Journal of Chromatography, 232 (1982) 225—235
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1386

GAS CHROMATOGRAPHIC DETERMINATION OF FREE AMINO ACIDS AND AMINO ACIDS ATTACHED TO TRANSFER RNA IN BIOLOGICAL SAMPLES

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(First received April 13th, 1982; revised manuscript received June 4th, 1982)

SUMMARY

A method was developed to analyze quantitatively free amino acids and amino acids attached to transfer RNA (tRNA) in tissue samples by gas chromatography. Free amino acids were purified by ion-exchange chromatography after deproteinization. Total cellular aminoacyl-tRNA was extracted from rabbit reticulocytes and liver by a modified phenol extraction method under conditions which were designed to prevent deacylation of the attached amino acids. After deacylation and separation from tRNA by pressure ultrafiltration, eighteen amino acids were determined by gas chromatography as their N-heptafluorobutyryl isobutyl derivatives.

INTRODUCTION

Alterations in the levels of specific transfer RNA (tRNA) species have been observed in association with changes in the rate of specific protein synthesis, e.g. in silk gland of silkworms [1], in avian liver during yolk protein synthesis [2], and in rabbit reticulocytes specialized for hemoglobin synthesis [3, 4]. The method generally used to determine the abundance of specific tRNA species in a purified sample of tRNA is that of acylation of radioactive amino acids, one at a time, to the tRNA in vitro. There are several potential sources of error, however, when this method is used. They include variation in the activity and purity of the synthetase, purity of the tRNA sample, nature and composition of the acylation buffer, salt and substrate concentration, etc. It is thus possible that the maximal level of acylation is not achieved in vitro for a given amino acid. On the other hand, the level of aminoacylation achieved in vivo may be lower than what is found in vitro, depending on the physiological state of the cell.

0378-4347/82/0000—0000/$02.75

We have been interested in developing a direct method for determination of amino acids attached to tRNA in vivo. The gas chromatographic method used in this study is sensitive enough to allow analysis of endogenous amino acids attached to a tRNA preparation derived from a single tissue sample (e.g. rabbit liver). Butler et al. [5] have reported results from a similar study. They determined twelve amino acids attached to rabbit liver tRNA as their trifluoroacetyl methyl derivatives. We have analyzed all twenty amino acids (glutamine is determined with glutamic acid and asparagine with aspartic acid) attached to rabbit reticulocyte and liver tRNA as their N-heptafluorobutyryl isobutyl esters using both packed and capillary columns.

MATERIALS AND METHODS

Reagents and standards

Cycloheximide was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), sodium heparin from Medica (Helsinki, Finland) and sodium pentobarbital from Abbot S.A. (Ottignies, Belgium). Triton X-100 was from BDH Chemicals (Poole, Great Britain), and phenylhydrazine, chloroform, phenol and most of the commonly used chemicals were from Merck (Darmstadt, G.F.R.). Sephadex G-100 was from Pharmacia Fine Chemicals (Uppsala, Sweden) and Diaflo ultrafiltration membranes, Type UM 10, from Amicon Co. (Lexington, MA, U.S.A.). Dowex-1 (1-X8-200 Cl^-) and Dowex-50W (50-X8-200, H^+) were purchased from Sigma.

An amino acid calibration mixture (type 1) was obtained from Beckman (Spinco Division, Palo Alto, CA, U.S.A.). It contained 2.500 ± 0.004 m*M* alanine, arginine, aspartic acid, phenylalanine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, proline, serine, threonine, tyrosine and valine, and 1.250 ± 0.004 m*M* cysteine. Norleucine and ornithine were purchased from Fluka (Buchs, Switzerland), and hydroxyproline, tryptophan and cystine were from Merck.

Isobutanol—HCl was prepared by bubbling anhydrous HCl through three traps containing concentrated sulphuric acid and then into isobutanol at 0—4°C. The amount of HCl (3 *M*) was determined by titration. Isobutanol (Merck) was dried and distilled before use. Methylene chloride and ethyl acetate were obtained from Merck and purified by fractional distillation. Heptafluorobutyric acid anhydride was from Fluka.

Gas chromatography

Gas chromatography was carried out with either a Varian Aerograph Model 1400 or a Varian Series 3700 gas chromatograph. Both were fitted with flame ionization detectors and temperature programmer units. The glass column (183 cm × 2 mm I.D.) was packed with Chromosorb W AW (80—100 mesh; Johns-Manville Celite Division, Denver, CO, U.S.A.) coated with 3% (w/w) SE-30 stationary phase (Varian Aerograph, Walnut Creek, CA, U.S.A.). The glass capillary column (25 m × 0.39 mm I.D.) SE-30 (WCOT) was purchased from Varian. Nitrogen was used as a carrier gas. The flow-rate was 20 ml/min in the packed column and 1 ml/min in the capillary column. The temperature program was from 80°C (4°C/min). The pre time was changed from 0 to 8 min.

Attenuation was 32×10^{-11} with the packed column and 32×10^{-12} with the capillary column. With the capillary column, a splitless injection with a delay of 30 sec was used. Direct manual measurement of peak heights was used as the basis for all quantitative calculations. A Servogor 210 potentiometric strip chart recorder was used (Goerz Electro, Vienna, Austria).

tRNA preparation

The rabbits were injected subcutaneously with 2.5% phenylhydrazine solution (pH 7.0) according to a standard schedule [6]. On the seventh day, the rabbits were anesthesized by injection of 60 mg/kg of sodium pentobarbital intraperitoneally. An intraperitoneal injection of cycloheximide (40 mg/kg in saline) was given and after about 5 min the rabbits were bled via direct cardiac puncture. The blood was collected with a heparinized needle and syringe and immediately frozen in liquid nitrogen. All subsequent steps were carried out at 0—4°C.

The frozen blood was weighed, pulverized in a cooled mortar and mixed with one volume of a buffer containing 0.1 *M* potassium acetate, pH 5.0, 25 m*M* KCl, 5 m*M* $MgCl_2$, 0.2 mg/ml sodium heparin, 2% (w/v) Triton X-100 and 1 m*M* cycloheximide. After homogenization in a glass—Teflon homogenizer with ten strokes using a tight pestle, an equal volume of cold, water-saturated phenol was added, and the mixture was shaken in a mechanical shaker at 4°C for 60 min. The phases were separated by centrifugation for 10 min at 27,000 *g*, and the phenol phase re-extracted with one volume of 5 m*M* sodium acetate, 5 m*M* $MgCl_2$, pH 5.0, and one volume of chloroform. The resulting aqueous phase was combined with the aqueous phase from the first extraction and one additional phenol extraction was performed for 30 min at 4°C. The nucleic acids were precipitated from the aqueous phase by addition of 0.1 volume of 20% (w/v) potassium acetate, pH 4.9, and 2.5 volumes of cold ethanol. After mixing, the nucleic acids were allowed to precipitate overnight at —20°C. Livers from untreated, anesthesized rabbits were exposed, cut out and immediately frozen in liquid nitrogen. Extraction of the nucleic acids from frozen tissue was as explained above for frozen blood.

tRNA was purified by passing the RNA sample through a column of Sephadex G-100 (2.5 × 100 cm) in a buffer containing 0.5 *M* NaCl, 10 m*M* $MgCl_2$ and 5 m*M* sodium acetate, pH 5.0. The absorbancy at 254 nm was monitored and the major A_{254}-absorbing peak corresponding to tRNA was precipitated with ethanol, redissolved in 5 m*M* formic acid, pH 5.0, and the absorbance units measured at 260 nm. One A_{260} unit of tRNA is defined as that quantity which, when dissolved in 1 ml of distilled water, gives a solution having an absorbance of 1 at a path length of 1 cm and a wavelength of 260 nm.

Deacylation of aminoacyl-tRNA

The tRNA sample was dialyzed overnight against 5 m*M* formic acid, pH 5.0. After dialysis, the endogenous amino acids were released from the tRNA by adjusting the pH to 9—10 with dilute ammonium hydroxide. The mixture was incubated at 37°C for 120 min and the released amino acids were separated from the tRNA by pressure ultrafiltration using a UM 10 Diaflo membrane.

Free amino acids

Ten grams of frozen rabbit liver were pulverized in a cooled mortar, mixed with 25 ml of 20% trichloroacetic acid and homogenized with a glass—Teflon homogenizer. After centrifugation for 30 min at 32,000 *g*, the supernatant and 2.5 μmol of the internal standard, norleucine, were evaporated with a rotary evaporator and dried with a stream of nitrogen. The residue was dissolved in 0.1 *M* HCl and passed through a cation-exchange resin (Dowex-50W). The resin was eluted with 3 *M* ammonium hydroxide and the eluate dried and further purified by passing through an anion-exchange resin (Dowex-1). The resin was eluted with 3 *M* acetic acid and the eluate evaporated with a rotary evaporator and dried with a stream of nitrogen. The free amino acids were determined either by an automatic amino acid analyzer (Kontron, Zürich, Switzerland) or by gas chromatography.

Derivatization of amino acids

Derivatization of amino acids for gas chromatography was performed essentially as described by MacKenzie and Tenaschuk [7, 8]. Ten nmol of the internal standard, norleucine, was added and the amino acid sample was evaporated at 50° C with a stream of nitrogen. The standard amino acid mixture contained 100 nmol of each amino acid plus norleucine with the exception of cysteine (50 nmol). Isobutanol—HCl (0.25 ml) was added and the solution was heated at 120° C for 30 min in an oven. After cooling to room temperature, excess reagent was evaporated with a stream of nitrogen; 50—75 μl of heptafluorobutyric anhydride were added and the tubes were closed tightly and placed at 150° C for 10 min. After cooling, the contents of the tube were evaporated to dryness with a stream of nitrogen. The amino acid derivatives were dissolved in ethyl acetate and immediately analyzed by gas chromatography. Methylene chloride was used in each evaporating step to dry the sample completely. Co-injection of the sample and acetic anhydride for the on-column acylation of histidine [7, 8] was tested and it resulted in increased yields of histidine in some columns. However, in the analyses presented in this study, the yields of histidine were similar with and without co-injection of acetic anhydride.

RESULTS

tRNA isolation

Cycloheximide treatment of the rabbits prior to liver removal or reticulocyte collection, rapid freezing of the tissue sample in liquid nitrogen, and handling of the tRNA throughout the purification scheme in ice-cold solutions buffered to pH 5.0 were designed to inhibit elongation reactions on ribosomes during tissue collection and also non-enzymatic deacylation of tRNA during purification. The phenol-extracted tRNA eluted as a symmetric peak on Sephadex G-100 gel filtration (not shown). RNA samples were chromatographed in two or three portions to ensure that RNA species of higher molecular weight were not mixed with the tRNA peak during gel filtration. The average yield of tRNA obtained from 100 ml of blood was 3.1 ± 0.5 mg (n = 7) and from 100 g of

rabbit liver 12.3 ± 2.3 mg (n = 6). The latter figure compares well with the value of 11.6 mg reported by Butler et al. [5] for rabbit liver.

Gas chromatography of standard amino acids

Cystine, hydroxyproline, norleucine, ornithine and tryptophan were added to the standard amino acid mixture containing seventeen amino acids (Beckman Amino Acid Calibration Mixture). A representative elution profile on a glass capillary column is shown in Fig. 1. Each peak represents about 50 pmol of injected amino acid with the exception of cysteine (25 pmol). The relative molecular responses (RMR) for individual amino acids were calculated on the basis of peak heights. Results from samples containing unknown quantities of amino acids were calculated using the molecular responses relative to norleucine internal standard. Retention times and elution profiles from the standard amino acid mixture were used as a reference. In uncertain cases,

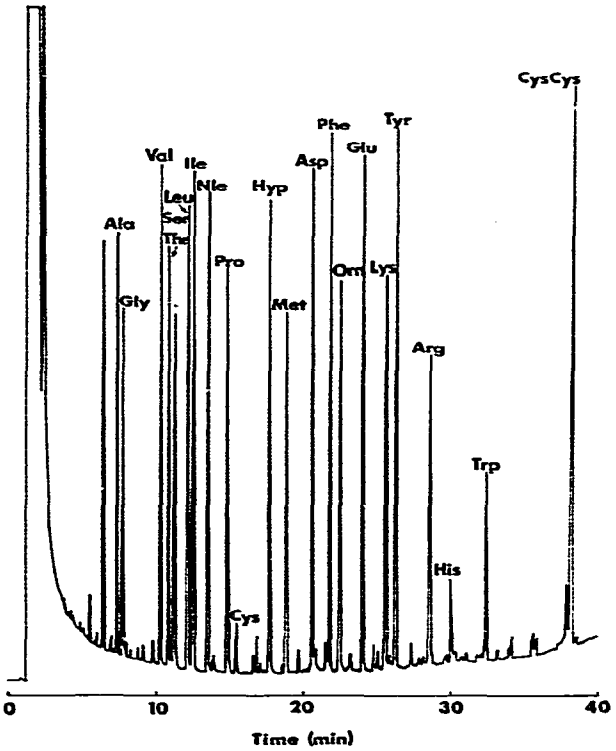


Fig. 1. Gas chromatographic separation on a glass capillary column of N-heptafluorobutyryl isobutyl esters of standard amino acids. Temperature program rate was 4°C/min. Other chromatographic conditions are given in the text.

spiking with amino acid standard injected with the sample was used to identify an unknown peak. All amino acids except glutamine and asparagine were well separated and could be quantitated. Glutamine and asparagine eluted with glutamic acid and aspartic acid, respectively. N-Heptafluorobutyryl n-propyl derivatives were also tested but the isobutyl derivatives gave better yield for

methionine and better separation of ornithine and glutamic acid (results not shown).

Amino acids attached to reticulocyte and liver tRNA

Representative elution profiles of amino acid derivatives from reticulocyte and liver aminoacyl-tRNA are shown in Figs. 2—4. There were a number of unknown peaks when biological samples containing amino acids from both types of tRNA were analyzed. The impurities made an accurate quantification with packed column difficult (Fig. 3). With the capillary column, separation of the individual peaks was much better and quantification of sixteen amino acids and Asn + Asp and Gln + Glu was possible (Table I). The correlation between determinations on packed and capillary columns was good ($r = 0.947$). The results obtained with amino acids derived from reticulocyte and liver tRNA were qualitatively similar. However, with respect to quantities of individual amino acids, certain differences were observed. The relative scarcity of leucine and isoleucine in aminoacyl-tRNA derived from reticulocytes was especially noteworthy (Figs. 2 and 4).

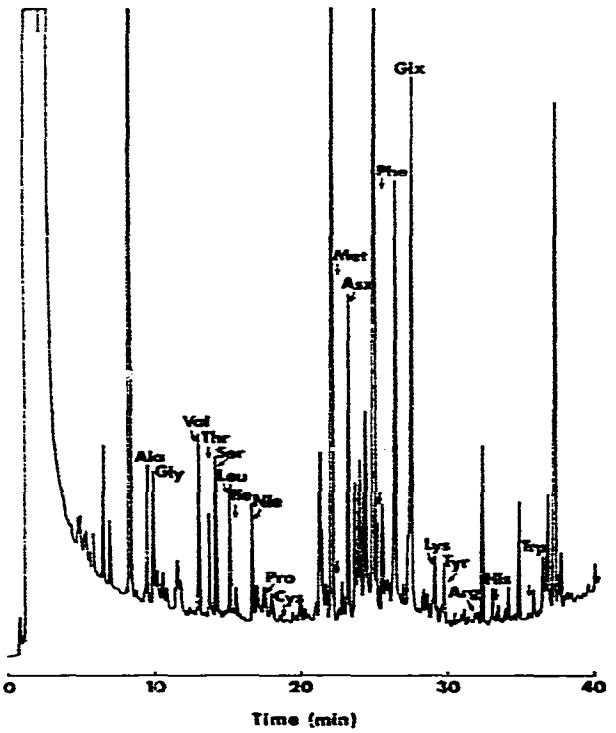
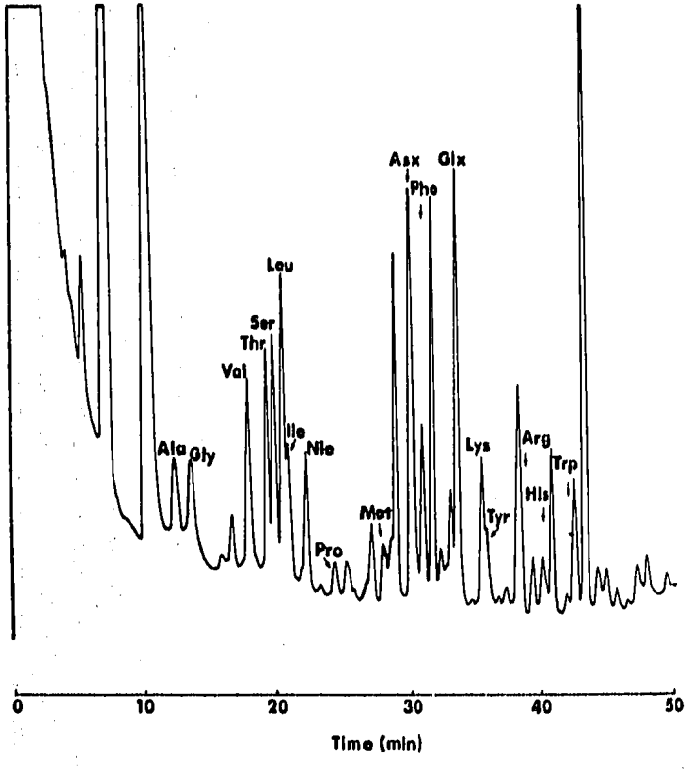


Fig. 2. Gas chromatographic separation on a glass capillary column of N-heptafluorobutyryl isobutyl esters of amino acids derived from rabbit reticulocyte tRNA. Conditions for chromatography are as in Fig. 1.



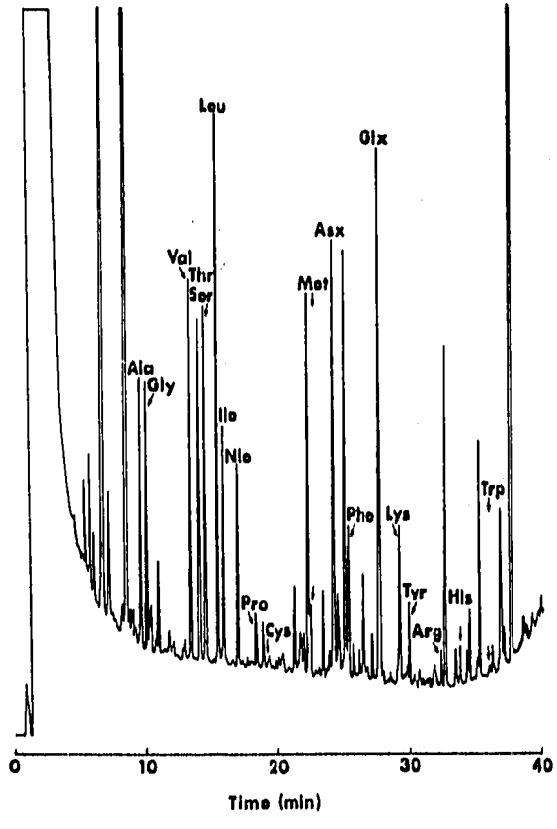


Fig. 3. Gas chromatographic separation on a packed column of N-heptafluorobutyryl isobutyl esters of amino acids derived from rabbit liver tRNA. Conditions of chromatography are given in the text.

Fig. 4. Gas chromatographic separation on a glass capillary column of N-heptafluorobutyryl isobutyl esters of amino acids derived from rabbit liver tRNA. Conditions of chromatography are as in Fig. 1.

TABLE I

AMINO ACIDS ATTACHED TO RABBIT LIVER tRNA IN VIVO

Comparison of results from gas chromatography of a tRNA sample on packed and glass capillary columns (pmol/nmol tRNA, mean ± S.E.M., n = 3). The detection limit for the method was 3 pmol/nmol tRNA.

Amino acid	Packed column	Glass capillary column
Ala	31.9 ± 6.5	45.9 ± 2.4
Arg	—*	6.0 ± 1.4
Asn + Asp	47.8 ± 1.8	39.8 ± 1.9
Cys	—*	—*
Gln + Glu	49.0 ± 3.5	48.4 ± 2.0
Gly	33.6 ± 5.5	49.5 ± 2.7
His	23.5 ± 3.9	23.9 ± 1.4
Ile	20.8 ± 0.8	22.7 ± 0.7
Leu	48.3 ± 2.9	53.5 ± 0.8
Lys	18.7 ± 2.1	15.8 ± 1.0
Met	11.3 ± 1.0	12.3 ± 1.0
Phe	16.6 ± 0.3	12.2 ± 0.8
Pro	5.8 ± 1.0	6.3 ± 0.4
Ser	44.3 ± 1.4	43.6 ± 1.3
Thr	37.9 ± 1.3	38.0 ± 0.5
Trp	6.6 ± 2.4	3.5 ± 0.2
Tyr	8.1 ± 0.7	7.1 ± 0.6
Val	36.5 ± 3.3	44.3 ± 1.4
Total	440.7	472.8

*Value below the detection limit.

Free amino acids

A representative elution profile of free amino acid derivatives from rabbit liver is shown in Fig. 5. Separation and quantification with glass capillary column was relatively simple due to the preliminary purification with ion-exchange resin and the abundance of free amino acids in the liver. Determination of the same samples was also made with a conventional amino acid analyzer (Table II). The results correlated well (r = 0.979). No significant correlation was observed with quantities of free amino acids in liver and amino acids attached to tRNA (r = 0.450) (Tables I and II).

DISCUSSION

There are only a few reports in the literature of a direct determination of amino acids attached to tRNA in vivo. Yegian et al. [9] determined sixteen amino acids attached to *Escherichia coli* tRNA with an automatic amino acid analyzer. They also estimated the acceptor capacity of the tRNA with and without periodate oxidation of tRNA. Smith and McNamara [3, 4] determined the extent of aminoacylation of rabbit reticulocyte tRNA in vivo indirectly by using periodate treatment and enzymatic acylation in vitro with

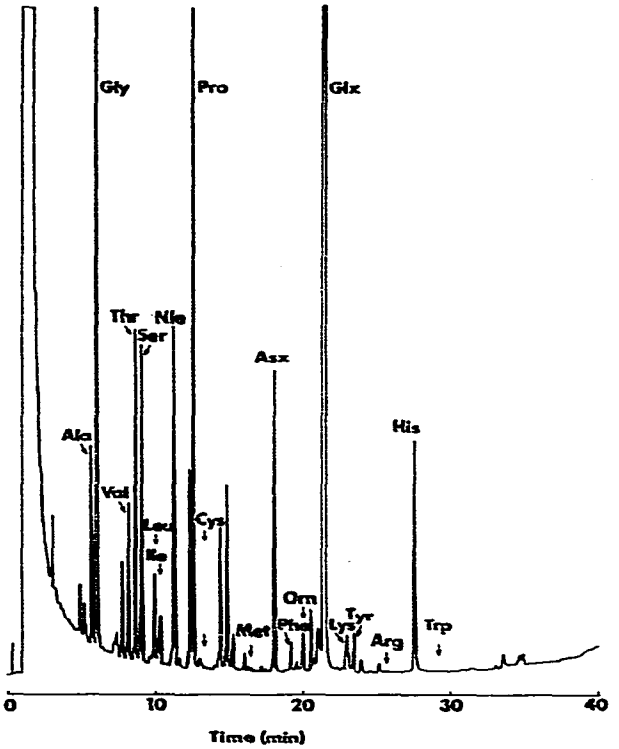


Fig. 5. Gas chromatographic separation on a glass capillary column of N-heptafluorobutyryl isobutyl esters of free amino acids from rabbit liver. Conditions for chromatography are as in Fig. 1.

radioactive amino acids. Butler et al. [5] reported results from a study where twelve amino acids attached to rabbit liver tRNA were determined directly as their trifluoroacetyl methyl derivatives using gas chromatography on packed columns. We have shown here that eighteen amino acids (Gln is determined with Glu, and Asn with Asp) attached to rabbit reticulocyte and liver tRNA can be separated and quantified as their N-heptafluorobutyryl isobutyl esters using gas chromatography on capillary columns. The method also allows analysis of free amino acids in tissue samples.

Determination of amino acids attached to tRNA in vivo involves a number of problems. One is preservation of the aminoacyl-tRNA ester bond during isolation and purification of tRNA. Another is separation and quantification of small amounts of amino acids obtained from tRNA by deacylation. A third factor is interpretation of the results in terms of different forms of tRNA present in the cell. Cycloheximide was given to rabbits prior to collection of reticulocytes or removal of liver to "freeze" the elongation process on ribosomes and thus to maximize the proportion of tRNA that would be in aminoacylated form. Rapid freezing of tissue samples in liquid nitrogen and maintenance of low pH until deacylation in vitro were further efforts to protect the aminoacyl-tRNA ester bond.

Transfer RNA is cycled in the cell through several stages during translation.

TABLE II

FREE AMINO ACIDS IN RABBIT LIVER

Comparison of results from determination of an amino acid sample with gas chromatography on a glass capillary column or with an amino acid analyzer (μmol/g liver, mean ± S.E.M., n = 3).

Amino acid	Gas chromatography	Amino acid analyzer
Ala	0.19 ± 0.007	0.18 ± 0.009
Arg	—*	—*
Asn + Asp	0.21 ± 0.003	0.32 ± 0.02
Cys	—*	—*
Gln + Glu	3.55 ± 0.16	3.20 ± 1.60
Gly	4.21 ± 0.11	5.31 ± 0.75
His	0.60 ± 0.06	0.39 ± 0.03
Ile	0.03 ± 0.001	0.04 ± 0.003
Leu	0.07 ± 0.002	0.07 ± 0.003
Lys	0.03 ± 0.001	0.02 ± 0.001
Met	0.01 ± 0.001	0.01 ± 0.002
Orn	0.03 ± 0.002	0.04 ± 0.003
Phe	0.02 ± 0.001	0.10 ± 0.003
Pro	0.83 ± 0.01	0.32 ± 0.07
Ser	0.29 ± 0.003	0.38 ± 0.007
Thr	0.28 ± 0.001	0.33 ± 0.01
Trp	—*	—*
Tyr	0.02 ± 0.002	0.03 ± 0.003
Val	0.12 ± 0.003	0.14 ± 0.007
Total	10.49	10.88

*Value below the detection limit.

These include free tRNA, tRNA attached to its specific aminoacyl-tRNA synthetase, free aminoacyl-tRNA, aminoacyl-tRNA attached to its specific codon on the ribosome and finally peptidyl-tRNA. Smith and McNamara [3, 4] have estimated by in vitro acylation with and without periodate oxidation that the level of tRNA aminoacylation in rabbit reticulocytes ranges from 71% for tRNA accepting leucine and phenylalanine to most values being close to 90%. The remaining 10% consists of free tRNA and peptidyl-tRNA. The proportion of peptidyl-tRNA probably varies according to the rate of protein synthesis and the amount of ribosomes in the cell. There may also be situations where tRNA aminoacylation is not maximal due to a limited supply of amino acids or energy although high levels of aminoacylation have been found in rat liver during starvation [10]. Some potential sources of error have probably been avoided in the present study by using only well-fed animals and the precautions outlined above during collection of the cells and handling of the aminoacyl-tRNA during preparation. Determination of amino acids attached to tRNA by gas chromatography on capillary columns under these conditions gives an estimate also of the abundance of amino acid specific tRNA species in the cell although a small source of error is included in the form of free and peptidyl-tRNA which are not measured by this method.

Smith and McNamara [3, 4] found that only about half of the material absorbing at 260 nm and eluted as a single symmetrical peak from the Sephadex G-100 column accepts amino acids in vitro. This was confirmed in the present study, since only about 47% of liver tRNA contained attached amino acids (Table I). The nature of the "inactive" tRNA is not known, but it may consist of incomplete tRNA molecules lacking the CCA end or being partly degraded, tRNAs with altered modifications, or RNA molecules other than tRNA.

Taken together, separation and quantification of free amino acids and amino acids attached to tRNA in vivo from a single tissue sample is shown by a direct gas chromatographic method. The method is rapid and sensitive and yields data for all amino acids in a single chromatographic run. Further, it gives information on tRNA which may not be possible to obtain from in vitro experiments. Other uses of the method include studies of in vivo acylation of tRNA in situations where the supply of amino acids or energy may become limiting for protein synthesis.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Signe and Ane Gyllenberg Foundation, the Sigrid Jusélius Foundation and the Research Council for Natural Sciences, Academy of Finland. Hanna Heikkinen and Maija Hiltunen provided excellent technical assistance. We wish to thank Dr. André Darbre and Dr. Jasbir Chauhan for their advice. Part of this study was performed during a stay of L.L. at the Department of Biochemistry, King's College, University of London (EMBO Short Term Fellowship). Dr. Matti Laitinen performed the analysis of free amino acids with an automatic amino acid analyzer.

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