The Enzymic Estimation of Glutamate and Glutamine

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A method of estimating glutamic acid is described, based on its dehydrogenation by glutamate dehydrogenase coupled, by means of *N*-methylphenazine methosulphate, to the reduction of tetrazolium salts. The method is suitable for the estimation of 0.0–0.3 μ mole of glutamic acid. The response is linear, but not stoichiometric: possible reasons for this are discussed. If suitable precautions are taken, the use of a partially purified preparation of glutaminase makes it possible to estimate glutamine also.

No convenient and specific micro-method for the estimation of glutamate and glutamine exists, except that developed by Holzer & Söling (1962) which uses glutamate dehydrogenase and the rather expensive acetyl-NAD. The present paper describes a method in which the oxidation by glutamate dehydrogenase is coupled to the reduction of suitable tetrazolium salts. Glutamine can be estimated after hydrolysis to glutamate. Preliminary reports of the method have appeared (Sowerby & Ottaway, 1961, 1962).

EXPERIMENTAL AND RESULTS

Materials

Glutamate dehydrogenase as a solution in 50% (v/v) glycerol was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. It was stored at -15° .

Glutaminase I (L-glutamine amidohydrolase, EC 3.5.1.2) was prepared from pig-kidney mitochondria by the method of Klingman & Handler (1958). Purification was taken only to the stage of the second precipitation with 4M-phosphate, since further purification gave poor yields of enzyme which very quickly lost activity, even at -15° . The precipitate from the second phosphate treatment was dissolved in a small volume of 0.03M-borate–0.02M-phosphate buffer, pH 8.1, which stabilizes the enzyme (Klingman & Handler, 1958), to give a solution containing about 12 mg. of protein/ml. This was dialysed in the cold against the same borate–phosphate buffer to remove bound glutamate and dispensed into 5 ml. vials, which were stored at -15° . This preparation was stable for 6–12 months at this temperature and for several weeks at 0° . Occasional preparations were even stable for 24 hr. at room temperature, much longer than the time reported by Klingman & Handler (1958).

Glutamic acid and glutamine were obtained from Roche Products Ltd., Welwyn Garden City, Herts. Stock solutions (0.05M) are stable for 1–2 weeks in the refrigerator.

NAD was obtained from Mycofarm, Delft, The Netherlands. A stock solution containing 0.5% was stable for several weeks in the refrigerator.

PMS* was obtained from Sigma Chemical Co. Ltd. (London). A 1% (w/v) stock solution was kept in the refrigerator in an opaque bottle. It was stable indefinitely in such conditions but it, and any dilute solution prepared from it, must be rigorously protected from light. *N*-Ethylphenazine methosulphate was prepared by the method of Dickens & McIlwain (1938). MTT was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and INT from British Drug Houses Ltd., Poole, Dorset. Some samples of INT from this and other sources could not be reduced at all by the assay system, even after recrystallization from hot water. Solutions (0.4%) of these compounds were stable indefinitely at room temperature, when kept in the dark. The INT solution was filtered before use.

The formazans corresponding to INT and MTT were most conveniently prepared by the enzymic reduction of the tetrazolium salts, with glutamate as hydrogen donor. Alternatively, they could be reduced by ascorbate in the cold at pH 8.0 if PMS was also added. The insoluble formazans were filtered off, washed well with water, recrystallized from aqueous acetone or methanol and dried to constant weight *in vacuo*. Fig. 1 shows that the absorption spectra of the formazan produced by chemical reduction of INT with more vigorous reagents, e.g. dithionite, was not identical with that of the enzymically produced compounds. Their millimolar extinction coefficients were: in aq. 40% (v/v) acetone, INT 12.5 at 460 $m\mu$, MTT 11 at 560 $m\mu$; in ethyl acetate, INT 8.1 at 490 $m\mu$ and MTT 8.8 at 560 $m\mu$.

Acetone was purified by distillation from $KMnO_4$. An efficient vapour trap was necessary since the addition of acetone containing traces of manganese salts to INT dissolved in phosphate buffer gave rise to formazan even in the absence of other hydrogen donors.

Tris was purified by recrystallization from methanol.

Phosphate was purified by preparing a saturated solution, at room temperature, of NaH_2PO_4 in water containing

* Abbreviations: PMS, *N*-methylphenazine methosulphate; MTT, 3-(4,5-dimethylthiazol-1-yl)-2,5-phenyltetrazolium bromide; INT, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride.

Fig. 1. Absorption spectra of formazan produced by the reduction of INT. The solvent was aq. 40% (v/v) acetone. A, Enzyme reduction with glutamate as hydrogen donor; B, reduction with ascorbate, pH9, in the presence of PMS; C, reduction with dithionite.

0.1% (w/v) of EDTA or 8-hydroxyquinoline, warming slightly, and adding the calculated quantity of NaOH to form Na_2HPO_4 . The precipitated salt was washed with ethanol-water (1:1, v/v) and then with ethanol, and dried *in vacuo*. Phosphate analysis showed the product to be $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$.

All other chemicals were A.R. grade. Glass-distilled water was used throughout.

Deproteinization of tissue samples

Tissues were frozen in an acetone-solid CO_2 mixture and pulverized in a percussion mortar cooled to -70° . Either of the methods of deproteinization described below could then be used.

(1) A weighed amount of the still-frozen powder was homogenized with 2 ml. of cold acetone/g. of tissue in a centrifuge tube, by means of a loose-fitting power-driven Teflon pestle, which was subsequently washed with 1 ml. of water/g. of tissue. The solution was immersed in a water bath at 70° for 3 min., care being taken not to let the mixture boil over. Most of the acetone was removed by a stream of air, and 2 ml. of water/g. of tissue was added, together with a few drops of *N*-acetic acid to bring the pH of the solution to the isoelectric point of the proteins. The mixture was left for at least 15 min., then centrifuged, and the supernatant removed. The precipitate was washed with water and recentrifuged, and the combined supernatants were made up to a suitable volume.

(2) The frozen powdered tissue (0.2–0.5 g.) was homogenized with 2.5 ml. of cold ethanol-*N*-HCl (1:1, v/v), the pestle was washed with 1 ml. of water and the suspension heated in a water bath at 60° for 3 min. After cooling, the solution was neutralized with 1.25 m-moles of KOH and allowed to stand at 4° for 3 hr. The volume was then made up to 10 ml., the protein centrifuged down and the supernatant used for analysis.

Estimation of glutamate

The enzyme solution, made up on the day of use, consisted of: 3 ml. of 0.2M-tris buffer, pH7.6, containing 0.025% of EDTA, 2 ml. of 7 mM-NAD and 0.1 ml. of glutamate dehydrogenase solution (i.e. 2 mg. of protein). The coenzyme was added to the enzyme solution, since dilute solutions of glutamate dehydrogenase rapidly lose activity in the absence of NAD (Frieden, 1959). The dilute enzyme solution is stable for at least 24 hr. at 0° .

To a test tube were added the following reagents: up to 1 ml. of sample containing 0–0.3 μ mole of glutamate, 1 ml. of 0.2M-phosphate buffer, pH7.6, 0.5 ml. of 8 mM-INT or 4 mM-MTT, 0.5 ml. of enzyme solution and water to make a total volume of 3.5 ml. The reaction was started by adding to each tube 0.1 ml. of a 1:100 dilution of the stock PMS solution. (Alternatively, stock PMS could be added to INT, or MTT, solution to give a 1:500 dilution of PMS. In this case 0.5 ml. of freshly made up PMS-INT reagent was added to start the reaction.)

The tubes, including a blank containing all reagents except glutamate, were incubated for 30 min. at 37° and, if INT was being used, each tube then received 0.5 ml. of 2N-HCl, which prevents turbidity due to precipitation of protein and salt on the addition of acetone, and also inhibits the slow production of formazan induced by slightly impure acetone in the presence of phosphate. The formazan of MTT is decolorized even in slightly acid solutions, so that HCl could not be added when this reagent was used. When INT was used, 5 ml. of purified acetone was then added to each tube; with MTT turbidity could be avoided by using the same volume of aq. 60% (v/v) acetone. The contents of the tubes were mixed and allowed to stand for 5 min. to reach room temperature. The colours, which are stable for at least 12 hr., were then read against the reagent blank at $460\text{ m}\mu$ (INT) or $560\text{ m}\mu$ (MTT). Beer's law is obeyed to about 0.2 μ mole of glutamate with INT and up to about 1 μ mole with MTT. The final mixture of acetone and water dissolves only about 0.3 μ mole of INT formazan; samples containing more than 0.3 μ mole of glutamate could be estimated by diluting the formazan suspension with aq. 50% (v/v) acetone.

The reaction could be made about twice as sensitive by extracting the formazan into 3–4 ml. of ethyl acetate; it was not necessary in this case to add acid. The absorption maximum of INT in ethyl acetate is at $490\text{ m}\mu$, and that of MTT at $560\text{ m}\mu$. Extraction was almost quantitative, but it was usually necessary to centrifuge the tubes to remove water droplets from the upper layer. The ethyl acetate technique was advantageous with very small amounts of glutamate, or when there was so much salt in the sample that it was precipitated on the addition of acetone.

Estimation of glutamine

Up to 1.5 ml. of the sample was added to 1 ml. of 0.2M-phosphate buffer, pH7.6, containing 1–10 μ moles of Mg^{2+} and 0.05 ml. of glutaminase I solution. The total volume was made up to 2.5 ml. with water. The mixture was incubated at 37° for 30 min., by which time hydrolysis was complete. It was desirable to boil the samples at this stage, to destroy enzymes associated with the glutaminase preparation. Then 0.5 ml. of INT or MTT solution containing 20 μ g. of PMS/ml. was added, followed by 0.5 ml.

of glutamate dehydrogenase-NAD solution. The samples were subsequently treated as described for the glutamate assay. It was necessary to run a blank containing all reagents since the glutaminase contains some tetrazolium-reducing substances, even after dialysis to remove bound glutamate (Klingman & Handler, 1958). Carrying out the estimation sequentially, as described above, gave better yields of formazan than if the GAD and glutaminase were added at the same time. A separate glutamine standard curve was prepared. If the sample contained preformed glutamate, a duplicate set of tubes without glutaminase was run and the glutamine content was calculated by difference. We have found no evidence for inhibition of glutaminase by glutamate in amounts up to $0.3 \mu\text{mole/tube}$. Also, NH_4^+ does not inhibit the reaction in amounts up to $1 \mu\text{mole/tube}$.

Factors affecting the estimation

Substrate specificity. At pH 9.6 acids other than glutamate are oxidized by glutamate dehydrogenase, notably norvaline and L- α -aminobutyrate (17 and 3% as fast as glutamate respectively). At pH 7.6, however, Struck & Sizer (1960) found that no amino acid other than glutamate is oxidized, thus confirming the original report of Olson & Anfinsen (1953). Under our conditions only 1% of $1 \mu\text{mole}$ samples of norvaline and L- α -aminobutyrate were oxidized in 30 min. All other amino acids tested, including aspartate and glutamine ($1 \mu\text{mole}$), produced no detectable amount of formazan in 30 min. Alanine was tested in amounts up to $37 \mu\text{moles}$.

Suppression of non-enzymic reduction of PMS. Direct reduction of PMS by substances present in tissue extracts may occur. This could be prevented by a preliminary oxidation of interfering substances by oxygen (incubation for 10–30 min. of the sample in buffer with PMS but without glutamate dehydrogenase-NAD and final hydrogen acceptor). The assay was then completed by adding fresh PMS, INT and glutamate dehydrogenase-NAD in that order. Fresh PMS must be added because it is oxidized in air to phenazine, 2-oxophenazine and other products. Under these conditions direct non-enzymic reduction of PMS is negligible.

Effect of pH. The optimum pH for the estimation is 7.6. Five buffers were tested: tris, imidazole, phosphate, glycylglycine and N-ethylmorpholine. Formazan production was poor with both glycylglycine and N-ethylmorpholine, satisfactory with tris if cyanide was also added, and satisfactory with phosphate (with this buffer the presence of cyanide did not affect formazan production) and with imidazole (addition of cyanide depressed formazan formation). Since, when assaying glutamine, it is necessary to activate glutaminase with phosphate, this buffer was chosen for routine assays of

glutamine; it has the added advantage that cyanide need not be used. Better results were obtained when A.R.-grade phosphate was recrystallized from a solution containing a metal-sequestering agent.

Effect of temperature on the yield of formazan. The optimum temperature is between 30 and 37°. The enzyme is rapidly inactivated at temperatures above 50°. The amount of formazan formed at temperatures lower than 30° could not be increased by lengthening the incubation time; at all temperatures tested, formazan formation practically ceased after 20 min. incubation. The low percentage formation of formazan at low temperatures did not appear to be due to inactivation of the enzyme before all the glutamate had been dehydrogenated. Table 1 shows that the amount of formazan formed from a known amount of glutamate after 30 min. was much less at 22° than at 37°. However, continued incubation at 22° after the addition of more glutamate dehydrogenase or PMS only increased the yield slightly, and not up to that obtained from a 30 min. incubation at 37°. Other experiments of this type, in which a second addition of glutamate was made after a 30 min. incubation, showed that all the components of the reaction were still active at this time. These observations are further discussed below.

Concentration of reagents. The percentage production of formazan increased with the concentration of enzyme, and it was not possible to compensate for low concentrations of glutamate dehydrogenase by prolonging the incubation time. Recovery of formazan did not increase further when more than 200 $\mu\text{g.}$ of glutamate dehydrogenase was added to each tube. The percentage formation of formazan became independent of NAD concen-

Table 1. *Production of formazan from glutamate under various conditions*

Each tube contained $0.18 \mu\text{mole}$ of glutamate, $1.75 \mu\text{moles}$ of EDTA, $20 \mu\text{moles}$ of KCN, $1.4 \mu\text{moles}$ of NAD, $200 \mu\text{g.}$ of glutamate dehydrogenase, $33 \text{ m}\mu\text{moles}$ of PMS and $4 \mu\text{moles}$ of INT, together with 1 ml. of 0.15 M -tris buffer, pH 7.6. The total volume was 3.5 ml. The reaction was stopped by the addition of 0.5 ml. of 1.5 N -HCl, and the formazan was estimated spectrophotometrically after the addition of 5 ml. of acetone.

Conditions	Formazan produced (% of theoretical amount)
Incubation for 30 min. at 37°	84
Incubation for 30 min. at 22°	54
Incubation for 60 min. at 22°	62
Glutamate dehydrogenase ($100 \mu\text{g.}$) and PMS ($10 \mu\text{g.}$) added after 30 min. at 22°, then incubation for further 30 min. at 37°	63

tration when this was greater than $1.5\mu\text{moles}$ of NAD/tube. Dilute solutions of glutamate dehydrogenase are said to split into inactive sub-units in the absence of some nucleotides, of which NAD is one (Frieden, 1959). However, a dilute enzyme solution made up as described in the section on 'Estimation of glutamate' was perfectly stable for at least 14hr. when the NAD concentration was as low as $1\mu\text{mole/ml}$.

The optimum concentration of INT was $4\mu\text{moles/tube}$, and that of MTT $2\mu\text{moles/tube}$. Fig. 2 shows the optimum concentrations of PMS for use with INT and MTT. These concentrations ($33\text{m}\mu\text{moles/tube}$ or less) are much lower than those usually employed (see Ells, 1959). When the final hydrogen acceptor is oxygen, the use of high concentrations of PMS is understandable, since the rate constant for the reaction:

is relatively low (see Ottaway, 1966). However, the reaction of PMSH_2 (reduced PMS) with tetrazolium salts is fast, and the rate-limiting step in the oxidation of glutamate is the primary dehydrogenation. Since PMS and PMSH_2 readily decompose to give, in particular, phenazine and 2-oxophenazine, both of which are reddish compounds, high concentrations of PMS give high and variable blanks, and should therefore be avoided. A concentration of $33\text{m}\mu\text{moles/tube}$ was generally used.

Fig. 3 shows that the presence of a small concentration of EDTA or other chelating agent increased the percentage yield of formazan. Higher concentrations depressed the yield almost to zero, possibly because glutamate dehydrogenase contains zinc (Adelstein & Vallee, 1958), which can be removed by EDTA. A concentration of $0.5\text{--}2.0\mu\text{moles/tube}$ was used. If the reaction is carried

out in non-sequestering buffers, Ca^{2+} and Mg^{2+} interfere; they can be removed by a suitable increase in the concentration of EDTA. When phosphate buffer is used, Mg^{2+} (in amounts up to $1\mu\text{mole/ml}$ of sample) no longer interferes, and Ca^{2+} interferes only slightly. No advantage was gained by including either zinc salts or Zn-EDTA in the reaction mixture.

Effect of cyanide on the reaction. Cyanide ($20\mu\text{moles/tube}$) increased the yield of formazan considerably, but not to 100%, when tris was used (Fig. 4). The effects of EDTA and cyanide were additive (Fig. 3). On the other hand, cyanide was without effect in phosphate buffer, and definitely diminished the yield in imidazole buffer.

Two effects of cyanide, on different stages of the complete reaction, may be responsible for these observations. Spectrophotometric estimation of

Fig. 3. Effect of EDTA concentration on the production of formazan from glutamate. The amount of EDTA per tube was varied in the absence of KCN (●) and in the presence of $20\mu\text{moles}$ of KCN (○). In other respects the reaction mixture was as given in Table 1 and the procedure was that described in the text.

Fig. 2. Effect of PMS concentration on the production of formazan from glutamate. Each tube contained the amount of PMS indicated and $4\mu\text{moles}$ of either MTT (○) or INT (●). Otherwise the reagents were as given in Table 1 and the procedure was that described in the text.

Fig. 4. Effect of cyanide concentration on the production of formazan from glutamate. Each tube contained the amount of KCN indicated and $4\mu\text{moles}$ of either MTT (○) or INT (●). Otherwise the reagents were as given in Table 1 and the procedure was that described in the text.

the equilibrium between glutamate and NAD catalysed by glutamate dehydrogenase showed that cyanide both increased the rate of oxidation of glutamate and shifted the equilibrium in favour of NADH₂. These results are compatible with the removal of α -oxoglutarate as cyanhydrin. On the other hand, a kinetic study of the reactions:

suggests that cyanide both lowers the effective PMS/PMSH₂ redox potential and prevents the formation of an intermediate PMSH₂-NAD compound, which is not readily oxidized. On this view, the buffer-cyanide interactions would reflect the differing tendencies of the buffer ions to stabilize the non-reactive intermediate. The varying percentage yields of formazan from glutamate found with the different buffers with and without cyanide were very similar to the yields from NADH₂ added directly to similar reaction systems, which suggest that in practice the effects of cyanide on the NADH₂ $\rightarrow$ PMS reaction are more important than those on glutamate oxidation itself. This is also suggested by the very large effects of cyanide on yield of formazan in the succinate-oxidizing system described by Nachlas, Margulies & Seligman (1960).

Interfering substances. Hydrazine, hydroxylamine and to a smaller extent ascorbate interfere by reducing PMS directly, and hence INT or MTT. Semicarbazide, cysteine and glutathione do not reduce PMS at this pH. α -Oxoglutarate and NH₄⁺ decrease the yield of formazan slightly at concentrations of 1 μ mole/tube; below this their effect is negligible. Interference from ammonia can be removed by making the phosphate buffer 0.01M with respect to Mg²⁺. No interference has been found from any amino acid other than norvaline or α -aminobutyrate, or from citrate, fumarate, succinate, β -hydroxybutyrate, pyruvate or lactate at a concentration of 1 μ mole/tube. Oxalate at this concentration decreases the yield slightly.

Deproteinization of tissue extracts. Most protein precipitants such as tungstic acid, molybdic acid and trichloroacetic acid, even when neutralized, inactivate glutamate dehydrogenase. Saturated (0.06M) potassium perchlorate does not do so, but forms an insoluble salt with INT. Two methods of deproteinization with organic solvents have been described in the present paper. That with acid ethanol is the less satisfactory, since Grisolia, Fernandez, Amelunxen & Quijada (1962) have reported that glutamate dehydrogenase is inhibited by high concentrations of salts, including potassium chloride. We found that formazan formation from glutamate was inhibited by about 10% when 1 ml. samples of the supernatant (containing about

Table 2. *Effect of oxygen concentration on yield of formazan from INT*

Each tube contained 0.18 μ mole of glutamate, and the other reagents were identical with those reported in Table 1. The anaerobic experiments were carried out in evacuated Thunberg tubes; the reaction was started by the addition of enzyme from the side arm.

Concn. of O ₂ (μ mole/ml. of reaction mixture)	Formazan produced (% of theoretical amount)
0.0 (anaerobic)	99.5
0.0 (reagents made up in freshly boiled water)	96
0.2 (in air)	70.5
1.0 (moist O ₂ gas bubbled through)	48.5

120 μ moles of potassium chloride) were used for analysis. With 0.5 ml. samples there was no inhibition. The concentration of ethanol in the final supernatant was about 12%; this had no effect on the reaction, even with 1 ml. samples.

The samples should not be heated too long during deproteinization, since glutamate and glutamine (the latter very rapidly) form pyrrolidonecarboxylate on being heated in aqueous solution at neutral pH. Re-formation of glutamate from this compound is very slow. Under the standard assay conditions the loss of glutamate or of glutamine is less than 5%.

Effect of oxygen. The yield of formazan from glutamate or NADH₂ could be increased to the theoretical amount by carrying out the reaction anaerobically in Thunberg tubes. Yields of about 95% could be obtained by using reagents made up in freshly boiled water and stored under nitrogen. Conversely, the yields were much lower than usual if the reagents were saturated with oxygen (Table 2). Direct measurement with an oxygen electrode by using air-saturated reagents failed, however, to show any significant disappearance of oxygen to account for the less-than-theoretical yield of tetrazolium salt.

A mitochondrial factor increasing the yield of formazan. When the glutaminase preparation from kidney mitochondria was present in the assay system the yield of formazan from INT or MTT was theoretical, with glutamate as hydrogen donor. The effect was independent of the concentration of oxygen, and independent of cyanide when tris buffer was used. Glutaminase activity could be lost from the extract without simultaneous loss of ability to enhance the yield of formazan. There is reasonable evidence that the active factor is a protein (Sowerby & Ottaway, 1962), but it is not clear that it is truly enzymic, since it withstands boiling for 5 min. in dilute (but not con-

centrated) solution. It does not replace PMS, and is therefore not a tetrazolium reductase (Slater, 1959). Scaife (1964) has described a coupling factor, with very similar properties to our material, which is lost from thymus mitochondria on irradiation. We were unable to separate the formazan factor from glutaminase by chromatography or electrophoresis.

Since oxygen depresses the yield of formazan in the assay, but does not disappear in equivalent amounts, it seemed possible that its effect is due to the production of hydrogen peroxide, which might, for example, inactivate glutamate dehydrogenase. The glutaminase preparation is very rich in catalase, but its property of enhancing the yield of formazan is not due to this enzyme, because it is effective in the presence of cyanide. Further, added catalase does not affect the yield of formazan.

The mitochondrial factor does not affect either the position, or the rate of attainment, of equilibrium between glutamate and NAD. Kinetic studies show that, in the presence of EDTA, the mitochondrial factor increases the rate of reduction of PMS by NADH_2 . In the presence of 1 mM-cyanide this capability is largely lost, and catalase and the mitochondrial factor now have very similar effects on the reaction $\text{NADH}_2 \rightarrow \text{PMS} \rightarrow \text{O}_2$. The ability of the mitochondrial factor to increase the yield of formazan is not, however, inhibited by cyanide. These results may be interpreted to mean that PMSH_2 , and possibly also PMS, are bound to a non-haem site on a protein, to form essentially the prosthetic group of an enzyme catalysing the transfer of hydrogen from NADH_2 to tetrazolium salts. The binding need not be very specific, since boiled mitochondrial factor appears to be at least partially effective as an 'apo-enzyme'.

Use of phenazine ethosulphate as carrier. The *N*-methyl group of PMS is very readily oxidized to formaldehyde, but other *N*-alkylphenazines are much more stable. Most of the present experiments were repeated with *N*-ethylphenazine ethosulphate, but no differences were found in the rates of reaction or in percentage yields of formazan.

Enzyme specificity of the glutaminase preparation

The glutaminase preparation contains no α -oxoglutarate-specific transaminase activity (at least in the absence of added pyridoxal phosphate), no L-amino acid-oxidase activity and no detectable lactate-, succinate-, malate-, β -hydroxybutyrate- or ethanol-dehydrogenase activity, but it does contain some so far unidentified NAD-requiring dehydrogenase. Most biological samples do not contain the substrate(s) for this enzyme or enzymes, but it was found advisable to check this. Alternatively, interference from dehydrogenase can be

removed by placing the samples in a boiling-water bath for 2 min. after incubation with glutaminase. This treatment causes about 10% of the glutamate present to cyclize, and for accurate estimations internal standards should be used.

DISCUSSION

The methods described above have been in routine use in this Laboratory for over 2 years and have given no trouble other than occasional decrease of sensitivity caused by a batch of impure INT. In view of the unpredictable variations in this tetrazolium salt, and the difficulty of purifying it, we recommend the use of MTT, although the molar extinction coefficient of its formazan is rather smaller than that from INT. We have not used the factor enhancing the yield of formazan described in the test, because of the danger of incomplete inactivation of glutaminase. Although the assay could be carried out under anaerobic conditions, we have preferred aerobic conditions and have compensated for the failure to obtain quantitative yields of formazan by using internal standards, particularly when estimating glutamine.

The incomplete yields of formazan in the absence of the mitochondrial factor may be interpreted in terms of a PMSH_2 derivative that does not react with tetrazolium salts. The amount of reducing equivalents in the intermediate could not itself be great enough to account for the apparent loss of formazan, but if its breakdown to free PMS were assisted by NADH_2 (see Ottaway, 1966), and particularly if the intermediate were stabilized by some of the ions present, the concentration of free PMS might fall to very low levels after the first few minutes of the reaction. This would mean that the rate of oxidation of NADH_2 would become immeasurably small, i.e. a false equilibrium would be reached. Such an explanation would account for the fact that, particularly at room temperature (see Table 1), the reaction comes to a halt before completion, and yet, on the addition of more glutamate, all the components of the system are found to be still active. It would also provide an explanation for the effects of cyanide on the reaction.

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