for inhibiting responses was 0.9 ± 0.2 mg/kg, n = 11). The potency rank order and ID_{50} values were similar in these tests to values obtained for inhibition of NMDA responses: Mrz 2/502 1.0 ± 0.3 mg/kg (n = 10), Mrz 2/576 3.3 ± 0.4 mg/kg (n = 5).

Mrz 2/576 was compared with morphine in behavioural tests on Sprague-Dawley rats. Paw pressure, thermal (Hargreaves) and rotarod (accelerating) tests were performed on rats with normal paws, and thermal and paw weight-bearing tests on rats with acute (4 h) carrageenan-induced inflammation of one hindpaw. Dose-response curves were established (8 rats per point) and data analysed as differences from baseline. In non-inflamed animals Mrz 2/576 increased paw pressure thresholds at a dose of 5 mg/kg i.p. that did not impair rotarod performance; at 10 mg/kg rotarod performance was affected. Morphine at 5 mg/kg enhanced paw pressure threshold but also decreased rotarod performance. In animals with an acutely inflamed paw, a reversal of both thermal and mechanical hyperalgesia was produced by Mrz 2/576, with the same potency as in normal animals, whereas the potency of morphine in the thermal test was twice that found in normal animals.

All Mrz compounds had a rapid onset and a short duration of action. Following i.v. administration the recovery $t_{½}$ of NMDA responses or of nociceptive reflexes was 8–12 min. Following i.p. administration the effects peaked at 5 min.

These compounds are therefore selective NMDA antagonists with ready systemic availability. They produce antinociception at doses somewhat below those causing motor impairment and with a potency comparable to morphine.

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Novel glycine$_B$ antagonists show neuroprotective activity *in vivo*

Opening of the NMDA channel initiates either neuroplasticity or cytotoxicity. Effective neuroprotection against NMDA receptor stimulation can be achieved by non-competitive antagonists so that the degree of inhibition could not be diminished by high levels of an agonist for the NMDA recognition site. Such agents include NMDA receptor channel blockers, e.g., memantine and MK-801, and the recently developed antagonists at the glycine binding site. Acetylcholinergic neurons within the NBM are vulnerable to excess stimulation of NMDA receptors, e.g., by local injection of NMDA. We investigated the effects of three glycine$_B$ antagonists (MRZ 2/570, 2/571, 2/576) and a novel NMDA channel blocker (MRZ 2/579) on the toxicity produced by an NMDA injection into the NBM. MRZ 2/579 (30 min prior to NMDA) and MRZ 2/570, MRZ 2/571 or MRZ 2/576 (given 15 min before and 15 min after NMDA) provided significant neuroprotection starting at 10, 20, 10 and 5 mg/kg, respectively. Also implantation of an osmotic minipump immediately after the NMDA injection, prefilled with MRZ 2/570 (20 and 40 mg/kg/day x 14 days), provided significant neuroprotection against NMDA. Therefore, both types of NMDA receptor antagonists were able to provide neuroprotection from overstimulation of NMDA receptors.

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The role of glycine recognition site of NMDA receptors in the control of motor behavior

Motor behavior depends on the integrity of basal ganglia nuclei. The glutamatergic system is critically involved in its control. In this respect, the function of the NMDA receptor has been studied intensively. The characteristics of its allosteric glycine site are not analyzed in depth in the past due to a limited number of drugs passing the blood brain barrier. Now, there are ligands available. Using this opportunity, the effects of several glycine site antagonists (7-chlorokynurenate, ACEA 1021, M-576, felbamate) and a partial agonist ((+) HA-966) on sniffing stereotypy, locomotion, prepulse inhibition and pharmacological-induced catalepsy were tested in rats.

It was found that each glycine binding site ligand had its individual motor behavioral profile. Some of them induced a sniffing stereotypy, reduced D2- but not D1-receptor-mediated catalepsy and produced a prepulse inhibition deficit, whereas others had minor effects on these behavioral parameter. However, it seems to be a general finding that in non-sedative doses locomotion was unaffected by all ligands.

The results suggest that the glycine binding site allows a specific control of motor behavior via the NMDA receptor complex. This control is however not a simple blockade of glutamatergic effects but a structure- and receptor subtyp-depending control of specified motor behavior. The results also indicate therapeutic benefit of glycine site antagonists and partial agonists in neurodegenerative disorders which are often accompanied by an overactive glutamate system. The allosteric glycine binding site might offer the possibility for symptom-specific drug-design.

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Glycine$_B$ antagonists as potential therapeutic agents – previous hopes and present reality

Although a number of uncompetitive and competitive NMDA receptor antagonists are already used clinically or are at a late phase of development, less is known about the therapeutic potential of antagonists acting at the glycine site of the NMDA receptor (glycine$_B$) since most of the studies to date have utilised either partial agonists or agents with doubtful brain penetration.

Several MERZ glycine$_B$ antagonists and L-701,324 as reference agents have been tested in a range of screening tests. They showed anticonvulsive activity in mice and much improved penetration to the brain (Parsons et al., this symposium). At doses within the anticonvulsive range, myorelaxation (traction test) and ataxia (rotarod test) were observed. In rats in the open field test the same glycine$_B$ antagonist produced sedation and attenuated the hyperlocomotion induced by both PCP and amphetamine. However, they neither changed prepulse inhibition on their own nor antagonised the deficit produced by PCP. In line with that observation they also did not produce vacuolisation in the cingulate/retrosplenial cortex. These data indicate a lack of psychotomimetic and antipsychotic potential. The glycine$_B$ antagonists tested attenuated haloperidol-induced catalepsy but failed to produce antiparkinsonian-like activity in rats after reserpine treatment or nigrostriatal system lesion. Also no clear-cut potentiation of L-DOPA action was observed in the latter two test. No reliable anxiolytic activity was observed either in the elevated plus maze or the Vogel test. In global ischaemia in gerbils a neuroprotective action was observed which was connected with a short-lasting hypothermic effect. Moreover, the glycine$_B$ antagonists tested attenuated both physical dependence and motivational aspects connected with morphine treatment as evidenced by naloxone precipitated withdrawal in mice and place-preference test in rats. They failed to produce place-preference on their own. Hence, glycine$_B$ antagonist seem to have different behavioural profile from uncompetitive NMDA receptor antagonists.

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The role of kynurenines in normal and compromised brain function: Novel concepts

As endogenous ligands of excitatory amino acid (EAA) receptors, the kynurenine pathway metabolites quinolinic acid (QUIN) and kynurenic acid (KYNA) have long been speculatively linked to the pathogenesis of excitotoxic brain diseases. In addition to the substantial increase in brain QUIN levels which occur when the immune system is compromised, recent studies have identified brain region-specific increase in KYNA levels in the kindled brain, after the administration of chemoconvulsant stimuli, and in the brain of schizophrenic patients. In contrast, reductions in KYNA levels are observed in the neostriatum of Huntington's disease victims.

Systemic administration of C-amphetamine or dopamine (D1 or D2) receptor agonists also results in decreases in brain KYNA. In particular, reductions down to 30% of control levels occur when these drugs are given during the first two postnatal weeks. A critical role for brain kynurenines in the perinatal period is also indicated by the precipitous drop in brain kynurenine, 3-hydroxy-kynurenine and KYNA levels on the day of birth.

The regulation of KYNA production has been studies *in vivo* and, more extensively, using tissue slices *in vitro*. Several mechanisms, some specific to the brain, were found to control the *de novo* production of KYNA from kynurenine. For example, depolarizing conditions, or interference with energy metabolism through inhibition of glycolysis, Krebs cycle or oxidative phosphorylation, results in a substantial reduction in KYNA formation. In several instances, this effect can be reversed by lactate or, more effectively, by pyruvate and other 2-oxoacids that can also act as co-substrates for kynurenine transamination.

Taken together, these data suggest that multiple factors can influence kynurenine pathway metabolism in the brain. Separately or in concert, these mechanisms are likely to control the extracellular concentrations of QUIN and KYNA. This, in turn, will determine the role of neuroactive kanurenines in physiological and pathological processes which are mediated through EAA receptors.

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Expression of the kynurenine enzymes in macrophages and microglial cells: Regulation by immune modulators

Several lines of evidence suggest a relevant role for brain macrophages in the overproduction of neuroactive kynurenines in brain inflammation. To investigate the specific role of activated macrophages and brain microglia, we studied the regulation of the expression of indoleamine 2,3-dioxygenase (IDO) and other enzymes of the kynurenine pathway by interferon-γ IFN-γ) and other immune modulators in cloned murine macrophages (MT2) and microglial (N11) cells. Both cell lines expressed IKO activity after IFN-γ-stimulation. The regulation of the enzyme expression, however, appeared to differ in the two cell lines. Thus, nitric oxide (NO) production negatively modulates the expression of IDO activity in IFN-γ-primed macrophages, suggesting the existence of a cross-talk between the IFN-γ-activated kynurenine and nitridergic pathways in murine macrophages. Conversely this down-regulation of IDO activity by NO did not occur in microglial cells. Kynureninase activity was much higher in MT2 macrophages than in N11 microglial cells and IFN-γ markedly stimulated (~ 10-fold) the activity of this enzyme only in MT2 cells. Kynurenine aminotransferase, kynurenine 3-hydroxylase (KH) and 3-hydroxyanthranilate dioxygenase were constitutively expressed to a similar extent and, in both cell lines, only KH activity appeared to be up-regulated (~ 3-fold) by IFN-γ. Altogether, our results add weight to the concept that cells of phagocytic lineage from different organs substantially diverge in the kynurenine pathway functional reactivity upon immune stimulation and consequently point out to a distinct role of mycrophages and microglial cells in the production of potentially neurotoxic kynurenine metabolites in neuroinflammation.

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Excitotoxic actions of quinolinic acid: Modulation by endogenous antagonists

Focal injections of quinolinic acid (QUIN), a pyridine dicarboxylic acid derived from the kynurenine pathway of tryptophan metabolism, produce depletion of biochemical or histochemical markers for brain cholinergic, dopaminergic, gagaergic, enkephalinergic and NADPH diaphorase neurons. Dose response studies show that the dopaminergic, enkephalinergic and NADPH-d neurons (core site) are highly sensitive to QUIN toxicity. The QUIN precursor, 3-OH anthranilic acid (3-OHAA), at higher doses, also produces dopaminergic neuron damage. However, unlike QUIN the effect of the precursor is not blocked by an NMDA receptor antagonist, suggesting that its toxicity does not result from the production of QUIN. The metabolic pathway yielding QUIN also yields a number of other neuroactive metabolites which can antagonize QUIN action. Thus, one of these metabolites, kynurenic acid (KYNA), acts as a non-selective glutamate receptor antagonist and effectively blocks QUIN-induced toxicity. The brain levels of *endogenous* KYNA can be elevated by agents which inhibit activity of kynureninase or kynurenine 3-hydroxylase. Ad-

ministration of nicotinylalanine, one such inhibitor, prior to a neurotoxic dose of QUIN significantly elevates brain KYNA and attenuates the action of QUIN on dopamine and NADPH-d neurons. Another tryptophan metabolite, picolinic acid (PIC), also attenuates QUIN action in models of dopamineregic, NADPH-d and cholinergic neuron toxicity. The mechanism underlying the protective action of PIC is not clear but appears to differ from that underlying KYNA action. PIC is known to chelate zinc and this property may contribute to its anti-QUIN action. This mechanism is favoured by the observation that other agents known chelate zinc indeed mimic the neuroprotective action of PIC against QUIN-induced toxicity. PIC itself does not exert a toxic action on the cholinergic, dopaminergic or NADPH-d neurons. Endogenous excitotoxins such as QUIN have been implicated in the neuron loss associated with a number of neurodegenerative disorders. The occurrence of endogenous factors which inhibit QUIN toxicity presents opportunities for harnessing their neuroprotective potential by pharmacological approaches.

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Excitatory amino acid release and NMDA-receptor activity studied with positron emission tomography

Release of excitatory amino acids and activation of the NMDA-receptor complex are involved in a number of physiologic and patophysiological processes. Opening of the NMDA-receptor controlled calcium channel has been implicated in pain transmission and in the consolidation of memory. Excessive excitatory amino acid release has been related to neurodegeneration, in the generation of epilepsia pathology and in the phenomenon of "wind-up" in pain transmission.

Interest has been focussed to develop radiotracers for positron emission tomography, PET, with the ability to trace NMDA receptor activation and ongoing degenerative processes. Ketamine is an anesthetic drug which bind to the PCP-site of NMDA-receptor controlled channels and was chosen as radioligand for studies on NMDA-activation following ischemic pain [2] and in epilepsia patients.

The regional brain kinetics of the two enantiomers of the NMDA-channel blocker ketamine rediolabelled with ^{11}C was studied in the Rhesus monkey using PET [1]. Radioactivity distribution correlated with brain regions with a high density of NMDA-receptor complexes. The uptake was blocked in a dose-dependent manner for both ^{11}C-labelled enantiomers with simultaneous doses of both *(S)*- and *(R)*-ketamine, respectively. Bindung of *S*-[N-methyl-^{11}C]ketamine was selective and displaceable by the *(R)*-enantiomer and by MK-801.

Plasma concentrations, maximum regional brain concentration and specific binding in the brain after administration of 0; 0.1 and 0.2 mg/kg doses of *(S)*-ketamine were measured in a randomized, double blind cross-over study in volunteers and correlated to induced effects such as analgesia, amnesia and mood changes [2]. Specific binding in the brain was assessed by simultaneous administration of *(S)*-[*N*–methyl-^{11}C]ketamine and quantified by PET. A significant and dose-dependent reduction of binding was measured as a result of displacement of radioactivity. Memory impairment and psychomimetic effects were related to dose, plasma concentration and decreased regional binding of *(S)*–ketamine in the brain. In further studies, the cerebral blood flow increase due to ischemic pain hided effects of excitatory amino acid release on *(S)*-[*N*–methyl-^{11}C]ketamine binding. Binding decreased in epileptic brain tissue probably as an effect of neurodegeneration and decreased blood flow.

A new option of PET is the quantitation of binding in relation to the activation of the receptor. Ligands for the PCP-site, bind rapidly to NMDA-receptor complexes with activated receptors. Such a use-dependency can be used to quantitate synaptic activity in humans with use of PET and selective tracers and is thus a new prinicple for functional studies in the brain.

References

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Modulation of cerebellar granule cells $GABA_A$ receptors by phosphorylation/dephosphorylation events

$GABA_A$ receptors of the rat cerebellum granule cells were studied by the patch clamp recording technique in the whole cell configuration.

The function of these receptors is regulated by phosphorylation in several fashions. First, a run-down phenomenon was found for $GABA_A$ receptors which can be avoided adding Mg^{++}/ATP in the recording pipette. The phosphorylation state of tyrosine residues appears to be critical in this phenomenon.

Second, the function of $GABA_A$ receptors is negatively modulated by activators of the serine/threonine protein kinases, A and G. In particular, the negative modulation of the receptor's activity by nitric oxide is mediated by protein kinase G activity stimulation.

Third, a negative modulation of $GABA_A$ receptors by previous NMDA receptor activation is caused by activation by intracellular calcium of NO synthase and calcineurin. The first pathway eventually implies protein kinase G mediated phosphorylation of serine/threonine residues of the $GABA_A$ receptor. The second one implies dephosphorylation at different serine/threonine residues. Evidently, the two sites involved are differently situated in the receptor and differently available to PKG and calcineurin.

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Regulation by Ca^{++} of Deiters' neuron $GABA_A$ receptors

The function of classical $GABA_A$ receptors of the rabbit Deiters' neurons has been studied at the single membrane level by a biochemical micromethod involving the study of labelled chloride permeation. In particular, labelled chloride permeation across microdissected single membranes was studied in a microchamber system. The stimulation of $^{36}Cl^-$ out→in permeation by "extracellular" GABA was determined under different conditions in the respect of Ca^{++}. When the conditions were such that "intracellular" Ca^{++} was between 0.02 and 2 μM there appeared to be an optimal effect by GABA on chloride passage. Conditions presumably resulting in an increase of $[Ca^{++}]_i$, beyond the limit reported above led to a decreased GABA effect, especially at the highest GABA concentrations used ($\geq 10^{-4}$ M).

However, complete removal of Ca^{++} by a high (12 mM) intracellular EGTA concentration erased completely the GABA effect.

These results indicate that in these neurons an optimal $GABA_A$ receptor function requires $[Ca^{++}]_i$ levels below micromolar.

The high $[EGTA]_i$ effect seems to imply that too low a $[Ca^{++}]_i$ is also harmful to the proper function of these $GABA_A$ receptors.

Neurochemistry

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Evidence against increased glycoxidation in Alzheimer disease

Neuropathological findings of Alzheimer disease (AD) are intracellular (neurofibrillary tangles) and extracellular (senile plaques) filamentous protein aggregates. Non-enzymatic glycation has been proposed as a primary factor in this pathogenesis, leading to increased insolubility of tau protein and amyloid beta. The aim of our study was to test the hypothesis that increased glycoxidation, i.e. oxidised products from non-enzymatic glycation could be found in brains of patients with AD and of aged Down syndrome (DS) subjects with abundant AD-like neuropathological lesions. Frontal cortex specimens were assayed for pentosidine (Pent) and N-epsilon-carboxymethollysine (CML), well-documented markers for glycoxidation, by reversed phase high performance liquid chromatographical methods. Pentosidine and N-epsilon-carboxymethyllysine levels in AD (n = 10; Pent: 35.5 ± 4.84 µmol/g wet-weight tissue; CML: 135.2 ± 5.0 µmol/g wwt) were comparable to DS (n = 9; Pent: 36.4 ± 3.21; CML: 133.5 ± 4.7) and controls (n = 10; Pent: 35.2 ± 3.55; CML: 136.9 ± 3.3). We conclude that the results are not compatible with the concept of increased glycoxidation in AD compared to normal ageing.

Neuropharmacology and Neurobiology

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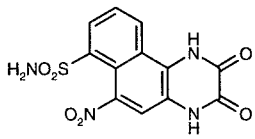
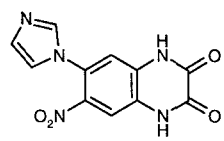
Novel water-soluble and highly potent AMPA-antagonists from the quinoxalinedione series

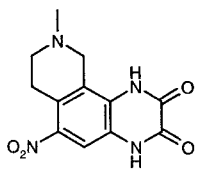
Glutamate-mediated excitotoxicity plays a key role in a number of acute and chronic neurodegenerative disorders such as spinal cord and head trauma, cerebral schemia and Parkinson's disease. Consequently, competitive glutamate receptor antagonists are presently in therapeutic focus as potentially useful neuroprotective agents. Selective competitive antagonists of



NBQX



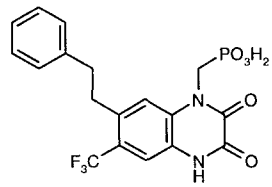
YM 90K



PNQX

the AMPA-glutamate receptor subtype like NBQX, YM 90K, and PNQX have preclinically demonstrated pronounced neuroprotective efficacy in a variety of *in vitro* and *in vivo* models of neurodegeneration. In addition, intriguing and stimulating results regarding the therapeutic time window have been obtained. In spite of these findings, up to now the poor solubility of these substituted quinoxalinediones and the concomitant risk of kidney failure have prevented their further clinical development.

By introduction of an additional acidic function a major breakthrough in the design of water-soluble, selective and potent AMPA-antagonist has been achieved.



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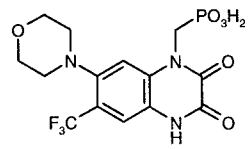

ZK 200775

MPQX

Structural optimization resulted in development candidates ZK 202 000 and MPQX (ZK 200 775) of which the latter is presently in clinical evaluation for stroke therapy.

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Local influence of endogenous excitatory amino acids on basal and stress-induced 5-HT release in the locus coeruleus

Among other neurons, serotonergic and excitatory amino acid utilizing neurons innervate the locus coeruleus (LC). We used the push-pull superfusion technique to investigate whether endogenous amino acids influence basal and sensory stimuli-induced release of serotonin (5-HT) in the LC.

The basal release rate of 5-HT in the LC was 7.4 ± 1.1 (mean value ± S. E. M., n = 36). Superfusion with the NMDA receptor antagonist AP5 (10 μM) decreased the release rate of 5-HT in the LC. Superfusion with AP5 in the presence of the nMDA/glycine receptor antagonist kynurenic acid (1 mM) further decreased the amine release rate. NMDA enhanced 5-HT release, while in the presence of AP5, the releasing effect of NMDA was abolished. Tetrodotoxin (1 μM) did not prevent the NMDA-induced release of 5-HT. Like AP5 and kynurenic acid, superfusion with the kainate/AMPA antagonist DNQX (10 μM) inhibited 5-HT release. The agonist kainic acid (50 μM) caused a DNQX-sensitive increase in 5-HT release.

Noise stress and pain elicited by tail pinch enhanced the release of 5-HT. Superfusion with AP5 (10 μM) attenuated tail-pinch-induced 5-HT release, while AP5 in the presence of kynurenic acid (1 mM) diminished the effects of both, tail pinch and noise, on the 5-HT lelease rate. On the other hand, superfusion with DNQX (10 μM) failed to influence 5-HT release by sensory stimuli.

The findings show that the activity of serotonergic neurons in the LC is modulated by endogenous excitatory amino acids. Furthermore, excitatory amino acid utilizing neurons, via NMDA/glycine receptors, are implicated in the stress-induced activation of serotonergic neurons within the LC.

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Effects of chlormethiazole on convulsions produced by excitatory amino acid agonists in mice

Chlormethiazole is a sedative, hypnotic and anticonvulsant drug used clinically to treat ethanol withdrawal, eclampsia and status epilepticus. It is suggested that this agent potentiates τ-aminobutyrate (GABA) and glycine-activated currents. In this study, a possible link between excitatory amino acid (EAA)-mediated neurotransmission and mechanisms of anticonvulsant activity of chlormethiazole was investigated.

EAA agonists: α-amino-3-hydroxy-5-methyo-isoxyzole-4-propionate (AMPA), kainic acid (KA) and N-methyl-D-aspartate (NMDA) were delivered into the lateral brain ventricle of unanesthetized mice at CD97 doses for the clonic convulsions. Chlormethiazole (Heminevrin, Astra, Sweden; given subcutaneously) protected mice against seizures elicited by EAA agonist in the following rank order of potency: AMPA > KA > NMDA. The respective ED50 values were 50 (33–75), 72 (54–99) and 107 (87–130) mg/kg.

As evaluated in the chimney and rotorod tests, chlormethiazole produced an impairment of motor coordination with ED50 values of 112 (103–120) and 117 (107–127) mg/kg, respectively.

The effects of EAA agonists on the anticonvulsant activity of chlormethiazole were estimated in maximal electroshock (alternating current 50 Hz, 25 mA, 0.2 s). NMDA administered intraperitoneally at the dose of 50 mg/kg impaired the protective action of chlormethiazole enhancing its ED50 value from 127 (116–139) to 155 (139–172). KA (9 mg/kg) was ineffective in this respect.

Our results suggest an involvement of EAA-mediated events in the mechanism of anticonvulsant activity of chlormethiazole.

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The role of NMDA receptors in the mechanism of neuroleptic action

It is generally assumed that typical neuroleptics such as haloperidol induce their antipsychotic effect via blockade of dopamine D_2 receptors. On the other hand, the mechanism responsible for the therapeutic effect of the atypical neuroleptic clozapine is still unclear. Recently it has been found that neuroleptics interact with the glutamatergic system. Moreover, it has been suggested that an abnormal dopaminergic-glutamatergic interaction may be involved in the pathophysiology of schizophrenia.

The aim of the present study was to investigate the influence of chronic treatment with haloperidol and clozapine on NMDA receptors in the rat brain cortex using an autoradiographic analysis. Additionaly, we examined the influence of those drugs on the level of the mRNA encoding NMDAR1 subunit of NMDA receptors in different cortical areas using an in situ hybridization method. The drugs were given to animals in drinking water for 3 months: haloperidol in a dose of 1 mg/kg/day, and clozapine in a dose of 30 mg/kg/day. On day 5 of the withdrawal, the rats were killed by decapitation. [^{3}H]CGP 39653 was used for labelling the recognition site of the NMDA receptor complex. An oligonucleotide probe (cDNA), corresponding to the sequence encoding the NMDAR1 subunit of NMDA receptors, was labelled with [^{35}S]dATP using terminal transferase. An analysis of autoradiograms was carried out by a computer-assisted image analysing system.

The [^{3}H]CGP 39653 binding was significantly enhanced in the frontal, parietal and insular cortices after haloperidol administration. Chronic treatment with clozapine increased the binding of [^{3}H]CGP 39653 in the parietal and insular cortices. Moreover, clozapine raised the level of the mRNA encoding NMDAR1 subunit of NMDA receptors in the insular cortex of rats. On the other hand, haloperidol significantly lowered the level of the mRNA encoding NMDAR1 subunit of NMDA receptors in the frontal and parietal cortices.

The obtained results suggest that the up-regulation of NMDA receptors (measured by an increased binding of [^{3}H]CGP 39653) in different cortical regions may contribute to the antipsychotic effect of haloperidol and clozapine. Moreover, it seems that some of the changes in NMDA receptors induced by neuroleptics may be due to their influence on the NMDAR1-mRNA expression.

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The influence of acute and chronic treatment with tetrahydroisoquinoline (TIQ) on muscle tone in rats

1,2,3,4-Tetrahydroisoquinoline (TIX) as well as the related compounds sansolinol and N-methyl-sansolinol are regarded

as putative endogenous toxins which induce Parkinson's disease in humans. It has been shown that these substances evoke certain behavioural and biochemical symptoms similar to those found in parkinsonian patients and in monkeys and rats. Recently we have demonstrated that treatment with model substances inducing parkinsonian symptoms in animals, such as reserpine, haloperidol, and 6-hydroxydopamine (6-OHDA destroying ca. 90% of nigral dopamine cells) increase the muscle resistance and the EMG reflex response to passive movements in rats. The latter effects resemble those seen in parkinsonian patients. The aim of the present study was to find out whether TIQ injected acutely or chronically evokes muscle rigidity and causes degeneration of dopamine nigral cells. Muscle tension was measured as resistance (torque, mechanomyogram, MMG) of a rat's hind foot to passive flexion and extension. Simultaneously with the MMG, fine-wire electrodes recorded the electromyographic (EMG) response to movements. Degeneration of catecholamine cells was assessed by immunostaining of frontal brain slices for thyrosine hydroxylase. TIQ injected both acutely (50 and 100 mg/kg, ip) and chronically (50 and 100 mg/kg/day, 19 days, ip), as well as after a 3-day withdrawal period induced an increased torque and late EMG reflex responses to movements. Preliminary inspection of the brain slices immunostained for tyrosine hydroxylase did not reveal any marked neurodegeneration. Our preliminary results show that, like in other model substances (reserpine, haloperidol, 6-OHDA), TIQ induces parkinsonian-like muscle rigidity, however, its mechanism of action remains unknown.

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Glutamate's and glutamine's adaptive functions in plants and animals under ammonia intoxication

Detoxication of ammonia in plants and animals in case of its toxical effect and in intoxication at the expense of catabolism in stress conditions and diseases takes place by inclusion of glutamate (Glu) and glutamine (Gln) into amino acids. Glu is formed from 2-oxoglutarate with participation of glutamate-dehydrogenase (GDH) and Gln is formed from Glu by glutamine synthetase (GS). For some plants species (Ceratophyllum submersumi, Potamogetol pectinatus, Lemna minor, Elodea canadensis) established that with increased ammonia concentrations slightly above norm glutamine is accumulated in their organisms (activated GS), repression synthesis of which is caused by ammonia concentrations to ~ 50 mM. In these conditions its detoxication is accomplished by binding into glutamate GDH. Primary low significance correlation of Glu/Gln increases. The change in suggested correlation is a switching factor of metabolic ways of ammonia detoxication, which acts through regulative enzyme GS. The transition from glutamine to glutamate way of ammonia detoxication is expedient for adenilate charge preservation, breaching of which occurs in Gln synthesis with preservation of high rate protein synthesis.

In animals (we studied Cyprinus carpio L., Salmo gairdneri Rich.) the first ammonia assimilation enzyme is GDH and formed by its reaction glutamate is a substrate for GS. The latter is activated after GCH repression with ammonia level 0.07 mg/l. Synthesis switching with ammonia concentration increase (from 0.014 mg/l to 0.07 mg/l and 0.10 mg/l) correspond to correlation change of Glu/Gln : 1.52, 1.58, 1.40. Biological expediency of Glu/Gln accumulation in detoxicating system in this case determined by the fact that it is namely its $-NH_2$ group is nitrogen donor in urea synthesis in ureotelic organisms and is the glutaminase substrate in ammoniotelic organisms. Moreover, Gln different to Glu does not display neuromediatoric action in animals and penetrates through membranes escaping active transport systems.

Thus, the mechanism of ammonia detoxication in plants and animals using the same reactions, has adaptive differences caused by habitation conditions and by metabolic peculiarities. Glu/Gln correlation may used as an indicative coefficient criterion of detoxication organism's abilities estimation.

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Homeostasis regulation in glutamine-glutamate-gamaaminobutirate in fish brain under intoxication

The Gln-Glu-GABA system components inclusion into plastic/synthetic/processes including ammonia detoxication, into energy metabolism, or orientation towards neuromediators pool is determined by metabolism direction in nerve cells depending on outward effects.

In connection with temperature and seasonal dependence of fish organism tolerance to toxicants research has been made in different months of the year. It has been established that under the influence of exogenous ammonia concentrations there is no certain presence of it in fish brain but there is a clear tendency of glutamine level increase. By this glutamine acid contents does notably change in all months with the exception of October. Glutamine synthetase activity level increase in December confirms Glu taking part in ammonia detoxication. In October glutamine branch metabolic activity is insignificant. In autumn months glutamine branch activity is predominant. By this glutamate formation can also have a neuromediator function. Gln synthesis from Glu and its desintegration probalby effects HXBA-shunt and as a result Glu concentration increase does not happen even in interaction of 2-oxoglutarate with ammonia. Intensive HXBA-shunt functioning under ammonia intoxication is confirmed by a three times growth of HXBA-transaminase activity and by 1.5 times growth of glutamatedecarboxylase activity. By this acetylcholineesterase activity decreases by 2 times. Besides that, HXBA-transaminase activity increase is indicative of primary HXBA transformation into GABA, the latter having stronger inhibition effect. Glu usage energy branch should not be excluded because in fish, glutamate oxidizes faster than glucose.

Thus, under ammonia glutamate directed use as the Gln-Glu-GABA system centre depends on seasonal conditions, macroergs provision and neuromediators concentration, protective inhibition development which contributes to fish brain's homeostasis energy maintainance.

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Developmental aspects of traumatic brain injury. Potentiation by NMDA receptor antagonists

Mechanical trauma to 7-day-old rat brain produces an acute local lesion and delayed lesions seen 24 hrs later. To investigate delayed traumatic damage during development anesthetized 3–30 day old Wistar rats were subjected to mechanical injury to the right parietal neocortex. A 40 cm long cylinder guided a 10 g weight, left to fall from a height of 16 cm onto a footplate which rested on the infant skull. In rats aged 3–10 days the skull remained unopened. Animals were transcardially perfused 24 hours later and the brains stained according to the DeOlmos cupric silver and the TUNEL protocols. Lesions were visualized in the frontal, parietal, cingulate and retrosplenial neocortices, the caudate, thalamic nuclei and the subiculum, mainly ipsilaterally to the site of impact. Densities of degenerating cells were calculated in these areas

using the stereological method of optical dissector. Three- and seven-day-old rats showed most severe lesions which spread to the contralateral hemisphere. At the age of 10 days, lesions were limited to ipsilateral cortical areas and thalamic nuclei, and at 14 days to the area of primary impact. MK-801 (0.5 mg/kg/dose given four- or eight-hourly for 24 hrs; beginning 1 hr after trauma) and CPP (15 or 30 mg/kg/dose i.p. q50 minX3 followed by 8-hourly injections; beginning 1 hr after trauma) potentiated brain damage in 7-day-old rats. Thus, vulnerability to delayed posttraumatic damage in the rat brain is age-dependent with a peak between 3 and 7 days. NMDA receptor antagonists have a unique ability to potentiate this injury.

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Glutamate receptor dysfunction and Alzheimer's disease

Excessive activation of NMDA receptors by endogenous glutamate causes excitotoxic neuronal degeneration in acute CNS injury syndromes such as stroke and trauma. It has been suggested that excessive NMDA receptor activation may also play a role in Alzheimer's disease (AD). We propose that if excessive NMDA receptor activation plays a role in AD, it is an indirect role in which excessive NMDA receptor activation destroys NMDA receptors, thereby rendering the NMDA receptor system hypofunctional. According to our hypothesis, when the NMDA receptor system becomes sufficiently hypofunctional it unleashes a complex excitotoxic process (fueled both by acetylcholine and glutamate) which is a major contributing factor to the widespread pattern of neuronal degeneration in AD. This hypothesis is derived in part from evidence that the NMDA receptor system may, in fact, be hypofunctional in AD, and that experimental induction of NMDA receptor hypofunction in rat brain (by administration of NMDA receptor antagonist drugs) triggers a pattern of neuronal degeneration resembling that seen in AD.

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Stress-protective effect of the novel psychotropic compound, dilithium N-acetyl-L-aspartate on a rat model of depression

Previous studies have provided evidence that excitatory amino acids (EAA) play an important role in the central regulating of cardiovascular and behavioral responses to stress. However poor penetration NAA through blood-brain barrier don't allow to use native NAA structures with pharmacological purposes. Conjugation amino acid structures with lipophilic radicals is one of the wide-spread ways for creation effective therapeutic drugs.

By means of hydrophosphoril compounds homolytic reactions was carried out synthesis of new acetyl- and phosphonate aspartate amino acid derivatives. These compounds produce a wide range of psychotropic effects. Most pronounced effect was obtained on dilithium N-acetyl-L-aspartate. This compound displayed nootropical, antidepressant and stress-protective activity.

The effects of AKF-94 on the behavior and function of monoamines were tested in Wistar rats during free behavior and emotional stress caused by single 2-hour immobilization. The stress-protective effect of AKF-94 preparations was rated by the indices of individual and social behavior of the rats, as well as by the level of dopamine (DA) in striatum. Exogenous analogues of aspartate on the behavioral and neurochemical manifestations of the stress-reaction in the rats were found, which suggests that the use of EAA is promising as protective agents of emotional stress.

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Changes in temperature of the brain and the rectum after intracerebroventricular AMPA and kainate in rats

Recent studies suggest that brain temperature can be a factor which determines the outcome after brain insults including cerebral ischemia and cerebral trauma in patients, as well as experimental animals. We recently found that stimulation of N-methyl-D-aspartate (NMDA) receptors, a subtype of glutamate receptors, which are thought to contribute to brain damage after the above insults, caused an increase in temperature of the brain and the rectum in rats, though the increase in the former prededed that in the latter (Hara et al., Brain Res. 737: 339, 1996). In this study, we examined whether stimulation of non-NMDA glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate (KA) receptors, caused an increase in temperature of the brain and the rectum in rats. The brain temperature was measured with a thermocouple (0.25 mm in diameter) located in the ventral hippocampus. AMPA (5 nmol, i.c.v.) caused an increase in the temperature of the brain and the rectum, though the rate of the increase in brain temperature was higher than that in the rectum. KA (2 nmol, i.c.v.) induced comparable increases in brain and rectal temperatures. An AMPA/KA receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 and 20 nmol, i.c.v.), suppressed the AMPA- and KA-induced increases in brain and rectal temperatures, although it enhanced the initial increase in rectal temperature after KA. MK-801 (250 nmol, i.c.v.), an NMDA receptor antagonist, also suppressed the AMPA-induced increases in brain and rectal temperatures in a similar manner to DNQX, but it transiently suppressed the KA-induced increases in brain and rectal temperatures, and thereafter, both temperatures, expecially brain temperature, increased and reached comparable levels to those without MK-801. Indomethacin (500 nmol, i.c.v.), a cyclooxygenase inhibitor, almost completely suppressed the AMPA-induced increases in brain and rectal temperatures and the KA-induced increase in rectal temperature, and it partly suppressed the KA-induced increase in brain temperature. These findings suggest that stimulation of AMPA or KA receptors leads to an increase in temperature of the brain and the rectum and that distinct mechanisms may be involved in the temperature increase mediated through the two receptors.

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Anterograde amnestic effects of NMDA receptor antagonist MK-801 and nitric oxide inhibitor L-NAME

Long-term potentiation (LTP), a physiological correlate of learning and memory, has been suggested to consist of two steps – an induction step and a maintenance step. The induction step may occur at the postsynaptic neuron, and the maintenance step may occur at the presynaptic neuron. Investigations of excitatory amino acid receptors and synaptic transmission indicate that the induction of LTP may be mediated by postsynaptic receptors that are selectively sensitive to N-methyl-D-aspartate (NMDA). Administration of NMDA antagonists to block NMDA receptor function has been shown to block the induction of LTP. Recent evidence suggests that nitric oxide

(NO), a retrograde messenger carrying signals backward from the postsynaptic to the presynaptic neurons, may initiate the maintenance of LTP. Blocking the synthesis of NO prevents the expression of LTP. The present study investigated whether MK-801, an NMDA receptor antagonist, and L-NAME, an NO inhibitor, produced anterograde amnesta (AA) in goldfish using active avoidance conditioning as the learning paradigm. The study also investigated whether the drugs impaired performance processes that were necessary for learning to occur. Goldfish were trained and tested semiweekly in fish shuttleboxes. In Experiment 1, fish received intracranial (i.c.) injections of water or various doses of MK-801 or L-NAME 30 minutes before three training sessions and were tested without drug treatments in Session 4. One-way ANOVA with multiple comparisons on the percentage of avoidance responses during test showed that both MK-801 and L-NAME produced AA in a dose-dependent manner. In Experiment 2, fish received several training sessions and an i.c. injection of water or various doses of MK-801 or L-NAME 30 minutes before testing. One-way ANOVA on the test scores showed that both drugs did not impair the expression of learned avoidance responses except the highest dose of MK-801. Thus MK-801 and L-NAME produced AA at doses that did not impair performance processes necessary for learning to occur. Further studies are needed to investigate whether MK-801 and L-NAME produced AA by disrupting learning and memory consolidation differently.

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Effects of some protein and non-protein amino acids on experimental models of epilepsy

In recent years protein and non-protein amino acids have attracted considerable interest from a pharmacological point of view.

The aim of the present study was to elucidate the effect of L-arginine (L-arg), L-ornithine (L-orn), L-canavanine (L-cav), L-citrulline (L-cit) and L-canaline (L-can) on pentylenetetrazol (PTZ) (85 mg/kg s.c.) seizure model in male mice. The effect was evaluated by the differences between control and experimental groups of mice in seizure intensity determined by a six point scale, latency to first seizure, percentage of mice with tonic seizures and mortality. Amino acids was injected intracerebroventricularly (i.c.v.) at a dose of 20 µg/mouse.

L-Canavanine exerted a strong anticonvulsive effect, decreased markedly the seizure intensity, mortality and the percentage of mice with tonic seizure.

L-Arginine, L-ornithine and L-citrulline had no effect on PTZ-seizure, whereas L-canaline increased a significant convulsive effect of PTZ-seizure.

The data suggest that L-canavanine can modulate seizure reactivity.

(This work has been supported by Grant L-421 of the National Science Fund to the Ministry of Education, Science and Technologies of Republic of Bulgaria.)

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Effects of some new synthesized TRH-analogues on behavioural despair in mice

Thyrotropin releasing hormone (**TRH**, *p*Glu-His-Pro-NH2) is known to cause a stimulating action on the central nervous system (CNS) in addition to its endocrine action as a thyrotropin agent. Its central effects including antidepressive activity is short lasting because of its rapid degradation.

Having in mind these data we aimed to synthesized a new more stable analogues of TRH, where labile amide bond between the first two residues is replaced by thiazole ring and substitution of *p*Glu with non-protein amino acid Canavanine (Cav). To this end peptides with a common formula were prepared:

Xaa-His-Pro-NH2 where **Xaa - (Pro)Thz, Cav**

The new (Pro)Thz[1]-TRH and Cav[1]-TRH analogues were synthesized using TBTU and EDCI condensation methods.

(Pro)Thz[1]-TRH was investigated for eventual antidepressive activity, using the Porsolt's method of behavioural despair in mice. The mice were placed individually in cylinders (height 20 cm, diameter 12 cm) containing 6 cm of water at 23 °C. The behaviour during 4 min testing was quantified by measuring the time they spent floating: remained passively in the water, rests, keeping its head at the surface of the water in a horizontal or vertical posture. The horizontal and vertical locomotar activity was measured in "Opto Varimex" apparatus. (Pro)Thz[1]-TRH at a dose of 1 and 3 mg/kg injected i.p. 15 min before testing significantly decreased the floating time in comparison with controls. In these doses (Pro)Thz[1]-TRH did not influence the locomotor activity.

These results suggest that the observed antidepressive activity of (Pro)Thz[1]-TRH is not connected with the changes in the locomotor activity.

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Plant Amino Acids

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Inhibitors of histidine biosynthesis

In the last decade two enzymes in histidine biosynthesis, imidazoleglyerolphosphate dehydratase (IGPD) and histidinol dehydrogenase (HDH), have attracted significant attention in the design of novel herbicides.

IGPD (EC 4.2.1.19) catalyses the conversion of imidazoleglycerolpholphate to imidazoleacetolphosphate. The proposed reaction mechanism involves a diazafulvene intermediate. Triazoles are competitive inhibitors of the enzyme which are thought to mimic this intermediate. IGPD has long been implicated in the mode of action of the herbicide 3-amino-1,2,4-triazole ("amitrole"). Although amitrole is a relatively potent inhibitor of IGPD, this is not the only target site for this

compound and it is unclear to what extent inhibition of histidine biosynthesis contributes to the observed herbicide activity. More recently, triazolyl phosphonate herbicides have been identified as potent inhibitors of the enzyme. Unlike amitrole, the herbicidal effects of these compounds can be attributed to inhibition of IGPD.

The final enzyme in the pathway, HDH (EC 1.1.1.23) has also been a target for herbicide design. HDH converts L-histidinol to L-histidine in an irreversible two step oxidation with the concommitant reduction of two molecules of NAD^+. Potent inhibitors of HDH have been achieved by attaching a lipophile to the reaction centre of a histidinol mimic. This strategy was based on known inhibitors of the mechanistically related enzyme HMG CoA reductase.

It is significant that inhibitors of IGPD are promising herbicides while inhibitors of HDH with similar potencies have only very weak herbicidal activity and it may be that HDH is an intrinsically poor herbicide target.

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Amino acid composition of nontraditional plants for low input, sustainable forage systems

Nontraditional plants are being evaluated for forage production on acid, infertile, and droughty hill-land soils in the northeastern United States where performance of traditional forage species is limited by the environmental constraints. The stress tolerant, perennial legumes *Lathyrus sylvestris* L. (flatpea) and *Lathyrus tuberosus* L. (tuberous vetchling) produce herbage that is high in crude protein (> 20%, dry matter basis), but nonprotein amino acids are prevalent in the tissues. In vegetative flatpea herbage produced during a period of summer drought, the concentration of α,γ-diaminobutyric acid (DABA) was 2- to 3-fold higher than its level in vegetative tissue produced during the spring or fall when rainfall was plentiful. Signs of neurotoxicity developed rapidly among sheep consuming the conserved summer herbage. Neither DABA nor β-N-oxalylamino-α-aminopropionic acid, a toxic amino acid that occurs in other *Lathyrus* species, was prominent in tuberous vetchling herbage. A major peak in amino acid profiles of tuberous vetchling extracts also did not correspond to the lathyrogenic compounds β-cyanoalanine or β-aminopropionitrile. Tuberous vetchling has not been studied agronomically. Accessions of this species exhibit phenotypic differences. Analyses of the free amino acids and protein hydrolyzates from herbage from these various accessions provide early insights into forage quality and antiquality characteristics and the potential of the accessions for forage use.

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Signals and mechanisms regulating lysine synthesis and catabolism in plants

The accumulation of the essential amino acids lysine in plants is regulated both by its synthesis and catabolism (Galili, 1995). We have recently cloned from Arabidopsis cDNAs and genes encoding several enzymes in lysine biosynthesis, as well as a cDNA encoding two linked enzymes in lysine catabolism, namely lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH). *In situ* mRNA hybridization and promoter-GUS analyses have shown that genes encoding enzymes in lysine synthesis and catabolism follow a special expression pattern (Zhu-Shimoni et al., 1997), and are also coordinately regulated during plant development. In addition, we have found that excess accumulation of free lysine in plant cells stimulates its own catabolism via a signaling cascade mediated by Ca^{2+} and protein phosphorylation (Karchi et al., 1995). To address this issue further, we have tested whether the activity of soybean LKR, as well as SDH, are modulated by direct phosphorylation of the bifunctional polypeptide containing both of these linked enzymes. In vitro dephosphorylation of the bifunctional polypeptide with alkaline phosphatase significantly inhibited the activity of LKR, but not of its linked enzyme SDH. Our results suggest that LKR represents a major regulatory step in lysine catabolism in plant cells, and that its activity may be stimulated or inhibited by a counter-balanced action of protein kinases and phosphatases. Moreover, these results also propose that the up and down regulation of LKR activity is subject to compound auto-regulation by lysine.

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Regulation of cysteine biosynthesis in plants

Cysteine biosynthesis plays a crucial role in sulfate assimilation in plant cells. Cysteine is the first organic precursor for the formation of sulfur-containing metabolites in plants such as methionine and glutathione. In plants, cysteine is formed by the conjunction pathway of reductive metabolism of sulfate ion and activation of serine.

Two regulatory mechanisms for cysteine formation have been clarified by our recent studies; one at the level of enzyme activity and the other at the transcriptional leve. The enzyme activity of serine acetyltransferase catalyzing the formation of *O*–acetylserine from serine and acetyl-CoA is inhibited by L-cysteine at the concentration of less 10 μM. This inhibition was observed in the isoforms from watermelon, spinach and *Arabidopsis thaliana*. However, two isozymes from *A. thaliana* were insensitive to L-cysteine, suggesting different regulation among isozymes. Northern hybridization analysis of sulfate starved plants indicated that the steady-state mRNA level of an isoform of sulfate transporter (AST68) increased specifically in roots up to ~ 9 folds by sulfate starvation. Among all the structural genes encoding the proteins for sulfate assimilation, sulfate transporter (AST68), APS reductase (APR1) and serine acetyltransferase (SAT1) are inducible by sulfate starvation in *A. thaliana*. The sulfate transporter (AST68) exhibited the most intensive and specific response in roots, indicating that AST58 plays a central role in the transcriptional regulation of sulfate assimilation in plants.

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Expression analysis and subcellular localization of cysteine synthase isoforms from *Arabidopsis thaliana*

Cysteine synthase, a key enzyme for the fixation of reduced sulfur catalyses the formation of cysteine from O-acetylserine and inorganic sulfide as substrates. Based on the isolation of respective enzyme activities from various subcellular compartments from spinach leaves, pea roots and cauliflower flowering parts, the presence of three isoforms of cysteine synthase has been suggested. However, no data are available concerning the interaction and specific function of the various isoforms hypothesised for various cell compartments. In order to get a deeper

insight into the regulation and compartmentation of sulfate assimilation we isolated three cDNAs encoding three different cysteine synthase isoforms from *Arabidopsis thaliana*. The cDNA clones, designated ASL-A, ASL-B and ASL-C, were 1.2, 1.4 and 1.6 kb in length. While the level of ASL-A expression appeared to be similar in leaves and roots, ASL-B mRNA was more abundant in leaves while ASL-C showed a higher expression in roots. All three isoforms were transcriptionally inducible by sulfur starvation, though to different extents. However, enzyme activities increased only marginally. Two strategies were used to verify the subcellular localization of cysteine synthase isoforms of *Arabidopsis thaliana*: 1. in vitro uptake systems and 2. epitope targeting using a human c-myc epitope. We could show that ASL-B and ASL-C encode chloroplastic and mitochondrial forms of cysteine synthase, respectively.

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Biosynthesis of the non-protein amino acid δ-aminolevulinate in plants

δ-aminolevulinate is produced as an intermediate during biosynthesis of chlorophyll, heme, phycobilin and vitamin B_{12}. There are two ways by which this is made. In animals and fungi succinyl CoA and glycine condense in a single step catalyzed by the enzyme δ-aminolevulinate synthetase and produce δ-aminolevulinate for biosynthesis of heme while for the synthesis of chlorophyll and heme in plants, glutamate is converted to δ-aminolevulinate by a multienzyme pathway. Glutamate is activated at α-carboxyl by ligation to $tRNA^{Glu}$ with an aminoacyl tRNA synthetase. An NADPH dependent reductase converts glutamyl $tRNA^{Glu}$ to glutamate 1-semialdehyde which is finally converted to δ-aminolevulinate by an aminotransferase. In plants and algae the $tRNA^{Glu}$ is encoded in chloroplast DNA while the enzymes are encoded in nuclear DNA. The $tRNA^{Glu}$ has a hypermodified $mam^5s^2U\Psi C$ anticodon and probably plays a role in the light-dark regulation of δ-aminolevulinate synthesis. Ligation of glutamate to $tRNA^{Glu}$ requires ATP and Mg^{2+} and proceeds via a ternary intermediate. Glutamate 1-semialdehyde aminotransferase contains vitamin b_6. The enzyme is converted to spectrally different forms by treatment with 4,5-diaminovalerate or 4,5-dioxovalerate. Pyridoxamine 5' phosphate form of the enzyme converts (S)-glutamate 1-semialdehyde to δ-aminolevulinate via 4,5-diaminovalerate.

Sports and Exercise

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Amino acids in sports and exercise – state of art 1997

A protein supplementation of 0.8 g/kg weight up to a maximum of 2 g/kg weight depending on kind, duration and intensity of the sport done can be considered to be sufficient. Questions regarding the amino acid metabolism under physical exertion remain open since alterations in the amino acid pool have been clarified only partially: 1. During and after physical exertion of varying duration and intensity. 2. After the application of amino acids (AA). 3. Effects on physical performance and capacity of regeneration. 4. Predictor of an overload syndrome. 5. Effects on the immune system.

The amino acids named in the following had been subect of scientific investigations which have led to partly contradictory results or produced hypotheses which need to be verified yet.

Arginine and ornithine stimulate the release of the somatotropic hormone (STH) when applicated intravenously. The hypothesis that orally applicated arginine and ornithine increase STH values beyond the physiological plasma level and thus have a favourable influence on the building up of muscles, has not been proved. Investigations on arginine carried out recently show an immunomodulating effect.

Branched-chain-amino acids (BCAA) can be used to give energy during physical exercise when a depletion of carbohydrates occurs. A decrease of the BCAA values in serum during physical exertion and an increase of free fatty acids in blood lead consecutively to an increase of free tryptophane. An increased entrance of tryptophane into the brain is regarded as being responsible for central fatigue. The application of BCAA to achieve improved physical performance has produced contradictory investigation results.

Glutamine: Maintaining a normal plasma-glutamine level is essential for the immune system. The plasma concentration of glutamine is reduced with endurance athletes. For this reason, a glutamine supplementation is recommended in sports with long duration and/or high intensity levels.

Taurine is regarded as a conditionally essential AA. Test results describe a favourable influence on physical performance after the application of taurine. The hypothesis that taurine leads to improved haemodynamic parameters of the heart and that it economizes the heart's physical performance in a digitaloid way has to be proved yet.

Tryptophane is a precursor of serotonin, a neurotransmitter, and is being held responsible for central fatigue. The ammonium value which increases under intensive workload also influences the brain's serotonin synthesis. This leads to alterations in the rate of transportation of large-neutral-amino acids (LNAA) and free tryptophane. Investigations on improved physical performance through alterations of the plasma-tryptophane and LNAA-levels have produced contradictory results.

Tyrosine is known to be a precursor of catecholamines and has an influence on the intracerebral synthesis of these neurotransmitters. A dopamin-agonist induced favourable effect on physical performance has been described. After the application of tyrosine this effect has been demonstrated only partially, however. Intensive investigations on the metabolism of amino acids under exertion can offer insights into the complex alterations of these processes, show deficiencies under physical exercise and give thus useful recommendations regarding supplementation.

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Influence of carbohydrate intake on the concentration of plasma amino acids during exercise and the recovery period

The relation between carbohydrate (CH) intake and plasma amino acid concentration ([AA], cubital venous blood) was

investigated in 3 series. **I)** 8 endurance athletes performed an incremental test to exhaustion, followed by an endurance test at 60% of maximum performance. This test took place twice, once after a ten day period having almost carbohydrate free meals combined with daily training of low intensity and secondly 3 days later after carbohydrate loading. After the carbohydrate deficit [Leu], [Ileu], [Val] were 2 to 2.5 times higher ($p < 0.01$) at rest and decreased during exercise whereas these concentrations remained unchanged after glycogen loading. **II)** 6 endurance trained athletes performed 2 endurance tests at 50% of VO_{2max} for 150 minutes, one after an overnight fast, drinking only mineral water during exercise, the other after having breakfast and drinking a carbohydrate solution (10% Maltodextrin 19, CPC, Heilbronn; in mineral water). There were only small differences between the two trials. [Gly] and [Ala] tended to be higher when carbohydrates were given during exercise, whereas [Val], [Leu] and [Ileu] tended to be lower. [Val], [Leu], [Ileu] decreased during exercise with time under both conditions. 1 hour after exercise under both conditions most [AA] were higher than during exercise. This increase was not due to haemoconcentration indicating an ongoing protein degradation presumably in the muscle after exercise. **III)** 7 subjects performed 2 endurance tests to exhaustion. After one the athletes had to eat their normal food, after the other additionally Maltodextrin (3 g/kg body weight) was given immediately after termination of exercise and within the next 2 hours. The CH-ingestion lead to a decrease of most [AA] within the first hour after exercise. [AA] remained low for at least 4 hours indicating a rapidly starting protein synthesis. This might be due to the increasing insulin concentration (more than 4-fold compared to normal food, $p < 0.02$) after CH-ingestion. *Conclusion:* CH-intake before and during exercise reduces protein breakdown and CH-intake after exercise stops protein degradation and stimulates protein synthesis. Thus CH-intake shortens recovery time and should enhance training adaptations.

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Changes of the plasma amino acids profile during acute exhaustive exercise of short and long duration and significance for tolerance of training loads

Introduction. The small pool of amino acids (AA) contributes to many mechanisms of adaptation, repair and regeneration during and after training. Since changes of plasma AA were related to signs of fatigue and overstrain we studied changes in 18 plasma AA

1. during a stepwise increasing treadmill exercise before and after intensified training
1. before, during and after a 2.5 hour run of moderate intensity

Methods. 1. 15 runners increased training load. By a psychophysiological questionnaire they were subdivided into a group with overstrain (OS) or without (nOS). Before and after treadmill exercise (1% incline, 8 km/h + 2 km/h every 3.5 min + 0.5 min pause) until exhaustion venous blood was collected. 2. 13 runners ran 2.5 h with controlled HF in the range of blood lactate levels of 1.5–1.8 mM. Venous blood was collected before, after 75 and 150 min of running and after 1, 2, 6 and 24 h of regeneration.

AA were measured by HPLC with fluorescence detection after derivatisation with OPA.

Results. During short exhaustive treadmill running Ala and Arg increased, Glu only in nOS. Trp, Asn and Ser decreased, but without sign. in nOS for Asn and Ser. Gly was lowered after intensified training. Gly, Ser and Hxl showed group specific differences. Psychophysiological scores correlated with Lys, Trp and Ileu. Pretraining decreases of Hxl, Tyr, Threo, Val were found in those who did not tolerate well the intensified training, not in nOS. After the 2.5 h Glu, Arg and Tyr were elevated, Meth, Asp and Hxl unchanged and all others decreased, 2 h later Asp and Hxl unchanged, Glu elevated, all others sign. decreased.

Discussion. Acute exercise of high intensity or long duration activates AA metabolism. Up to 2 hours after long distance running essential and glucoplastic AA decrease i.e. by needs of gluconeogenesis, but even after short intensive exercise the plasma AA profile changes. Thereby decreasing levels of Ser, Val, Threo, Hxl and Tyr or Leu and Lys may be predictors of awaiting overstrain. Mechanisms may be concurrence between energy metabolism and precursors of hormons, neurotransmitters and free radical absorption.

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Effects of ingesting a taurine-enriched, caffeine-containing drink on performance and haemodynamics in acyclic trained athletes

Introduction. Changes in cardiovascular parameters during exercise after taurine application are described. These effects could be due to an improvement of haemodynamic data similar to the characteristics of digitalis. The purpose of this study is to verify the above mentioned results in acyclic trained athletes and to answer questions about effects of taurine on haemodynamics by direct spirometric measurement during cycling.

Methods. Twenty healthy male athletes completed two experimental trials with 60 minutes of submaximal cycling at approximately 50% VO_2max and a subsequent incremental protocol until subjective exhaustion. 20 min before cycling and after 25 min of submaximal cycling the following drinks were applicated in a randomized order with double blind administration:

U1: "Red Bull" (500 ml) without taurine and glucoronolacton, with caffeine (160 mg), glucose (10.5 g) and saccharose (43 g)

U2: "Red Bull" (500 ml) original drink containing taurine (2 g), glucuronolacton (1.2 g), caffeine (160 mg), glucose (10.5 g) and saccharose (43 g).

Respiratory parameters were determined breath by breath using an open ergospirometric system. The heart rate was recorded every fifth second using a bipolar chest wall electrocardiogram. Data were analysed using the Student-t-test for paired data, significance level ($P < 0.05$).

Results. The mean endurance time to exhaustion on the maximal intensity level was 153.5 sec (SD 101) in U1 vs. 217.1 sec (SD 106) in U2. This difference is statistically significant ($p = 0.012$). Concerning the maximal oxygen uptake (VO_2max) significant differences could also be determined between the trials ($p = 0.001$). The mean value relative to body weight was 48.6 ml/min/kg (SD 4.5) in U1 and 54.1 ml/min/kg (SD 6.9) in U2. The mean respiratory exchange ratio at exhaustion showed a tendency of lower values in U2 but no significant differences (1.04 ± 0.08 in U1 and 0.99 ± 0.08 in U2). No significant differences were observed concerning heart rates. Levels of blood lactate did not show any significant differences between the groups. Catecholamines showed significantly higher values in U2 at the 45th submaximal min (noradrenaline: U1 599.1 ± 138.6, U2: 1105.1 ±604.2, $P = 0.002$, adrenaline: U1 160.1 ± 27.1, U2: 281.7 ± 123, $P = 0.03$).

Discussion. The major result of this study is the significant increase of performance during the maximal intensity level in the original taurine-containing drink with a concurrent significant higher maximal oxygen uptake. The increased cardiac out-put after ingesting taurine seems to be the explanation for the better performance. The higher VO_2max could be a sign of an increased contractility of the myocard, caused by taurine. The special composition of both drinks with glucose/saccharose and caffeine causes metabolic effects which are independent of taurine. Caffeine is known to increase the lipolysis and glycolysis, decreases on the other hand the catabolism of catecholamines which blood levels rise after the fast increase of blood glucose. However, the different catecholamine levels between the two trials as a possible effect of taurine remain unclear and need to be investigated.

Synthesis

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Influence of α-fluoralkyl substituted amino acids on secondary structure and properties of peptides

One of the major disadvantages for the application of peptides as pharmaceutics is their considerable conformational flexibility leading to undesired interactions with different receptors. Therefore, some sort of restraints are needed to limit the number of conformers that can adopted by linear backbones. Investigation of the biological properties and three-dimensional structure of peptides produced by microbial sources rich in the conformationally restricted $C^{\alpha,\alpha}$-disubstituted amino acids such as Aib is of current interest. Due to the electronical properties of the fluorine substituents α-trifluoromethyl substituted amino acids (Tfm amino acids) constitute a special class of $C^{\alpha,\alpha}$-disubstituted amino acids. Therefore, incorporation of Tfm amino acids is a new approach to design biologically active peptide sequences with increased metabolic stability [1] and stable secondary structure.

The crystal structure of (*S,S,S*)-Z-Phe-(α-Tfm)Ala-Ala-NH_2 was determined by X-ray diffraction and compared to that of the corresponding Aib substituted peptide. Both peptides are folded in a type II β-turn conformation stabilized by an intramolecular H-bond. NMR measurements on the temperature dependence of the NH chemical shift values of these tripeptides suggest a backbone conformation involving a hydrogen bond between the NH proton of Ala as hydrogen donor and the carbonyl function of the Z-group as acceptor in the case of the Aib and the (*S,R,S*)-diastereomer. *Ab initio* MO calculations and molecular dynamicx simulations were performed to examine the influence of the Tfm group on the β-turn formation tendency of peptides. It was found that Tfm amino acids generally favour β-turn formation both at the 2- and 3-position. Depending on the configuration various β-turns can selectively be influenced.

Furthermore, several peptides were designed to examine the effects of the positioning and the total number of the α-Tfm groups on the peptide conformation

Reference

Koksch B, Sewald N, Hofmann H-J, Burger K, Jakubke H-D, Proteolytically stable peptides by incorporation of α-TFM amino acids. J Pept Sci (in press)

R. Pires, J. Spengler, H. Schedel, and K. Burger

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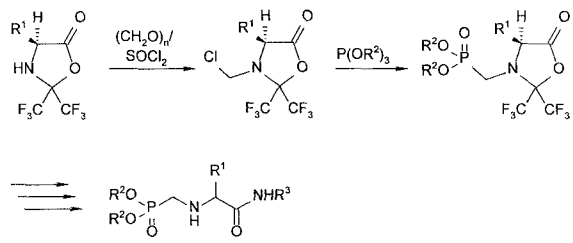
New routes to natural and non-natural amino acids

Hexafluoroacetone (HFA) represents a new type of protecting and activating agent offering an alternative approach to the synthesis of natural and non-natural amino acids. In a single step, the α-functionality and the carboxylic group of α-functionalized carboxylic acids (e.g. α-amino acids, α-hydroxy and α-mercapto acids) are protected simultaneously. Moreover, the carboxylic group is already activated towards nucleophiles.

N-Chloromethylation of bis(trifluoromethyl) substituted oxazolidinones followed by reduction with triethylsilane/trifluoroacetic acid affords N-methyl-amino acid derivatives in a one pot procedure in excellent yields (> 90%). Building blocks for potential neutral endopeptidase inhibitors are obtained upon reaction of N-chloromethylated amino acid derivatives with phosphites. The reaction sequence seems to be suitable for combinatorial chemistry.



Starting from ω-carboxylic α-amino acids, N-methyl-amino-ω-acid chlorides are obtained. Upon further reaction with trimethylsilylazide, isocyanates are formed, which on addition of equimolar amounts of alcohols yield fully protected, carboxy-activated ω-amino-α-N-methylamino acid derivatives.

Poteintial GABA-analogues have been synthesized by two new routes. Chlorine elimination of HFA-protected N-chloromethylated amino acid with N-methylmorpholine provides 1,2-dipoles, which can be trapped in situ with various types of multiple-bond systems stereoselectively furnishing highly substituted proline derivatives as promising GABA-analogues and building blocks for the synthesis of peptidomimetics.

Furthermore, a completely different route to GABA analogues is described starting from (S)-malic acid, (S)-citramalic acid or thiomalic acid derivatives.

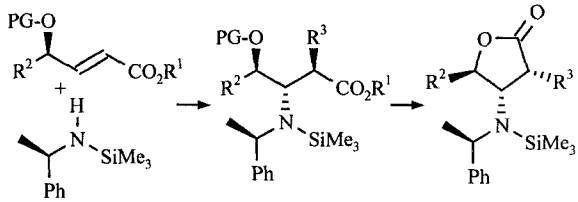
N. Sewald[1], M. Körner[1], A. Müller[1], F. Schumann[1], and M. Koksch[2]

[1]Department of Organic Chemistry and [2]Medical Clinic I, University of Leipzig, Federal Republic of Germany

β-Amino acids: asymmetric synthesis and incorporation into physiologically active peptides

The synthesis of enantiomerically pure β-amino acids can be achieved by Michael addition of homochiral amidocuprates or by Arndt-Eistert chain elongation of protected α-amino acids. Conjugate addition of homochiral amidocuprates or lithium amides, based on N-[(R)-1-phenylethyl]trimethylsilylamine to α,β-unsaturated esters provides efficient methodology for the asymmetric synthesis of β-amino acids [1]. Pronounced

matched/mismatched pair effects are observed upon addition to α,β-unsaturated esters with an additional hetero substituted chiral centre in γ-position. Polyfunctional derivatives, e.g. polyhydroxy α-deuterio β-amino acids and the corresponding γ-lactones can be synthesized stereoselectively via this route. The



skeleton atom pattern of a peptide is modified considerably on replacement of α-amino acids by β-amino acids resulting in conformational changes and potential modulation of metabolic stability as well as physiological activity. The amino acid sequence Arg-Gly-Asp (*RGD*) is known as a universal binding and recognition sequence involved in cell-cell and cell-matrix interactions. Some cyclic penta- and hexapeptides [c-(RGDfV), c-(RGDfVG); f corresponds to D-Phe] with the partial sequence RGD (Arg-Gly-Asp) are powerful RGD-antagonists [2] with varying selectivity towards different receptors depending on the ring size and the local RGD conformation. Several cyclic peptides have been synthesized by SPPS (Fmoc protocol) allowing a "fine tuning" of the ring size between 15 [e.g. c-(RGDXY), no β-amino acid] and 21 [e.g. c-(RGD$^{\beta}$X$^{\beta}$Y$^{\beta}$Z), three β-amino acids]. The antiaggregatory activity of the peptides was determined by aggregometry [3].

References

1. Sewald N, Hiller KD, Helmreich B (1995) Liebigs Ann Chem 925
2. Pfaff M et al (1994) J Biol Chem 269: 20233
3. Müller A, Schumann F, Koksch M, Sewald N. Lett Pept Sci (submitted)

T. Pajpanova

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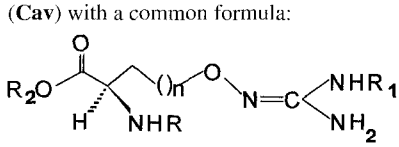
Amino acid analogues of non-protein amino acid canavanine: synthesis and biological activity

Optically-active natural and unnatural α-amino acids are important precursors for the synthesis of pharmaceuticals, agrochemical, and food ingredients. For example, guanidine-containing amino acids are a group of important modified amino acids which have been widely used in medicinal chemistry and biochemistry to change the conformation, restrict the flexibility and enhance the potency of molecules. Moreover, the synthesis of new non-protein amino acids and their incorporation into devours natural biologically active peptides might become a powerful method for the design of peptide and peptidomimetics therapeutics.

In the course of our methodological studies on non-protein amino acids in the present work we have set as an objective to obtain several new α-amino acids containing guanidinooxy function, as structural analogues of arginine and canavanine (**Cav**) with a common formula:



1	a	b	c
n	1	2	3

1 a,b,c (D,L)

where: R = H, Boc, Z, Fmoc; R_1 = H, Boc, CH_3, NO_2;
R_2 = H, CH_3, Bzl, NH_2, $NHNH_2$, $NHN(CH_2CH_2Cl)_2$.

Their effect on the growth of microorganisms, model plant systems, cultured tumor cells as well as their antitumor activity *in vivo* was evaluated.

Preliminary results showed that Cav and its new analogues exerted significant antibycteria activity *in vitro* against some microbial strains.

(This work has been supported by Grant L-421 of the National Science Fund to the Ministry of Education, Science and Technologies of Republic of Bulgaria.)

M. Stanchev, T. Pajpanova, and **E. Golovinsky**

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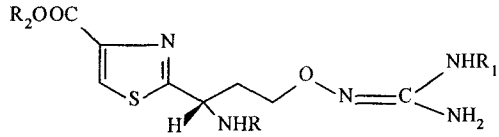
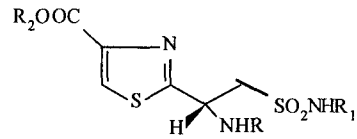
Synthesis and antibacterial activity in vitro of some new non-protein amino acids containing thiazole residue

The intensive development of combinatorial chemical libraries has drawn more attention to the preparation of non-protein amino acids which are useful tools in medicinal chemistry and biochemistry. Among further applications are their incorporation in active peptides or peptidomimetics aimed at changing the conformation, restriction of the flexibility and potency enhancement of the molecule.

Following our current interest in synthetic application of unnatural amino acids we report now the synthesis of some new Canavanines and S-cysteinesulfonamides containing thiazole residue (**1, 2**).



1 (D,L)



2 (D,L)

R=H, Boc, Fmoc, Z R_1=H, Boc, Fmoc, Tmob R_2=H, Et

For the synthesis of (Cav)Thz and ($CySO_2HN_2$) Thz suitable protected Canavanine and S-cysteinesulfonamides amides were easily converted into thioamides by Lawesson's reagent in high yields. The following cyclization of the thioamide to thiazole was achieved by using ethyl brompyruvate according the modified Hantzch reaction. Resolution of the racemates was achieved by using alkaline protease from *Bacillus subtilis* DY strain.

Preliminary results showed that (Cav)Thz and ($CySO_2NH_2$)Thz derivatives exerted antibacterial activity *in vitro* against various gram-positive (*Staphylococcus aureas, Bacillus cereus* etc), gram-negative microbial strains.

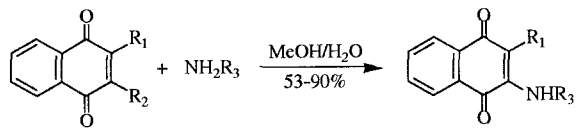
(This work has been supported by Grant X-619 of the National Science Fund to the Ministry of Education, Science and Technologies of Republic of Bulgaria.)

S. Bittner[1] and **M. Fridkin**[2]

[1]Department of Chemistry, Ben-Gurion University of the Negev, Beer-Sheva, and [2]Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel

N-Quinonyl amino acids; potential structural components for the synthesis of cytotoxic peptides

L-Histidine, L-lysine, N^{α}–cbz-L-histidine, L-tryptophan and L-proline react with 1,4-naphtoquinone or 2,3-dichloro-1,4-naphtoquinone to afford novel modified N-quinonyl amino acids. Such compounds are potential components in cytotoxic peptides conjugates. The modified amino acids can be employed as building blocks in stepwise peptide synthesis or for post-peptide chain-assembly modifications.



With free α-amino acids, the quinone moiety is attached to the α-amino group. With blocked α-amino acids the quinone moiety is attached to the heterocyclic nitrogen atom. Synthetic methods and spectroscopical data will be discussed. The ability of these quinonic derivatives to produce semiquinone radicals using chemical (i.e. $NaBH_4$) and enzymatic (i.e. NADPH-cytochrom P-450 reductase) routes, was evaluated employing electron spin resonance spectroscopy.

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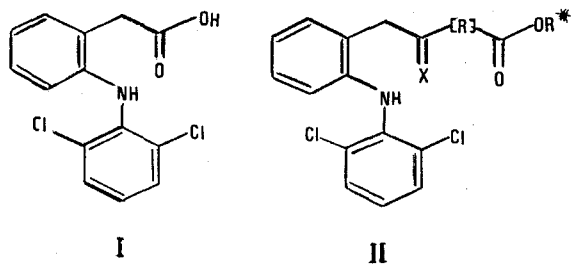
M. H. Abo-Ghalia, A. M. Shalaby, W. I. El-Eraqi, and **H. M. Awad**

National Research Centre, Dokki, Cairo, Egypt

Synthesis and comparative anti-phlogistic potency of some new nonproteinogenic amino acid conjugates of "Diclofenac"

In search for potent, particularly less ulcerogenic substitutes for the universal antiphlogistic drug "Diclofenac" [2-(2,6-dichlorophenyl-1- amino) phenylacetic acid (**I**)], its conjugates with some nonproteinogenic amino acids and their thio mimics (**II**), were newly synthesized by a methodology relevant in organic and peptide syntesis.

While, were herein confirmed practically nonulcerogenic in rats, eleven of the yet synthesized twelve candidates, retained considerable comparative anti-inflammatory (% induced oedema, pleural fluid and mean gain in pellet weight tests) and analgesic activities (electric-shock test).



I II

R = nonproteinogenic amino acid residue
(D-phe, D-Leu β-Ala . . .)
R* = H or Me
X = O or S

Thio amide candidates (II,X = S, R = Me) were generally moderately more potent than the corresponding amides (II, X = O,R, = Me).

The followed methodology, structure/activity relationships and perspectives will be discussed.

Determination of toxicity profile, therapeutic and gastrointestinal tolerability indices for the candidates are in progress.

M. Panteva, T. Varadinova, S. Shishkov, S. Hrisafi, and **P. Bontchev**

Laboratory of Virology, Faculty of Biology, Faculty of Chemistry, Sofia University, Bulgaria

Complexes of amino acids with biometals – influence on virus-cell interaction

Amino acids (AAs) being natural ligands of biometals are their main transporters through the body and regulators of metal homeostasis within the cells. On the other hand changes in the levels of biometals, like zinc and copper are closely related to different pathological processes and virus infections in particular.

The aim of the present study was to evaluate the role of the AAs lys, arg, his and ser on the interaction between Herpes simplex viruses and host cells. For this purpose complexes of the above AAs with Zn(II) and Cu(II) were used. The cytotoxicity of complexes, their effect on free virions, as well as on virus replication into host cells were studied. The experiments were done using MDBK cells and Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2).

The data showed that: 1) The AAs used did not influence the cytotoxicity of the particular metal ion measured as maximal nontoxic concentrations (MNC). The MNC of all complexes tested was 100 MM calculated as amount of Zn(II) and Cu(II). 2) The effect of Zn(II) and Cu(II) complexes on the infectivity of both HSV-1 and HSV-2 virions strongly depended on the AA. 3) The complexes of AAs with Zn(II) and Cu(II) moderately inhibited the replication of HSV-1 with up to 75%. It was interesting to note that ser complexed with Cu(II) – $Cu(ser)_2$, inhibited HSV-1 replication in the highest degree – 93%. In respect to the replication of HSV-2, ser also predetermined the inhibitory effect of Zn(II) and Cu(II). Contrary, the effect of complexes of Zn(II) and Cu(II) with lys, arg and his strongly depended on the particular AA and the metal ion bounded to it. Thus, lys and arg promote a potential stimulatory effect of Zn(II) complexes, while $Zn(his)_2$ moderately inhibited (up to 70%) HSV-2 infection. The opposite effect of complexes of these AAs with Cu(II), as compared to that with Zn(II), was found. Thus, $Cu(lys)_2$ and $Cu(arg)_2$ inhibited HSV-2 replication with 83% and 50% respectively, while $Cu(his)_2$ potentiated HSV-2 replication with 27%. The data obtained also show that the effect of complexes of lys, arg, his and ser with Zn(II) and Cu(II) on HSV infection in cell cultures depended on the specificity of the virus type as well.

These results are a part of a broad investigation of our team on the effects of Zn(II), Cu(II) and Co(II) with different bioligands on the interactions between HSV and host cells (J Chemother, 1993, MBD 1996, 1997).

F. Bordusa, S. Gerisch, M. Hänsler, S. Thust, A. Stein, and **H.-D. Jakubke**

Department of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Federal Republic of Germany

Protease-catalyzed coupling of natural and unnatural amino acids with minimum and even without protection

The application of proteases in peptide synthesis provides an attractive alternative to chemical synthetic methods [1]. Enzymes catalyze reactions with high stereo- and regiospeci-

ficity. Therefore, they reduce the need for protecting groups and give enantiomerically pure products.

This paper describes recent progress in the development of novel strategies for the protease-catalyzed coupling of natural and unnatural amino acids which cannot be performed by chemical methods. Based on specificity studies on the cysteine protease clostripain we could establish an incredible broad specificity of the S' subsite. In contrast to other cysteine and serine proteases, we could show that amino acid esters, unnatural amino acid derivatives and even free amino acids were accepted as nucleophilic amino components. These findings underline that the endopeptidase clostripain is capable to act as a reverse carboxypeptidase.

Furthermore, medium engineering offers interesting possibilities of reversing the equilibrium of protease-catalyzed reactions towards synthesis. Since it is known from literature that freezing decreases the rate of proteolysis we have studied enzymatic prptide bond formation in frozen aqueous systems [2]. It is surprising that endopeptidases under this reaction conditions are capable to couple even free amino acids in the sense of a reverse carboxypeptidase. In addition, chymotrypsin-catalyzed reactions using N^{α}-unprotected amino acid esters as carboxyl components underline an unexpected substrate specificity of a reverse aminopeptidase. Using chymotrypsin, trypsin etc. peptide synthesis in frozen aqueous systems at – 10 °C compared with those at room temperature were studied. N^{α}-unprotected amino acid esters with Phe in P_1-position and also β-phenyllactyl-OMe were accepted as acyl donors for the coupling with amino acid derivatives. A synthesis of kyotorphin H-Tyr-Arg-OH from H-Tyr-OMe with unprotected Arg catalyzed by chymotrypsin is without doubt the simplest type of a dipeptide synthesis which cannot be performed by chemical methods at all.

References

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(This work was supported by DFG (INK 23/A1 and A2) and the Fonds der Chemischen Industrie.)

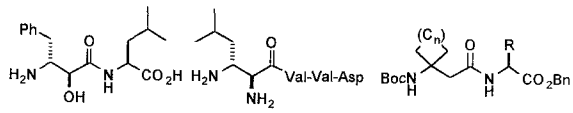
C. Palomo and **M. Oiarbide**

Departamento de Química Orgánica. Facultad de Química, Universidad del País Vasco, San Sebastián, Spain

A non-conventional β-lactam route to short peptide segments containing β-amino- and α,β-diamino acids

The concept of structural modification in peptide fragments to confer on them specific properties is of current interest in the study and design of new bioactive targets [1]. Wilst the majority of the investigations on this topic have dealt with the synthesis and use of α-amino acids, relatively less work has been done with either β-amino or α,β-diamino acids. In the present contribution we disclose our [2] β-lactam approach to such units, including the last findings on the application to the synthesis of constrained spiranic peptides.

The route is based on an effective preparation of the corresponding β-lactam intermediate either from a keteneimine [2+2] cycloaddition reaction or an alkene-chlorosulfonyl isocyanate cycloaddition reaction. In the first case, the reaction takes place with virtually absolute stereocontrol over the two new stereocenters. Further activation of the β-lactam ring for peptide coupling was done by N-deprotection and subsequent treatment with $(Boc)_2O$ and DMAP. The resulting N-Boc β-lactams reacted smoothly with representative α-amino acid esters to afford the corresponding peptides in excellent yields. On the other hand, the reactivity of the N-Boc β-lactam depends on the substitution pattern of the ring, being the reactions typically carried out in DMF and, in most cases, with the assistance of NaN_3 or KCN as the promoters. Under these reaction conditions no appreciable isomerization products were detected in the respective crudes, being the peptides isolated as single stereoisomers.



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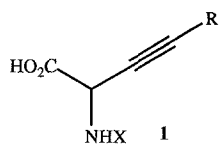
P. Meffre and **F. Le Goffic**

Laboratoire de Bioorganique et Biotechnologies, Ecole Nationale Supérieure de Chimie de Paris, France

New developments in the field of ethynylglycine derivatives: Synthesis of dipeptides containing an acetylenic residue



1

The synthesis of unusual amino acids is an interesting challenge because these amino acid analogues possess potential biological activities. Thus, naturally occurring L-ethynylglycine **1** (R=H, X=H) exhibits antimicrobial activity [1] and we have recently described the first synthesis of optically active β,γ-alkynylglycine derivatives **1** (R=H, Me, Bu, TMS; X=Boc) with high enantiomeric excesses [2].

Unprotected β,γ-alkynylglycine **1** (X=H) are notoriously labile molecules but their incorporation into peptides could lead to stable compounds that would be easily transported by biological systems and would show potent biological activity. Moreover, as new peptidomimetics, incorporation of such units in a peptide chain may influence the secondary and tertiary structure.

First results in the synthesis of dipeptides containing an optically active acteylenic residue of type **1** will be described.

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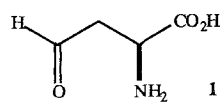
2. Meffre P, Gauzy L, Branquet E, Durand P, Le Goffic F (1996) Tetrahedron 52: 11215–11238

P. Meffre and **F. Le Goffic**

Laboratoire de Bioorganique et Biotechnologies, Ecole Nationale Supérieure de Chimie de Paris, France

Synthesis and uses of aspartaldehyde derivatives



1

Derivatives of (*S*)-2-amino-4-oxobutyric acid **1** (aspartic acid β-semialdehyde, 3-formyl alanine) are interesting chiral intermediates for the synthesis of biologically relevant mole-

cules of wide interest (nicotianamine and analogues, iron chelating agents; naturally occuring unusual α-amino acids; serine-phosphate peptides isosteres; penicillins or cephalosporins analogues) or compounds used in some enzymes studies. They have been synthesized from expensive allylglycine via ozonolysis or oxidative cleavage, from aspartic acid via reduction of the acid side chain to give homoserine derivatives or from methionine via an homoserine derivative and subsequent oxidation to the aldehyde, or directly from expensive homoserine. These strategies mostly suffer from low chemical and optical yields due to the number of steps or from expensive, unpractical starting material or reagents.

The poster will describe a new synthesis of protected derivatives of **1** with ee > 90% from L-methionine in a limited number of steps using cheap and easily handled reagents [1].

Some other new applications of the strategy used will be shown.

Reference

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G. Kokotos[1], J. M. Padrón[2], T. Martin[2], W. A. Gibbons[3], and V. S. Martín[2]

[1]Department of Chemistry, Laboratory of Organic Chemistry, University of Athens, Greece

[2]Instituto Universitario de Bio-Orgánica "Antonio González", Universidad de La Laguna, La Laguna, Tenerife, Spain

[3]Department of Pharmaceutical Chemistry, The School of Pharmacy, University of London, United Kingdom

A general approach to the asymmetric synthesis of unsaturated lipidic α-amino acids. The first synthesis of α-amino arachidonic acid

Nonproteinogenic, unnatural α-amino acids have increasingly attracted the attention of numerous disciplines in connection with the design and synthesis of potential constituents of pharmaceuticals, for example enzyme inhibitors, and of optically active starting materials for a variety of synthetic applications. As a consequence, a lot of effort has been devoted to the preparation of amino acids in enantiomerically pure form of either configuration, a subject already covered by general reviews.

On the other hand, particular attention has been focused on fatty acids ant their involvement in signal transduction. It has been demonstrated that fatty acids, e.g. arachidonic acid, act both as modulators and messengers, particularly of signals triggered at the level of cell membranes. Recently, it has been proposed that abnormal membrane phospholipid concentrations of the 20-carbon and 22-carbon essential fatty acids of the n-6 and n-3 series are the cause of vascular disease.

Trying to combine structural features of amino acids with those of fatty acids, we have prepared chiral lipidic α-amino acids which are unnatural α-amino acids with saturated or unsaturated long aliphatic side chains.

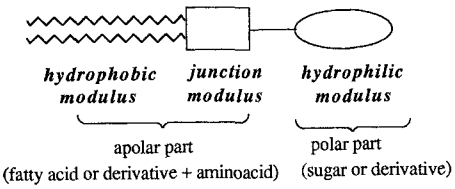
In this communication we report a simple, efficient and general method to synthesize unsaturated lipidic α-amino acids in their enantiomeric forms which eventually can be transformed into the saturated products by a simple hydrogenation.

V. Alliot, A. Meyers, C. Gérardin-Charbonnier, J. Amos, and C. Selve

LESOC. URA CNRS 406, Laboratoire de Chimie Physique Organique, Faculté des Sciences, Université Henri Poincaré-Nancy I, Vandoeuvre-lès-Nancy, France

Synthesis of hydrophobic part of surfactants on the basis of lysine or glutamic acid

The aim of this work is to propose simple and effective methods to prepare trimodular surfactants such as:



The interest is to valorise three kinds of substances which are extracted from agricultural products:

- fatty acids or derivatives
- amino acids (in particulary lysine or glutamic acid)
- sugars or derivatives (lactose, glucose, lacto bionic acid, . . .)

The difficulty is to control the reactivity of these polyfunctional molecules under the easiest conditions. We use the method of modular synthesis.

The original preparation of the apolar group, proposed, is to carry out the condensation of one or two fatty acids or derivatives (amines or alkohols) on a trifunctional amino acid (lysine or glutamic acid) by amidification or esterification.

The length of the chain varies between ten and eighteen carbon atoms. This methodology is by now under control.

The fixation of the sugar on the apolar part – which still contains a reactive function – is under study. It will be made either by the Amadori reaction or by reaction with an acid or amine function of a sugar derivative.

The present results are satisfactory. Further experiments will permit the validation of the protocols.

Physical properties are being evaluated. These results and the biological properties will allow to select the most interesting molecules and to determine the potential applications of these.

C. Gérardin-Charbonnier[1], C. Selve[1], A. Labrosse[2], and A. Burneau[2]

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Synthesis, caracterisation and evaluation of material on the basis of grafted silica by amino acids or peptides

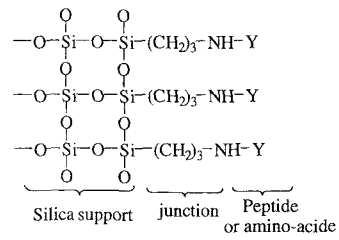
There is considerable interest in the synthesis of pure silica but the studies of material on the basis of grafted silica has been booming for at least ten years ago. Indeed, this kind of material presents a lot of potential applications in various domains: chemistry, biology, non linear optics, environment . . .

We propose a synthesis of silica by copolymerisation of a tetraalkoxysilane with a modified alkoxysilane with the aim to obtain silica which can complex metallic cations and has antioxydant properties.

The organic groups grafted are peptides – like carnosine (β-alanine-histidine) – or amino acids.

We present the synthesis and the characteristic of some of the silica obtained.

Scheme of structures



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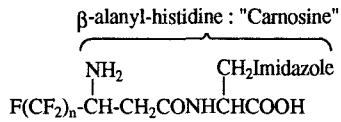
Synthesis of 2-perfluoroalkylated analogous of β-alanine and peptidoamines derivatives

Organofluorine compounds are of great interest in synthetic and medicinal chemistry owing to their unique physical and biological properties imparted by fluorine atoms. So, it has been shown that fluorocarbons have a great capacity to dissolve oxygen, and their use as blood substitutes has become a major objective in biomedical research.

However, because of their insolubility in biological aqueous media, it was necessary to prepare them in the form of emulsions, which implies the use of surfactants.

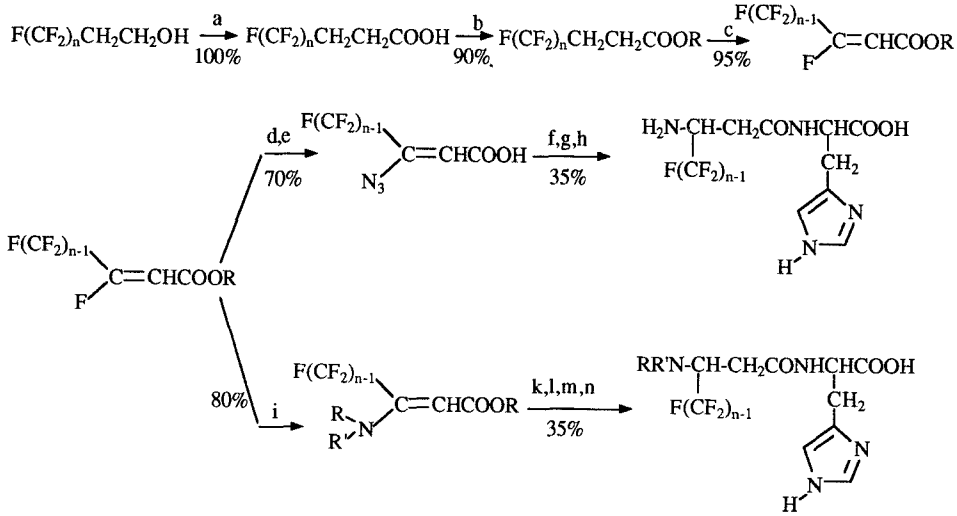
Furthermore, massive oxygen delivery results in the appearance of peroxydes and toxic radicals.

Therefore, our purpose is the synthesis of a new type of perfluorinated surfactants A:



This kind of compounds contains a perfluorinated chain as hydrophobic part and a hydrophilic part such as "Carnosine" which can form antioxydant complexes with Cu^{2+}. We hope that they present surfactant properties. The synthesis is described by Scheme 1.

The stereoselectivity of the reductive step is under investigation and the capacity of the terminal peptide to complex copper will be evaluated.



Scheme 1. **a** CrO_3,H_2SO_4,acetone; **b** ROH,H_2SO_4,toluene; **c** NaOH 2N,THF; **d** NaN_3,H_2NCHO; **e** NaOH 2N,MeOH; **f** BOP,HCl.HisOMe,Et_3N,CH_2Cl_2; **g** NaOH 2N,MeOH; **h** H_2/Ni Raney, 80 °C, 80 bars; **i** RR'NH, Et_2O,reflux **k** NaOH 2N,MeOH; **l** BOP,HCl.HisOMe,Et_3N,CH_2Dl_2; **m** NAOH 2N,MeOH; **n** H_2/Pd/C, 80 °C, 80 bars

Taurine

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Identification of a specific protein in the mitochondrial fraction of the rat heart whose phosphorylation is inhibited by taurine

In 1989 it was first reported from our laboratory that taurine has an effect on the phosphorylation of proteins present in the rat heart. In subsequent studies it was determined that rat heart mitochondrial preparations contained a specific protein with a molecular weight of approximately 44 kDa whose phosphorylation was inhibited by taurine (IC_{50} = 9.1 ± 3.5 mM) in a dose-dependent manner [J Mol Cell Cardiol 26: 1675–1689, 1994]. Structure-activity-relationship experiments using a wide variety of taurine analogues demonstrated that the N-C-C-S structure of the taurine molecule was critical for activity and could be placed within certain ring structures with retention of activity. Interestingly, when the N-C-C-S moiety was located within an unsaturated ring the compounds stimulated the phosphorylation of the ~44 kDA protein rather than acting as inhibitors.

Isolation of the ~44 kDa phosphoprotein was achieved by first separation on a preparative (no wells) one-dimensional polyacrylamide gel and then re-electrophoresing the part of the gel containing the ~44 kDa phosphoprotein on an analytical tricine-buffered gel. The band of protein obtained from the first 1-D gel was further separated on a tricine-buffered gel into several additional bands, one of which contained radioactive phosphate. It was this portion of the tricine-buffered gel containing the phosphoprotein that was cut out and sent to a commercial facility for digestion, HPLC separation of the various peptides, and sequence analysis. The sequence analysis of two peptides revealed that the isolated ~44 kDa phosphoprotein is pyruvate dehydrogenase.

Identification of the ~44 kDa phosphoprotein as pyruvate dehydrogenase appears reasonable since both the unknown rat heart phosphoprotein and authentic pyruvate dehydrogenase have similar properties such as the following: 1) pyruvate dehydrogenase, depending upon the source, has a molecular weight of 41–43 kDa. Commercial pig heart pyruvate dehydrogenase migrates on 1-D polyacrylamide gels in the same region as the rat heart radioactive ~44 kDa phosphoprotein; 2) pyruvate dehydrogenase is a known phosphoprotein; 3) the ~44 kDa protein is phosphorylated only in a serine residue (not threonine or tyrosine) which is consistent with the reported serine phosphorylation sites for the mouse liver, mouse testis, human somatic, and human testis pyruvate dehydrogenases; and, 4) the phosphorylation of pyruvate dehydrogenase is cAMP independent. This observation is also consistent with our data that cAMP has no effect on the phosphorylation of the ~44 kDa protein.

The pyruvate dehydrogenase complex converts pyruvate to acetyl-CoA in a series of reactions important to aerobic energy metabolism in the cell. Pyruvate dehydrogenase is known to be regulated by phosphorylation which in turn is affected by numerous low molecular weight metabolites. For instance, the pyruvate dehydrogenase kinase is sensitive to fluctuations in the ratios of ATP/ADP, acetyl-CoA/CoA, and NADH/NAD and in the changes in the concentrations of Ca^{2+}, Mg^{2+}, K^{+}, and pyruvate. The pyruvate dehydrogenase phosphatase is also sensitive to fluctuations in Mg^{2+}, Ca^{2+}, NADH and polyamines, primarily spermine. Thus the data presented in this study that taurine also inhibits the phosphorylation of pyruvate dehydrogenase implies an additional possibility that taurine has an effect on energy metabolism in the cell.

(This work was supported in part by grants from the RGK Foundation of Austin, TX, and the Taisho Pharmaceutical Co., Ltd. of Tokyo, Japan.)

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Taurine and some carboxylic acids in the rodent olfactory system

Since the axons of the olfactory neurons project directly into the olfactory bulb the olfactory epithelium may serve as a portal of entry for compounds to the brain. We have studied the uptake of radiolabelled taurine (a β-sulfonic amino acid) in the olfactory bulb in adult mice. After an unilateral intranasal instillation of radiolabelled taurine in the right nostril the level of radioactivity was markedly higher in the right olfactory bulb (> 12-fold) than in the left olfactory bulb. Still 24 hours after a single intranasal administration of radiolabelled taurine radioactivity occurred in the olfactory bulb. In mice lacking olfactory neurons and axons in the dorsomedial part of the olfactory region (due to pretreatment with the olfactory toxicant dichlobenil) there was a markedly decreased level of radioactivity in the olfactory bulbs as compared to vehicle-treated controls given radiolabelled taurine intranasally. Preliminary studies did not indicate that the uptake of taurine in the olfactory bulb was related to the presence of a transport/binding protein. Similar studies have been performed after intranasal administration of radiolabelled carboxylic acids including the tryptophan metabolite picolinic acid, in mice. The results demonstrate that taurine and some carboxylic acids are rapidly migrating in the axoplasmic flow of olfactory neurons into the olfactory bulb. Taurine has been suggested to play a role in axonal sprouting and/or synapse formation. Hence, the selective localization of taurine in the olfactory system may possibly be related to the continuous regeneration of olfactory neurons in adult animals.

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Precursors of taurine in female genital tract of mammals: Effects on gametes and embryo

The importance of protecting preimplantation embryos from oxidative damages when they are cultured *in vitro*, is being increasingly recognized. Nevertheless acrosome reaction and fertilization are associated with reactive oxygen species generation: a "balance of benefit and risk" probably exists. Hypotaurine and taurine are antioxidant compounds present in genital secretions and synthesized by oviduct cells [Guérin et al. (1995) Zygote 3: 333–343]. Cysteamine (CSH) provides considerable protection against ionizing radiations *in vitro* and is a scavenger of hydroxyl radical (OH°). The present study was conducted to quantify CSH and cystamine (CSSC) in oviduct and follicular fluids of mammals and to assess the effect of hypotaurine on *in vitro* production of bovine embryos.

Cysteamine in oviduct and follicular fluids were determined by ion-exchange chromatography (Amino acid analyzer Beckman 6300). Glucosaminic acid (10 nM) was used as an internal standard. CSH and CSSC were detected by ninhydrin coloration and absorbance at 570 and 440 nm.

Effects of hypotaurine on bovine embryo production. Antral follicles were aspirated from bovine ovarics and matured on granulosa cell monolayers in M199 (+ 20% fetal bovine serum [FBS], 10 μg/mL FSH/LH, 1 μg/mL 17β-estradiol and 0.5 ng/mL epidermal growth factor). Oocytes were cultured 24 h (39 °C, 3% CO_2). Frozen semen from a single bull was thewed in 37 °C and layered under 1 ml of capacitation medium (modified Tyrode's calcium-free medium). After swim-up the spermatozoa were added to fertilization tube (10^6 cells/mL). The fertilization medium was modified Tyrode's (+ heparin [10 μg/mL], penicillamine [20 μM], hypotaurine [10 μM] and epinephrine [1 μM]). Oocytes were washed in fertilization medium and transferred in 500 μL of this medium. Oocytes and spermatozoa were incubated 18 h (39 °C, 5% CO_2). Vero cell monolayers were prepared according to Ménézo (Biol Reprod 42: 301–306). Zygotes were transferred to co-culture droplets under mincral oil (20–25 zygotes/50 μL). Embryos were cultured 6 days in B_2 medium (10% FBS) with either 0, 1, or 10 mM hypotaurine. Blastocyst yield were scored 7 days after insemination.

Cysteamine is present in follicular fluid of all species tested, and absent in oviduct fluid (Table 1).

Table 1. Mean cysteamine concentrations in rabbit, cow, sow, goat and dog follicular fluids (nmol/ml; mean ± SEM). *Flushing

Rabbit	Cow	Sow	Goat	Dog*
(n = 8)	(n = 10)	(n = 7)	(n = 6)	(n = 2)
79 ± 11	54 ± 4	45 ± 10	58 ± 10	Presence

Nevertheless, follicular fluid is present in the oviduct after ovulation. Then cysteamine is certainly present in embryo environment. Blastocyst percentage formation is higher ($p < 0.01$; Chi square test) in the medium supplemented with 1 mM hypotaurine when compared with 0 and 10 mM (Table 2).

Table 2. Effect of hypotaurine addition to culture medium on bovine blastocyst development (partial results)

Hypotaurine (mM)	Inseminated oocytes	Blastocysts yield	
	n	n	(%)
0	454	108	23.8
1	351	125	35.6
10	510	125	24.5

Fertilization and acrosome reaction occur in presence of follicular fluid. On the opposite capacitation occurs in oviduct fluid. Hydrogen peroxide has a promoting action in sperm capacitation *in vitro*. SCH reacts with H_2O_2 but hypotaurine does not. Presence of CSH in follicular fluid and its absence in oviduct secretion suggest that neutralization of H_2O_2 is important for acrosome reaction and fertilization. CSH oxidation to hypotaurine and taurine could participate to the neutralization of ROS or to the maintainance of high level of GSH/GSSG ratio. Furthermore CSH is a cationic thiol that condense near DNA at pH 7, leading to high concentrations of cations near DNA, which allow them to scavenge hydroxyl radicals and repair DNA.

Addition of hypotaurine improves bovine embryo development *in vitro*. Consequently hypotaurine should be systematically added in culture media used for *in vitro* fertilization and embryo culture. The presence of significand amounts of CSH in follicular fluid, and the important direct and indirect protective properties of this thiol suggest that its addition to culture media could permit to ameliorate the embryo production and viability.

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Taurine and glutamate release from brain tissue during simulated ischemia

Taurine (Tau) and glutamate (Glu) both occur in high concentrations in brain tissue, although their role in physiologic and pathophysiologic states are very different. Both amino acids (aa) are released from brain tissue during cerebral ischemia. This aa release has often been accounted for as non-specific processes as "overflow", "leakage" or "carrier reversal" secondary to energy depletion. Most of the large ischemic release of aa occurs after the rapid anoxic depolarisation (or "spreading depression"). At this time sodium, potassium, calcium and chloride gradients over the cell membrane are dissipated. In the present study we simulate the effect of ischemia on brain tissue by manipulating the ion gradients in different ways, and measure the resulting release of Tau and Glu.

Methods. Transverse slices of rat hippocampus were incubated for 20 minutes in artificial cerebrospinal fluid (ACSF) containing 10 mM glucose, 0.1 mM Ca^{++} (to avoid vesicular release) and saturated with 95% O_2/5% CO_2. In four sets of experiments (each n = *10*) the content of the ACSF was manipulated in various ways: **1**) Ion gradients of sodium and potassium were reduced (*Con:* normal ACSF; *LoNa:* Sodium substituted with N-methyl-D-glucamine (NMDG); *HiK:* 50 mM potassium, substituted for sodium; *Ver:* 50 µM veratridine added to open sodium channels; *ED:* Energy deprivation, 0 mM glucose and $=_2$ substituted by N_2). **2**) Sodium and chloride was substituted or reduced (*Con; LoNa; LoCl:* Chloride substituted by isethionate; *LoNaCl:* NaCl concentration of the ACSF reduced by 75 mM). **3**) Sodium influx was prevented (*Con; TTX;* Acsf with 1 µM tetrodotoxin (TTX) added to block sodium channels; *ED; ED/TTX:* ED, 1 µM TTX; *ED/LoNa:* ED, sodium substituted by NMDG). **4**) Chloride substituted by isethionate during 35 min of ED, fractions of ACSF being sampled every 5 min. Aa content of the ACSF was measured by means of a sensitive HPLC method. In experiments 1–3, values are given as pmol aa released per mg protein, average ± standard error. In experiment 4, values are expressed as change in aa release in % of plain ED (*Con*).

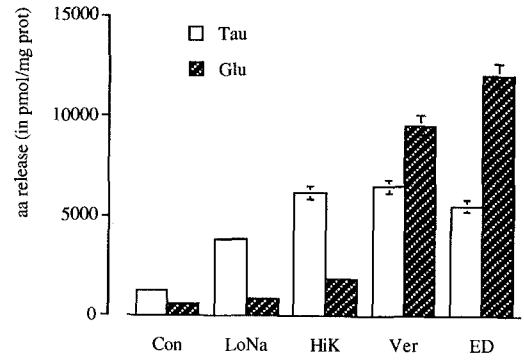

Fig. 1. Manipulation of sodium and potassium gradients

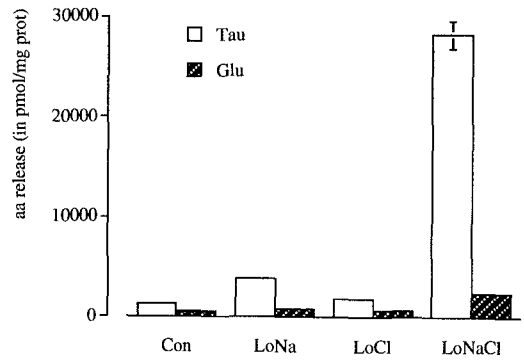

Fig. 2. Substitution/reduction of sodium/chloride

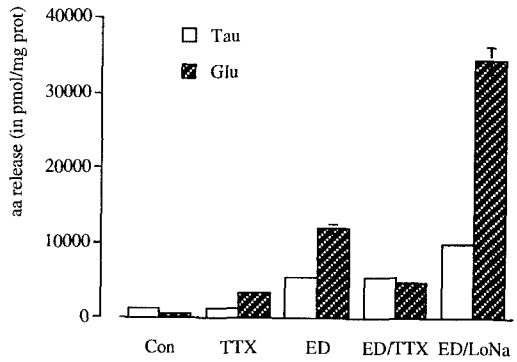

Fig. 3. Preventing sodium influx

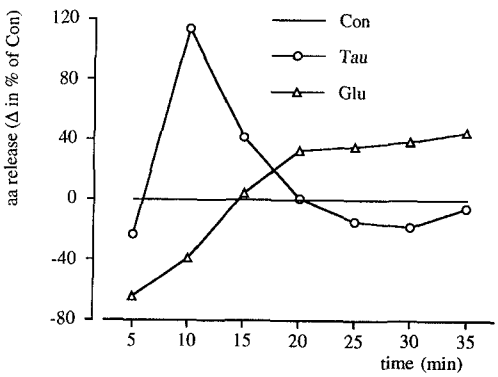

Fig. 4. Chloride substitution during ED

Results. Low sodium and high potassium increased Tau release more than Glu, whereas sodium influx and ED increased Glu more than Tau (Fig. 1). Sodium and chloride substitution both increased Tau more than Glu. Omitting 75 mM NaCl generated a large Tau release and a small Glu release (Fig. 2). TTX reduced Glu but not Tau release during ED, whereas preventing ED-induced sodium influx by omitting sodium in the ACSF increased both Glu and Tau release, Glu >> Tau (Fig. 3). Chloride deprivation during ED increase Tau release in early fractions, whereas Glu was greatly reduced. In later fractions this pattern reversed (Fig. 4).

Conclusions. The results show that calcium independent Tau and Glu release is modulated in different ways when different aspects of the ion gradient perturbation that occur during cerebral ischemia is mimicked in this *in vitro* model. They also demonstrate that aa efflux does not depend on sodium influx (Fig. 3). The results shown in Fig. 4 indicate that chloride dependent and chloride independent release mechanisms for Tau and Glu are activated at different times as ischemia develops. Taken together, the results contradict the common notion that ischemic aa release is a non-specific process. Rather, release of different amino acids seems to be modulated actively in a differentiated manner.

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Adenosine A_2 receptor activation enhances high K^+-evoked taurine release from rat hippocampus

We have recently reported that the increase in endogenous adenosine by adenosine transport inhibitors enhances the high K^+-evoked taurine release from the rat hippocampus [1, 2]. Furthermore, it has been shown that exogenous adenosine increases taurine levels in the brain areas including the hippocampus [3]. The present study was designed to examine which type of adenosine receptors was involved in the enhancement of taurine release from the *in vivo* rat hippocampus using microdialysis. Adenosine at 0.5 mM increased the high K^+-evoked taurine release. A non-selective adenosine receptor antagonist, theophylline (1 mM), did not modulate the taurine release. A selective adenosine A_1 receptor agonist, R(-)-N^6-2-phenylisopropyladenosine (PIA), at 2 μM did not modulate the taurine release, but a selective adenosine A_{2a} receptor agonist, CGS21680, at 20 μM enhanced it. A selective adenosine A_1 rceptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 1 μM), increased the taurin efflux, but a selective adenosine A_2 receptor antagonist, 3,7-dimethyo-propagylxanthine (DMPX, 1 mM), did not. These results demonstrate that adenosine enhances the high K^+-evoked taurine release via the activation of adenosine A_{2a} receptors from neurons and glial cells in the *in vivo* rat hippocampus.

References

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Taurine and aspartate both show swelling-induced release from astrocytes and aspartate shows significant release by reversal of the transporter, in response to raised extracellular $[K^+]$

When primary astrocyte cultures are exposed to media where Na^+ was replaced with K^+, there was a marked release of preloaded [^{3}H] taurine or [^{3}H]-D-aspartate. This release was large, reaching a values of 3 and 5% respectively, as a percent of the amount of labeled compound present at each time, a value we term percent fractional release. The time course of this release showed an increase with a few minutes latency and then a progressively increasing release. This profile was similar to that shown for swelling of the astrocyte monolayer when measured by an extracellular electrical impedance method. The transport system involved appears to be the VSOAC or voltage sensitive organic anion channel (Strange et al. Am J Physiol 270: C711–C730, 1996). It was inhibited by DIDS, NPPB, extracellular ATP and an anion transport inhibitor we have shown is neuroprotective, L644,711. Efflux was also inhibited by reducing intracellular ATP by treating the cells with iodoacetate.

The progressive release of [^{3}H]-D-aspartate was preceded by a small transient peak of release. We hypothesized this may be due to reversal of the amino-acid transporter since this transporter had been described to reverse when extracellular $[K^+]$ is raised. This is because aspartate/glutamate transporters co-transport K^+ and alkalinizing anions from the cell, as well as Na^+ and the amino acids into the cell. In contrast taurine is transported on a different transporter which co-transports Na^+ and Cl^- and no initial peak was detectable for taurine. A further prediction is that if intracellular $[Na^+]$ as well as extracellular $[K^+]$ is raised, the release due to reversal of the transporter should be increased. Treatment for even 10 min. with ouabain which is known to raise intracellular Na^+ in these cells, presumably due to inhibition of the $(Na^+ + K^+)$, pump caused the predicted increase in the initial transient peak with no effect on the second progressive increase. Furthermore the initial peak was inhibited by loading the cell by prior treatment with the non selective competitive inhibitor of the EAA transporter threo β hydroxyaspartate. In contrast the non-competitive inhibitor dihydrokainic acid (DHK) had no effect. This, however, corresponds to the report that primary astrocyte cultures have the astrocytic GLAST isoform rather than the predominant GLT1 form found in astrocytes in the cortex, striatum and hippocampi of adult rats. In addition the initial peak was more sensitive to raised extracellular K^+ than the second component. The initial peak showed an EC_{50} of 25 mM K^+ while the second component was only activated at $[K^+]_o$ of > 50 mM.

These *in vitro* results suggest that under pathological states such as ischemia, when brain extracellular $[K^+]$ and intracellular Na^+ rises, a significant portion of the raised extracellular EAAs glutamate and aspartate could occur by reversal of the astrocytic transporter unaccompanied by an increase in the inhibitory and neuroprotective taurine. During the phase of astrocytic swelling, however, taurine may be equally or even freferentially released through the VSOAC.

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Are alterations in the balance between taurine uptake and efflux important in the regulation of cell volume in cerebral cortical minislices prepared from hyponatraemic rats?

When slices prepared from the cerebral cortex of normal rats are subjected to acute hyposmotic shock (122 mM Na) there is marked acceleration of efflux of taurine from pre-loaded cells, accompanied by limited swelling (Law, RO (1994) Biochim Biophys Acta 1221: 21–28). Conversely, cells from rats whose plasma Na had been reduced to (mean) 112 mM over 4 days by the provision of Na-free diet, 5% dextrose in lieu of drinking water, plus LVP (i.p.) maintained normal cell volumes, and a rate of taurine efflux typical of normonatraemic control animals. Hyponatraemic cell volume maintenance may depend in part on establishment of a new set-point in the balance between reduced cellular efflux and re-uptake of taurine from the slice interstitium. In addition, sensitivity to the inhibitory effects on efflux of the anion transport inhibitor DIDS is attenuated by chronic hyponatraemia.

Cells from normal rats show a moderate further enhancement of taurine efflux, plus small (< 10%) additional cell swelling, when exposed to media containing 102 mM Na. Cells from hyponatraemic animals, however, swell by > 30%, without any increase in the rate of taurine efflux. The swelling response is blocked by the NaCl cotransport inhibitor bumetanide (50 μM).

When slices from hyponatraemic rats are acutely exposed to media containing normonatraemic levels of Na (142 mM) there is a marked (> 20%) paradoxical increase in cell volumes, with no change in the rate of cellular taurine efflux. This swelling response is entirely abolished by bumetanide, and is not observed in cells that have not been pre-loaded with taurine.

Cell volume regulation in response to external hyposmolality in pre-loaded slices may be assumed to depend, at least in part, on osmotically (or volume) dependent taurine efflux moderated by re-uptake. The interpretation of relevant data in whole tissue (e.g. slices) is complicated by the fact that whereas taurine loss is probably primarily neuronal, glial cell reaccumulate taurine by channel diffusion down a favorable concentration gradient. Neuronal re-uptake, however, might also occur. The present results using bumetanide suggest that such re-uptake may be partly due to transport of anionic taurine by the Na/anion cotransporter. The fact that in cells from hyponatraemic rats net rates of taurine efflux in media of 102, 122 and 142 mM Na remain unchanged (unlike the responses of cell from normal rats) may indicate that during hyponatraemia the taurine efflux mechanism(s) remains "locked" in a down-regulated mode: this might be functionally associated with the diminished sensitivity to DIDS. The enhanced swelling observed in 102 mM Na might reflect failure of taurine efflux to accelerate in response to increased hyposmolality, while the swelling in 142 mM Na might be due to unaltered rates of taurine efflux coupled with enhanced bumetanide-sensitive taurine re-uptake.

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Hypothalamic mechanisms underlying taurine-induced prolactin secretion

Taurine (Tau), a putative inhibitory amino acid neurotransmitter, has been shown to stimulate prolactin (PRL) release. Using ovariectomized adult rats we investigated initially the effect of this amino acid, injected by different routes, on PRL secretion *in vivo*. Tau (100–500 mg/kg), given i.p., had no effect on PRL release; 15 min after i.c.v. injection of Tau (3 μmoles), a significant increase in serum PRL levels was observed (78 ± 9 ng/ml over basal levels, $p < 0.01$ vs. controls). *In vitro* (cultured anterior pituitary cells) PRL release was not affected by a 0.5 h incubation with Tau (10^{-3}–10^{-8} M). Basal dopamine (DA) or gammaaminobutyric acid (GABA) output from superfused mediobasal hypothalamic fragments (MBH) was not affected by Tau (10^{-3} M or 10^{-5} M). However, during stimulation with KCl (50 mM), Tau (10^{-3} M) significantly lowered DA release, and increased GABA output. It is concluded that Tau acts at a central level to increase PRL secretion, most probably by modulating the hypothalamic release of neurotransmitters controlling lactotroph function.

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Inhibition of the generation of neutrophil inflammatory mediators by taurine chloramine

We previously reported that taurine chloramine (TauCl), a product of activated neutrophils, inhibits the generation of macrophage inflammatory mediators such as nitric oxide, TNF-α, and PGE_2. Taurine, the most abundant free amino acid in the cytosol of neutrophils, is chlorinated to form taurine chloramine by the halide-dependent myeloperoxidase system, and under physiological conditions, reduces HOCl toxicity.

In this study, we investigated the influence of TauCl on generation of oxygen free radicals, catokines and eicosanoids by activated neutrophils. We have found that TauCl, but not taurine alone, inhibited the production of nitric oxide, interleukin 6, tumor necrosis factor-α and prostaglandin E_2, in a dose dependent manner. Taurine chloramine also reduced the level of inducible nitric oxide synthase (iNOS) mRNA and TNF-α mRNA in activated neutrophils. On the contrary, the products of respiratory burst, as measured by luminol-dependent chemiluminescence, were reduced by both reagents; taurine and taurine chloramine. The results of these studies suggest that TauCl decreases production of tissue-damaging inflammatory mediators and may regulate the balance between protective, microbicidal and harmful effect of neutrophils.

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Antidiuretic hormone (ADH; arginine vasopressin; aVP) administration in taurine and hypertonic saline-induced hypernatremia

Ingestion of a solution containing 0.1 M taurine and 1.8% NaCl (T+S) induces hypernatremia in rats within a few days, and death within one to three weeks. The basis of this effect is not known. Our earlier studies established that rats drinking T+S are unable to concentrate their urine (*i.e.*, to retain "free water") sufficiently to maintain either a normal plasma osmolality or even the mild hyperosmolality and hypernatremia observed in rats drinking 1.8% NaCl (S) alone. Animals with access only to hypertonic solutions, who are unable thus to rely upon the thirst mechanism to maintain body fluid osmolality, are critically dependent upon free water retention during the formation of urine. That the hypernatremic potential of the T+S combination is peculiar to taurine is suggested by the absence of significant hypernatremia when

β-alanine (a structural analog of taurine) was substituted for taurine in the solution. Also, our findings that rats drinking β-alanine+S (or, indeed, β-alanine+T+S) retained significantly more urine concentrating ability than those drinking T+S argues against an effect due simply to an increased solute load. The current set of experiments was designed to test whether the hypernatremia induced by T+S ingestion can be minimized by the simultaneous administration of aVP. Subcutaneously implanted osmotic minipumps containing 1 mg of aVP per 10 ml of distilled water induced a frank antidiuresis and maintained it through the 8-day course of the experiment in a group (n = 6) of adult rats drinking tap water (TW+aVP). Metabolism cages were used to obtain data from that group which then were compared with those from rats drinking T+S, one group (n = 6) with implanted minipumps containing aVP (T+S+aVP) and one group (n = 6) with implanted minipumps containing distilled water only (T+S w/o aVP). Rats were placed into metabolism cages on Day 2 and allowed to stabilize for two days while being monitored with no treatment. Osmotic minipumps were implanted and administration of the selected drinking regimens were begun following sample collection on Day 0. Daily measurements were made of body weight, solution and food consumption, urine volume, urine sodium concentration, and urine osmolality. Daily sodium balance, daily "visible" water balance, cumulative sodium balance (ComNaal) and cumulative water balance (CumWatBal) were calculated. Alternate day (0, 2, 4, 6, 8) measurements were made of plasma sodium concentration (P_{Na}), plasma osmolality, and hematocrit. Mean P_{Na} in the T+S w/o aVP group was 147 mmol/L on Day 2, 157 on Day 4, and 170 on Day 8. (One animal in this group died on Day 5.) By contrast, in the T+S+aVP group P_{Na} remained below 145 mmol/L through Day 6 and was still < 150 on Day 8. The T+S+aVP group was able to maintain a CumWatBal which was consistently higher, albeit modestly so, than that of the T+S w/o aVP group. However, the CumNaBal/CumWatBal ratio suggests little uniform advantage to the T+S+aVP group; nor does comparison of the urine osmolality in the two groups. While the nature of the advantage conferred by aVP administration upon rats drinking T+S remains unclear, we believe the hypothesis that T+S somehow interferes with the ADH system of extracellular osmolality and volume regulation remains viable.

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The effect of taurine on chelerythrine inhibition of ATP-dependent Ca^{2+} uptake and ATPase activity in rat retina

Chelerythrine (CHT), a benzophenanthridine alkaloid, is a well-known, selective and potent inhibitor of protein kinase C (Biochem Biophys Res Commun 172: 93–99, 1990). We reported (Adv Exp Med Biol 403: 343–350, 1996) that CHT paradoxically stimulates the phosphorylation of an ~20 kDa protein present in the mitochondrial fraction of the rat retina but inhibits the phosphorylation of an ~44 kDa protein in rat heart mitochondria, and that these effects apparently have no relationship to the effects of CHT on protein kinase C.

In the present studies, we investigated the effects of CHT on both ATP-dependent Ca^{2} uptake and ATPase activity in rat retinal preparations in the presence and absence of taurine. Taurine had been shown to have a significant stimulatory effect on Ca^{2+} uptake and an inhibitory effect on the phosphorylation of an ~20 kDa protein present in the rat retina. This inverse relationship of taurine prompted these studies to determine if there was a common underlying mechanism between Ca^{2+} uptake and the phosphorylation of the ~20 kDa protein and if the use of chelerythrine coul help towards determining this long-term goal.

CHT (0–100 μM) was tested on Ca^{2+} uptake activity under three different conditions: ATP-independent (basal) uptake, ATP-dependent uptake, and taurine-stimulated ATP-dependent uptake. CHT had no effect on ATP-independent but inhibited ATP-dependent Ca^{2+} uptake. With taurine-potentiated (32 mM taurine) ATP-dependent Ca^{2+} uptake, CHT inhibited uptake with the same potency as non taurine-stimulated uptake but to a greater degree with respect to control values.

When Ca^{2+} uptake was measured in the presence of varying concentrations of ATP (0–4.2 mM) it was observed that CHT (100 μM) had an increased effect at saturating ATP concentrations. CHT exhibited the same increased effect at saturating ATP concentrations with taurine present. These results suggest that CHT and ATP have a common site or are close to each other spatially in the Ca^{2+} transporter. When ATP-dependent Ca^{2+} uptake was assayed in the presence of varying concentrations of taurine (0–80 mM), the maximum stimulatory effect of taurine was reached at 32 mM taurine. CHT (100 μM) inhibited the taurine stimulation at all concentrations of taurine and decreased the Ca^{2+} uptake activity to a constant basal level.

The effects of taurine and CHT were also tested on total ATPase activity. When assaying ATPase activity under the same experimental conditions as the ATP-dependent Ca^{2+} uptake assay it was observed that taurine (32 mM) did not produce a significant change in activity. The presence of taurine, though, shifted the inhibition curve of CHT to the right, indicating lesser potency. In kinetic experiments in which ATP was varied, a fixed concentration of CHT (100 μM) was used to inhibit ATPase activity in the presence and absence of taurine. In the control ATPase assay, CHT was non-competitive with respect to ATP while in the presence of taurine the inhibition was competitive. Thus, it appears that in the ATPase enzyme, the binding sites for both ATP and CHT are situated close to each other, as suggested in the Ca^{2+} uptake experiments, and that taurine alters the spatial relationship of these sites bringing them closer together in a competitive mechanism.

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Renal excretory responses of taurine-depleted rats to hypotonic and hypertonic saline infusion

Intracellular accumulation of organic osmolytes, i.e., taurine, by renal tubular cells serves as an adaptive mechanism to cope with increased interstitial osmolality; the latter is essential for the kidney to concentrate urine. The aim of this study was to determine whether taurine depletion affects renal excretion of saline solutions of different tonicity. Seven-week old male Wistar-Kyoto (WKY) rats were given either tap water (control; n = 6) or 3% β-alanine (taurine-depleted; n = 5) for three weeks. Thereafter, animals were implanted with femoral vessels and bladder catheters. Two days after surgery, each rat was given an intravenous infusion of saline at the rate of 50 μl/min and urine samples collected at 15 minute intervals. Isotonic saline solution (0.9% NaCl) was infused for determination of baseline parameters followed by

infusion of hypotonic saline solution (0.45% NaCl). Two days later, the infusion protocol was repeated in the same animals; however, a hypertonic saline solution (1.8% NaCl) was substituted for a hypotonic saline solution. Renal excretion of sodium, but not fluid, increased in the control rats during the hypotonic saline infusion. In contrast, renal excretion of fluid and sodium were not affected in the taurine-depleted rats by the hypotonic saline infusion. Interestingly, diuretic and natriuretic responses were similar between the groups during hypertonic saline infusion. The results suggest that taurine-depletion in rats affects renal excretory responses to a hypotonic, but not a hypertonic, saline solution.

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Molecular cloning, sequence analysis and genomic expression of cysteine sulfinate decarboxylase (CSD)

The taurine biosynthesis enzyme, cysteine sulfinate decarboxylase (CSD), was purified to homogeneity from rat liver. The final protein isolated through preparative SDS-electrophoresis appeared homogeneous on reverse-phase HPLC, showed a molecular weight of 51 ± 2 kDa, and led when used as immunogen to an antiserum that quantitatively immunoprecipitated CSD enzymatic activity.

Following tryptic digestion three peptides could be isolated through HPLC and partially sequenced according to the automated Edman degradation. Two of them showed a marked homology with glutamate decarboxylase and their respective position on the amino and carboxy terminal side of CSD amino-acid sequence was postulated accordingly. Corresponding sense and an antisense degenerated primers were synthetized and used to generate an amplified CSD-cDNA fragment from rat liver poly(A+) mRNA through RT-PCR. This fragment was cloned and used as a probe to screen a rat liver cDNA library.

Three cDNAs, long around 1800 bp, were isolated and sequenced. They all contained an open reading frame (ORF) encoding a 493 amino-acid protein with a calculated molecular weight of 55.2 kDa close to the experimental values for CSD. The encoded protein contained the known consensus sequence for fixation of pyridoxal phosphate (NHPK) that was found in many decarboxylases as well as the sequence of the three peptides isolated from homogenous liver CSD. The fusion protein obtained by cloning the ORF in an expression vector decarboxylated cysteine sulfinate in hypotaurine and cysteate in taurine but did not decarboxylate homocysteine sulfinate, homocysteate or glutamate. This is consistent evidence that the cloned cDNA is CSD-cDNA.

Through Northern blot an mRNA of 2.5 kb was labelled with the CSD-cDNA used as a probe in poly(A+) mRNA from liver, brain and kidney. Using different pairs of appropriate primers overlapping RT-PCR amplified cDNA fragments were generated from brain poly(A+) mRNA and sequenced. The results establish the molecular identity of brain and liver CSD suggested by our previous investigations.

Quantification of CSD-mRNA was achieved through competitive RT-PCR using as competitor a modified CSD-mRNA synthesized *in vitro* from mutated CSD-cDNA. The absolute content was similar in kidney, about three-fold higher in liver and below reliable detection in heart RNA extracts.

We showed previously that in brain CSD is strictly localized in astrocytes. We are presently investigating the regulation of the genomic expression of CSD in primary culture of astrocytes in various experimental conditions.

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Dual effect of taurine on nigrostriatal dopaminergic neurons, an in vivo microdialysis study

The amino acid taurine (2-aminoethanesulphonic acid) has long been known to be present in high concentrations in the central nervous system, but so far its function remains unclear. Taurine is suggested to act as a neurotransmitter or neuromodulator in brain (Della Corte et al., 1990). The present series of studies was conducted to elucidate a possible involvement to brain dopaminergic neurons in the central effects of taurine in basal ganglia using microdialysis technique. In addition, the effects of taurine were compared with those of the major cerebral inhibitory neurotransmitter GABA, which is structurally related to taurine. In anaesthetised rats intracerebrally infused taurine (2 µl/min, 50 to 450 mM for 4 hours) was found to have a dual action on nigrostriatal dopaminergic neurons. Intrastriatally infused taurine elevated striatal extracellular dopamine, whereas intranigrally infused taurine decreased dopamine concentration in the ipsilateral striatum. Dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) was increased both after intrastriatal and intranigral taurine, whereas the changes in homovanillic acid (HVA) were opposite to those found in dopamine. In the freely moving rats both taurine and GABA when administered locally were found to elevate striatal extracellular dopamine. Addition of tetrodotoxin (1 µM) into the perfusion fluid blocked the increase of dopamine caused by the amino acids (150 mM, 2 hours). This implies that both taurine and GABA increase dopamine release impulse flow dependently. This view is confirmed by finding that omission of Ca^{2+} in the perfusion fluid also blocked the GABA-induced elevation of extracellular dopamine. However, the elevation caused by taurine was not blocked by omission of Ca^{2+}. In conclusion, the finding that intranigral taurine decreased dopamine in the ipsilateral striatum agrees with the earlier suggestions of inhibitory role of taurine on dopamine release based on post mortem measurements (Panula-Lehto et al., 1992). Although local taurine seems to stimulate striatal dopamine release in a similar way to GABA, the differences in the Ca^{2+}-sensitivity of their effects suggest that these amino acids act on nigrostriatal dopaminergic neurons via more than one mechanism.

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Taurine: An important determinant of myocardial ischemic injury

Two of the most important myocardial functions of the amino acid, taurine, is osmoregulation and Ca^{2+} modulation. Since both processes dramatically affect cardiac function, it was felt that reductions in intracellular taurine levels should significantly impact the ischemic heart. To test this idea, cardiac taurine pools were progressively reduced by feeding rats with water containing 3% β-alanine. Hearts from these

and control animals were then subjected to 45 minutes of regional ischemia followed by 2 hours of reperfusion. Infarct size, as a percentage of risk zone, was linearly related to the degree of taurine depletion, with a 40% drop in taurine levels reducing infarct size from 57 ± 3% to 24 ± 5%. Similarly, isolated cardiomyocytes incubated for 3 days with medium containing 5 mM β-alanine were found resistant to hypoxic induced cell death. Since the β-alanine-treated myocytes exhibit changes in both Ca^{2+} transport and cell size, it is unclear which factor contributes to the observed cardioprotection. Nonetheless, the results indicate that intracellular taurine content is an important determinant of ischemic and hypoxic myocyte injury.

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Role of taurine in neuronal migration and expression of certain splice variants of NCAM

Depletion of taurine in dissociated microcultures of early postnatal (P6) mouse cerebellum by exposure of the cultures to the taurine uptake inhibitor guanidinoethane sulfonate (GES) leads to reduced neuronal migration. Moreover, the number of cell processes was found to be decreased. As neuronal migration may be related to expression of cell adhesion molecules (DAMs), effects of taurine depletion on expression of neuronal CAM (NCAM) was investigated. Exposure of cultures to GES had no effect on overall expression of NCAM isoforms A, B and C as revealed by Western blot analysis. Northern blot analysis of expression of different mRNA splice forms of NCAM, however, revealed that the expression of the VASE containing form was decreased. This effect could be reversed by addition of taurine to the culture medium. Exposure to GES was additionally found to have a slight stimulatory effect in expression of c-fos in the cells, but no adverse effects of GES was observed with regard to survival of function of the cultured neurons. It is proposed that taurine is essential for processing of NCAM mRNAs and that this, in turn, may affect neuronal migration.

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Expression and localization of cysteine dioxygenase mRNA in various tissues of rat

Cysteine dioxygenase (CDO) catalyzes the oxygenation of cysteine to cysteine sulfinate that is a key intermediate of cysteine metabolism to taurine in mammals. We have isolated a cDNA from rat liver CDO and characterized it. The cDNA, designated rCDO-39, contained a 1,458 b insert, and encoded a polypeptide of 23,025 Da consisting of 200 amino acids. In this work, we examined the expression of CDO gene in liver, skeletal muscles, and lung of rats by using *in situ hybridization*. The 433 b fragment generated from rCDO-39 by PCR was inserted into the insertion site of pGEM T-vector (Promega). A linearized plasmid DNA was transcribed with SP6 or T7 RNA polymerase using DIG-labelled UTP as a substrate to prepare sense and antisense probes. *In situ hybridization* was performed in rat tissues fixed with 4% buffered paraformaldehyde and embedded in paraffin. After hybridization, the labelled probes were detected with anti DIG antibody conjugated with alkaline phosphatase, and the hybridization products were visualized with NBT and X-phosphate. Significant expression of the CDO gene was detected in the liver and the lung, but not in the skeletal muscle. These results were in good agreement with that of Northern blot analysis. In the lung, intensive signal was found in the bronchiolar epithelium. In the liver, the signal was confined in the cytoplasm of the hepatocytes. Furthermore, the signal was stronger around the periportal area than that around the perivenous area.

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The effect of a soluble fibre source on taurine bioavailability in cats

Cats conjugate their bile acids exclusively with taurine and have a limited ability to synthesise taurine when the dietary level is reduced. Previous studies have suggested that the inclusion of soluble fibre sources in canned cat foods may encourage modifications to the feline gut flora which cause increased conversion of primary to secondary bile acids and an increase in taurine degradation (Anantharaman-Barr et al., 1994). This study was designed to investigate the effect of different levels of soluble fibre on taurine status in cats. Twelve healthy adult cats (6 neutered males, 6 entire females, mean age 3.35 ± 1.46 y) participated in the study. Six canned diets containing 0%, 0.5% or 1.0% carrageenan, each level with and without taurine supplementation, were fed to each cat for 3 weeks. All 6 diets were fed in rotation over a 36-week period with washout periods of 2 weeks between test diets. Food and water intakes were measured daily and during the final 7 days of each 3-week feeding period full faeces collections were made. Plasma biochemistry, haematology, taurine and whole blood taurine were measured on days 1 and 21 of each feeding period.

Food intakes were similar for all diets and all the cats maintained bodyweight throughout the course of the study. There was no effect of dietary level of carrageenan on either plasma or whole blood taurine status. The mean plasma taurine levels for unsupplemented diets were significantly lower than for the taurine-supplemented diets. Plasma taurine values showed a positive linear correlation with whole blood taurine ($r = 0.69$, $n = 72$, $SE = 39.9$). The relationship is described by the equation:

plasma taurine = (0.23 x whole blood taurine) – 24.38

The inclusion of carrageenan at 1% of the diet tended to depress fat digestibility although digestibility coefficients remained within normal ranges. There was no effect on the apparent digestibility of the other nutrients. Wet faeces output increased significantly as the level of carrageenan in the diet increased. In conclusion, carrageenan included in a canned cat food at levels of up to 1% did not affect taurine status in healthy adult cats. Unsupplemented diets depressed circulating taurine levels indicating that commercial canned cat foods should be supplemented in order to maintain normal plasma and whole blood levels.

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Immunohistochemical localization of taurine in various organs

We have already reported that ^{3}H-taurine is incorporated extensively into the nuclei and cytoplasm of the endothelial cells and smooth muscle cells of the capillaries, venules and arterioles. In this study, we investigated the localization of taurine using an anti-taurine antibody. [Materials and Methods] Mice were perfused through the heart with 4% paraformaldehyde solution and dissected several organs: brain, heart, lung, liver, kidneys, stomach and intestine. The organs were post-fixed, rinsed, dehydrated, embedded in paraffin and cut into 5 microns. The sections were mounted on the glass slides, deparaffinized, dehydrated in ethanol, and boiled for 5 minutes in the microwave of an oven-range. Thereafter the sections were incubated in a blocking solution of 1.5% normal goat serum for 60 minutes followed by mounting with anti-taurine antibody for 16 hrs in a cold room. The primary antibody against taurine was purchased from Serotec Co. (Oxford, England), diluted in 1.5% normal goat serum, 1:900, 1800 and 5400. Then the sections were mounted by biotinized rabbit IgG produced in goat diluted 1:200 for 60 minutes, rinsed, and mounted with avidin-biotin-complex diluted 1:4000 for 60 minutes. These sections were reacted with 0.02% 3.3-diaminobenzidine 4HCl solution containing 0.0045% H_2O_2 for 10 minutes. The control sections were not reacted by the primary antibody. [Results] The control sections were translucent. The best contrast of staining was obtained by taurine antibody solution diluted 1:1800. The sections were stained brown. In all the tissues, the nuclei and cytoplasm of the endothelial cells of the capillaries as well as the venules and arterioles were stained a deep dark brown. The nuclei of all the cells, the matrices of connective tissues, cardiac muscles, the epithelial cells of the proximal tubules of kidneys, stomach and small intestine were stained a medium dark brown. The cytoplasm of the skeletal muscle cells were faint brown. The red blood cells were not stained. [Conclusion] The immunolocalization of taurine in the tissues corresponds to the localization of ^{3}H-taurine intake. The immunohistochemical localization which was observed using our methods is in agreement with the localization found by other investigators. This method was uncomplicated and presented the stable results.

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Vectorial transport of neurotransmitter amino acids and taurine across the blood-brain barrier

The characteristics of vectorial transport of amino acids including taurine at the blood-brain barrier were studied by using primary cultured bovine brain capillary endothelial cells (BCECs). The uptakes of [^{3}H]glutamate, [^{3}H]β-alanine and [^{3}H]taurine by cultured cells showed that the active transporters function on both the luminal and antiluminal membranes of BCECs. The kinetic parameters for the saturable transport of these amino acids were estimated. For the luminal uptake of taurine, the Michaelis constant, Kt, was 12.1 ± 0.5 μM and the maximum uptake rate, Jmax, was 4.32 ± 0.05 nmol/30 min/mg protein; for the antiluminal uptake, Kt was 13.6 ± 2.4 μM and Jmax was 4.32 ±0.05 nmol/30 min/mg protein. The luminal and antiluminal uptakes of [^{3}H]taurine were each dependent on both Na^+ and Cl^-. Stoichiometric analyses suggest that two Na^+ and one Cl^- are associated with the luminal uptake of one taurine molecule. β-Amino acids such as β-alanine and hypotaurine strongly inhibited the uptake of [^{3}H]taurine, whereas α- and γ-amino acids had little or no effect. Furthermore, by *in situ* brain perfusion and *in vivo* brain capillary depletion methods, the carrier-mediated transport found by *in vitro* experiments was confirmed to function for the translocation of taurine mulecule from the vascular space into the brain. From these results, it was concluded that Na^+ and Cl^- gradient-dependent transport (uptake) system for β-amino acids sensitive for taurine exists in both the luminal and the antiluminal membranes of brain capillary endothelial cells.

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In vivo production of taurine and sulfate in rats

Introduction. Sulfate and taurine are main metabolites of L-cysteine in mammals and are excreted in the urine, and sum of these metabolites constitute over 90% of the total sulfur excreted. We have studied the effect of protein contents in the diet on the excretion of sulfate and taurine in rats. As discussed previously [Amino Acids (1995) 8: 345–352], the relation of intake and excretion of sulfur in rats seems to be in the state of sulfur equilibrium. Therefore, in the present study, we evaluated in vivo production of sulfate and taurine by determining urinary excretion of these metabolites.

Methods. Groups A, B and C of rats (five each) were fed with sulfate- and taurine-free synthetic diets containing 10, 25 and 40% casein, respectively. Single intraperitoneal injection of 5 mmol of L-cysteine/kg of body weight was given on 15th and 22nd day after the start of the synthetic diets. Taurine and hypotaurine contents in the 24-h urine were determined by RP-HPLC after dabsylation, and total sulfate (inorganic plus ester sulfate) by ion chromatography after hydrolysis.

Results. Excretions (μmol/kg of body weight per day) of total taurine (taurine and hypotaurine) and total sulfate after adaptation to synthetic diets (mean ± SD of days 6–14) were: A: 14.2 ± 13.4 and 122.3 ± 39.6; B: 280.4 ± 93.8 and 943.2 ± 144.8; C: 553.4 ± 124.5 and 2975.0 ± 391.0. The mean excretions (μmol/kg of body weight per day) after L-cysteine injection on the 15th and 22nd day were: A: 684.6 ± 192.3 and 2318.5 ± 164.0; B: 818.6 ± 117.0 and 2910.4 ± 107.4; C: 587.8 ± 94.0 and 3149.2 ± 54.6, respectively. Thus, the excretion ratios of taurine to taurine + sulfate (= T) in groups A, B and C were 0.103 ± 0.077, 0.225 ± 0.045 and 0.174 ± 0.044. By cysteine administration, T changed to 0.221 ± 0.059, 0.218 ± 0.019 and 0.162 ± 0.013, respectively.

Thus, when rats were fed with 25 or 40% casein diet, the excretion ratio of taurine (T) was approximately 0.2 (0.174–0.225). This ratio of taurine excretion decreased to 0.1 when rats were fed with 10% casein diet. However, the high ratio of taurine excretion (0.22) was attained when additional cysteine was given to 10% casein diet group.

Summary. The excretion ratio of taurine / (taurine + sulfate) was 0.2 when enough sulfur amino acids were contained in the diet, and when sulfur amino acid content was reduced, the decrease in taurine excretion was bigger than that of sulfate excretion. These results suggest that the ratio of in vivo production of taurine / (taurine + sulfate) is presumably 0.2 when enough sulfur amino acids are supplied, and deficient supply of sulfur amino acids influences the rate of taurine production more strongly than that of sulfate production.

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Both taurine transport and the transport of other amino acids appear to regulate cellular taurine content and hence the beneficial effect of taurine on preimplantation mouse embryo development

Several nonessential amino acids including taurine increase the proportion of 1- and 2-cell mouse embryos that develop into blastocysts in culture. The presence of taurine in the culture medium also increases the number of cells in the resultant blastocysts [Doumoulin et al. (1992) J Reprod Fert 94: 373–380]. The taurine content of embryos developing *in vivo* correlates well with their Na^+-dependent system β transport activity. Moreover, the taurine content of embryos increases both *in vivo* and *in vitro* in the presence of 6.0 mM taurine, whereas the taurine content of embryos decreases when they develop in medium with no added taurine. Hence, uptake of taurine via system β appears to produce the beneficial effects of taurine in preimplantation development. Furthermore, when embryos develop *in vitro* in the presence of 1.0 mM glycine, 0.4 mM L-alanine or 1.0 mM L-glutamate the taurine content of the resultant blastocysts is reduced both in the presence and in the absence of 6.0 mM taurine. Taurine does not compete detectably with α-amino acids for transport via Na^+-dependent and Na^+-independent systems known to be present in preimplantation embryos. Moreover, apparently nonsaturable taurine channels are expressed throughout preimplantation development. These channels open in response to osmotic swelling of the cells in embryos. We propose that the Na^+-dependent uptake of glycine, alanine or glutamate results similarly in the swelling of cells of embryos in culture and that as a result of this swelling, taurine channels open. The open channels would allow taurine that is accumulated via the Na^+-dependent system β to leave the cells of embryos thus reducing their taurine content in the presence of glycine, alanine or glutamate. Furthermore, system β activity is decreased in cleavage-stage embryos as a result of hypotonic swelling of cells and such also may be the case for the swelling proposed to occur as a result of the Na^+–dependent accumulation of glycine or alanine. Na^+-dependent amino acid transport may help the cells of preimplantation embryos to maintain an anabolic state associated with cell swelling when faced with the hypertonic environment in the oviduct *in situ*. If metabolism is controlled in these ways in early embryos, then regulation of their taurine content through the opening or closing of taurine channels and the activation or inactivation of system β coul contribute also to regulation of cell volume and hence to regulation of embryonal metabolism.

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The increase of striatal dopamine concentration by intrastriatal taurine is blocked by diazepam but not altered by β-CCM

It has been suggested that taurine subserves a neurotransmitter or neuromodulator role in the striatonigral pathway (Della Corte et al., 1990). Intranigral infusion of taurine decreases the extracellular striatal concentration of dopamine (DA) in anaesthetised rats, which confirms the inhibitory role of taurine in the control of nigrostriatal Daergic neurons (Ruotsalainen et al., 1996). In contrast, intrastriatal taurine increases striatal DA release in freely moving rats in a tetrodotoxin sensitive manner (Ruotsalainen and Ahtee, 1996). In the present work we studied the effect of diazepam on taurine induced elevation in striatal DA ba in vivo microdialysis. The changes in the extracellular concentrations of DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) during two-hour taurine infusion (2 µl/min; 150 mM) were analyzed by HPLC with electrochemical detection. Taurine administered to the left striatum via a microdialysis probe increased the striatal DA maximally ba 190% of freely moving rats. Extracellular DOPAC was increased maximally by 90% and that of HVA by 30%, respectively. Diazepam (DZP) (5 mg/kg i.p.) given at the beginning of the taurine infusion completely abolished the elevation of striatal DA concentration caused by taurine, but did not alter the increase of DOPAC and HVA. The inhibitory effect of DZP on the elevation of DA concentration caused by taurine was antagonized by flumazenil (10 mg/kg i.p.) given one hour after DZP. DZP and flumazenil alone at the used doses did not affect the striatal DA. The effects of a benzodiazepine inverse agonist, β-CCM (methyl β-carboline-3-carboxylate) were also studied. It had no effect on striatal extracellular DA when administered alone at the dose of 10 mg/kg i.p. Neither had it any effect on the elevated DA concentrationi caused by intrastriatal taurine. However, in combination with β-CCM DZP did not anymore antagonize the taurine-induced elevation of extracellular DA. These results indicate that in the striatum GABA-benzodiazepine receptor complex is at least partially involved in the mediation of the effects of intrastriatal taurine on DA release. Further, the mechanism by which taurine elevates the striatal DA metabolites seems to differ from the one involved in dA release.

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Structure-activity determinants of the effects of taurine (2-aminoethanesulfonic acid) and related sulfur-containing compounds on ethanol-induced oxidative stress in the rat

Experimental evidence is available to indicate that ethanol-induced liver injury may be linked, at least partly, to an oxidative stress caused by excessive free radical production and/or decreased antioxidant reserve. Antioxidant disturbance by an acute ethanol load is invariably manifested by lower hepatic reduced glutathione (GSH) and higher thiobarbituric acid-reactive substances (TBARS) concentrations. Based on the fact that some sulfur-containing compounds (i.e., L-cysteine, hypotaurine, taurine) have demonstrated antioxidant properties in various free radical- and lipid peroxidation-generating systems, the present study was designed to assess the effects of taurine and 22 other structurally related sulfur-containing compounds (2.4 mM/kg, intraperitoneally, IP) on hepatic GSH and TBARS of rats treated with an acute, 4 g/kg, (IP) dose of ethanol. The results have been correlated with certain structural features of the test compounds (i.e., type of sulfur functionality, chain length, N-substitution, and esterification). Sulfinic

acids (hypotaurine, cysteinesulfinic acid) effectively preserved hepatic GSH (> 50%) and lowered TBARS (> 30%). All sulfonic acids (taurine, N-methyltaurine, N-(carbamoylmethyl)taurine, pantoyltaurine, homotaurine, aminomethanesulfonic acid, propanesulfonic acid, propanedisulfonic acid, 3-mercapto-propanesulfonic acid, cysteic acid, homocysteic acid, α-sulfo-β-alanine, isethionic acid) exhibited good antioxidant actions (TBARS decreases 13–69%, GSH increases 9–146%), with a longer chain length increasing the effect. Most sulfhydryl compounds (cysteine, cysteine methyl ester, cysteine ethyl ester, N-acetylcysteine, homocysteine, cystine, cysteamine, N-acetyl-D-penicillamine, thioglycolic acid) decreased TBARS by 46–70% and elevated GSH by 13–125%, excepting cystine and thioglycolic acid, which lowered the GSH content. Among these compounds, the antioxidant action was enhanced by chain lengthening, esterification and N-substitution.

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The effect of clenbuterol on taurine in the heart: Studies *in vivo* and *in vitro*

The importance of taurine in the heart is well known but not well understood [1]. Exposure to β-agonists have been reported to decrease heart taurine levels [2–5]. In order to determine whether this was due to a direct receptor mediated effect on the heart or an extra cardiac effect such as in the liver, two studies were carried out:

i) isolated heart perfusion was carried out to determine if clenbuterol caused an efflux of taurine *in vitro*. Perfusion of the isolated rat heart with medium containing clenbuterol resulted in an increase in efflux of taurine from the tissue. This indicates a direct effect of clenbuterol on cardiac taurine levels occurs.

ii) various β-blockers were used *in vivo* to determine the effect on cardiac taurine depletion by clenbuterol.

The specificity of the effect of β-adrenergic agonists on heart taurine levels was studied by means of administration of specific β-adrenergic antagonists, 1 h before the treatment with either clenbuterol (β_2-selective adrenergic agonist) or isoproterenol (nonspecific β-adrenergic agonist). Female Random-Hooded (RH) rats were divided into groups of similar body weight (150–200 g), consisting of four animals per group. The animals were housed singly in metabolism cages at a constant temperature of 21 ± 1°C on a 12 h light/dark cycle. Food and water were available *ad libitum*. After acclimatisation and the collection of predose urine samples, rats were injected intraperitoneally either with saline solution or with an equivalent volume of saline vehicle containing one of the following β-blockers: propranolol, butoxamine or atenolol (20 mg.kg^{-1} body weight). 60 min after the administration of one of the above β-blockers, each animal was injected subcutaneously either with saline or with an equivalent volume of either clenbuterol (250 μg.kg^{-1} body weight) or isoproterenol (80 mg.kg^{-1} body weight). At 12 h post clenbuterol or isoproterenol administration, the post-dose urine sample was collected over ice and the animals were killed and heart, liver and serum were removed and taken for analysis.

Taurine levels in the heart were significantly reduced by both clenbuterol and isoproterenol by approximately 30% and 43% respectively. While clenbuterol had no effect on taurine in the liver, isoproterenol reduced taurine level by approximately 64%. Clenbuterol and isoproterenol had no effect on serum taurine levels.

Propranolol (20 mg.kg^{-1}; nonspecific β-adrenergic antagonist) prevented totally the reduction in heart taurine levels caused by clenbuterol. However, the specific β_1-adrenergic antagonist, atenolol, prevented up to 13% of this reduction in taurine caused by clenbuterol, whereas the specific β_2-antagonist butoxamine, had no effect on the reduction of heart taurine caused by clenbuterol. Butoxamine had no effect on the reduction in heart taurine caused by isoproterenol but atenolol reduced this effect by about 14%. In the liver neither atenolol or butoxamine affected the reduction in taurine levels caused by isoproterenol.

These data indicate that the decrease in cardiac taurine levels caused by clenbuterol may be mediated by β-receptors, whereas the decrease in the liver is not.

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Is there a correlation between taurine levels and xenobiotic-induced perturbations in protein synthesis? A study with tetracycline in rats

We have previously shown that many hepatotoxic compounds such as those which cause necrosis (eg. carbon tetrachloride, thioacetaminde and galactosamine) and those which cause fatty liver (steatosis) (eg. ethionine and hydrazine) result in elevated levels of urinary taurine [1]. All of these compounds inhibit protein synthesis; a feature of hepatotoxicity. Subsequently we have shown that after dosing with the β-agonists salbutamol and clenbuterol [2] (which increase protein synthesis), or cycloheximide [3] (which reduces protein synthesis) levels of urinary taurine were significantly decreased or increased respectively. There were significant correlations between protein synthesis measured as the incorporation of [^{3}H]-leucine into acid precipitable proteins and taurine levels in serum and urine. We have suggested that measuring urinary taurine levels could be a useful non-invasive method for monitoring changes in protein synthesis. These studies have been continued by following the urinary excretion of taurine after the administration of different dosages of tetracycline to rats.

Study 1. Urinary taurine excretion was monitored before and after dosing rats (male, Han Wistar, 4 groups, 4 rats/group) with 0, 50, 150 or 200 mg.kg^{-1} (free base) tetracycline HCl (saline, pH adjusted to 5.5, i.p.) in order to find the time of maximum elevation of urinary taurine. Clinical signs of toxicity were seen after the top two doses (body weight loss and reduced food consumption). Blood and tissues were taken for biochemical analysis at post mortem 24 h after dosing. Serum albumin, total protein, triglycerides and cholesterol were all lowered significantly by the top two doses, and creatinine and urea were slightly raised suggesting there may have been kidney damage. Urinary taurine was maximally elevated in the 8–12 h urine collection. The lowest dose of tetracycline (50 mg.kg^{-1}) produced no effects.

Study 2. The experiment was repeated with doses of 0, 100, 150 or 200 mg.kg^{-1}. [^{3}H]-Leucine (100 μCi/rat, i.p.) was administered (5 mM leucine in 4 ml.kg^{-1}) to rats 8 h after dosing with tetracycline. Serum and tissues were taken for biochemical analysis at post mortem 10 h after dosing with tetracycline. [^{3}H]-Leucine was measured in acid precipitable proteins from serum, muscle and liver and correlated with taurine levels in urine, serum and liver. There was an increase in urinary taurine and a dose dependent increase in serum taurine 5–10 h and 10 h respectively after dosing with tetracycline. [^{3}H]-Leucine incorporation into liver and muscle, but not serum protein, was reduced. There was a negative correlation between the total

amount of urinary taurine excreted post-dose, serum taurine at the time of post-mortem and [^{3}H]-leucine incorporation into liver and muscle protein taken at post mortem.

The data suggest that urinary taurine levels may be a useful non-invasive marker of perturbations in protein synthesis.

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Characterisation of taurine uptake in MDR and non MDR tumoral cell lines in culture

Clinical cancer treatments are encountering two types of tumours: those which are sensitive to the usual chemotherapeutic agents and those which are resistant. One of the most common mechanism of resistance is related to the Multidrug Resistance phenotype (MDR), caused by the overexpression in the plasma membrane of resistant cancer cells of P-glycoprotein (P-gp), a transporter which extrudes many chemotherapeutic agents from the cells. In order to know if the presence of P-gp could affect other transport functions, we have studied taurine transport by two human MDR carcinoma cell lines (KB-V-1 and LoVoDx) and their chemosensitive counterparts (KB-3–1 and LoVo^s).

Our results show that for all the cell lines, taurine uptake is mediated respectively by a high affinity and a low affinity taurine transporter, and by diffusion of taurine. The two taurine transporters share dependence on sodium, chlore and on hyperosmolarity. They show a high selectivity for β-amino acids. A difference in the transport parameters (Km, Vmax) was observed between chemosensitive and MDR cells.

Chemotherapeutic agents like vinblastine and doxorubicine do not affect taurine uptake by the two KB cell lines, whatever they are present in the incubation medium or in the culture medium. The same observation was made for verapamil, an agent which reverses MDR.

Our results suggest that according to the cell type, the functioning of some other transporters may be altered upon the emergence of the MDR phenotype.

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