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A Radiometric Technique for Measuring L-Asparagine in Picomole Quantities

By DAVID A. COONEY and HARRY A. MILMAN
Laboratory of Toxicology, National Cancer Institute, Bethesda, Md. 20014, *U.S.A.*

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A radiometric technique for measuring L-asparagine in small samples of biological origin is presented. After removal of L-glutamate and L-aspartate by enzymic decarboxylation, L-asparagine in the sample is hydrolysed to L-aspartate with L-asparaginase from *Escherichia coli*. The L-aspartate so generated is then enzymically transaminated with 2-oxo[1-^{14}C]glutarate yielding L-[1-^{14}C]glutamate. After specific chemical removal of unchanged 2-oxo[1-^{14}C]glutarate, the L-[1-^{14}C]glutamate generated is determined by scintillation counting of the radioactivity. This technique can measure the concentration of L-asparagine present in approx. 1 μl of mouse blood, and is capable of detecting as little as 25 pmol of the amide. Illustrative applications of the method are presented and discussed, including measurement of the L-asparagine content of the mouse hypophysis.

A variety of techniques are available for the measurement of L-asparagine in physiological specimens (Krebs, 1950; Tower *et al.*, 1963; Cooney & Handschumacher, 1968; Mistretta *et al.*, 1968; Kojima & Wacker, 1969; Cooney *et al.*, 1970). Enzymic spectrophotometric techniques permit the determination of 5 nmol of L-asparagine in a 1-ml sample; fluorimetric micro-modifications extend the sensitivity to 500 pmol (Cooney *et al.*, 1970). Nevertheless, because the concentration of L-asparagine is low in many biological materials, a still more sensitive assay was required for situations where only one small sample at a time could be procured, e.g. in the mouse bled repetitively from the ophthalmic plexus. In the present paper, a radiometric assay for L-asparagine is described, which is specific by virtue of the specificity of the crystalline enzyme used as the principal reagent, and sensitive by virtue of the purity and specific radioactivity of the labelled compound.

Experimental

Materials

Enzymes. L-Aspartate 4-decarboxylase (EC 4.1.1.12) was purified (Tate & Meister, 1968) and provided by Dr. Suresh Tate of Cornell University. The enzyme exhibited a specific activity, with L-aspartate as substrate, of 77 units/mg of protein, and was stored in individual portions of 0.1 ml at −100°C in 50% (v/v) glycerol (pH 5.0), 20 μM with respect to pyridoxal phosphate. This solution contained 300 units/ml. L-Glutamate decarboxylase (EC 4.1.1.15) was purified from an acetone-dried powder of *Escherichia coli* procured from Sigma Chemical Co., St. Louis, Mo., U.S.A. The enzyme was extracted and purified by the method of Shukuya & Schwert (1960). After the second DEAE-cellulose chromatography the most active fractions were pooled, and the enzyme was precipitated by the addition of $(NH_4)_2SO_4$ to 50% saturation. The precipitate was collected by centrifugation at 2000 g for 30 min and redissolved in 5 ml of water. Glycerol (5 ml) was added and the pH was carefully adjusted to 5.0 with 1 M-HCl. Pyridoxal phosphate was then added to a concentration of 20 μM and the glycerol-treated enzyme stored at −20°C. This solution exhibited an activity of 10 units/ml. L-Glutamate–oxaloacetate transaminase (EC 2.6.1.1) (180 units/mg of protein) was the product of Boehringer (Mannheim) Corp., New York, N.Y., U.S.A. To remove 2-oxoglutarate and any contaminating amino acids, the enzyme solution was dialysed against four changes of 100 vol. of water containing 10 μM-pyridoxal phosphate, then adjusted to 50% (v/v) glycerol and stored at −20°C. Malate dehydrogenase (EC 1.1.1.37) (720 units/mg of protein) from Boehringer (Mannheim) Corp. was processed in the same way as L-glutamate–oxaloacetate transaminase except that pyridoxal phosphate was omitted from the dialysis steps. L-Glutamate–pyruvate transaminase (EC 2.6.1.2) and lactate dehydrogenase (EC 1.1.1.27) were also obtained from Boehringer (Mannheim) Corp. L-Asparaginase (EC 3.5.1.1) from *Escherichia coli* (340 units/mg of protein) was purified at the Merck Institute for Therapeutic Research, West Point, Pa., U.S.A. and provided by the Drug Development Branch of the National Cancer Institute. Pronase was procured from Calbiochem, Los Angeles, Calif., U.S.A.

Radiochemicals. 2-Oxo[1-^{14}C]glutarate (specific radioactivity 26μCi/μmol) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. This was the only commercial product available that was approx. 95% decarboxylated by 1% (v/v) H_2O_2 in 1 M-HCl, and was thus the only radiochemical suitable for the assay described here. L-[1-^{14}C]Glutamate was also purchased from New England Nuclear Corp. and was 95% decarboxylated by purified L-glutamate decarboxylase.

Chemicals. All amino acids were purchased from Mann Research Laboratories, Orangeburg, N. Y., U.S.A. NADH was purchased from Sigma Chemical Co. and freeze-dried before use. Pyridoxal phosphate was a generous gift of Sigma Chemical Co. All other chemicals were reagent grade.

Vessels. Eppendorf 1500μl disposable plastic centrifuge vessels were used throughout and were purchased from the Brinkman Instrument Co., Silver Spring, Md., U.S.A.

Animals. BALB/c white, male laboratory mice supplied by the Texas Inbred Mouse Co., Houston, Texas, U.S.A., were used throughout.

Methods

Procurement of samples. The ophthalmic plexus of the unanaesthetized mouse was entered at the medial canthus of the eye with a disposable glass micropipette (Riley, 1960). When a drop of blood welled up, a 20μl sample was taken with an Eppendorf pipette and immediately dispensed into 100μl of ice-cold 0.9% NaCl in a 1500μl Eppendorf conical vessel. The mixture of blood and 0.9% NaCl was immediately centrifuged for approx. 5s at ambient temperature in the Eppendorf Zentrifuge, after which the supernatant was immediately transferred to new Eppendorf vessels being maintained at 95°C in an Eppendorf automatic hot-plate. Speed in this last step, which served to inactivate any enzymes present in the sample of diluted plasma, was especially important when the mice had received L-asparaginase. The closed vessels were heated at 95°C for 5min and then placed in ice.

Samples of human plasma were obtained and processed as previously described (Cooney *et al.*, 1970).

Assay procedure. A reagent mixture was prepared by mixing 15μl of glutamate–oxaloacetate transaminase in glycerol, 15μl of malate dehydrogenase in glycerol, 4.9mg of NADH, 900μl of 0.5M-tris–HCl buffer, pH8.4, and 3.8×10^6c.p.m. of 2-oxo[1-^{14}C]-glutarate in a volume of 20μl. This reagent mixture was then divided into two parts: to the first part was added 20μl of water, and to the second part was added 20μl of L-asparaginase (2 units/ml). The former reagent mixture was designated 'Cocktail A'. It was used to measure L-aspartate plus L-glutamate (see the Results section). The latter, containing dilute L-asparaginase, was called 'Cocktail B'. It was used to measure L-aspartate plus L-glutamate plus L-asparagine. When L-glutamine was to be measured (see the Results section) the 'cocktail' was fortified with 20μl of L-asparaginase (2000 units/ml). This was designated 'Cocktail C'. Each 'cocktail' contained 40000c.p.m./10μl.

Portions (10μl) of the saline-diluted heat-inactivated plasma, representing 1.6μl of mouse blood, were pipetted into four Eppendorf 1500μl conical vessels. Then 10μl of 'Cocktail A' was added to the first two vessels and 10μl of 'Cocktail B' was added to the second two vessels. The reagents and samples were driven together by a brief centrifugation (approx. 2s at 12000g) in the Eppendorf Zentrifuge and incubated at 37°C for 20min. Then 10μl of 1% (v/v) H_2O_2 in 1M-HCl was added to the vessels to remove any unchanged 2-oxo[1-^{14}C]glutarate and the contents were evaporated to dryness at 95°C. Then 20μl of water was added to dissolve the residue; the covers of the vessels were cut off, and the conical tubes dropped into glass scintillation vials containing 15ml of a suitable scintillant (Cooney *et al.*, 1971) and counted for radioactivity in a Beckman model LS-230 liquid-scintillation spectrometer at 50% efficiency. Any numbering on the vessels was removed with a towel dampened with toluene.

Other samples. When samples containing high concentrations of L-aspartate or L-glutamate were being processed, it was necessary to remove these interfering amino acids from the samples before the incubation with 2-oxo[1-^{14}C]glutarate (see the Results section). To do this, the samples were preincubated with purified preparations of L-aspartate 4-decarboxylase and L-glutamate acid decarboxylase, as follows: a reagent mixture, the 'Decarboxylation Mix', was prepared by combining 9 units of L-aspartate 4-decarboxylase and 1.2 units of L-glutamate decarboxylase in 0.66M-sodium acetate buffer, pH4.2, to a final volume of 360μl. Then 5μl of the 'Decarboxylation Mix' was added to each of the plasma samples, and the two solutions were driven together by a brief centrifugation in the Eppendorf Zentrifuge. The two decarboxylations were allowed to go to completion at 37°C for at least 2h. Then the decarboxylation enzymes, which would interfere with the subsequent step of the assay, were inactivated by heating the closed reaction vessels at 95°C for 5min. Then 'Cocktail A' or 'Cocktail B' was added to the appropriate vessels, and the assay was carried out as described above for mouse plasma.

Use of N-bromosuccinimide. To circumvent the problem of high blank values, when it was necessary to measure amounts of L-asparagine less than 50pmol, an alternative method had to be adopted. In this alternative technique, the reaction vessels were incubated and unchanged 2-oxo[1-^{14}C]glutarate was removed as described above. Then, to the dry residue,

now containing L-[1-^{14}C]glutamate, was added 50μl of a solution of 1% (w/v) *N*-bromosuccinimide in 0.1M-sodium citrate buffer, pH4.0, which decarboxylated the radioactive L-glutamate (Chappelle & Luck, 1957). The $^{14}CO_2$ so generated was collected in small droplets of alkali deposited on the under-surface of the top of the reaction vessel as previously described (Cooney *et al.*, 1971).

Calculations. The counts remaining in the experimental vessels were corrected by subtracting the blank counts remaining in vessels that had received only 10μl of deionized water and 10μl of either 'Cocktail A' or 'Cocktail B'. 'Cocktail A' blanks were virtually identical with 'Cocktail B' blanks. The corrected counts in the experimental vessels receiving 'Cocktail A' were then subtracted from the corrected counts in the experimental vessels receiving 'Cocktail B' and the difference in c.p.m. was compared with a standard curve to yield a value of pmol of L-asparagine/sample. It was necessary to plot a standard curve for each new batch of 2-oxo[1-^{14}C]glutarate. In practice a set of standards was also included in each assay.

Measurement of the dicarboxylic acid decarboxylases. L-Aspartate 4-decarboxylase was measured radiometrically with L-[4-^{14}C]aspartate as substrate, or spectrophotometrically as follows. Samples (10μl) of enzyme were incubated at 37°C with 500μl of 10mM-L-aspartate plus 1mM-2-oxoglutarate in 0.1M-sodium acetate buffer, pH5.0. After 15min the reaction was terminated by adding 50μl of 2M-HCl and then 10min later 50μl of 2M-NaOH. The L-alanine produced by the decarboxylation of L-aspartate was measured as follows. Portions (100μl) of the reaction mixture were pipetted into glass cuvettes; 900μl of water was added, followed by 100μl of a 'cocktail' prepared by dissolving 10mg of 2-oxoglutarate in 5ml of 0.5M-tris–HCl buffer, pH8.4. The contents of the cuvettes were mixed and the E_{340} was read. Then 10μl of L-glutamate–pyruvate transaminase (60 units/ml) was added and the reaction allowed to proceed to completion. Under these conditions a decrease in E_{340} of 0.115 is equivalent to 20nmol of L-alanine.

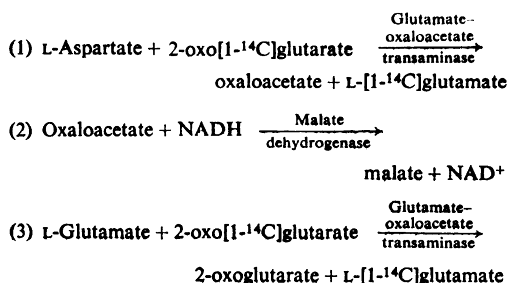
L-Glutamate decarboxylase was measured radiometrically in a substrate solution of L-[1-^{14}C]glutamate fortified with 0.15M-NaCl and 1mM-pyridoxal phosphate. Together, these additives were found to triple the activity of this decarboxylase at pH5.0, in confirmation of the results of Shukuya & Schwert (1960). $^{14}CO_2$ was trapped and counted for radioactivity as previously described (Cooney *et al.*, 1971).

Results

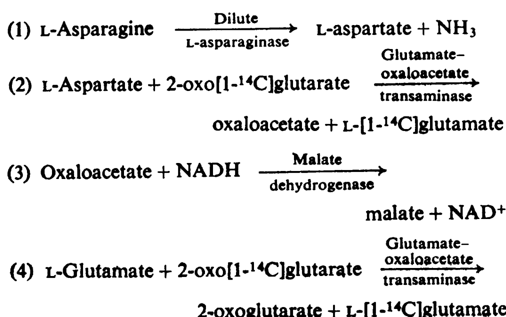
Validation of the individual reactions

In the technique described here, the following series of reactions take place.

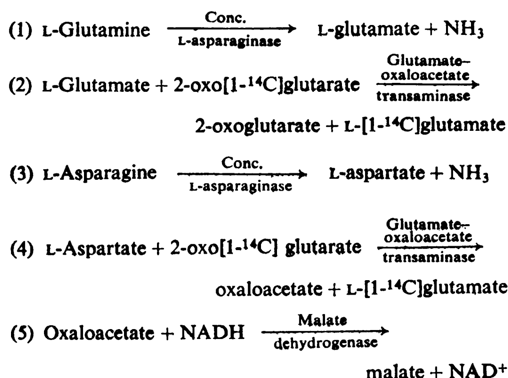
In those vessels receiving 'Cocktail A':



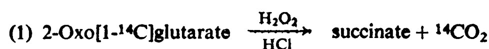
In those vessels receiving 'Cocktail B' ['Cocktail A' plus L-asparaginase (2 units/ml)]:



In those vessels receiving 'Cocktail C' ['Cocktail A' plus L-asparaginase (2000 units/ml)]:



For the decarboxylation of excess of 2-oxo[1-^{14}C]glutarate:

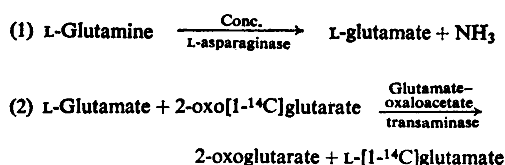


If the technique is to be a quantitative one, it is necessary to verify that each of these reactions goes to completion. The stoicheiometry and specificity of each step will be considered in turn.

Specificity for L-asparagine

Of the 20 naturally occurring amino acids only L-aspartic acid, L-asparagine and L-glutamic acid gave rise to peroxide-stable radioactivity when assayed

with 'Cocktail B', as described above under the conditions of the standard assay. Neither β-cyano-L-alanine (Lauinger & Ressler, 1970; Giza *et al.*, 1963; Ressler *et al.*, 1969) nor L-glutamine, both feeble and naturally occurring substrates of L-asparaginase, interfered. Moreover, even 4nmol of L-glutamine did not interfere with the measurement of 200pmol of L-asparagine. However, when high concentrations of L-asparaginase were included in the reaction mixture (i.e. when 'Cocktail C' was used), L-glutamine did cause peroxide-stable radioactivity. In exploring this effect further, it was found that L-glutamate was the interfering species and that an exchange transamination was taking place:



Thus the reagent enzymes were capable of detecting both dicarboxylic acids and their amides under appropriate conditions. When this feature was understood, it was hoped that the technique could be applied to the measurement of all four amino acids in small biological samples. Towards this end, standard curves of L-aspartate, L-asparagine, L-glutamate and L-glutamine were obtained under the conditions described in the legend to Fig. 1.

L-Aspartate and L-asparagine, alone and in combination, gave linear standard curves and the peroxide-stable radioactivity caused by the L-[1-^{14}C]glutamate formed was proportional to the specific radioactivity of the 2-oxo[1-^{14}C]glutarate used. On the other hand, the standard curves for both L-glutamate and L-glutamine were curved (Fig. 1). An examination of the reaction (see above) shows why this was the case. When L-[1-^{12}C]glutamate is transaminated with 2-oxo[1-^{14}C]glutarate, 2-oxo[1-^{12}C]glutarate is produced; this non-radioactive 2-oxoglutarate will dilute the specific radioactivity of the 2-oxo[1-^{14}C]glutarate. Therefore the standard curve will diminish in slope as the quantity of L-[1-^{12}C]glutamate is increased. One other conclusion followed from the studies illustrated in Fig. 1: any quantity of L-glutamate (greater in practice than approx. 50pmol/sample) would interfere with the determination of either L-aspartate or L-asparagine by generating 2-oxo[1-^{12}C]-glutarate, which in its turn would appear to alter the stoicheiometry of the subject reactions. It therefore became clear that most of the L-glutamate would have to be removed from physiological samples before the measurement of L-asparagine. When the first organ extracts were assayed, it was also found that the concentration of L-aspartate in most physiological specimens (but not in normal plasma) far exceeded the concentration of L-asparagine. This is in keeping with published results (Stein & Moore, 1967; Oreskes *et al.*, 1965). Since the radiometric measurement of L-asparagine depends on the difference in peroxide-stable radioactivity generated in samples treated with 'Cocktail A' (L-asparaginase-free) and 'Cocktail B' (containing dilute L-asparaginase), this preponderance of L-aspartate over L-asparagine would cause a small difference in radioactivity of uncertain significance. For this reason it was also necessary to remove L-aspartate from tissue samples before the measurements of L-asparagine were carried out. Systematic studies to establish the optimum conditions for the removal of L-glutamate and of L-aspartate verified that 200pmol of L-aspartate and L-glutamate could be removed within 60min at pH4.2 in the presence of 0.016 unit of L-glutamate decarboxylase and 0.023 unit of L-aspartate 4-decarboxylase. L-Asparagine and L-glutamine were unaffected by this preincubation. Control of pH in the enzymic decarboxylation of L-glutamate was critical; for this reason, sodium acetate buffer at pH4.2 was finally selected for the adjustment of pH during the decarboxylation step, even though acetate ions are known to inhibit L-glutamate decarboxylase from *Escherichia coli*. Nevertheless, only sodium acetate buffer was suitable to adjust samples of physiological fluids to the requisite acidity in a reproducible way. Moreover, L-aspartate 4-decarboxylase was nearly fully active under these circumstances, so that both decarboxylations could be carried out simultaneously.

Transamination of L-*aspartate*

L-Aspartate, either preformed or generated *in vitro* from L-asparagine by the action of dilute L-asparaginase, was quantitatively transaminated in 10min under the conditions of the standard assay described in the Experimental section. If malate dehydrogenase was omitted from the reaction, the transamination reached an equilibrium position after approx. 180pmol of L-aspartate had been transaminated. For this reason malate dehydrogenase and its cofactor, NADH, were included in all reagent mixtures as a routine.

Optimum conditions for the oxidative decarboxylation of 2-oxo[1-^{14}*C*]*glutarate*

Pilot studies with 2-oxo[1-^{14}C]glutarate showed that 1% (v/v) H_2O_2 in 1M-HCl was an efficient means of α-decarboxylating this acid. However, before the routine adoption of acid peroxide as a reagent in the assay it was considered necessary to characterize the pH optimum of this decarboxylation and to demonstrate conclusively that L-glutamate would resist α-decarboxylation under such circumstances. It was

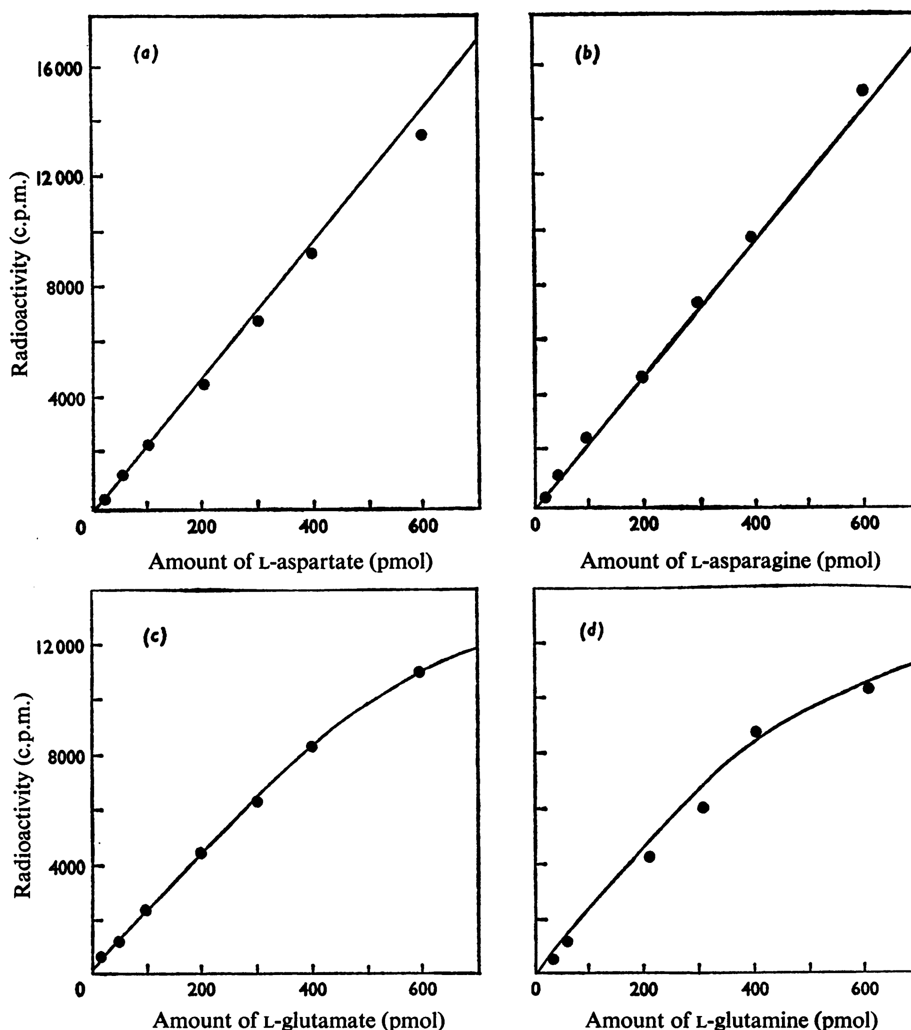


Fig. 1. *Standard curves for* L-*aspartate*, L-*asparagine*, L-*glutamate and* L-*glutamine*

Portions (25, 50, 100, 200, 400 or 600pmol) of L-aspartate (*a*), L-asparagine (*b*), L-glutamate (*c*) or L-glutamine (*d*) in a total volume of 10μl of water were dispensed into 1500μl Eppendorf conical vessels and incubated at 37°C for 20min with 'Cocktail A' (for the determination of L-aspartate and L-glutamate), 'Cocktail B' (for L-asparagine) or 'Cocktail C' (for L-glutamine). The reaction was terminated and unchanged 2-oxo[1-^{14}C]glutarate dissipated by the addition of 10μl of 1% (v/v) H_2O_2 in 1M-HCl and heating at 95°C in open vessels for 30min. The resulting radioactive L-glutamate was dissolved by the addition of 20μl of water. The caps of the vessels were then removed and the vessels immersed in a suitable scintillant and counted for radioactivity in a liquid-scintillation counter. Appropriate corrections for blanks were made in all cases. The ordinate represents c.p.m. of L-[1-^{14}C]glutamate generated.

found that at pH7, H_2O_2 is able to release significant radioactivity from L-[1-^{14}C]glutamate, presumably by α-decarboxylation. At pH1.0, however, the oxidative attack by H_2O_2 in 1M-HCl was specific for α-oxoglutarate, and L-glutamate totally resisted decarboxylation.

The problem of blank radioactivity

As mentioned in the Experimental section, the best commercially available preparation of 2-oxo[1-^{14}C]-glutarate was only 95% decarboxylated by 1% H_2O_2; this in turn was responsible for a radioactive blank of approx. 2000c.p.m. in the standard assay system.

Such a blank is prohibitively high, from a statistical point of view, when it is necessary to measure low concentrations of L-asparagine. To circumvent this problem, therefore, the quantity of 2-oxo[1-^{14}C]-glutarate added to the reagent 'cocktails' must be appropriately diminished when it is expected that only low concentrations of amide will be encountered in a given sample. Decreasing the concentration of 2-oxo[1-^{14}C]glutarate in the reagent 'cocktail' did not decrease the blank linearly, which would appear to be an argument against the presence of a fixed amount of impurity. However, a substantial decrease in the blank could be obtained by adding only 4000c.p.m. per vessel. The K_m of glutamate–oxaloacetate transaminase for 2-oxoglutarate is sufficiently low (Velick & Vavra, 1962) that this decrease in the concentration of one of the prime reactants in the transamination reaction does not slow the reaction unduly, i.e. it still will go to completion in the 20min of incubation in the standard assay when malate dehydrogenase is present to drive the reaction. It should be pointed out in this context that the fewer counts of 2-oxo[1-^{14}C]-glutarate added per vessel the more disproportionate and troublesome the diluting effect of an exchange transamination between L-[1-^{12}C]glutamate and 2-oxo[1-^{14}C]glutarate will become. In other words, when less radioactive oxoglutarate is used, it becomes imperative to remove all L-glutamate present in a sample by its total enzymic decarboxylation. This fact is illustrated in Fig. 2, which shows that as the concentration of 2-oxo[1-^{14}C]glutarate is decreased the curvature of the standard curve is increased.

Features of the assay

Before the radiometric technique described here could be adopted as reliable for L-asparagine, it was necessary to assess its sensitivity and reproducibility. It was found that the standard method could readily detect 25pmol of L-asparagine with acceptable precision (Fig. 3). If *N*-bromosuccinimide is used to α-decarboxylate L-[1-^{14}C]glutamate, the absolute sensitivity of the method can be extended to about 5pmol. It should be noted, however, that with *N*-bromosuccinimide the yields of $^{14}CO_2$ are lower by 20% than the yields of residual radioactivity found in the standard assay. We think this may be because a fraction (20%) of the L-glutamate formed by transamination is cyclized or otherwise rendered resistant to oxidative decarboxylation by *N*-bromosuccinimide in the course of the drying step at 95°C. Thus the standard curves generated with the standard technique and the *N*-bromosuccinimide technique are not coincident (Fig. 3). The reproducibility of the method can be appreciated from the results of ten determinations made by the standard technique; 200pmol of L-asparagine was repetitively assayed with a mean of 199.9pmol and a S.D. of approx. 5pmol. With the *N*-bromosuccinimide technique ten determinations yielded a mean of 199.9 and a S.D. of 4.7pmol.

It was considered important to compare the radiometric assay with the spectrophotometric assay for L-asparagine, to make sure that only L-asparagine was in fact being measured. The results of this study, with human plasma samples, are illustrated in Fig. 4. In nearly every instance the radiometric assays yielded measurements of L-asparagine comparable with those made spectrophotometrically.

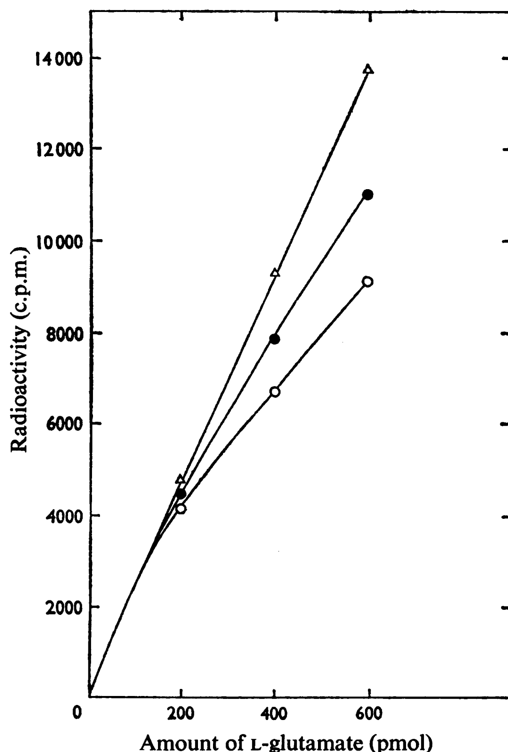


Fig. 2. *Effect of 2-oxo[1-^{14}C]glutarate concentrations on the recovery of L-glutamate*

Portions (100, 200, 400 or 600pmol) of L-glutamate in a total volume of 10μl of water were dispensed into 1500μl Eppendorf conical vessels and incubated at 37°C for 20min with 'Cocktail A'. The concentration of 2-oxo[1-^{14}C]glutarate in the 'cocktail' was systematically varied as follows: △, 130000c.p.m.; ●, 86000c.p.m.; and ○, 41000c.p.m. of 2-oxo[1-^{14}C]-glutarate added per vessel. The reaction was terminated by adding 10μl of 1% (v/v) H_2O_2 in 1M-HCl and heating at 95°C in open vessels for 30min. The radioactive residue was dissolved in 20μl of water and counted for radioactivity in a suitable scintillant. The ordinate represents c.p.m. of L-[1-^{14}C]glutamate generated.

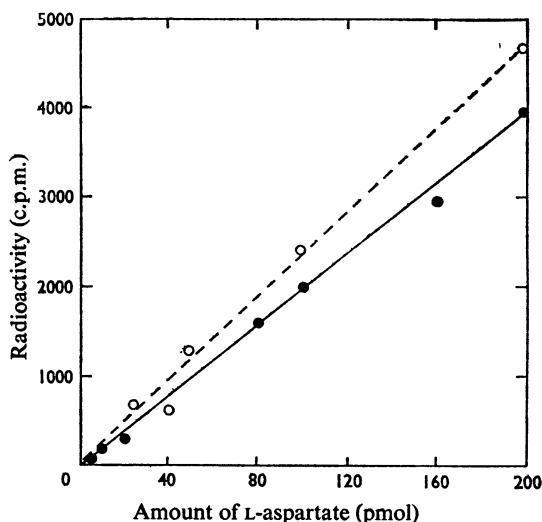


Fig. 3. *Comparison of standard and N-bromosuccinimide techniques for measuring L-aspartate*

Portions (10, 50, 80, 100, 160 or 200pmol) of L-aspartate in a total volume of 10μl of water were assayed in duplicate by either the standard technique (○) or the *N*-bromosuccinimide technique (●). The ordinate represents c.p.m. of L-[1-^{14}C]glutamate generated.

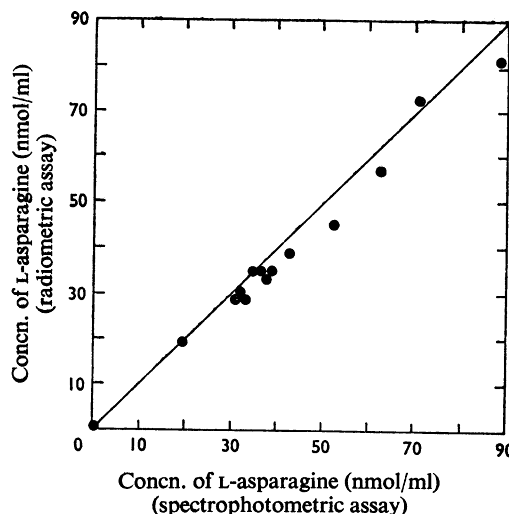


Fig. 4. *Comparison of radiometric and spectrophotometric assays for L-asparagine*

Samples of human plasma were boiled for 20min in polycarbonate tubes, centrifuged for 30min at approx. 176000*g*, and then 1-ml samples were assayed spectrophotometrically for L-asparagine by the technique of Cooney *et al.* (1970). Samples (10μl) of the same boiled plasma samples were dispensed into quadruplicate 1500μl Eppendorf conical vessels and preincubated with 5μl of the 'Decarboxylation Mix' for 3h as described in the Experimental section. These reactions were terminated by heating the closed vessels at 95°C for 5min. The vessels were cooled and assayed by using 'Cocktail A' and 'Cocktail B' as described in the Experimental section.

Applicability

To test the applicability of this radiometric technique to the measurement of L-asparagine in minute biological samples, a number of illustrative experiments were carried out. In the first of these, the concentration of the free amide was measured in mouse pituitary. For these studies, the whole hypophysis was removed and transferred to a 10μl droplet of 1M-HCl. The droplet, contained in a 1500μl Eppendorf conical vessel, was frozen to −70°C, melted, vigorously stirred on a vibrator, and centrifuged in the Eppendorf Zentrifuge at 12000*g* for 3min. This process, which was repeated 12 times, served to liberate the intracellular fluids of the gland and was used instead of conventional homogenization because of the small volumes involved. The whole droplet was then neutralized with 10μl of 1M-NaOH and the concentration of L-asparagine was measured radiometrically in the supernatant. The pellets were then washed well with 5% (v/v) $HClO_4$, ethanol and acetone and digested with 0.1% (w/v) Pronase. The results of these pilot studies are given in Table 1, which shows that mouse pituitary contains a high concentration of free L-asparagine. Treatment of the donor mice with L-asparaginase practically eliminated the presence of any free L-asparagine in the gland but did not alter the composition of the L-asparaginyl residues in its protein.

When the quantity of L-asparagine was sequentially measured in the blood of six mice at various times of the day, no marked circadian periodicity was detected. A mean of 36nmol/ml (S.D. 19.6) was detected at 10.00h, 27nmol/ml (S.D. 16.9) at 11.00h, and 36nmol/ml (S.D. 18.3) at 18.30h. It should be stressed that these determinations were made on 1.6μl samples of blood taken repeatedly from individual mice.

Discussion

The radiometric technique for measuring L-asparagine described in the present paper is about 100 times more sensitive than previously published enzymic spectrophotometric techniques and roughly 20 times more sensitive than most automatic amino acid analysers. If the specific radioactivity and purity

Table 1. *Recovery of* L*-asparagine from mouse pituitary after a single intravenous injection of* 10000 *units of E. coli* L*-asparaginase/kg*

Twelve BALB/c mice, maintained on an L-asparagine-free diet for 3 weeks, were divided into two equal groups. Six mice were injected intravenously with L-asparaginase (10000 units/kg) and the other six were injected with 0.9% NaCl. Then 5h later, all mice were killed and their pituitaries were removed and immersed in 10 μl of 1 M-HCl in 1500 μl Eppendorf conical vessels, then frozen at −70°C, thawed, and centrifuged at 12000*g* for 3 min. This process was repeated 12 times. Before the radiometric assay for free L-asparagine, 10 μl of 1 M-NaOH was added to the pituitary sample, followed by 10 μl of 0.66 M-sodium acetate buffer, pH 4.2, to adjust the pH. The samples were vortex-mixed for 5 min, then centrifuged at 12000*g* for 3 min. Portions (5 μl) of the supernatant were pipetted in quadruplicate into Eppendorf vessels. These samples were preincubated with 5 μl of the 'Decarboxylation Mix' for 2h, as described in the Experimental section, and were then incubated with 'Cocktail A' or 'Cocktail B'. Suitable blanks and standards were included. The pituitary pellets were then washed with 5% (v/v) $HClO_4$, ethanol and acetone and digested overnight with 50 μl of 0.1% (w/v) Pronase in 1 mM-$CaCl_2$. The resulting digest was assayed spectrophotometrically for L-aspartate and L-asparagine (Cooney *et al.*, 1970). Suitable blanks were included. The results are means of duplicate measurements of glands from six animals/group; S.D. values are given in parentheses.

Treatment	Mean free L-asparagine (pmol/pituitary)	Mean L-aspartyl residues (nmol/pituitary)	Mean L-asparaginyl residues (nmol/pituitary)	Mean ratio of L-asparaginyl to L-aspartyl residues
10000 units of L-asparaginase/kg	17.5 (27.4)*	23.5 (11.3)	34.3 (12.7)	1.58
Control (0.9% NaCl)	226.8 (51.9)	32.0 (6.3)	42.3 (6.5)	1.33

* Free L-asparagine was detected in only two out of six of the pituitary glands in this group.

of the 2-oxoglutarate used for the assay can be increased, then the sensitivity of the method as described will also be extended. The technique is readily applicable to microlitre samples of plasma or other biological fluids and can be employed even on turbid specimens. The only interfering substance known is L-glutamate, which is measured in much the same way as L-aspartate, although with different stoicheiometry. Here, cold L-glutamate transaminates with labelled 2-oxoglutarate, producing unlabelled 2-oxoglutarate, which participates in an exchange transamination with unlabelled L-glutamate. The effects of this exchange transamination between L-glutamate and 2-oxo[1-^{14}C]glutarate can be minimized by increasing the concentration of 2-oxo[1-^{14}C]-glutarate. This is expensive, however, and brings forth the problems of increasing blanks. Thus it is noteworthy that a satisfactory means of removing L-glutamate has been provided, so that this source of interference has largely been eliminated from the standard technique.

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