15N Investigation into the Effect of a Pollutant on the Nitrogen Metabolism of *Tetrahymena pyriformis* as a Model for Environmental Medical Research

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The impact of chemical, physical, and biological environmental factors on human health must be addressed by scientific risk assessment, a process that requires knowledge of exposure, dose response, and mechanisms. Suitable cell models are frequently used for basic research into effect monitoring at the cellular level. The cell system used for this study was the ciliated protozoan *Tetrahymena pyriformis*. *Tetrahymena*, a typical eukaryotic cell, and mammalian cells have much in common in regard to their nutritional requirements, cell compartmentation, metabolic pathways, and sensitivity to cytotoxic substances (1-5).

Cell culture has been used frequently as a test system for toxicity assessment in pharmacology (4,6,7) and ecotoxicology (8,9). By observing end points such as growth impairment (10), modification of motility (7,11), and inner and outer morphology (6,12,13), only the end result of changes in metabolism caused by harmful substances can be pinpointed. The purpose of this investigation was to identify metabolic changes in normal cell metabolism (biological effect monitoring), namely, at the level of amino acid and protein metabolism. The *Tetrahymena* cells generally require 11 essential amino acids, including arginine (14). Because these eukaryotes lack the urea cycle present in mammals, the final product of nitrogen metabolism is ammonia (15,16).

The harmful substances we are interested in are volatile organic compounds (VOCs) with toluene as the representative compound. As air is the main exposure route for most VOCs, effects on target cells such as alveolar macrophages (17) and bovine bronchoepithelial cells (18) have already been studied. Exposure often occurs repeatedly over a longer period. This may result in accumulation of VOCs in fatty tissue and, after mobilization, in higher blood concentrations (19), which lead to increased exposure of other organs such as the liver. Although various adverse effects of toluene *in vivo* are described in the literature (20-22), the effect of toluene on amino acid metabolism in humans has not yet been satisfactorily clarified (23).

To date only a few articles have been published on 15N measurements of amino acids by gas chromatography/combustion interface–isotope ratio mass spectrometry/mass spectrometry (GC/C-IRMS/MS) coupling (24-28). None of these studies was designed to observe the use of an essential amino acid under the impact of a harmful substance using a 15N-labeled tracer. Therefore, the aim of our study was to determine changes in the use of the 15N-labeled amino acid L-arginine by *Tetrahymena pyriformis* under the impact of toluene using 15N emission spectrometry and a novel GC/C-IRMS/MS coupling for 15N/14N analysis.

We intended to analyze the quantity and 15N concentrations of the end product ammonia, as well as 15N distribution in amino acids and related metabolites. Ultimately, such a system characterizes the impact of specific environmental pollutants on specific steps of the protein metabolism and serves as an early-effect monitoring method.

**Materials and Methods**

*Cultivation*. *Tetrahymena pyriformis* strain W (1630/1W) was obtained from the Culture Collection of Algae and Protozoa (CCAP; Ambleside, UK). The cells were grown in a chemically defined medium with salts, trace salts, and vitamins in accordance with Szablewski et al. (29). The medium contained the 11 essential amino acids (6), albeit at a fourfold concentration to ensure short generation times and high cell numbers to meet the requirements for isotope analyses. Glucose was added as a carbon source after separate autoclaving to a final concentration of 1%. Cells were transferred twice weekly to fresh medium, taking into account a surface-to-volume ratio of 2:1. The generation time was determined to be 7.5 hr. Stock cultures were maintained in conformity with CCAP information.

In the 15N-labeling experiments, we used 24-hr cultures (late exponential growth phase). The labeled defined medium contained 40 mg/l [guanidino-15N₂]l-arginine (Fig. 1) and 1,768 mg/l unlabeled arginine. The 15N enrichment of arginine, calculated by isotope dilution formula, is 2.4 at%. The unit at% expresses the relative 15N-abundance in a sample, which is the ratio of the amount of 15N (mol)/total amount of N (mol). The resulting 15N enrichment of freeze-dried medium was determined to be 0.765 at% by means of elemental analyzer–emission spectrometric coupling (FAN Fischer Analysen Instrumente, Leipzig, Germany). The cells were grown in 500-ml screw-capped glass bottles containing a total volume of 30 ml medium, glucose solution, and inoculated cell suspension. Toluene concentration in toluene-exposed cultures was calculated as 989 µM, but the detected

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exposure concentration was lower. By means of headspace GC (Perkin Elmer, Ueberlingen, Germany), we detected 270 µM toluene at the beginning of the experiment and 150 µM after 24 hr of cultivation in the nutritional medium. The bottles were rotated. After the 24 hr cultivation period, the cultures were centrifuged at 1,000g at 4°C for 10 min. The growth temperature was 28°C. Cells were counted with a Coulter Counter (model ZM; Coulter Electronics GmbH, Krefeld, Germany). Depending on cell number, the sample was diluted with Isoton II (Coulter Euro Diagnostics GmbH, Krefeld, Germany).

**Chemicals.** All chemicals were of analytical grade and were obtained from Serva (Heidelberg, Germany) and Merck (Darmstadt, Germany). Trifluoroacetic anhydride (TFA) was purchased from Merck and [guanidino-15N2]-arginine (95 at%) from Berlin Chemie (Germany).

**Figure 1.** [Guanidino-15N2]-arginine.

**Figure 2.** Ammonia formation during cultivation. (A) Cell proliferation (Z2 = cell enumeration at the beginning of the experiment (t2); Zt = cell enumeration at t; x hours [10]). (B) total ammonia formation (10⁶ cells/ml); (C) 15N ammonia formation (10⁶ cells/ml); (D) ratios of 15N ammonia/total ammonia (values of nonexposed and exposed cells differ significantly according to Wilcoxon test, 0.01≤α >0.001).

**Determination of total ammonia and its 15N enrichment.** Ammonia was isolated from the supernatant by microdiffusion (30). To perform quantitation, the isolated ammonium sulfate was titrated with 0.01 N HCl and evaporated to dryness. An aliquot of 15 µg nitrogen dissolved in 30 µl doubly distilled water was converted to N2 using the Rittenberg procedure for emission spectrometric 15N isotope analysis (31) (NOI-6PC; FAN Fischer Analyisen Instrumente).

**Isolation of nitrogen-containing pools.** Cells were harvested by centrifugation for 10 min at 800g at 4°C after the addition of ice-cold 10% perchloric acid (PCA) to a final concentration of 1% (v/v), washed twice with cold 0.6% PCA (v/v) and with doubly distilled water, and frozen at -80°C. Then frozen cells and frozen medium were freeze-dried (Lyvac GT2; FINNAQUA, Huerth, Germany). For separation into protein and nonprotein nitrogen (NPN), the freeze-dried cells were resuspended in 20 ml of 10% trichloroacetic acid (TCA; w/v), heated in a boiling water bath for 10 min, and allowed to stand overnight at 4°C. The sample was centrifuged for 30 min at 10,000g, and the sediment (total protein) was washed three times with doubly distilled water and freeze-dried.

The supernatant (NPN) was filtered through a glass-fiber filter, and the clear solution was cleaned up by cation exchange (DOWEX 50Wx8, 60–170 mesh, Merck, Germany) (32). The freeze-dried medium was dissolved in 0.1 N HCl and purified in the same way. The eluates containing the amino acids were evaporated on a rotary evaporator and dried first under a gentle stream of helium in a water bath at 60°C and then with methylene chloride. The NPN fraction yielded 1–2 mg as dry weight for analysis. The proteins (10 mg) had to undergo acidic hydrolysis (32) before further preparation.

**Derivatization.** The isolated amino acids were esterified with acidic isopropanol for 1 hr at 110°C, and the residue was dried after evaporation of the solvent with CH3Cl2.

The residue was derivatized with trifluoroacetic anhydride overnight at room temperature using a modified procedure (33). It had been previously demonstrated that this technique does not cause any isotope fractionation (D. Hofmann, personal communication), enabling the isotopic measurement of arginine. Finally, the sample was concentrated under a gentle stream of helium in an ice bath. The amides glutamine and asparagine were hydrolyzed under the acidic conditions of derivatization to glutamic and aspartic acid. The cooling of the autosampler to 5°C improved the reproducibility of the isotopic analysis of the derivatives.

**Isotope and organic mass spectrometry of amino acids.** We analyzed the nitrogen-specific isotopes [15N/14N] of the amino acids of all isolated pools by means of GC/C-IRMS/MS coupling (Finnigan MAT, Bremen, Germany). After gas chromatographic separation of derivatized amino acid mixtures, the eluate was divided for the purpose of combustion and subsequent isotopic measurement of nitrogen and for organic mass spectrometry including MS/MS analyses [for more details, see Metges et al. (26)]. This enabled both the distribution of 15N into the individual amino acids during metabolism as well as the structural elucidation of the metabolites to be determined.

The isotopic composition of the individual amino acid is defined as

\[
8^{15}N = \frac{[^{15}N/^{14}N]_{\text{Sample}} - [^{15}N/^{14}N]_{\text{Standard}}}{[^{15}N/^{14}N]_{\text{Standard}}} \times 1,000\% ,
\]

where [15N/14N] is the ratio of the number of 15N atoms to the number of 14N atoms in the sample or standard. Delta units of 15N/14N ratios are commonly given relative
to a standard ratio, which is 3676.5 ± 8.1 for air, in terms of parts per thousand (per mil, ‰). The standard generally used is atmospheric air, defined as ‰.

**GC/C-IRMS/MS measurements.** The amino acid derivatives were separated on a capillary GC (HP 5890; column: SGE BPX 5, 50 m × 0.32 mm × 0.5 μm). The following temperature program was used: 50°C; held 1 min; ramp in 10 min to 100°C, held 10 min; ramp in 3 min to 175°C, held 5 min; ramp in 7 min to 300°C; held 15 min; injector: 280°C. The GC is connected to a combustion interface (type II, Finnigan MAT; oxidation reactor 980°C, reduction reactor 600°C) coupled to an IRMS (MAT 252) for isotopic analysis. Another part of the GC eluate was applied to an organic mass spectrometer (GCQ, MAT 252, Finnigan MAT).

**Results**

Both total ammonia and its 15N content were enhanced in the toluene-exposed cultures by 30% and 43%, respectively (Fig. 2). The amounts reflect a cultivation period of 24 hr. All detectable amino acids of the cell proteins (except threonine and lysine) showed an increase in 15N enrichment in both the control and toluene-exposed cultures. However, in the toluene-exposed cells the amino acids alanine, glutamic acid, aspartic acid, and tyrosine were additionally enriched by 10–25 delta units (Fig. 3). In a number of chromatograms, additional peaks of nonproteinogenic amino acids such as Nα-acetylarginine and pyrrolidonecarboxylic acid were observed. The structure of Nα-acetylarginine was elucidated using MS/MS technology (data not shown). The mass spectrum of pyrrolidonecarboxylic acid is shown in Figure 4.

In the NPN pool, which contains the sample after cleanup, mainly the free amino acids of the cells, differences were detected in the 15N-enrichment in various amino acids, but so far these differences are not reproducible because of the very low amino acid concentrations. The presence of the metabolites ornithine and aminoadipinic acid was established using organic MS.

Medium samples examined at the end of each cultivation period indicated no significant differences in most amino acid compositions and 15N enrichment (Fig. 5). There were detectable amounts of high enriched glutamic acid in the medium of the toluene-exposed culture. Ornithine was released into the nutritional medium by both control and toluene-exposed cultures.

**Discussion**

This is the first stable isotope (nonradioactive) study into changes induced by pollutants in the amino acid utilization of a cell system related to mammalian cells. With *Tetrahymena pyriformis*, conventional toxicity tests are usual and are performed using certain complex media (6,10–13). In this investigation a combination of the chemically defined media (20) regarding salts, trace salts, and vitamins and of certain amino acid composition (14) was chosen to ensure defined conditions for reproducible isotopic and mass spectrometric analyses. Our working hypothesis was that the impact of a pollutant should be reflected in changes in both the amount and the 15N abundance of nitrogen in the various pools.

The growth of *Tetrahymena pyriformis* is accompanied by the secretion of ammonia as the end product of nitrogen metabolism (which includes many metabolic processes). An increase in ammonia production may be caused by gluconeogenesis and the formation of glycogen from amino acids (34,36), or alternatively by protein degradation under conditions of oxygen deficiency (35). In our case the ammonia production under toluene exposure indicates intracellular metabolic changes with increased deamination activity and increased use of arginine. L-Arginine is an essential amino acid for *Tetrahymena pyriformis* and is a precursor for proline synthesis (37). In the mammalian liver cells, the guanidino group is split off from arginine, as urea and amino acids

**Figure 3.** δ15N values of the individual amino acids of protein hydrolysate (values are means ± standard deviations of five measurements of one derivatized sample of one cultivation experiment; the results were reproducible in three parallel experiments).

**Figure 4.** Mass spectrum of 2-pyrrolidonecarboxylic acid.
The influence of toluene owing to the more greatly enriched tyrosine. The amounts of amino acids in the protein hydrolysate of nonexposed and exposed cells seem to be equal. This will have to be confirmed by quantitative analyses with an internal standard.

Attempts have already been made in occupational medical research (23,42) to identify specific effects on amino acid and protein metabolism resulting from workplace exposures by measuring plasma amino acid concentrations. Due to the lack of isotopic markers, this has only been partly successful because amino acids are involved in many metabolic processes.

To our knowledge, the presence of the metabolite pyrrolidonecarboxylic acid and of N0-acetylated arginine has not yet been reported in publications dealing with Tetrahymena pyriformis. Pyrrolidonecarboxylic acid presents the ring condensation product of glutamic acid and has been proposed as a protective protein end group against proteolytic degradation (43). We assume that this result from intracellular reactions because of the noticeably higher amounts of pyrrolidonecarboxylic acid compared to its formation in standard mixtures. In the arginine pathway of both mammalian cells and Tetrahymena pyriformis, pyrrolinecarboxylic acid is normally formed from glutamic semialdehyde. The existence of the keto group was established by calculations from mass spectrometric data.

Our MS fragmentation study permits the conclusion that arginine is acetylated in the guanidino group. So far only N0-acetylated amino acids are well known compounds in biochemical reactions and have specific functions in metabolism; the most frequently observed acetylated residues are amino acids other than arginine (44). N0-acetylated arginine can be detected in a rare hereditary disorder of the urea cycle, which causes hyperargininemia (45).

The aminoacidic acid detected in the pool of free amino acids (NPN) is a known intermediate of the lysine pathway and ornithine of the arginine pathway. The formation of the latter is caused by physiological activity, as during the derivatization procedure of arginine alone, only traces of ornithine developed. The formation of unlabeled ornithine can take place in arginine catabolism or the formation of labeled ornithine can take place by transamination of glutamic semialdehyde.

The amino acids in the nutritional medium showed no differences. Ornithine and glutamic acid were detected, which conforms with the findings of other authors (14,40). The high 15N enrichment of glutamic acid may point out that this compound is a secretion whose purpose is to release ammonia from the cell. But when there are also detectable amounts of glutamic acid in the control culture, it may be that this indicates 15N abundances similar to those in the toluene-exposed culture. Due to the high concentrations of added amino acids, it is difficult to detect the low concentrations of amino acids secreted by the cells. This problem will have to be addressed in further experiments.

Conclusions

The impact of toluene is reflected in metabolic changes in the use of arginine. Qualitative analysis of amino acids must be augmented by quantitative analysis to assess changes in amino acid synthesis rates. Because of the possibility that arginine serves as an energy source in Tetrahymena pyriformis, examining the use of other essential amino acids would be of interest. In addition, the effect of other harmful substances (including mixtures) could be studied.

Environmental medicine requires effect-monitoring methods to detect effects of exposure to harmful substances in addition to determining internal exposure by analyzing biotransformation products. This pilot study is a first step. We believe that the use of the 15N tracer technique in combination with the sensitive GC/C-IRMS/MS coupling provides a powerful tool for the development of such diagnostic methods.

REFERENCES AND NOTES

1. Elliott AM, ed. Biology of Tetrahymena. Stroudsburg, PA: Dowden, Hutchinson & Ross, 1973.
2. Hausmann K, Braddock PH, eds. Giliates: Cells as Organisms. Stuttgart, Germany: Gustav Fischer Verlag, 1996.
3. Bagley D, Boorman KA, Bruner LH, Casterton PL,
Environmental Health Perspectives • Volume 106, Number 8, August 1998

G. Emissionsspektroskopische 15N-Analytik 1986. Isotopenpraxis 22:181–194 (1986).

Kaiser FE, Gehre C, Lehnert K, Kus KC. Amino acid analysis-hydropolysis, ion-exchange clean-up, derivatization, and quantitation by gas-liquid chromatography. J Chromatogr 94:113–133 (1974).

Siller W, Huthner M, Macko SA, Jung EJ. Stable carbon isotope analysis of amino acid enantiomers by conventional isotope ratio mass spectrometry and combined gas chromatography/isotope ratio mass spectrometry. Anal Chem 63:370–374 (1991).

Ryley JF. Studies on the metabolism of protozoa. 3. Metabolism of the ciliate Tetrahymena pyriformis (Glaucocoma pyriformis). Biochem J 52:483–492 (1952).

Levy MR, Scherbaum OH. Glycogenogenesis in growing and non-growing cultures of Tetrahymena pyriformis. J Gen Microbiol 38:221–230 (1965).

Larsen J, Svensmark B, Nilsson JR. Variations in the growth medium during the culture cycle of Tetrahymena thermophila with special reference to ammonia (NH3), ammonium (NH4+), and pH. J Protozool 35:541–546 (1988).

Hill DL, Chambers P. The biosynthesis of proline by Tetrahymena pyriformis. Biochem Biophys Acta 148:435–447 (1967).

Cynober L, Le Boucher J, Vasson M-P. Arginine metabolism in mammals. Nutr Biochem 6:402–413 (1975).

Deibel RH. Utilization of arginine as an energy source for the growth of Streptococcus faecalis. J Bacteriol 87:988–992 (1964).

Porter P, Blum J, H Erod. Subcellular distribution of aspartate transaminase, alanine amino transferase, glutamate dehydrogenase and lactate dehydrogenase in Tetrahymena. J Protozool 19:375–378 (1972).

Blum J, H Erod. On the regulation of tyrosine transaminase, glutamic dehydrogenase and asparaginase transaminase in Tetrahymena. Exp Cell Res 77:335–346 (1973).

Franzen E. Changes in the amino acid household in connection with industrial medicine. Z ges Hyg 24:12–20 (1978).

Jakubke H-D, Jeschke H, eds. ABC-Chemie. Leipzig: VEB F. Brockhausverlag, 1977.

Lee F-J, Lin L-W, Smith J. A N-acetylcysteine transferred to N-acetyltransferase selectively transfers an acetyl group to NH2-terminal methionine residues: purification and partial characterization. Biochem Biophys Acta 1338:244–252 (1997).

Exmans EL, Alderdieck FC, Maresca BA, Lowenthal AA. Desorption chemical ionization mass spectrometry of guanidino compounds. Anal Chem 56:693–695 (1984).

Reynolds H. Effect of type of carbohydrate on amino acid accumulation and utilization by Tetrahymena. J Bacteriol 104:719–725 (1970).