Estimation of Amino Acids by an Isotopic Dilution Procedure Using the Enzymatic Synthesis of Aminoacyl Transfer Ribonucleic Acid

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ABSTRACT: A new isotopic dilution procedure for the estimation of amino acids, using the enzymatic synthesis of aminoacyl transfer ribonucleic acid (AA-tRNA) is presented. The effect of addition of an unknown amount of cold amino acid (*c*) on the radioactivity of the AA-tRNA formed is measured upon incubation of a mixture containing a crude source of rat liver enzyme, liver tRNA, adenosine triphosphate (10 mM), magnesium acetate (5 mM), KCl (25 mM), Tris-Cl, pH 7.5 (0.1 M), mercaptoethanol (1 mM), a known amount of the amino acid in question labeled with ^{14}C, ^{3}H, or ^{35}S (*r*) ($1-2 \times 10^{-4}$ M), and a mixture of the other unlabeled amino acids. After incubation the AA-tRNA is isolated by acid precipitation followed in some cases by alkaline hydrolysis or by phenol extraction followed by ethanol precipitation and counted. With an incubation volume of 0.1 ml, amounts of 5–20 mμmoles are estimated with maximum accuracy. The procedure has been standardized for the determination of leucine, and it is applicable to the L form of the 20 amino acids normally incorporated into protein. Suitable samples should have no effect other than isotopic dilution. This is tested by changing *r*. When curves are extrapolated to values of *r* infinitely large no inhibition should be obtained. As an example, the preparation of extracts of rat liver for the measurement of leucine is discussed.

In the course of a study on the control of protein synthesis in mammalian cells, we required a technique for determining, in the livers of large numbers of mice, the concentration of leucine in the amino acid pool. We were unable to separate leucine from isoleucine by standard paper chromatography and, since an amino acid analyzer was not available to us, we developed a new isotopic dilution procedure for the estimation of leucine, based upon the enzymatic synthesis of Leu-tRNA. In principle, we observe the effect of the addition of a suitably prepared extract of liver containing an unknown amount of nonradioactive leucine to an incubation mixture composed of tRNA, Leu-tRNA synthetase, Mg^{2+}, K^{+}, ATP, Tris-Cl, mercaptoethanol, a known amount of radioactive leucine, and an excess of the other amino acids. We incubate the mixture until the reaction is completed and measure the specific activity of the Leu-tRNA formed. The procedure taking advantage of the high specificity of the AA-tRNA synthetases is accurate, sensitive, attractively simple, and generally applicable.[1]

Experimental Procedure

Incubation Mixture and Preparation of Components. The following components were incubated at 37° in a final volume of 0.1 ml. A blank in which ATP and/or incubation was omitted was included in each assay.

(*1*) *Aminoacyl-tRNA Synthetase.* The fraction precipitated from whole rat liver supernatant at pH 5.1 (Hoagland *et al.*, 1956) was used as a source of enzymes. The amount of enzyme is expressed in gram-equivalents, that is the grams of liver from which the pH 5 precipitate was derived. Thus, if 1 ml of the enzyme preparation was obtained from 4 g of liver it was said to contain 4 equiv/ml. Livers were homogenized in two volumes of ice-cold medium (0.25 M sucrose, 5 mM magnesium acetate, 25 mM KCl, and 0.1 M Tris-Cl, pH 7.5 at 0°) and centrifuged first at 15,000*g* for 10 min and then 4 hr at 60,000*g*. The postmicrosomal supernatant was diluted with two volumes of water and brought to pH 5.1 with 1 M acetic acid. The pH 5 precipitate was collected by centrifugation, washed with cold water, redissolved in medium at a concentration of 4 equiv/ml, and brought to pH 7.5 with KOH. It was kept frozen at −20° and remained active for at least 1 month. A typical preparation contained 17 mg of protein and 0.28 mg of RNA per equiv. The concentration of enzyme for each amino acid is listed in Table I.

(*2*) *tRNA.* Ribonucleic acid extracted by the phenol procedure (Kirby, 1964) from whole liver supernatant was used. Supernatant prepared as described above was stirred for 30 min in the cold with one volume of 90% phenol (Baker) containing 1 g/l. of 8-hydroxyquinoline (Eastman). The mixture was centrifuged at 15,000*g* for 15 min and the water and phenol phases separated. The phenol phase was reextracted with one volume of medium for 10 min and the procedure repeated. Both aqueous phases were then combined and extracted once with one volume of ether. The remaining ether was removed from the aqueous phase by bubbling air through it. One-tenth volume of potassium acetate, 2 M, pH 5.1, and

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[1] A preliminary report of this procedure was presented at the Latin American Congress of Physiological Sciences, Mexico City, August 1967.

TABLE I

Amino Acid	Enzyme (equiv/ml)	tRNA (mg/ml)	min at 37°	Isolation Procedure[a]	Amino Acid 10^{-4} M	AA-tRNA (cpm/0.1 ml) Incorporation	
						Blank	Minus Blank
Leucine	0.7	0.35	20	I	1.0	20	1000
Lysine	1.0	0.35	45	I	2.0	42	661
Valine	1.0	0.35	45	I	2.0	47	650
Alanine	0.9	0.31	60	I	1.8	46	828
Proline	0.9	0.31	60	I	1.8	35	266
Threonine	0.9	0.31	60	I	1.8	51	730
Serine	0.9	0.31	30	I	1.8	41	957
Arginine	0.9	0.31	60	I	1.8	34	478
Aspartic	0.7	0.29	10	I	1.7	25	292
Glutamic	0.7	0.29	10	I	1.7	23	584
Asparagine	0.8	2.30	45	I	1.7	8	418
Glutamine	0.8	2.30	45	I	1.7	6	835
Isoleucine	0.8	3.15	30	I	1.7	75	370
Glycine	1.0	1.85	40	I	1.7	150	750
Phenylalanine	0.7	0.60	30	II	1.8	90	250
Tyrosine	0.7	1.20	45	II	1.8	100	233
Histidine	1.3	0.27	60	II	1.6	62	323
Methionine	0.8	1.85	45	III	1.5	46	480
Cysteine	0.8	2.15	60	III	1.5	154	446
Tryptophan	0.8	2.60	30	III	1.5	177	1530

[a] Conditions in which theoretical isotopic dilution curves for the twenty amino acids were obtained. In addition to enzyme, tRNA, and the radioactive amino acid the following components were present in the mixture: ATP, 10 mM; magnesium acetate, 5 mM; KCl, 25 mM; Tris-Cl, pH 7.5, 0.1 M; 2-mercaptoethanol, 1 mM; cold amino acids mixture minus the radioactive one, 0.1 mM each. Isolation procedures I (cold trichloroacetic acid precipitation), II (cold trichloroacetic acid precipitation followed by alkaline hydrolysis), and III (phenol extraction) are described in the Procedure section.

two volumes of ethanol were added and RNA was allowed to precipitate overnight at −20°. The RNA was collected by centrifugation, redissolved in water at an approximately calculated concentration of 10 mg/ml, dialyzed against water in the cold for 3 hr, and cleared by a low-speed centrifugation. The actual concentration of RNA was determined by absorption at 260 mμ ($A_{1\,cm}^{1\%}$ 28.5) and it was stored at −20°. The yield was usually 0.2 to 0.3 mg per g of liver. The concentration of tRNA for each amino acid is indicated in Table I.

(*3*) *Adenosine Triphosphate.* Na_2ATP (Sigma) was dissolved to a concentration of 0.1 M in water containing 1 equiv of magnesium chloride and brought to pH 7.0 with potassium hydroxide. One-tenth volume of this stock solution was added to the incubation mixture, to a final concentration of 10 mM.

(*4*) *Salts.* The concentration of magnesium acetate (5 mM exclusive of the Mg added with the ATP), potassium chloride (25 mM), and Tris-Cl (0.1 M) of the medium were maintained in the final mixture.

(*5*) *2-Mercaptoethanol* was included at a final concentration of 1 mM.

(*6*) *Radioactive Amino Acids.* L-Cysteine-^{35}S and L-asparagine-^{14}C (uniformly labeled) were obtained from the Radiochemical Center (Amersham, England). L-Glutamine-^{14}C (uniformly labeled), L-methionine-^{14}C (labeled in the methyl group), and L-tryptophan-*t* were purchased from Schwartz BioResearch (Orangeburg, N. Y.). The remaining 15 amino acids (uniformly labeled) were acquired, also from Schwartz BioResearch, as their "^{14}C L-amino acid kit." The specific activity of all of the amino acids in the incubation mixture was 10 μCi/μmole, except for tryptophan-*t* which was used at a final specific activity of 50 μCi/μmole. The concentration of each amino acid in the incubation mixture will be found in Table I.

(*7*) *Nonradioactive Amino Acids.* Unlabeled glycine was obtained from Sigma and isoleucine from Schwartz BioResearch. The remaining amino acids were purchased from Mann Research Lab. In each case, a mixture of the 19 unlabeled amino acids other than the one whose concentration was being determined was added to the incubation mixture at a final concentration of 10^{-4} M each.

Separation and Counting of the AA-tRNA. At the end of the incubation it was necessary to separate the labeled AA-tRNA from the large excess of radioactive free amino acid. In the case of leucine and 13 other amino acids this could be accomplished by a simple precipitation with cold trichloroacetic acid (procedure I). With phenylalanine, histidine, and tyrosine however, high blanks were obtained with simple trichloroacetic acid precipitation and further alkaline hydrolysis of the precipitate was introduced in addition (procedure II). Finally in the case of cysteine, tryptophan, and methionine we found it necessary to isolate the AA-tRNA from the

incubation mixture by phenol extraction followed by ethanol precipitation (procedure III). In every case the blank was processed simultaneously and its value subtracted from the radioactivity of each sample.

Procedure I. Tubes were chilled and 1 ml of an ice-cold solution of 5% trichloroacetic acid, containing 20 μg/ml of unlabeled amino acid, was added. Subsequent steps were carried out in an ice bath. After a 15-min period 4 more ml of trichloroacetic acid–amino acid solution was added. The precipitate was then collected by centrifugation and washed twice with the trichloroacetic acid–amino acid solution. When ^{14}C was the label the precipitate was dissolved in 1 ml of 1 N ammonium hydroxide, plated on stainless steel planchets, and dried under a lamp for counting in a gas-flow counter. (Particular care should be taken in this last step; certain amino acids, leucine for instance, will sublime if the planchette is overheated.) When ^{3}H-labeled amino acids were used, the precipitate was dissolved in 0.5 ml of 1 N Hyamine hydroxide in methanol (Packard) and added to 20 ml of toluene scintillation fluid (Packard) for counting in a scintillation counter. On occasion we tried to collect the trichloroacetic acid precipitate on a Millipore or a glass fiber filter, but it offered no appreciable advantage and although the filters were extensively rinsed through with fresh trichloroacetic acid–amino acid solution the blanks were consistently higher than in the procedure here described.

Procedure II. Procedure I was followed as described above. The final precipitate was suspended in 0.3 ml of 0.1 N KOH containing 0.15 μmole/ml of unlabeled amino acid. The AA-tRNA was hydrolyzed for 30 min at 37°. The tubes were then chilled and 0.4 ml of ice-cold 0.3 N perchloric acid was added. Reprecipitated protein and crystals of potassium perchlorate were centrifuged and the supernatant was dried on a stainless steel planchet for counting.

Procedure III. The incubation was stopped by the addition of 1 ml of a cold solution of potassium acetate, 0.2 N, adjusted to pH 5.2, containing 1 mg/ml of yeast RNA (Mann Research) as carrier. Phenol (1 ml, 90%) was next added and phenol extraction and ethanol precipitation were followed essentially as described in the preparation of tRNA above, except that the operation was carried out in a 12-ml conical centrifuge tube which permitted the simultaneous handling of a large number of samples. The ethanol precipitate was washed once with 75% ethanol at −20° and finally dissolved in ammonium hydroxide or Hyamine for counting.

Preparation of Liver Extracts for the Estimation of Leucine. A liver extract, free of factors that could affect the assay other than by isotopic dilution, was prepared as follows. Rats or mice were killed by decapitation. Their livers were chilled and homogenized in seven volumes of distilled water. The homogenate (4 ml), mixed with 0.25 ml of 80% trichloroacetic acid, was centrifuged at low speed. The pellet was resuspended in 3 ml of 5% trichloroacetic acid and recentrifuged. The combined supernatants were extracted three times with ether and once with ethanol–ether–chloroform (2:2:1). The aqueous phase was incubated at 50° for about 1 hr until the residual ether and chloroform had evaporated. It was then passed by gravity through a column (4.5 × 0.6 cm) of the sulfonic resin AG 50W − X 8, 200–400 mesh (Bio-Rad), prepared in the H^+ form and suspended in water. The column was successively rinsed with 15 ml of water, 5 ml of 0.1 N ammonium acetate buffer, pH 9, and 4 ml of water. The leucine was then eluted with 6 ml of 1 N ammonium hydroxi The eluate was dried *in vacuo* (Evapo Mix, Buchler Inst ments), redissolved in 0.25 ml of distilled water, and neutr ized with 1 N HCl.

As a check on the recovery of leucine throughout the p cedure, 0.01 μCi of leucine-^{14}C (10 μCi/μmole) was added each 4 ml of homogenate before the trichloroacetic acid p cipitation and a 0.025-ml aliquot of the final solution w counted. (The resulting specific radioactivity of the leuci in the sample was calculated to be only about 0.01 of t of the leucine in the assay incubation mixture; the rad activity of the sample was thus ignored in the final ass alternatively leucine-*t* could be used.) Recoveries rang between 60 and 80%.

Results

Equations. Incubation of tRNA with excess amino ac ATP, and enzyme for a sufficient time allows the reaction proceed to completion. The tRNA is then saturated and t radioactivity, *a*, of the AA-tRNA is directly proportional the specific radioactivity of the amino acid in the incubati mixture. If a quantity, *c*, of cold amino acid is added to t mixture already containing an amount, *r*, of radioacti amino acid, the isotopic dilution may be determined by t equation: $a = K[r/(r + c)]$, where K is the amount of A tRNA synthesized and $r/(r + c)$ the resulting specific activi The relation of *a*, *c*, or *r* can be expressed in many ways. T equations will be used here:

$$\log a = 2 - \log \frac{r + c}{r}$$

and

$$\frac{100 - a}{a} = \frac{c}{r}$$

where *a* is expressed as per cent of the control; that is, wh $c = 0$, $a = 100$. In this procedure *r* is known, *a* is measur and *c* is calculated.

Determination of Leucine. It is apparent that theoreti isotopical dilution curves will be obtained only if conditic are properly selected for saturation of the tRNA. T concentration curves of enzyme and amino acid, and ti curves for each amino acid were required. Such curves leucine are shown in Figure 1. Since the reaction is reversi and the other components are in excess, the acceptor capac of the tRNA should not be affected by the presence of en genous amino acid esterified to it. In one experiment compared the course and extent of the incorporation leucine into a tRNA as obtained from the liver (presuma largely charged) and an aliquot of the same tRNA strip of endogenous amino acids by dialysis *vs.* Tris 0.2 M, 9.4, for 1 hr at 37°. We observed no difference. In all experiments reported here, untreated tRNA was used. O the conditions for each amino acid are established the ope tion is facilitated by mixing all the components (except source of cold amino acid) in a batch, from which microl amounts of the mixture can be dispensed into each as tube.

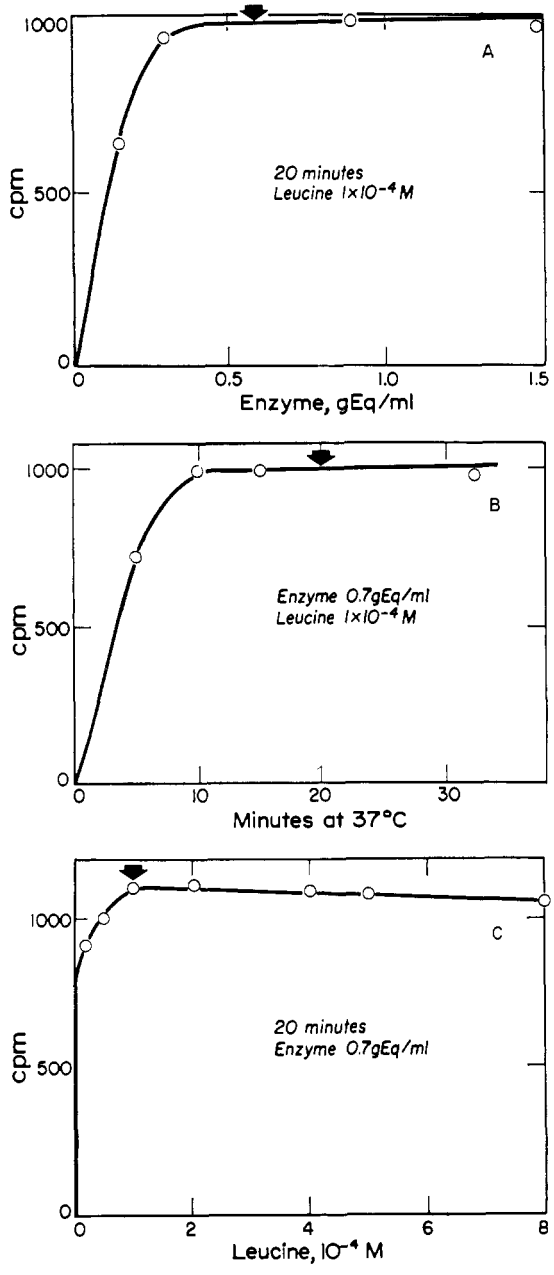

FIGURE 1: The enzymatic synthesis of Leu-tRNA. Shown is the dependence of the reaction on the concentration of enzyme (A) and leucine (C), and its time course (B). In addition to enzyme and leucine the incubation mixture contained, in a final volume of 0.1 ml, the following components: tRNA, 35 μg: ATP-Mg, 1 μmole; magnesium acetate, 0.5 μmole; KCl, 2.5 μmoles; Tris-Cl, 10 μmoles 2-mercaptoethanol, 0.1 μmole; cold amino acids mixture, minus leucine, 0.01 μmole each. The arrow in each curve indicates the parameter selected and corresponds to the values for leucine in Table I.

The procedure is simple and accurate estimation can be made. Figure 2 shows the effect of adding 10, 20, and 30 mμmoles of nonradioactive leucine. The line is the theoretical expression of eq 1.

Recognition of the proper amino acid and the corresponding tRNA is a property of each of the AA-tRNA synthetases essential for its function. This is illustrated in the case of

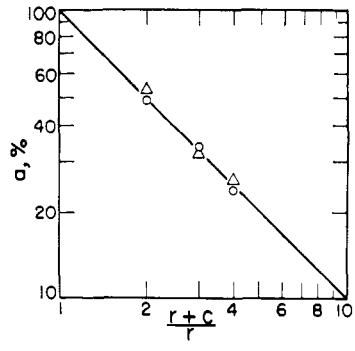

FIGURE 2: Isotopic dilution by nonradioactive leucine. Shown are the results of two separate experiments (○ and △) in which 10, 20, and 30 mμmoles of cold leucine (*c*) were added to an incubation mixture (0.1 ml) containing 10 mμmoles of leucine-^{14}C (*r*). The other components of the mixture as in Table I. The radioactivity of the Leu-tRNA (*a*) is expressed in per cent of a control in which the cold amino acid was omitted. The line is the theoretical expression of eq 1.

leucine by the experiment shown in Table II. In this case, the control lacked the usual mixture of amino acids minus leucine. The addition of an equivalent amount of leucine reduced the radioactivity of the AA-tRNA by half, as expected. It is clear the other amino acids had no effect. The small reduction in the radioactivity of the tRNA by a 20-fold excess of isoleucine could be explained if the isoleucine we used carried 0.9% of leucine with it; a likely possibility in view of the difficulty in separating these amino acids from one another.

A potential artifact in determining the amount of amino acid present in a crude sample is the occurrence of factors influencing the reaction by means other than isotopic dilution. If isotopic dilution by *c* is the only operative factor we should be able to reverse it in a predictable way by increasing *r* (eq 2). As an example the theoretical effect of a known amount of cold leucine or a suitably prepared extract from rat liver is shown in Figure 3 (left). In contrast a less purified extract of the same liver is seen to contain inhibitory factors even at infinitely large concentrations of *r* (Figure 3, right).

TABLE II[a]

Amino-^{12}C Acid	cpm (%)
None	100
Leucine (15 mμmoles)	49
All amino acids, minus leucine (15 mμmoles each)	103
Isoleucine (15 mμmoles)	102
Isoleucine (30 mμmoles)	106
Isoleucine (300 mμmoles)	85

[a] Effect of adding cold leucine or other amino acids to an incubation mixture (0.1 ml) containing 15 mμmoles of leucine-^{14}C. The composition of the mixture is as described in Table I, but for the cold amino acid mixture minus leucine except when indicated.

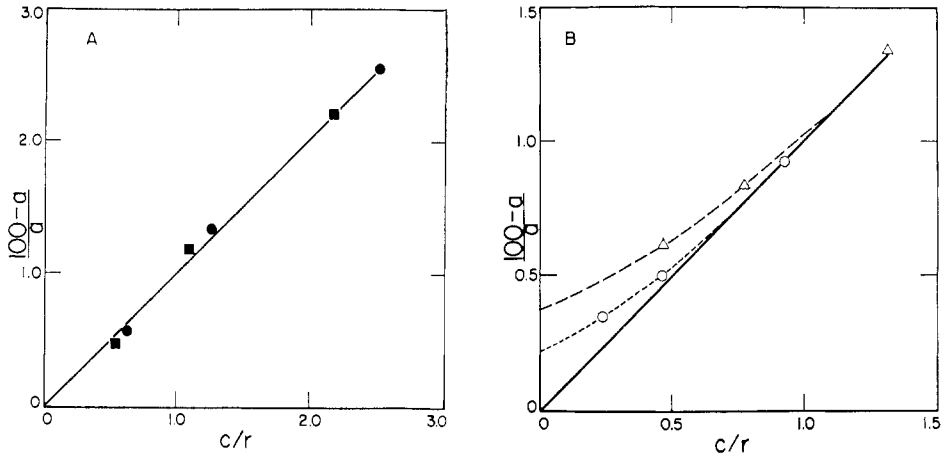

FIGURE 3: The effect of increasing r (10, 20, and 40 mμmoles of leucine-^{14}C) on the behavior of 10 mμmoles of cold leucine (●), 0.1 equiv of purified liver extract (■) (A), and 0.05 equiv (0), or 0.1 equiv (Δ) of a less purified extract (B). The composition of the incubation mixture was as in Table I. The purified liver extract was prepared as described in Experimental Procedure. An aliquot of the same preparation subjected to the same treatment except the fixation–elution of Dowex 50 was used as the less purified extract. At each r, the apparent value of c was determined from the observed a and the resulting c was plotted against $(100 - a)/a$. According to eq 2 the points should fall on a straight line intersecting the origin (the full line in both graphs). Note that while cold leucine and the purified liver extract follow this expectation, this is not the case with the less purified extract. The value extrapolated in the ordinate for 0.05 and 0.1 equiv of this extract corresponds to an inhibition of 18 and 28%, respectively, at an infinitely large concentration of r.

Conditions for the Determination of the Other Amino Acids. Conditions in which theoretical isotopic dilution curves were obtained for all of the amino acids are listed in Table I. Since we have extensive experience with leucine only, the conditions reported in the table refer to the particular preparations of enzyme and tRNA we used in each case and should not be taken as an attempt to standardize the procedure, but rather to show its feasibility with all the amino acids.

It was found convenient to enrich the incubation mixture with tRNA in addition to the amount present in the enzyme preparation. The amount of tRNA added to the incubation mixture for each amino acid is recorded in Table I. The quantity was selected for convenience of counting according to the acceptor capacity of the preparation for that particular amino acid and the value of the blank.

An unexpected difficulty was encountered in the case of aspartic and glutamic acids and their amides. Cells are known to have separate codons, tRNAs, and synthetases for each of them (Novelli, 1967). We were unable however to resolve these amino acid from their amides, as shown in Table III. No cross-contamination of the acids and the corresponding amides was detected by paper chromatography. This result could be due to an alteration of the specificity of the enzyme upon isolation, or more likely, to the presence of amidases in the crude preparation we use as a source of enzyme. That the incubation mixture does contain amidase activity was shown analyzing for radioactive asparagine in the incubation mixture by paper chromatography. A radioactive spot appeared in the position of aspartic acid after 45-min incubation at 37°, but not in an unincubated control. This point remains to be settled. It serves, however, to illustrate a potential source of error, namely, a crude preparation of enzyme may contain other enzymes capable of catalyzing a variety of reactions resulting in the interconversion of some amino acids during the assay. This possibility is limited, however, by the fact that in order for these reactions to occur other reactants or cofactors are often necessary (for example, the corresponding α-keto acid in transaminations, tetrahydrofolate in the interconversion of serine and glycine, tetrahydropteridine, and TPN in the hydroxylation of phenylalanine to tyrosine). Whenever the possibility is suspected it can be tested in various ways. One of them is the addition of the suspected precursor (labeled with a radioactive isotope) to the incubation mixture followed by chromatographic analysis of the product, as described above for asparagine. Also, if the measured amount of c is present from the beginning and none is generated during the incubation the isotopic dilution should be the same even at very short incubation times, as in the experiment of Figure 4. Furthermore, the omission of the mixture of unlabeled amino acids (presumably an important or sole source of the suspected precursor amino acid) from the incubation should have no effect on the isotopic dilution (as in Figure 4).

An obvious alternative is the use of a more purified enzyme preparation. This seems to be essential in the case of aspartic and glutamic acid and its amides. Purified enzymes would also facilitate the assay of phenylalanine, tyrosine, and cysteine where blanks tend to be high relative to the specific acylation in spite of changes in the isolation procedure. Highly purified aminoacyl-tRNA synthetases for many amino acids have been obtained from *Escherichia coli*, yeast, or rat liver (Lengyel and Söll, 1969). We see no reason why enzymes from this or other sources cannot be used, provided homologous tRNA is available [the recognition of tRNA by the enzyme is to some extent species specific (Novelli, 1967)].

Discussion

The method we have described has obvious advantages for routine analysis; it is simple, accurate, and inexpensive. It has been carefully studied for leucine; the incubation mixture has to be modified for each of the other amino acids

TABLE III[a]

Amino-^{14}C Acid	mμ-moles	Amino-^{12}C Acid	mμ-moles	cpm (%)
Aspartic	20	None		100
Aspartic	20	Asparagine	10	90
Aspartic	20	Asparagine	20	66
Glutamic	10	None		100
Glutamic	10	Glutamine	5	85
Glutamic	10	Glutamine	10	80
Asparagine	10	None		100
Asparagine	10	Aspartic	5	82
Asparagine	10	Aspartic	10	58
Asparagine	10	Aspartic	20	43
Glutamine	10	None		100
Glutamine	10	Glutamic	5	74
Glutamine	10	Glutamic	10	60

[a] Apparent competition between aspartic and glutamic acids and their respective amides. The incubation mixture (0.1 ml for aspartic acid and 0.06 ml for the others) was as described for each amino acid in Table II. The cold amino acid mixture lacked both the acid and the corresponding amide and they were added separately as indicated.

(Table I) and will require further standardization. Obviously, maximum accuracy is obtained when the amount of cold amino acid (c) is selected to be in the same range as the amount of radioactive amino acid (r), that is, when a is close to 50%. The sensitivity of the procedure is dependent on the amount of r. Theoretical behavior is attained at concentrations of r between 1×10^{-4} and 2×10^{-4} M. Thus, if the volume of the incubation mixture is 0.1 ml, the highest accuracy will be obtained when the quantity of amino acid to be measured is in the order of 10 to 20 mμmoles. This sensitivity compares favorably with that of paper or column chromatography and it can be increased severalfold by reducing the volume of the incubation mixture further.

The specificity of the enzymes restricts the application of this procedure to the L form of the twenty amino acids in Table I. Since the L form is generally the biologically significant isomer and since chromatographic techniques do not distinguish the isomers, the procedure has a further advantage. Its combination with chromatography may provide information on the degree of racemization of a crude preparation obtained by chemical synthesis or protein hydrolysis. It should also be noted that the procedure fails to detect amino acids that may occur in cells but are not incorporated into protein (such as ornithine) or amino acids in hydrolysates

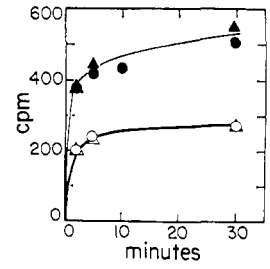

FIGURE 4: The time course of the incorporation of leucine-^{14}C into Leu-tRNA ni the absence (●) and in the presence (○) of a purified liver extract (0.03 g-equiv). The composition of the incubation mixture was as in Table I in a final volume of 0.05 ml. The liver extract was prepared as described in Experimental Procedure. In a parallel series of tubes the mixture of 19 cold amino acids (minus leucine) was omitted and the incorporation was observed in the absence (▲) and presence (△) of the liver extract.

that arise from modifications of residues after they have been incorporated into protein (such as hydroxyproline).

The most critical aspect of the determination is the nature of the sample. Fortunately the procedure allows us to detect any effect other than isotopic dilution, as shown in Figure 3. The preparation of liver extract described here for the estimation of leucine is fairly simple and will possibly serve with little or no modification for several neutral amino acids. Modifications of the conditions of fixation and elution will have to be determined for the others. Urine and other body fluids will require no deproteinization and lipid extractions and could probably be applied directly to the preparatory column.

In conclusion, the procedure seems particularly attractive for routine determination of amino acids particularly when one or a few at a time are to be determined in tissues and biological fluids. The commercial availability of tRNA and purified AA-tRNA synthetases will hopefully increase its attraction.

Acknowledgments

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