Amino Acids Attached to Transfer Ribonucleic Acid *in vivo*

By MICHAEL BUTLER,* ANDRÉ DARBRE and HENRY R. V. ARNSTEIN

Department of Biochemistry, University of London King's College, Strand, London WC2R 2LS, U.K.

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1. tRNA was extracted from rabbit liver by both the phenol and diethyl pyrocarbonate methods under conditions preventing deacylation of the amino acids attached *in vivo*. 2. After deacylation 12 amino acids were determined by gas-liquid chromatography, by using the flame-ionization and nitrogen-sensitive thermionic detectors. 3. Comparison of the distribution of 12 amino acids attached to tRNA with those contained in total tissue protein and in the free pool showed little correlation. 4. Results for the enzymic charging assay for tRNA *in vitro* did not correlate satisfactorily with the analysis of amino acids attached to tRNA *in vivo*. Marked differences were noted in comparisons made between our own and other published results.

Transfer RNA exists as different molecular species which are characterized by the amino acid capable of attachment or charging on to the 3-terminal adenylate residue (Hecht *et al.*, 1959; Staehelin, 1971; Deutscher, 1973). The concentrations of tRNA specific for the different amino acids may vary with different cells and tissues (Weiss & Kelmers, 1967; Marshall *et al.*, 1967; Caskey *et al.*, 1968; Yang & Novelli, 1968; Muench & Saffile, 1968; Gallo & Pestka, 1970), and also as a result of cell differentiation, viral infection and neoplasia. Merrick & Dure (1972) found no differences in the concentrations of cytoplasmic tRNA species between cotyledons and roots of cotton seed, but found increases of chloroplastic tRNA during early germination.

Itano (1965) first suggested that the pattern of tRNA could reflect the protein synthesized and, if this were true, then haemoglobin synthesis might be controlled by the limiting amount of any single tRNA type. Smith & McNamara (1971) suggested that in general the relative tRNA content of different kinds of cells in the same animal was similar, but that changes could occur in response to specific needs and their own results pointed to the specialization of the tRNA species in rabbit reticulocytes for haemoglobin synthesis. Similar specialization was indicated for tRNA in reticulocytes of mouse (Yang, 1971) and sheep (Litt & Kabat, 1972), the silk glands of silkworms (Garel *et al.*, 1970), fibroblasts of healing wounds (Lanks & Weinstein, 1970) and livers of roosters treated with oestrogenic hormones (Mäenpää & Bernfield, 1969).

The method generally used to determine the relative content of these tRNA types in a purified

sample of tRNA is by acylation of radioactive amino acids on to the tRNA *in vitro* (Bollum, 1959; Mans & Novelli, 1961; Scott, 1968). This involves incubation with an aminoacyl synthetase preparation. The extent of radioactive amino acid attachment to tRNA is measured by trapping the precipitated tRNA on a filter disc. There are serious limitations. The capacity of tRNA to accept amino acids *in vitro* is measured with one amino acid at a time, and also this may not reflect the degree of charging of tRNA *in vivo*. This is an important distinction because it has been shown that the extent of charging of tRNA *in vitro* depends on many variables, including pH, buffers, salts and substrate concentrations (Rubin *et al.*, 1971). Thus the maximum charging for a particular amino acid is not necessarily achieved and the efficiency of charging under one set of conditions is certainly not the same for each amino acid.

The direct analysis of amino acids attached to tRNA *in vivo* is difficult because of the small quantities normally obtainable. Methods were originally developed for bacteria. These were cultured in a medium with radioactive amino acids, and the radioactive content of extracted tRNA was determined for *Escherichia coli* (Lacks & Gros, 1959; Yamane, 1965) and three other bacterial species (Yamane, 1965). Such methods usually resulted in low yields when these were applied to mammalian tissues (Delihias & Staehelin, 1966). Davey & Manchester (1969) reported on the difficulties of measuring radioactive amino acid incorporation into tRNA of incubated rat diaphragm or perfused heart. These studies *in vivo* were limited to five amino acids which were used one at a time.

Only one report was found in the literature of the direct determination of more than one amino acid charged on to tRNA *in vivo* (Yegian *et al.*, 1966). The tRNA was extracted from cultures of *E. coli* and, after deacylation, 16 amino acids were deter-

* Present address: Department of Chemistry and Biology, Manchester Polytechnic, Chester Street, Manchester M1 5GD, U.K.

mined with an automatic ion-exchange amino acid analyser. These workers also determined the acceptor capacity of the tRNA with and without periodate treatment. Smith & McNamara (1974) deduced the extent of aminoacylation of tRNA *in vivo* in rabbit reticulocytes by making similar determinations, with and without periodate treatment. But they assayed acceptor capacity with labelled amino acids, one at a time.

In this paper we describe a method for directly determining the amino acids attached to tRNA *in vivo*. The amino acids deacylated from tRNA were converted into their trifluoroacetylated methyl ester derivatives and determined simultaneously by g.l.c. Our studies were limited to 12 amino acids. A preliminary report of some of this work has been given (Butler *et al.*, 1973).

Materials and Methods

Materials

Chemicals. Unlabelled amino acids, bentonite, 8-hydroxyquinoline, trifluoroacetic anhydride, specially dried methanol and A.R. grades of acetic acid, acetyl chloride, NH_3 , KOH and water were purchased from BDH Chemicals (Poole, Dorset, U.K.); trichloroacetic acid and phenol were from Fison's Scientific Apparatus (Loughborough, Leics., U.K.); ethanol (99.8%) was from James Burrough (60 Mountford Place, London S.E.11, U.K.); ATP was from Boehringer (Mannheim, Germany); diethyl pyrocarbonate (Baycovin) was from Bayer (Leverkusen, Germany); Dowex 50W (X8) and Dowex 1 (X8), mesh sizes 18–52, and salt-free, crystallized and freeze-dried ovalbumin were from Sigma Chemical Co. (P.O. Box 14508, St. Louis, Mo., U.S.A.); Sephadex G-100 was from Pharmacia (Uppsala, Sweden). Uniformly ^{14}C -labelled L-amino acids were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [L-alanine (75 mCi/mmol), L-aspartic acid (100 mCi/mmol), L-glutamic acid (125 mCi/mmol), L-glycine (50 mCi/mmol), L-isoleucine (150 mCi/mmol), L-leucine (150 mCi/mmol), L-methionine (25 mCi/mmol), L-phenylalanine (225 mCi/mmol), L-proline (125 mCi/mmol), L-serine (75 mCi/mmol), L-threonine (100 mCi/mmol), L-valine (125 mCi/mmol) and a protein hydrolysate (57 mCi/matom)]. Whole rabbit livers were obtained from Honee Bun Farm Products (Little Venn, Woolsey, Bideford, N. Devon, U.K.). They were frozen in liquid air immediately after excision from the animals and stored at -20°C until used.

Gas-liquid chromatography materials. Stationary phases and support materials were purchased as follows: XE 60 silicone gum (Applied Science Laboratories, P.O. Box 140, State College, Pa., U.S.A.), Silicone (fluoro)FS1265 (QF-1) and HP Chromosorb W, 80–100 mesh (F & M Scientific

Division, Hewlett-Packard, Avondale, Pa. 19311, U.S.A.), MS 200 100 cS silicone fluid (Hopkin and Williams Ltd., P.O. Box 1, Romford, Essex, U.K.).

Apparatus. G.l.c. was carried out with a Pye 104, model 24 gas chromatograph fitted with dual flame-ionization detectors and a similar one fitted with a Pye nitrogen-sensitive thermionic detector (rubidium chloride electrode) which was housed in a separate isothermally controlled oven. Honeywell-Brown 10mV, 1s (Honeywell Controls, 411 Taunton Road, Greenford, Middx., U.K.), and Speedomax W 1mV, 1s (Leeds and Northrup, Wharfedale Road, Tyseley, Birmingham, U.K.), strip chart recorders were used. Peak areas were determined with Kent Chromalog 2 (Kent Instruments, Luton, Beds., U.K.) and Vidar Autolab 6300 (Anachem, 636 Hitchin Road, Luton, Beds., U.K.) digital integrators. Nitrogen (99.9% 'white spot' from British Oxygen Co., East Lane, North Wembley, Middx. 8A9 7PT, U.K., and high-purity oxygen-free from Air Products, New Malden, Surrey, U.K.) was used as carrier gas. The glass g.l.c. column (3.25m $\times$ 2.5mm internal diam.) was packed with HP Chromosorb W 80–100 mesh, coated with 2.5% (w/w) of mixed silicone stationary phase consisting of XE 60, QF-1 and MS 200 (46, 27 and 27% respectively by weight) (Darbre & Islam, 1968). Modification of the instrument to allow for additional N_2 gas supply to the thermionic detector was as described by Butler & Darbre (1974).

Methods

tRNA preparation. Frozen rabbit livers were used throughout this work. Two methods were used for the extraction of nucleic acids from liver homogenate. One was a modification of published methods by the use of phenol (Kirby, 1956; Holley *et al.*, 1961) and the second made use of diethyl pyrocarbonate (Rosén & Fedorcsák, 1966; Solymosy *et al.*, 1968). Conditions were selected to minimize any possible deacylation of the tRNA during the stages of its isolation and purification. It has been shown that one of the most labile ester bonds (alanyl-tRNA) is stable at pH 5.1 (Judes & Jacob, 1972) and extraction was therefore carried out at pH 5.0 and as far as possible all operations were at 0–4°C.

Stage 1(a). Phenol method. Rabbit liver (100g) was homogenized for 10min in a Waring blender with 75ml of a solution containing 0.25M-sucrose, 10mM- MgCl_2 , 0.5mM-EDTA, 0.1M-potassium acetate buffer at pH 5.0 and 0.1% (w/v) bentonite and 75ml of water-saturated phenol containing 0.1% (w/v) 8-hydroxyquinoline. The homogenate was centrifuged at 23000g for 15min and the upper aqueous layer removed for stage 2.

Stage 1(b). Diethyl pyrocarbonate method. Rabbit liver (100g) was homogenized for 5min with 200ml of a solution containing 0.25M-sucrose, 10mM- MgCl_2 ,

0.5mM-EDTA, 0.1M-potassium acetate buffer at pH5.0 and 2.5% (v/v) diethyl pyrocarbonate. The homogenate was quickly brought to 37°C over a steam bath with stirring and maintained at 37°C for 10min. During this time expansion occurred owing to evolution of CO₂. The homogenate was centrifuged at 23000g for 30min and the clear yellow supernatant was used for stage 2.

Stage 2. The solution from stage 1 was added to 2.5vol. of ice-cold ethanol containing 2% (w/v) potassium acetate. The white flocculent material was allowed to precipitate for at least 2h or overnight at -20°C. After centrifugation at 23000g for 5min the supernatant was decanted and the precipitate dried with a stream of N₂. Traces of ethanol remaining caused difficulties in solubilizing the precipitate at the next stage.

Stage 3. The off-white solid in a large flask was shaken for 16h with 15ml of a solution containing 2M-LiCl, 10mM-MgCl₂ and 0.1M-potassium acetate buffer at pH5.0. After centrifugation at about 5000g the supernatant was removed for stage 4. When larger amounts of liver were used it was necessary to repeat the extraction with the LiCl-containing solution.

Stage 4. Fractions of the LiCl extract (10ml) were loaded on a column (100cm×1.8cm internal diam.) of Sephadex G-100 at room temperature (see Delilhas & Stachelin, 1966). The eluting buffer (0.1M-potassium acetate buffer at pH5.0 containing 10mM-MgCl₂) was passed through the column at 12–20ml/h and monitored with a Uvicord 1 u.v. absorptiometer at 254nm. Fractions (10ml) were collected. The volume of the pooled tRNA fractions was about 100ml. When ethanol (2.5vol.) was added to precipitate the tRNA recoveries were only 40–50%. The alternative method was to freeze-dry the pooled fractions. The residue was dissolved in about 3ml of eluting buffer and the excess of salt removed by dialysis against two changes of 1 litre of eluting buffer (0.01M-potassium acetate at pH5.0 containing 10mM-MgCl₂) for 4h each time.

Deacylation of the tRNA. The aminoacyl-tRNA species are alkali-labile. Sarin & Zamecnik (1964) used 2M-Tris buffer at pH8.0 for deacylation but Tris gave a large g.l.c. peak which interfered with the amino acid analysis. Instead, we added 2ml of 0.5M-NH₄Cl buffer at pH9.0 to each 3ml of tRNA solution and incubated this for 90min at 37°C, when ice-cold ethanol (2.5vol.) containing 2% (w/v) potassium acetate was added and left to stand at -20°C. Complete precipitation of the deacylated tRNA was achieved by centrifugation at 5000g for 10min. The deacylated tRNA was retained for enzymic aminoacylation studies. The supernatant was analysed for amino acids by g.l.c.

Aminoacyl-tRNA synthetase preparation. This was a modification of the method of Hoagland *et al.*

(1956). Fresh rabbit liver (10g) was homogenized in 25ml of cold 0.05M-NaCl. After centrifugation at 100000g for 90min, the supernatant was adjusted to pH5.0 with 0.1M-HCl. The resulting precipitate obtained by centrifugation at 38000g for 10min was dissolved in 5ml of Tris-HCl buffer (0.01M) at pH7.6 containing 5mM-2-mercaptoethanol and 20% (v/v) glycerol. Undissolved material was removed by centrifuging and discarded. The protein concentration of the supernatant was diluted to a concentration of 10mg of protein/ml, by reference to the *E*₂₈₀ of a standard solution of ovalbumin.

Aminoacylation assay of tRNA. This was a modification of the assay system commonly used for charging tRNA *in vitro* (Bollum, 1959; Mans & Novelli, 1961; Scott, 1968). Each assay (0.2ml final vol.) was carried out in a small glass vial which contained 0.1ml of a mixed solution [MgCl₂ (0.15M), ATP (0.1M), Tris-HCl buffer (0.5M) at pH7.6 (1:1:1, by vol.)], 10μl of deacylated tRNA (approx. 5mg/ml), 5μl of uniformly ¹⁴C-labelled L-amino acid (0.1M) and 75μl of double-glass-distilled water (or BDH Chemicals pure water). In the experiment on the effect of pH on the charging reaction (Table 4) the Tris-HCl buffer was replaced by 0.5M-Tris-maleate buffer. The final solution was incubated for 5min at 37°C and the reaction started by the addition of 10μl of aminoacyl synthetase (10mg of protein/ml). After incubation for 30min at 37°C, 1ml of cold trichloroacetic acid (5%, w/v) was added to stop the reaction. The contents of the reaction vial were filtered under suction through a glass-fibre filter (Whatman, GF/C) and washed with 5ml of trichloroacetic acid (5%, w/v) and 15ml of ether-ethanol (1:1, v/v). The filter disc was mounted on an aluminium planchet for radioactive counting with a Tracerlab gas-flow counter. Controls were carried out for non-specific adsorption of free radioactive amino acids on the filter disc, endogenous tRNA-aminoacylating activity of the enzyme preparation and non-enzymic adsorption of radioactivity on to tRNA.

Hydrolysis of liver protein. About 50mg of rabbit liver was hydrolysed in a closed Rotaflo stopcock (Darbre, 1971) with 3ml of 6M-HCl at 110°C for 24, 48 and 72h. Norleucine (0.5mg) was added as internal standard.

Extraction of free pool amino acids in liver. Rabbit liver (about 10g) was homogenized in 25ml of trichloroacetic acid (20%, w/v) and the solution stirred for 15min. Protein was removed by centrifugation at 38000g for 30min. The supernatant was removed and norleucine (0.3mg) added as internal standard.

Preparation of samples for g.l.c. This was a modification of the method used for blood and urine (Zumwalt *et al.*, 1970). The dried samples were dissolved in 5ml of HCl (0.1M) and this solution was passed through a glass column (4cm×1.1cm internal

diam.) packed with Dowex 50W cation-exchange resin in the H^+ form. The column was washed with double glass-distilled water (50–100ml) passed through the column at about 4ml/min by using pressure from a N_2 cylinder. The amino acids which were attached to the resin by their basic groups were eluted with 25ml of NH_3 (3M) at a flow rate of 4ml/min. The eluate was freeze-dried or taken to dryness with a stream of N_2 . The residue was dissolved in 5ml of KOH (0.1M) and passed through a similar column containing Dowex 1 anion-exchange resin in the acetate form. After being washed with water as before, the amino acids were eluted with 25ml of acetic acid (3M). The amino acid solution was taken to dryness over a hot-water bath with a stream of N_2 . Volatile solutes were removed in this way and the method proved better than freeze-drying or rotary evaporation.

G.l.c. of amino acids. Trifluoroacetyl amino acid methyl esters were prepared and separated on a mixed silicone stationary phase (Darbre & Islam, 1968). For the analysis of both total protein and free pool amino acids, the fully modified amino acids were dissolved in 500 μ l of ethyl methyl ketone and 1 μ l portions were injected on to the column by microsyringe. However, the tRNA amino acids were only available in limited quantities and from 100g of liver, all the modified amino acids obtained after deacylation of the tRNA were required for a single g.l.c. injection (Butler *et al.*, 1973). Islam (1970) originally described a method that combined the techniques of solid injection (Darbre & Islam, 1970) and gaseous trifluoroacetylation of amino acid methyl esters (Teuwissen & Darbre, 1970). This involved the transfer of amino acid methyl esters to a small glass capillary tube, evaporating excess of solvent and exposing the solid material to gaseous trifluoroacetylation for 30min at 30°C. The glass tube containing the fully modified amino acids was inserted on to the g.l.c. column (Darbre & Islam, 1970) for analysis with the flame-ionization detector.

At a later stage in our work the nitrogen-sensitive thermionic detector was available and, because of its greater sensitivity (Butler & Darbre, 1974), it was possible to inject smaller amounts of amino acids. From 100g of liver, the modified amino acids from the isolated tRNA were dissolved in 20 μ l of methyl ethyl ketone and 1 μ l samples were injected on to the g.l.c. column.

Results

tRNA isolation

The LiCl extract from rabbit liver, as described in the Materials and Methods section, was passed through a column packed with Sephadex G-100. The profile showing the peak for tRNA is shown in

Fig. 1. Small quantities at a time were chromatographed because Delihias & Staehelin (1966) obtained a slight shoulder on the leading edge of the tRNA peak when large amounts of material were used. The average yield of tRNA obtained from 100g of rabbit liver was $8.2mg \pm 0.3s.e.m.$ for seven diethyl pyrocarbonate extractions and $11.6mg \pm 0.9s.e.m.$ for five phenol extractions.

Total protein and free pool amino acid analysis

The g.l.c. separation of 12 amino acids from total liver protein and the free pool are shown in Figs. 2 and 3 respectively. The flame-ionization detector was used and the results were calculated by using the molar responses relative to norleucine internal standard (Islam & Darbre, 1972). The peak for trifluoroacetic acid resulting from the use of trifluoroacetic anhydride varied in size from sample to sample and because of its relatively high b.p. (72.4°C) it could not be removed by evaporation without danger of losing some of the more volatile amino acid derivatives (Darbre & Islam, 1968; Islam & Darbre, 1969). The separation of free pool amino acids (Fig. 3) showed a number of small peaks due to unknown compounds having retention times between 25 and 32min. The prior purification of the sample with cation- and anion-exchange resins used by Zumwalt *et al.* (1970) did not eliminate these peaks. In Table 1 the values are given for 12 amino acids present in total protein and free pool, both as mmol/100g and mol/100mol in whole rabbit liver. Methionine was present in small quantities and the error of determination was large. The very high values for free pool glutamic acid (24.9mol/100mol) and glycine (42.9mol/100mol) are noteworthy.

Fig. 1. Gel filtration of a LiCl extract of nucleic acids from rabbit liver on Sephadex G-100

For conditions see the Materials and Methods section. Peaks: 1, DNA and RNA; 2, tRNA; 3, low-molecular-weight contaminants.

Fig. 2. Separation by g.l.c. of trifluoroacetyl-amino acid methyl esters derived from total protein of rabbit liver

The liver (50mg) was hydrolysed with 6M-HCl at 110°C for 48h. The sample was purified with cation- and anion-exchange resins and subsequently modified and taken up in 500 μ l of ethyl methyl ketone. G.l.c. was carried out with the instrument and the column described in the Materials and Methods section. The flame-ionization detector was used. Inlet heater block temperature 230°C; N₂ gas flow rate 15ml/min; sample size 1 μ l; attenuation 1×10^{-9} A for full-scale deflexion; initial temperature 80°C programmed at 2°C/min to 140°C. Peaks: 1, solvent; 2, trifluoroacetic acid; 3, alanine; 4, valine; 5, glycine; 6, isoleucine; 7, threonine; 8, leucine; 9, norleucine; 10, serine; 11, proline; 12, aspartic acid; 13, methionine; 14, glutamic acid; 15, phenylalanine.

Fig. 3. Separation by g.l.c. of trifluoroacetyl-amino acid methyl esters derived from the free pool of rabbit liver

The extract prepared from 10g of rabbit liver was purified and analysed by g.l.c. as described in Fig. 2, except that attenuation was 2×10^{-9} A for full-scale deflexion. Peaks: 1, solvent; 2, trifluoroacetic acid; 3, alanine; 4, valine; 5, glycine; 6, isoleucine; 7, threonine; 8, leucine; 9, norleucine; 10, serine; 11, proline; 12, aspartic acid; 13, methionine; 14, glutamic acid; 15, phenylalanine.

Table 1. Amounts of 12 amino acids present in total protein and free pool of whole rabbit liver tissue

The amino acids were determined by g.l.c. with the use of the flame-ionization detector. Duplicate results are given for total protein after 48h hydrolysis with 6M-HCl at 110°C. Free pool amino acid results are the means of five independent extractions and determinations.

	Total protein		Free pool	
	mmol/100g	mol/100mol	mmol/100g	mol/100mol ($\pm$ S.E.M.)
Ala	13.1	13.6	0.26	14.0 $\pm$ 0.8
Asp	15.6	15.3	0.06	3.1 $\pm$ 0.3
Glu	19.2	17.6	0.46	24.9 $\pm$ 1.5
Gly	14.0	14.5	0.79	42.9 $\pm$ 1.4
Ile	8.9	9.2	0.02	1.0 $\pm$ 0.1
Leu	16.9	18.0	0.05	2.5 $\pm$ 0.2
Met	2.6	1.4	0.01	0.3 $\pm$ 0.1
Phe	7.7	7.3	0.03	1.6 $\pm$ 0.1
Pro	8.2	8.2	0.03	1.4 $\pm$ 0.4
Ser	8.3	8.6	0.06	3.4 $\pm$ 1.0
Thr	8.1	8.1	0.05	2.8 $\pm$ 0.4
Val	12.6	13.1	0.04	2.1 $\pm$ 0.1
Total	135.2	134.9	1.86	100.0

Amino acids attached to tRNA

The distribution of 12 amino acids attached to tRNA *in vivo* was determined by independent methods: tRNA was extracted, either with diethyl pyrocarbonate or phenol, and the deacylated amino acids were determined by g.l.c., by using either the flame-ionization detector or the nitrogen-sensitive thermionic detector.

Fig. 4 shows the g.l.c. separation of amino acids obtained from tRNA recorded with the flame-ionization detector. The exceptionally large tailing peak of trifluoroacetic acid resulted from trifluoroacetylation of the sample in the gaseous phase. The peak size increased with the amount of methanolic HCl which was used to transfer the amino acid methyl esters to the pre-column for exposure to trifluoroacetic anhydride vapour. The base line was not steady and there were some peaks due to carbon

Fig. 4. Separation by g.l.c. of trifluoroacetyl-amino acid methyl esters derived from tRNA

The amino acids were deacylated from 5 mg of aminoacyl-tRNA and purified by passage through cation- and anion-exchange resins as described in the Materials and Methods section. The entire amino acid sample was trifluoroacetylated and subsequently injected in a small capillary tube which was dropped into the inlet heater zone above the g.l.c. column. The g.l.c. conditions were as in Fig. 2, except that attenuation was 5×10^{-10} A for full-scale deflexion. Peaks: 1, solvent; 2, trifluoroacetic acid; 3, alanine; 4, valine; 5, glycine; 6, isoleucine; 7, threonine; 8, leucine; 9, norleucine; 10, serine; 11, proline; 12, aspartic acid; 13, methionine; 14, glutamic acid; 15, phenylalanine.

compounds of unknown origin, e.g. those with retention times at 15, 22, 38 and 43 min. The g.l.c. record in Fig. 4 should be compared with that in Fig. 5 where the amino acids from a different sample of tRNA were determined with the nitrogen-sensitive thermionic detector. The attenuation of detector response was the same, but the sensitivity of the thermionic detector was demonstrated because only 4% of the amount of amino acids used in Fig. 4 were injected to obtain Fig. 5. In Fig. 5 the solvent peak was negligible and the trifluoroacetic acid peak did not interfere with the more volatile amino acids. Peaks of unknown origin were negligible. In addition, trifluoroacetylation in the liquid phase and injection by microsyringe on to the g.l.c. column were more convenient. The thermionic detector is flow-sensitive and the drift in the baseline after 20 min was probably caused by column changes during temperature programming.

Table 2 presents the results for the mol percentage distribution of 12 amino acids attached to tRNA *in vivo*. These were obtained after

Fig. 5. Separation by g.l.c. of trifluoroacetyl-amino acid methyl esters derived from tRNA

The sample was obtained from tRNA as in Fig. 4. The entire amino acid sample was modified and taken up in 25 μ l of ethyl methyl ketone. The g.l.c. conditions were as in Fig. 2, except that attenuation was 5×10^{-10} A for full-scale deflexion. The nitrogen-sensitive thermionic detector was used. Peaks: 1, solvent; 2, trifluoroacetic acid; 3, alanine; 4, valine; 5, glycine; 6, isoleucine; 7, threonine; 8, leucine; 9, norleucine; 10, serine; 11, proline; 12, aspartic acid; 13, methionine; 14, glutamic acid; 15, phenylalanine.

Table 2. *Molar percentage distribution of 12 amino acids attached to tRNA in vivo*

The tRNA was extracted from liver by two different methods and the amino acids were determined by g.l.c. with both the flame-ionization detector and the thermionic detector. Each set of results represents the mean value ($\pm$ S.E.M.) for five independent extractions and analyses. The total charging for 12 amino acids (pmol of amino acid/nmol of tRNA) from which the molar percentage distribution was calculated is shown in the bottom line. For details see the text. Diethyl pyrocarbonate-extracted (flame-ionization detector) against diethyl pyrocarbonate-extracted (thermionic detector), correlation coefficient = 0.980. Diethyl pyrocarbonate-extracted (flame-ionization detector) against phenol-extracted (thermionic detector), correlation coefficient = 0.975. Diethyl pyrocarbonate-extracted (thermionic detector) against phenol-extracted (thermionic detector), correlation coefficient = 0.955. In all cases $P < 0.001$.

Amino acid	Method of determination		
	Diethyl pyrocarbonate-extracted		Phenol-extracted
	Flame-ionization detector	Thermionic detector	Thermionic detector
Ala	9.8 $\pm$ 1.8	8.7 $\pm$ 1.7	10.8 $\pm$ 1.1
Asp	7.2 $\pm$ 0.9	7.5 $\pm$ 0.5	7.4 $\pm$ 0.8
Glu	8.5 $\pm$ 2.4	10.1 $\pm$ 1.5	10.4 $\pm$ 2.2
Gly	25.0 $\pm$ 1.8	19.5 $\pm$ 2.0	18.7 $\pm$ 1.9
Ile	1.4 $\pm$ 0.2	3.8 $\pm$ 0.6	3.4 $\pm$ 1.0
Leu	2.5 $\pm$ 0.6	6.8 $\pm$ 1.4	3.4 $\pm$ 0.5
Met	1.2 $\pm$ 0.3	1.7 $\pm$ 0.5	1.6 $\pm$ 0.2
Phe	2.0 $\pm$ 0.5	3.5 $\pm$ 1.1	1.7 $\pm$ 0.4
Pro	1.9 $\pm$ 0.3	4.0 $\pm$ 1.0	3.4 $\pm$ 0.4
Ser	27.6 $\pm$ 2.3	19.8 $\pm$ 3.2	27.6 $\pm$ 2.8
Thr	6.2 $\pm$ 1.1	3.8 $\pm$ 0.6	6.3 $\pm$ 1.1
Val	5.3 $\pm$ 1.2	7.2 $\pm$ 0.6	5.2 $\pm$ 0.6
Amino acid (pmol/nmol of tRNA)			
Total	685.0	657.7	613.7

diethyl pyrocarbonate extraction of tRNA with subsequent g.l.c. determination by using either the flame-ionization detector or the thermionic detector and after phenol extraction with analysis by the thermionic detector. The correlation coefficients of any one set of results in Table 2 against another gave values between 0.955 and 0.980 ($P < 0.001$). This high degree of correlation indicated that the overall distribution of the amino acids was independent of the methods used for their extraction or their determination. The molar percentages of glycine and serine attached to tRNA were particularly high.

Comparison of enzymic charging of individual amino acids on tRNA prepared by different methods

By using tRNA prepared by both the diethyl pyrocarbonate and phenol methods for comparison individual charging assays at pH 7.6 were made for each uniformly ^{14}C -labelled amino acid described in the Materials and Methods section. To observe the effect of directly exposing tRNA to diethyl pyrocarbonate, 1.0mg of phenol-prepared tRNA dissolved in 200 μl of water was incubated with 5 μl of diethyl pyrocarbonate at 37°C for 10min. This was equivalent to the exposure made at the first stage of

tRNA extraction with 2.5% diethyl pyrocarbonate, but with the difference that protein was absent in this experiment.

Table 3 gives the values (pmol of amino acid per nmol of tRNA) for the enzymic charging of tRNA at pH 7.6 as described in the Materials and Methods section. Adsorption of radioactivity by tRNA, by the enzyme and by the filter disc, was non-selective and the total control values represented the equivalent of approx. 0.1 pmol of amino acid per nmol of tRNA for each amino acid. Phenol-prepared tRNA was charged to a greater extent than diethyl pyrocarbonate-prepared tRNA, with all the amino acids except leucine and methionine. One explanation is that diethyl pyrocarbonate may have altered the optimum pH for charging these two tRNA species, because it was shown later that both these amino acids were optimally charged at higher values than pH 7.6 used here. The effect of incubating phenol-prepared tRNA with diethyl pyrocarbonate was to decrease to some extent the charging value for 11 of the 12 amino acids. Although the total amounts of the amino acids charged by the aminoacyl synthetase preparation on to tRNA varied with the method of preparation of the tRNA there was a very significant correlation between the three sets of results as shown in Table 3. Charging the tRNA with a

Table 3. *Enzymic charging of amino acids on tRNA exposed to phenol and diethyl pyrocarbonate*

For details see the text. Phenol-prepared tRNA against diethyl pyrocarbonate-prepared tRNA; correlation coefficient = 0.799 ($0.01 > P > 0.001$). Phenol-prepared tRNA against phenol-prepared tRNA (diethyl pyrocarbonate-treated); correlation coefficient = 0.948 ($P < 0.001$). Diethyl pyrocarbonate-prepared tRNA against phenol-prepared tRNA (diethyl pyrocarbonate-treated); correlation coefficient = 0.814 ($0.01 > P > 0.001$).

	Amino acid (pmol/nmol of tRNA)		
	Phenol-prepared tRNA	Diethyl pyrocarbonate-prepared tRNA	Phenol-prepared tRNA (diethyl pyrocarbonate-treated)
Ala	22.5	17.2	7.2
Asp	1.8	1.4	1.2
Glu	5.2	0.5	4.0
Gly	20.8	0.3	10.5
Ile	4.2	0.7	2.7
Leu	0.9	4.4	1.0
Met	0.6	3.4	0.5
Phe	1.3	1.2	1.2
Pro	19.6	4.3	5.9
Ser	54.5	34.0	48.5
Thr	36.6	9.2	26.5
Val	21.0	0.8	15.3
Total	189.0	77.4	124.5
Protein hydrolysate	¹⁴ C (patom/nmol of tRNA)		
	290.0	100.0	206.0

uniformly ¹⁴C-labelled protein hydrolysate (Table 3, bottom line) also confirmed that phenol-prepared tRNA had the greatest acceptance with 290 pg-atoms of ¹⁴C per nmol of tRNA and the use of diethyl pyrocarbonate gave similar results to those for individual amino acids. There was good correlation between the values for phenol-extracted tRNA amino acids *in vivo* (Table 2) and those given in Table 3 obtained by enzymic charging (see below).

Effect of pH on enzymic charging of tRNA

The ability to charge 12 amino acids over a range of pH values was tested with phenol-prepared tRNA which was deacylated. The charging was carried out with individual radioactive amino acids. Fresh aminoacyl-tRNA synthetase preparations from fresh rabbit liver were used, because of the known differences of stability of these enzymes to heat (Yamane & Sueoka, 1964). Because of the known effects of many variable factors, including buffers (Rubin *et al.*, 1971), Tris-maleate buffer was used because it covered all the pH range under test. All other factors were kept constant. In Table 4 the quantity of amino acid charged on to tRNA is given for each of 12 amino acids over the pH range 5.8–8.6. The maximum charging was 316.8 pmol of amino acid/nmol of tRNA at pH 8.2. Glutamic acid showed optimum charging at pH 7.0. Five amino acids were optimally charged at pH 7.6 and five at pH 8.2. The optimum pH 8.6 for isoleucine was confirmed

Table 4. *Effect of pH on enzymic charging of phenol-prepared tRNA*

After extraction from liver by the phenol method, the tRNA was deacylated. Aminoacylation was carried out with individual radioactive amino acids, with an aminoacyl synthetase preparation as described in the Materials and Methods section.

pH ...	Amino acid (pmol/nmol of tRNA)					
	5.8	6.4	7.0	7.6	8.2	8.6
Ala	1.1	12.7	19.9	22.5	22.3	4.0
Asp	0.0	0.7	1.7	2.9	2.7	0.9
Glu	4.7	5.4	7.5	6.5	2.6	1.0
Gly	1.2	13.1	26.7	38.4	35.7	22.1
Ile	0.0	0.4	1.6	5.6	9.5	13.5
Leu	0.0	0.2	0.6	0.9	4.3	2.4
Met	0.0	0.5	0.2	2.0	7.9	3.6
Phe	0.0	0.5	0.6	1.2	2.6	1.4
Pro	0.4	8.7	16.7	32.7	29.8	14.0
Ser	0.1	11.8	19.8	55.4	109.1	62.5
Thr	1.7	14.5	22.9	54.4	68.7	60.9
Val	1.8	11.0	22.8	29.0	21.6	25.0
Total	11.0	79.5	141.0	251.5	316.8	211.3
Protein hydrolysate	¹⁴ C (pg-atoms/nmol of tRNA)					
	48	144	226	258	350	213

Table 5. Relationships between amino acids attached to tRNA *in vivo* and amino acids in protein and free pool

The last column gives the number of amino acid residues of haemoglobin per reticulocyte (Steiner & Vogel, 1973) divided by the number of molecules of corresponding tRNA species per reticulocyte (Smith & McNamara, 1972). See the text for details. Hb, Haemoglobin.

Present work			Smith & McNamara (1972)	
tRNA amino acids (pmol/nmol of tRNA)	tRNA amino acids (nmol/100g of liver)	$10^{-6} \times$ Amino acid residues of protein per tRNA	$10^{-3} \times$ Amino acid residues in free pool per tRNA	$10^{-6} \times$ Amino acid residues of Hb/tRNA molecules per reticulocyte
Ala	65.6	0.44	8.5	0.26
Asp	44.4	0.75	2.8	0.24
Glu	62.8	0.63	15.7	0.56
Gly	122.8	0.25	13.9	0.23
Ile	20.7	0.94	1.9	0.33
Leu	20.7	1.82	4.8	1.19
Met	9.9	0.43	1.1	0.05
Phe	10.2	1.59	6.2	0.50
Pro	20.6	0.85	2.7	0.31
Ser	166.5	0.11	0.8	0.48
Thr	38.0	0.46	2.9	0.32
Val	31.5	0.88	2.7	0.35
Total	613.7	—	—	—
Mean	—	0.76	5.3	0.40

by extending the pH range tested for this amino acid. If the optimum values for each amino acid in Table 4 are added a total of 339.1 pmol of amino acid was charged per nmol of tRNA, i.e. approx. 34% charging of the tRNA with 12 amino acids. The radioactivity in a uniformly ^{14}C -labelled protein hydrolysate acylated on to tRNA under the same conditions is given in Table 4, bottom line. The charging over the pH range showed a similar pattern to that of the totals for 12 individual amino acids, with maximum charging at pH 8.2.

Quantitative relationship between amino acid content of tRNA, total protein and free pool in liver

The average yield of 11.6 mg of tRNA (mol.wt. 25000) obtained from 100 g of liver was equivalent to $0.46 \mu\text{mol}$. The mol percentage values of amino acids attached to tRNA *in vivo* determined after phenol extraction (Table 2) were converted into the absolute values, pmol of tRNA amino acids per nmol of tRNA (Table 5, column 2) and per 100 g of liver (Table 5, column 3). A total of 613.7 pmol of amino acids was attached to 1 nmol of tRNA. This represented approx. 61% charging for 12 amino acids *in vivo*.

The amino acid values for 100 g of total protein (Table 1) were divided by the values in column 3 of Table 5 to give the number of amino acid residues in liver protein per aminoacyl-tRNA (Table 5, column 4). Thus the amount of alanine in the protein was 0.44×10^6 times greater than that attached to tRNA. Similarly, the amino acid values for free pool

in 100 g of liver (Table 1) were divided by the values in column 3 of Table 5 to give the number of amino acid residues in the free pool per aminoacyl-tRNA (Table 5, column 5). The amount of glutamic acid in the free pool was 15.7×10^3 times greater than glutamic acid attached to tRNA. This was the highest ratio for the 12 amino acids. The 12 free pool amino acids represented approx. 0.15% of the total weight of the liver. The correlation coefficients in Table 5 (i) for the amounts of amino acids attached to tRNA (column 3) against the amounts of amino acids in the protein (column 4), (ii) for the amounts of amino acids attached to tRNA (column 3) against the amounts of amino acids in the free pool (column 5) and (iii) for the amounts of amino acids in the protein (column 4) against the amounts of amino acids in the free pool (column 5) gave values of 0.22, 0.45 and 0.53 respectively. None of these was significant ($P > 0.05$).

Smith & McNamara (1972) showed the absolute molar content of tRNA for each amino acid per rabbit reticulocyte. If the assumption is made that the haemoglobin content of the mouse reticulocyte (Steiner & Vogel, 1973) is similar to that of the rabbit, a simple calculation is possible. From the haemoglobin content per reticulocyte (30 pg) the molecular weight of haemoglobin (68000) and Avogadro's number (6.023×10^{23}), the number of molecules of haemoglobin per reticulocyte = 2.66×10^8 . From the known amino acid composition of haemoglobin (Dayhoff, 1972) the number of molecules of any individual amino acid in haemoglobin per reticulocyte may be divided by the

Table 6. Comparison of results of enzymic charging of rabbit liver tRNA *in vitro*

Present work represents phenol-extracted tRNA charged at the optimum pH value for each amino acid, data taken from Table 4. Present work (mean of five determinations) against Gilbert & Anderson's (1970) results, correlation coefficient = 0.239 ($P > 0.1$). Present work (mean of five determinations) against Smith & McNamara's (1971) results, correlation coefficient = 0.485 ($P > 0.1$). Gilbert & Anderson's (1970) against Smith & McNamara's (1971) results, correlation coefficient = 0.730 ($0.01 > P > 0.001$).

	Present work		Gilbert & Anderson (1970)		Smith & McNamara (1971)		Igarashi & Dufresne (1972)
	(pmol of amino acid/ nmol of tRNA)	(mol/ 100mol)	(pmol of amino acid/ nmol of tRNA)	(mol/ 100mol)	(pmol of amino acid/ nmol of tRNA)	(mol/ 100mol)	(pmol of amino acid/ nmol of tRNA)
Ala	22.5	6.6	45.6	9.5	35.6	10.7	9.5
Asp	2.9	0.9	53.8	11.2	30.0	9.0	1.1
Glu	7.5	2.2	25.6	5.3	21.9	6.6	—
Gly	38.4	11.3	49.4	10.3	30.0	9.0	3.3
Ile	13.5	4.0	29.4	6.1	19.4	5.8	3.9
Leu	4.3	1.3	66.9	14.0	33.1	9.9	—
Met	7.9	2.3	30.6	6.4	33.8	10.1	9.5
Phe	2.6	0.8	18.8	3.9	11.9	3.6	14.5
Pro	32.7	9.6	26.9	5.6	24.4	7.3	6.7
Ser	109.1	32.2	51.3	10.7	38.8	11.6	17.5
Thr	68.7	20.3	41.9	8.8	30.6	9.2	22.5
Val	29.0	8.6	38.8	8.1	23.8	7.1	14.7
Total	339.1	100.1	479.0	99.9	333.3	99.9	103.2

number of molecules of the corresponding tRNA species per reticulocyte. The number of amino acid residues in liver protein per tRNA amino acid (Table 5, column 4) are of the same order of magnitude as the number of amino residues of haemoglobin per tRNA molecule in reticulocytes [Table 5, column 6, calculated from the data of Smith & McNamara (1972)]. It could be argued that these ratios represent the number of times each tRNA molecule is used in protein synthesis.

In Table 6 we present our results obtained by enzymic charging of phenol-extracted tRNA at the optimum pH value for each amino acid (data from Table 4). Results published by other workers on the enzymic charging capacities of rabbit liver tRNA are included in this Table for comparison. The values of Smith & McNamara (1971) were recalculated on the basis of 1 absorbance unit at 260nm $\equiv$ 40 μ g of tRNA (Abadom & Elson, 1970). The results obtained under standard acylating conditions by Gilbert & Anderson (1970) were similarly recalculated. The results of Igarashi & Dufresne (1972) do not represent saturation concentrations of the tRNA. They gave values for 15 amino acids, but these did not include glutamic acid and leucine. The determination of aminoacyl-tRNA was made on the one hand *in vivo* by deacylating tRNA (Table 5, column 2) and on the other hand *in vitro* by enzymic charging (Table 6, column 2). These two sets of values gave a correlation coefficient = 0.724 ($0.01 > P > 0.001$). If the values for serine are omitted from the calculation the correlation coefficient = 0.312 ($P > 0.1$) and if the values for both serine and glutamic acid are omitted, then

the correlation coefficient = 0.143 ($P > 0.1$). It should be noted that the total charging for 12 amino acids *in vivo* was approx. 61%, whereas *in vitro* this value was 34%. The latter may be low owing to incomplete charging of the tRNA under the assay conditions used here.

Discussion

The determination of amino acids attached to tRNA *in vivo* involves two major difficulties: (i) the isolation of tRNA from the source material under conditions that avoid hydrolysis of the aminoacyl-tRNA bonds; (ii) the separation and determination of small quantities of amino acids obtained after deacylation of the tRNA.

Methods were originally developed for the determination of aminoacyl-tRNA *in vivo* in bacteria by culturing them in the presence of radioactive amino acids one at a time, as used by Lacks & Gros (1959) and Yamane (1965). Attempts to apply these methods to mammalian tissues resulted in low yields (Delihas & Staehelin, 1966). A different approach to mammalian tissue was made by Davey & Manchester (1969), who incorporated radioactive amino acids into the tRNA of incubated rat diaphragm and perfused heart, but they could only study one amino acid at a time.

Yegian *et al.* (1966) appear to be the only workers to have directly determined in one experiment a number of amino acids attached to tRNA. The amino acids were deacylated from *E. coli* tRNA and 16 amino acids determined with the ion-exchange amino acid analyser. These workers did not discuss

any problems encountered in isolating and determining the small quantities involved. Our interest was the study of amino acids attached to mammalian tRNA and g.l.c. offered a rapid and sensitive method for their determination.

Two methods of tRNA extraction from frozen rabbit liver were used. Our mean yields of tRNA, 8.2mg for diethyl pyrocarbonate and 11.6mg for phenol extraction from 100g of liver were small when compared with 50mg (Cantoni & Richards, 1966) and 40mg (Igarashi & Dufresne, 1972) from 100g of rabbit liver, 80mg from 100g of rooster liver (Mäenpää & Bernfield, 1969) and 50mg from 100g of rat liver (Lanks & Weinstein, 1970). The difference may be due to the fact that we extracted the tRNA at pH 5.0. Lacks & Gros (1959) attempted to determine the size of the aminoacyl-tRNA pool *in vivo* in bacteria, but their work was criticized by Yamane (1965) because the procedure used for extraction caused the hydrolysis of aminoacyl-tRNA ester bonds. One of the most labile ester bonds (alanyl-tRNA) has been shown to be stable at pH 5.1 (Judes & Jacob, 1972) and extraction was carried out at pH 5.0 as used by Yegian *et al.* (1966) for bacterial tRNA. The pooled sample of frozen rabbit liver used in this work enabled results to be compared directly without the complication of variability in the source material. The aminoacyl-tRNA was extracted by both the phenol and diethyl pyrocarbonate methods and it was shown in Table 2 that independently of the method used for extraction, there was a high degree of correlation between the results ($P < 0.001$).

The g.l.c. determination of the deacylated amino acids with the flame-ionization detector was difficult because it was necessary to concentrate the sample prepared from 5mg of tRNA for a single injection on to the column. This involved the use of gaseous trifluoroacetylation of the amino acid methyl esters in a capillary tube followed by injection of this tube on to the g.l.c. column. Additional peaks due to unknown carbon compounds (Fig. 4) interfered with the amino acid analysis. The use of the nitrogen-sensitive thermionic detector for estimating amino acids by g.l.c. (Butler & Darbre, 1974) made possible conventional injection of 1 μ l samples on to the g.l.c. column because of the higher sensitivity of this detector. In addition spurious peaks were almost entirely eliminated (Fig. 5). Determinations of amino acids less volatile than those of the phenylalanine derivative (Darbre & Islam, 1968) were not carried out. In Table 1, the amino acid contents of total protein and the free pool of liver tissue are given. Hydrolyses of the protein for 24, 48 and 72h were carried out in duplicate, but only the results for 48h are reported because they represented the maximum values. There was no significant statistical correlation between total

protein and free pool amino acids (see below). The free pool (1.86mmol/100g) was less than 1.4% of the total protein (135mmol/100g).

A comparison of the amino acids attached to tRNA by using both phenol and diethyl pyrocarbonate extraction procedures and determinations by flame-ionization and thermionic detectors gave results (Table 2) that showed a high degree of correlation ($P < 0.001$). This finding indicated that there was no selective enrichment of certain tRNA species during isolation by the phenol method, a possibility that was considered and discounted by Yamane (1965). The correlation of these results also suggests that a quantitative, or at least representative, extraction of tRNA species was achieved (see Smith & McNamara, 1971). The proof that tRNA retained its acylated amino acids in the presence of diethyl pyrocarbonate is consistent with the view (Solymosy *et al.*, 1971) that diethyl pyrocarbonate can react with tRNA and thereby affect its recognition by enzymes, but that it does not cause breakdown or deacylation of the amino acid.

In Table 3 the enzymic charging (pmol of amino acid/nmol of tRNA) was compared between phenol-extracted and diethyl pyrocarbonate-extracted tRNA samples. Phenol-extracted tRNA showed a higher degree of acceptor activity, whereas a 10min period of contact between phenol-extracted tRNA and diethyl pyrocarbonate caused a marked lowering of acceptance values for 11 of the 12 amino acids. The reason for this effect is unknown, but may be related to the reaction of diethyl pyrocarbonate with certain nucleotides (Denić *et al.*, 1970; Leonard *et al.*, 1971) particularly in the absence of protein, which could serve to protect the tRNA (Denić *et al.*, 1970). Öberg (1971) reported on the reaction of diethylpyrocarbonate with tRNA bases. The lowered acceptance value of muscle tRNA that had been prepared by a phenol extraction method involving treatment with diethyl pyrocarbonate at 37°C for 5min was reported by Ortwerth (1971), who suggested that certain tRNA species were extremely sensitive to carbethoxylation. Enzymic charging of tRNA prepared by three methods (Table 3) showed good statistical correlation, however, and these results thus do not support the possibility that the different tRNA species showed differential sensitivity to diethyl pyrocarbonate.

There are at least 12 variable factors (Rubin *et al.*, 1971) which may affect the acceptance values determined with an aminoacyl-tRNA synthetase preparation based on the original method of Hoagland *et al.* (1956) and there are many modifications of the assay system reported in the literature. However, most workers used Tris-HCl buffer at pH 7.5. The effect of pH on the enzymic charging of phenol-prepared tRNA is shown in Table 4. Tris-maleate buffer was used because it covered the entire pH

range and all other variables were kept constant. Tests were carried out with different amounts of the enzyme preparation to ensure that the tRNA was saturated under these conditions. The pH for optimum charging of the amino acids varied from pH 7.0 for glutamic acid to pH 8.6 for isoleucine. In Table 4 the maximum charging of a protein hydrolysate (350 pg-atoms of ^{14}C per nmol of tRNA) as well as for the 12 amino acids (316.8 pmol of amino acid per nmol of tRNA) was achieved at pH 8.2.

A total of 285 nmol of amino acids attached to tRNA was found in 100 g of liver (Table 5). The average number of amino acid residues present in liver protein was 0.76×10^6 times greater than the corresponding aminoacyl-tRNA species. Half-life values of rat liver protein have been reported as 5–6.5 days (Shemin & Rittenberg, 1944), 4–5 days (Neuberger *et al.*, 1951), 2.2 days (Gan & Jeffay, 1971) and 1.9 days (Stephen & Waterlow, 1966). The half-life of rabbit liver tRNA was 4.9–5.3 days (Erdos & Bessada, 1966) and that of rat liver non-sedimentable RNA was 90 ± 11 h (Blobel & Potter, 1968). Because protein turnover had a similar half-life to that of the tRNA species, then this value would represent the number of times each tRNA species was used. There was on average 5.3×10^3 times more free pool amino acids than the corresponding tRNA species. We have found no statistical correlation between the aminoacyl-tRNA species, protein and free pool amino acids (see the Results section, $P > 0.05$). Some correlation has been claimed between aminoacyl-tRNA species and the protein composition of bacteria (Yamane, 1965), but more convincing evidence for tRNA specialization has been shown in special cases such as in rabbit (Smith & McNamara, 1971), mouse (Yang, 1971) and sheep (Litt & Kabat, 1972) reticulocytes, silk glands of silkworms (Garel *et al.*, 1970), granulation tissue (Lanks & Weinstein, 1970) and livers of roosters treated with oestrogenic hormones (Mäenpää & Bernfield, 1969). Nwagwu & Lianga (1974) showed quantitative differences between seryl-tRNA patterns in developing chick muscle. However, Merrick & Dure (1972) found a constant cytoplasmic tRNA population for 20 amino acids during development of cotton-seed cotyledons during germination.

Although the correlation between the values for all the amino acids attached to tRNA which were determined after deacylation (Table 5, column 2) and those determined by enzymic charging (Table 6, column 2) was satisfactory ($0.01 > P > 0.001$, see the Results section), omission of either serine or serine and glutamic acid resulted in a correlation for the remaining amino acids that was not significant ($P > 0.1$). We should expect a correlation to be meaningful only if it applied to the entire range of protein amino acids and conclude, therefore, that there are significant differences between charging

in vivo and *in vitro*. The total charging *in vitro* was consistently much less than the total charging determined *in vivo*. Our results obtained by enzymic charging at the optimum pH may be compared with those of Gilbert & Anderson (1970) and Smith & McNamara (1971) which are given in Table 6. Although values for the total charging of 12 amino acids were similar, there was no correlation ($P > 0.1$) between our set of results and that of either of these two groups of workers. The values of Igarashi & Dufresne (1972) are lower than those of other workers because they represent charging after 10 min incubation and not saturation values, but are included here for convenience. The data of Lanks & Weinstein (1970) recalculated on the basis $1E_{260}^{1\text{cm}}$ unit $\equiv 40 \mu\text{g}$ of tRNA indicated acceptance values for seven amino acids ranging from 2.9 (proline) to 28.1 (leucine) pmol of amino acid/nmol of tRNA from rat liver. They showed a total acceptance for 17 amino acids of 250–310 pmol/nmol of tRNA. Variations in the results for amino acids attached to tRNA by enzymic charging *in vitro* may be attributed to (i) differences in the source materials, (ii) methods of preparing tRNA and (iii) methods of measuring the extent of acylation of tRNA.

A method of deducing the state of charging of tRNA *in vivo* has been based on determinations of the enzymic charging of tRNA *in vitro*, both before and after periodate oxidation (Yegian *et al.*, 1966; Allen *et al.*, 1969; Folk & Berg, 1970; Lewis & Ames, 1972; Johnson & Chou, 1973; Smith & McNamara, 1974). Thus Smith & McNamara (1974) deduced that in rabbit reticulocytes, the tRNA aminoacylated was between 71 and 97% for 18 amino acids.

The results reported here show that it is possible to determine the amino acids attached to tRNA *in vivo* by a direct method. After deacylation 12 amino acids were modified and determined by g.l.c. By using the method of radioactive amino acid charging such a determination would normally require 12 parallel experiments and the cost would be much greater in time and money (see Igarashi & Dufresne, 1972). There is no theoretical reason why this study should not be extended to include all the amino acids, except asparagine and glutamine, but a number of problems would require solving. Arginine and histidine do not give g.l.c. peaks after trifluoroacetylation under the mild conditions described (see Darbre & Islam, 1968). Gaseous trifluoroacetylation was used here to inject on to the g.l.c. column the entire modified sample. This cannot be achieved under normal circumstances by using conventional injection by microsyringe. Gaseous trifluoroacetylation at higher temperatures than 30–40°C leads to large losses of the more volatile derivatives. The use of a temperature programme reaching about 220°C requires matched columns and it was not possible to have these in conjunction

with the thermionic detector. The determination of the state of charging of tRNA *in vivo* is in any case more relevant to problems of the possible participation of tRNA in the control of protein synthesis, or breakdown (Rafaeli-Eshkol *et al.*, 1974) than is the charging capacity *in vitro*.

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