Glutamine Synthetase in Muscle and Kidney

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1. Glutamine synthetase activity has been determined in extracts of rat cardiac and skeletal muscle and kidney, after treatment to ensure that the rate of synthesis was proportional to time of incubation and to amount of extract added. The activity was measured by two methods, with hydroxylamine as substrate. 2. No activity was detected in rat heart extract by either method. The activity in skeletal muscle was of the order of $20 \mu\text{mol}$ of glutamylhydroxamate synthesized/h per g of tissue under optimum conditions. The activity in kidney extracts was $180 \mu\text{mol}$ /h per g of tissue when measured as ferric hydroxamate. 3. The activity in both skeletal-muscle and kidney extracts was inhibited by P_i . The inhibition is competitive for the muscle enzyme, with a K_i of 12 mM . For the kidney enzyme the inhibition is non-competitive, and less marked. Possible enzyme mechanisms that would lead to these types of inhibition are discussed. 4. Several observations are reported that suggest that the enzymes from muscle and kidney are not identical. 5. Growth hormone, either *in vivo* or *in vitro*, did not affect the measured glutamine synthetase activity of tissue extracts.

Glutamine is one of the amino acids whose concentration is highest both intracellularly and in the blood plasma of vertebrates. In rat plasma its concentration is about 0.55 mM (Herbert, Coulson & Hernandez, 1966) which is higher than that of any other amino acid. In tissues its concentration is about $2.5 \mu\text{mol/g}$ of wet tissue, although it is much higher in liver, heart and brain. Kidney forms an exception to this general rule, since the concentration of glutamine in this tissue is only $0.3 \mu\text{mol/g}$, which is lower than that of many other amino acids.

In spite of this detailed information about the distribution of glutamine in the body, remarkably little is known about its function, except as a source of NH_4^+ in acid-base control by the kidney, and as a direct source of $-\text{NH}_2$ groups for the synthesis of some non-protein nitrogenous compounds, particularly purines. In many tissues of the body, however, purine nucleotide synthesis must proceed at a very slow rate, and it seems rather doubtful that such very high concentrations of glutamine are necessary to support these particular syntheses. It is widely supposed that glutamine is a detoxication product of ammonia (e.g. Cantarow & Schepartz, 1967), and that, by implication, the glutamine found in plasma is on its way from peripheral tissues to liver or kidney so that the $-\text{NH}_2$ group may be finally removed from the body as urea, uric acid, or NH_4^+ ions in urine. Experimental infusion of NH_4^+ ion into blood has been

shown to lead to a fall in glutamate concentration and a rise in glutamine (Takagaki, Berl, Clarke, Purpura & Waelsch, 1961), and this may well be a detoxication process. However, in certain circumstances muscle is capable of forming ammonia at a rapid rate [e.g. from the deamination of AMP (Parnas, 1935)], but it has never been clearly established that either skeletal or cardiac muscle can synthesize glutamine at all. Only two detailed reports on the synthesis of glutamine in these tissues in mammals have appeared (Trush, 1963; Wu, 1963). The former reported low levels of glutamine synthetase activity in both heart and skeletal muscle, but the experimental methods used were not above criticism, and the results cannot be accepted with complete confidence. Wu (1963) did not find any glutamine synthetase in cardiac or skeletal muscle of the rat. It was decided to re-investigate this problem, to help answer the question of whether the muscle mass is one of the sources of plasma glutamine, or whether muscle takes up glutamine from the plasma for a function that has not yet been established. Because we are interested in the relationship between pituitary growth hormone and nitrogen metabolism, some experiments on the concentration of glutamine synthetase in tissues after growth-hormone injections were also carried out and are reported here.

To establish beyond doubt the reliability and

limits of accuracy of the assay methods, it was thought desirable to include measurements on extracts of a tissue in which it has been well established that glutamine synthetase is present. Extensive studies have been carried out on the glutamine synthetase of brain, liver, retina and kidney (for reviews see Meister, 1965, 1968). We found that kidney was a satisfactory source of the enzyme (Reiner & Hudson, 1953; Richterich & Goldstein, 1958) for comparative purposes, although some evidence accumulated that the enzymes from muscle and kidney are not identical, at least in the rat.

METHODS

Two assay methods were used. One was based on the determination of the γ -glutamylhydroxamate formed when hydroxylamine is used as a substrate. The concentration of the hydroxamate may be measured by the extinction of its complex with Fe^{3+} ions. The sensitivity of the colour reaction was doubled by using $\text{Fe}(\text{NO}_3)_3$ in trichloroacetic acid instead of the FeCl_3 in HCl used by Lipmann & Tuttle (1945). We found that the nitrate gave a slightly more intense colour, and was less likely to give a high tissue blank, than the perchlorate in HClO_4 recommended by Goddu, LeBlanc & Wright (1955).

The reaction mixture was based on that of Elliott (1955). It contained $60\mu\text{mol}$ of MgCl_2 , $150\mu\text{mol}$ of imidazole-HCl buffer, pH 7.2, $300\mu\text{mol}$ of hydroxylamine, pH 7.2, $150\mu\text{mol}$ of sodium glutamate, $75\mu\text{mol}$ of 2-mercaptoethanol and $30\mu\text{mol}$ of ATP (sodium salt). The pH of the last three solutions was adjusted to 7.2 when they were made up. The hydroxylamine was made up as a 2M solution of the hydrochloride; a small portion of this was neutralized with 2M-NaOH immediately before use, and the volume was adjusted with water.

Water was added with the reagents so that when a suitable volume of tissue extract containing the enzyme was added to the reaction mixture the total volume was 3ml. The enzyme was added last, and the mixture was then incubated at 37°C , usually for 10min. The amount of tissue extract was adjusted so that less than $3\mu\text{mol}$ of hydroxamate was formed during the incubation: in these conditions we confirmed Elliott's (1955) finding that the amount of hydroxamate produced is proportional to the amount of extract added (except as described below).

The reaction was stopped by adding 2ml of ferric reagent [consisting of equal volumes of 40% (w/v) trichloroacetic acid and of $0.6\text{M-Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$]. After being rapidly mixed and allowed to stand for about 10min, the precipitated protein was removed by centrifuging in a bench centrifuge for about 10min, and the extinction of the supernatant was measured at 500nm. The concentration of γ -glutamylhydroxamate was determined from a calibration curve prepared from a sample of the pure substance [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.].

The method described above is reliable and convenient, but is not very sensitive, and has the major disadvantage that ADP, which is a powerful inhibitor of the enzyme, can accumulate from side reactions during the incubation, and may prevent the maximum activity from being measured. To overcome this difficulty a method based on

coupling the production of ADP, as the reaction proceeds, to the oxidation of NADH, by adding pyruvate kinase, phosphoenolpyruvate and lactate dehydrogenase to the system, was developed. The method described below allows a correction to be made for the activity of other ADP-producing reactions (adenosine triphosphatases) in the tissue extract; with muscle extracts these are often much more active than glutamine synthetase. Similar methods designed for use with purified glutamine synthetase have been published by Wellner, Zoukis & Meister (1966), Kingdon, Hubbard & Stadtman (1968), and Liess, Varricchio, Mecke & Holzer (1968).

The reaction mixture consisted of $60\mu\text{mol}$ of imidazole-HCl buffer, pH 7.2, $30\mu\text{mol}$ of MgCl_2 , $25\mu\text{mol}$ of hydroxylamine, $25\mu\text{mol}$ of mercaptoethanol, $50\mu\text{mol}$ of L-glutamate and up to $10\mu\text{mol}$ of ATP. The pH of the last four reagents was adjusted to 7.2 before addition. To this mixture was added $50\mu\text{l}$ of a solution containing $6\mu\text{mol}$ of NADH, $40\mu\text{mol}$ of phosphoenolpyruvate, 1.5mmol of KCl and $5\mu\text{mol}$ of EDTA (disodium salt)/ml, and $100\mu\text{l}$ of a solution containing approx. 240 units of lactate dehydrogenase, 250 units of pyruvate kinase and 120 units of adenylate kinase/ml. For the experiments described in this paper it was necessary to keep the reaction mixture free from NH_4^+ ions, and to this end the enzyme mixture was dialysed for about 3h before use against two changes of 1mM-EDTA solution, pH 7.0, in a cold-room at 2°C . In other circumstances NH_4^+ may be admissible as a substrate, in which case this dialysis may be omitted and the hydroxylamine in the reaction mixture replaced by up to $100\mu\text{mol}$ of NH_4^+ (preferably not as sulphate).

The volume at this stage was 0.8ml. When the lactate dehydrogenase, pyruvate kinase and adenylate kinase mixture was added, there was a rapid, though limited, decrease in E_{340} as ADP and AMP in the reagents were removed. The mixture was left for 3–5min until the E_{340} trace was steady, and at this point 0.2ml of tissue extract, suitably diluted with water, was added to the cuvette and rapidly mixed. The decrease in E_{340} was followed for about 3min; it was generally linear over this period. All measurements were made at 37°C .

Correction for adenosine triphosphatase activity could be made by running a parallel assay from which glutamate was omitted, but when measurements were being made at various ATP concentrations it was found to be more convenient to record the adenosine triphosphatase activity on the same sample. This was done by omitting the glutamate from the original reaction mixture, recording the decrease in E_{340} after the addition of tissue extract, and at a suitable time adding $50\mu\text{mol}$ of L-glutamate (usually in 0.1ml), and continuing to record the extinction change. It was necessary to make a correction for the change in concentration of both ATP and adenosine triphosphatase after adding the glutamate.

ADP was measured by the method of Adam (1963), and P_i by the method of Taussky & Shorr (1953).

Preparation of tissue extracts. This was based on the method of Trush (1963). The tissues were removed from a rat immediately after death and transferred into ice-cold $0.15\text{M-NaCl-5mM-NaHCO}_3$. The tissue samples were washed with an excess of this solution, blotted, weighed and then frozen at -20°C (for a minimum period of 45min). After being thawed, the tissue samples were chopped with scissors and homogenized in ice-cold NaCl-NaHCO_3 ,

(5 ml/g of tissue). A Potter-Elvehjem homogenizer with a Teflon pestle was used for heart and kidney, and an Ultra-Turrax (Janke und Kunkel K.G., Staufen i. Br., Germany) for skeletal muscle. About 2.5 g of muscle from the leg of each animal was used. The homogenate was left at 2°C for about 2 h, with frequent shaking, for extraction of the enzyme to reach completion.

As described in the Results section, the activity in the homogenates was increased by centrifuging the homogenate at 56000g for 45 min in the type 40 rotor of a Spinco preparative ultracentrifuge at 2°C. The supernatant was filtered through four layers of muslin to remove fat particles, and was then dialysed against 2×100 vol. of 0.15 M-NaCl-5 mm-NaHCO₃, containing 1 mm-EDTA and 5 mm-mercaptoethanol, for a total of 3 h at about 5°C. When the activity was to be assayed by the NADH method, a KCl-KHCO₃ solution of the same ionic strength was used. The dialysed supernatant was stored in the cold and used on the day of preparation, although it was possible to keep the frozen tissue samples at -20°C for up to 48 h without significant loss of activity.

In some instances interference by the adenosine triphosphatases of the centrifuged homogenates was removed by precipitating the glutamine synthetase with solid (NH₄)₂SO₄ (Enzyme grade; BDH Chemicals Ltd., Poole, Dorset, U.K.). The precipitate was left for about 15 min before being spun down on a bench centrifuge. The precipitate was redissolved in a small volume of KCl-KHCO₃ solution and dialysed as described above.

All chemicals used were AnalaR grade or the equivalent. P_i was recrystallized by adding the calculated quantity of 40% (w/v) NaOH to a hot concentrated solution of NaH₂PO₄ containing 5 mm-EDTA, and allowing the solution to cool. The crystals were filtered off, washed with ethanol and dried in a desiccator. They were found by analysis to consist of Na₂HPO₄·2H₂O. Enzymes were obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Bovine pituitary growth hormone, lot NIH GH-B11, stated potency 0.81 USP unit/mg, was a gift from the Endocrinology Study Section of the U.S. National Institutes of Health.

Experimental animals. All the animals used in this study were albino female rats of a local strain, fed *ad libitum* on a rat-cake diet (Oxoid Diet 86; Oxo Ltd., London S.E.1, U.K.). They were killed by ether anaesthesia. In the growth-hormone experiments, 1 mg of the hormone, dissolved in 0.9% NaCl by adding a trace of alkali, was injected subcutaneously each day over a 5-day period, with a final dose 2-3 h before the animal was killed. This amount of hormone produced a gain in weight of about 2 g/day in a slowly growing female of 200-225 g weight, as compared with controls injected with 0.9% NaCl.

RESULTS

Assay methods. (a) Hydroxamate method. Incubation of the reaction mixture at 37°C causes a slight increase in E_{500} after treatment with ferric reagent, even in the absence of glutamine synthetase activity. This is unimportant when measuring the enzyme concentration in active extracts, but it is essential to have an accurate value for the blank

extinction when assessing extracts of low or doubtful activity. Investigation showed that the components of the assay mixture that gave rise to most of the non-specific E_{500} on incubation were hydroxylamine and mercaptoethanol, and that the presence of the tissue extract, and increase in time of incubation, intensified the extinction. Addition of glutamate in the absence of ATP caused a slight increase in extinction. As a result of these tests, the control reaction was always a mixture from which ATP had been omitted, but to which the tissue extract was added. This was carried through the incubation procedure with the test samples.

(b) NADH method. Some trouble was experienced with a slow oxidation of NADH when the tissue sample was added, but before glutamate was added. The rate of oxidation was roughly proportional to the concentration of ATP present, but varied with different extracts. This artifact was traced to the presence of about 0.1% of AMP in the ATP that was being used, together with the presence of low and variable amounts of adenylate kinase in the tissue extract. The routine addition of adenylate kinase, in the concentration recommended, with the lactate dehydrogenase and pyruvate kinase, to the reaction mixture, abolished this slow oxidation of NADH without affecting the rate at which ADP was rephosphorylated by pyruvate kinase during the assay.

The method is not specific for glutamine synthetase, which is measured by the increase in the rate of NADH disappearance on the addition of glutamate. With muscle extracts, the rate of NADH oxidation caused by hydrolysis of ATP by adenosine triphosphatase was usually very great before the glutamate was added, and in consequence only small amounts of tissue extract could be added. This largely nullified the increase in sensitivity that could be gained by making measurements on NADH at 340 nm; the way in which this interference from adenosine triphosphatases was minimized is described in detail below.

With the NADH method it is possible to use either NH₄⁺ or hydroxylamine as substrate. We found that the activity was greater with NH₄⁺ than with hydroxylamine, (see Table 1), and that high concentrations of the latter were slightly inhibitory. However, in the investigations described here it was not possible to use NH₄⁺, since in the presence of the high concentrations of Mg²⁺ needed to activate pyruvate kinase, the P_i added as an inhibitor formed a copious precipitate of magnesium ammonium phosphate.

With muscle extracts, the activity in dialysed supernatants was found to be the same by the hydroxamate method as by the NADH method.

Optimum conditions for assay. (a) Substrates. The optimum pH range, except for very high

Table 1. Comparison between ammonia and hydroxylamine as substrates for glutamine synthetase

Substrate	Activity (measured by NADH assay) ($\mu\text{mol/h}$ per g of tissue)	
	Muscle	Kidney
Hydroxylamine (25 mM)	7.3	69.3
Hydroxylamine (0.1 M)	5.9	56.0
Ammonia (0.1 M)	9.6	114.6

concentrations of divalent cations, was found to be about 7–7.4 by Elliott (1951), Greenberg & Lichtenstein (1959) and Monder (1965). Apparent K_m values for most substrates are quoted by Meister (1962), but there are some differences of opinion about the optimum concentrations of ATP and Mg^{2+} . To some extent the different estimates of optimum ATP concentration (Speck, 1949a,b; Elliott, 1951; Baerle, Goldstein & Dearborn, 1957; Pamiljans, Krishnaswamy, Dumville & Meister, 1962; Levenbook & Kuhn, 1962; Trush, 1963) may have been due to the accumulations of different amounts of ADP, particularly from side reactions, in preparations of different purity. Elliott (1951) showed that glutamine synthetase from sheep brain is 50% inhibited when the $[\text{ADP}]/[\text{ATP}]$ ratio is 0.3. However, Levenbook & Kuhn (1962) and Trush (1963) reported that the activity of glutamine synthetase is inhibited by ATP at concentrations greater than 5 mM. We have re-investigated this question for our own experimental conditions, and since Speck (1949b), Trush (1963) and Wu (1964) have reported that an excess of Mg^{2+} depresses the activity, we have also redetermined the optimum concentrations of Mg^{2+} and ATP (Figs. 1 and 2). There was no sign of inhibition by an excess of ATP. Although the curves for muscle and kidney extracts are not quite identical, an ATP concentration of 10 mM appears to be saturating in both cases, and was used in the determination of enzyme activity. The optimum Mg^{2+} concentration for this concentration of ATP was found to be 15 mM (i.e. 5 mM-free Mg^{2+}), but in view of the rather higher requirements for full activity of the pyruvate kinase used in the NADH assay method, a concentration of 20 mM was in fact used. This is the concentration recommended by Elliott (1951) and Baerle *et al.* (1957).

(b) Extraction of enzyme from tissues. Extraction of the frozen and thawed tissue with sodium chloride–sodium bicarbonate solution, as described by Trush (1963), appeared to give optimum activities. This would be expected from the report by Wu (1963). In particular, no increase in activity of the extracts was found when homogenization was

Fig. 1. Variation of glutamine synthetase activity of rat tissue extracts with ATP concentration. ●, Skeletal muscle; □, kidney.

Fig. 2. Variation of glutamine synthetase activity of rat tissue extracts with total Mg^{2+} concentration in the assay system. ●, Skeletal muscle; □, kidney.

carried out in the presence of 2.5% sodium deoxycholate or 0.5% sodium dodecyl sulphate, as suggested by Wu (1961). This is in agreement with the later report by Wu (1963).

(c) Partial purification of crude extracts. The activity to be expected in muscle and heart extracts was very low (Trush, 1963). The molar

Fig. 3. Effect of treatment of tissue extracts on the glutamine synthetase activity detected by the hydroxamate assay. (a) Kidney extracts: ●, crude homogenate; ■, high-speed supernatant; ▼, dialysed supernatant. (b) Muscle extracts: ○, crude homogenate; □, high-speed supernatant; ▽, dialysed supernatant.

extinction coefficient of ferric glutamylhydroxamate at 500nm is only about 10^4 . This means that the increase in extinction after 10min of incubation, even with amounts of extract equivalent to 250mg of fresh tissue/tube, was only of the order of 0.1 for skeletal muscle, whereas the extinction of the control was about 0.05. For heart the values were even lower. To be certain that the observed increases in extinction were in fact due to glutamine synthetase activity, it was thought desirable to ensure that the amount of hydroxamate produced was proportional (i) to time of incubation and (ii) to the amount of tissue extract added. In addition, Reiner & Hudson (1953) reported that glutamine synthetase activity in rat kidney homogenates could be increased threefold by centrifuging at high

Fig. 4. Relation of activity to amount of supernatant added to assay system. (a) Non-dialysed supernatant from kidney homogenate. (b) □, Non-dialysed, and ▽, dialysed supernatant from muscle homogenate. The incubation time was 10min for kidney and 30min for muscle extracts.

speeds (about 40000g). It was clearly desirable to take advantage of this finding, if it could be confirmed.

As Figs. 3 and 4 show, the formation of glutamylhydroxamate was not linear with respect to either time or amount of extract when crude homogenates were used. When a homogenate (of kidney) was replaced by its high-speed supernatant the activity was approx. 3 times as great, and was linear for incubation times of up to 60min. It was also linear with respect to amount of extract added, up to an amount equivalent to 50mg of fresh tissue, when the incubation time was kept at 10min. It was found that centrifugation (in a Spinco model 40 rotor) for 45min at 52000g gave about 20% greater activity than centrifugation for 60min at 100000g as recommended by Reiner & Hudson (1953). When the precipitate from such a centrifugation was re-mixed with the original homogenate, the activity was decreased to its original value. There was no detectable glutamine synthetase activity in the precipitate at either speed.

The activity of high-speed supernatants from muscle extracts was only slightly greater than that of crude homogenates. The activity in the supernatants did not fall off so markedly with increase in incubation time, but it was still far from linear with respect to increase in tissue weight. On the grounds that the rather large amounts of supernatant that

Table 2. *Glutamine synthetase activity in muscle and kidney extracts*

Assay method	Activity ($\mu\text{mol/h per g of tissue}$, $\pm \text{S.E.M.}$)	
	Skeletal muscle	Kidney
Hydroxamate (12)	19.1 ± 3.0	183.3 ± 18.8
NADH (5)	20.8 ± 1.85	92.6 ± 5.5

it was necessary to use might contain an inhibitor (e.g. ADP) that would be additive with an inhibitor produced during the incubation, the effect of dialysing the supernatants for 3h at 2°C against sodium chloride-sodium bicarbonate buffer was tried. These dialysed supernatants of skeletal-muscle extracts were found to be linearly related to the time of incubation up to 60min, and to the amount of extract up to 250mg of tissue equivalent.

The activities determined, with these refinements, with both the hydroxamate and NADH methods, are summarized in Table 2. Heart extracts, even after centrifugation and dialysis, did not give any evidence of glutamine synthetase activity that satisfied the two criteria of linearity described above. To eliminate the possibility that purified heart extracts still contained enough of the inhibitor(s) whose existence has been inferred for muscle and kidney extracts, to prevent the detection of small amounts of glutamine synthetase activity, various amounts of heart extracts were added to cuvettes containing suitable amounts of purified rat liver extracts, which were being assayed by the NADH method. The latter contain very little adenosine triphosphatase activity, which makes slight alterations in synthetase activity easier to detect. An experiment in which skeletal-muscle extracts were added to cuvettes containing liver extracts was used as a control.

The effects of both heart and skeletal-muscle extracts on the adenosine triphosphatase activity were completely additive. Skeletal-muscle extract inhibited liver glutamine synthetase by about 10% when added in amounts equivalent to 10mg of fresh muscle. This might be expected from what has already been said about the effect of purification procedures on the activity of these extracts (cf. Fig. 3b). On the addition of heart extract containing no detectable activity there was a consistent potentiation of the activity of liver glutamine synthetase by about 25% when extract equivalent to about 10mg of heart was added. The potentiation did not increase when more heart extract was added. In one of the experiments shown in Fig. 5 the extract contained 130 units of glutamine synthetase activity/g and in the other, 285 units/g.

Fig. 5. Changes in rat liver glutamine synthetase activity on addition of rat heart and skeletal muscle extracts to the assay cuvette. The amounts added are expressed as the weights of fresh tissue equivalent to the volume added. ●, Heart extract added to a 5 μl sample of a liver extract containing 65 units/ml; ▲ (heart) and △ (skeletal muscle), extracts added to 5 μl samples of a liver extract containing 155 units/ml.

It is clear that this potentiation does not have the characteristics that would be expected if it were due to the addition of glutamine synthetase with the heart extract, and on the other hand, the latter does not contain an inhibitor that might mask the presence of small amounts of enzyme in a straight-forward assay. It was concluded that glutamine synthetase is either completely absent from rat heart or is present only in amounts too small to be enzymically detected.

It is noteworthy that for kidney, but not for muscle extract, the activity determined by the NADH method is only about 50% of that determined by the hydroxamate method. The reason for this discrepancy has not so far been discovered.

Minimizing the interference from adenosine triphosphatase. The adenosine triphosphatase/glutamine synthetase ratio found with kidney extracts was about 1:2, but with muscle extracts it was 3:1, and this made it very difficult to determine glutamine synthetase activity, by using the NADH method, except in optimum conditions. Various methods of partially inhibiting adenosine triphosphatase were tried. Azide (Vigers & Ziegler, 1968) proved ineffective, as did ethylene glycol bis-(β -aminoethyl ether)tetra-acetate (Martonosi, 1968). Pentachlorophenol (5mm) (Weinbach, 1957) inhibited the adenosine triphosphatase activity of dialysed kidney supernatants by 60%, but only inhibited glutamine synthetase by 10%. However, in muscle extracts glutamine synthetase activity was inhibited to about the same extent as adenosine triphosphatase (about 40%). Pentabromophenol (Weinbach &

Table 3. *Ammonium sulphate concentrations required to precipitate glutamine synthetase activity from supernatants prepared from skeletal muscle and kidney homogenates*

The solutions had been dialysed against 150 mM-NaCl-5 mM-NaHCO₃, as described in the Methods section.

Ammonium sulphate added (mmol/ml of supernatant)	Muscle		Kidney	
	Adenosine triphosphatase	Glutamine synthetase	Adenosine triphosphatase	Glutamine synthetase
None	36.5 (100%)	11.6 (100%)	69.0 (100%)	125.0 (100%)
1.5: supernatant precipitate	18.5 4.5 (12%)	1.2 7.0 (60%)	— 21.9 (32%)	— 60.5 (48%)
1.8: supernatant precipitate	— —	— —	37.0 23.0 (33%)	4.6 86.0 (69%)
2.0: precipitate	5.2	7.3	22.7	83.6 (67%)

Bowen, 1958) was less effective than pentachlorophenol. It was finally found possible to decrease the interference from adenosine triphosphatase by precipitating the glutamine synthetase in the supernatant solutions with ammonium sulphate. Most of the adenosine triphosphatase activity remained in solution. The optimum concentration of ammonium sulphate was different for kidney and muscle extracts. The results are summarized in Table 3.

This step was carried out as a routine when using the NADH method on both kidney and muscle extracts after centrifuging, but before the dialysis described above, unless quantitative assays were required. The precipitate was redissolved in a suitable volume of potassium chloride-potassium bicarbonate solution and dialysed until free of NH₄⁺ ions.

Inhibitors. (a) Sulphate. Glutamine synthetase activity was decreased by 15–30% when ammonium sulphate was used in place of ammonium chloride, at 0.1 M-NH₄⁺, in the NADH method. This inhibition is significant, but distinctly less than that reported by Gothoskar, Raina & Ramakrishnan (1960a) for the glutamine synthetase of chicken heart muscle.

(b) Phosphate. At first it seemed possible that the progressive inhibition found with crude tissue homogenates, described above, was due to accumulation of ADP produced by the combined action of glutamine synthetase and adenosine triphosphatases. However, direct measurement of the concentrations of ADP and P_i showed that although 45% of the two terminal phosphate groups of the added ATP were hydrolysed during a 30 min incubation with crude muscle homogenate (equivalent to 250 mg of tissue), there was only a slight increase in the concentration of ADP. This rose to approx. 1 mM when 10 mM-ATP was used. This is explicable if the extracts are supposed to be rich in adenylate kinase. AMP is known not to inhibit glutamine

synthetase (Elliott, 1951), a finding that we have confirmed.

It was also found that the supernatant from centrifuged muscle homogenate contained about 20 μmol of P/g of tissue extracted. Thus the total P_i concentration at the end of a long incubation could be as high as 10 mM. If P_i inhibits glutamine synthetase, this concentration would be enough to account for the observations shown in Figs. 3 and 4. Gothoskar *et al.* (1960a) found that chicken heart glutamine synthetase is inhibited completely by 90 mM-P_i. No studies on the mammalian enzyme have been published, but Meister (1962) quoted unpublished work by V. Pamiljans, P. R. Krishnaswamy & A. Meister, to the effect that phosphate is not inhibitory. It appeared that the question could profitably be reinvestigated.

When hydroxamate assays were carried out in the presence of 5 mM-P_i (added as sodium phosphate, pH 7.2), with incubation for 30 min at 37°C, the activity in dialysed supernatants of muscle extracts was found to be inhibited by 25%, and of kidney extracts by 10%. To confirm this result, and extend it to recognition of the inhibition type, it was thought best to use the NADH method, since in the hydroxamate assay, not only does P_i accumulate to a significant extent during the incubation, as we had already shown, but another product inhibitor, ADP, is also present in continuously changing concentration.

The type of inhibition was investigated by measuring the steady-state velocity of the reaction in the absence of P_i and in the presence of two concentrations of potassium phosphate, with ATP concentrations varying from 0.1 to 5 mM. The results were analysed by a computer programme based on that of Cleland (1967). It was necessary to make a correction for the amount of adenine nucleotide in the tissue extract that was converted into ATP by the reaction mixture. This correction

was obtained for each experiment, by making use of the results obtained from the preliminary measurement of adenosine triphosphatase activity in each assay, together with a measurement of adenosine triphosphatase activity in an assay in which no ATP was added to the cuvette. Then, assuming simple Michaelis-Menten kinetics for this enzyme, the rate v_0 in the absence of added ATP is given by:

$$v_0 = V_{\max} s_0 / (K_m + s_0) \quad (1)$$

where s_0 is the effective concentration of ATP added with the enzyme source. When a known concentration s of ATP has been added, the rate v is given by:

$$v = V_{\max} (s + s_0) / (K_m + s_0 + s) \quad (2)$$

Substituting from eqn. 1 into eqn. 2 gives:

$$v = [V_{\max} s + v_0 (K_m + s_0)] / (K_m + s_0 + s) \quad (2a)$$

which may be rearranged to give:

$$(K_m + s_0)(v - v_0)/s = V_{\max} - v \quad (3)$$

Thus on plotting $(v - v_0)/s$ against v for various values of s , a straight line is obtained whose intercept on the v axis gives $V_{\max}$ and whose slope is $(K_m + s_0)$. When these values are inserted in eqn. (1), s_0 may be calculated. The values obtained were $0.15 \pm 0.04/\text{g}$ of muscle extract and $0.20 \pm 0.12/\text{g}$ of kidney extract. These values refer to solutions containing an amount of glutamine synthetase, partially purified by ammonium sulphate precipitation, that had originally come from 1g of tissue. The amounts of purified extract normally used in each assay corresponded to 200mg of muscle and 50mg of kidney tissue.

The results of the inhibition studies with 5 and 20 mM- P_i are shown in Figs. 6 and 7, and the inhibi-

tor 'constants', calculated from the assumption that the slope or intercept terms of the reciprocal Michaelis-Menten equations were effected by a term $1 + 1/K_i$, are given in Table 4. The inhibition of the kidney enzyme by phosphate was essentially uncompetitive. Inhibition of the muscle enzyme was stronger, with a K_i calculated to be approx. 10mM, and the inhibition type was strictly competitive.

Effect of growth hormone. Pituitary growth hormone, either added to the assay systems *in vitro*, or injected for 5 days into the rats from which the tissues were taken, in amounts sufficient to cause a weight gain of 1-2g/animal per day, did not affect the glutamine synthetase activity, or cause activity to appear in heart-muscle extracts.

Fig. 7. Double-reciprocal plots for glutamine synthetase in kidney extracts; the NADH assay was used. ●, No P_i ; ■, 5mM- P_i ; ▼, 20mM- P_i .

Fig. 6. Double-reciprocal plots for glutamine synthetase in muscle extracts; the NADH assay was used. ●, No P_i ; ■, 5mM- P_i ; ▼, 20mM- P_i .

Table 4. Kinetic 'constants' for glutamine synthetase

Estimates of $1/V_{\max}$, $K/V_{\max}$, and K were obtained for each experiment with one extract by direct fitting of the experimental results to a hyperbola. Values for the phosphate inhibitor constants were obtained as described in the text. The values of K_m in the Table are means for the number of experiments shown in parentheses. The units are mM, $\pm$ S.E.M.

	Skeletal-muscle enzyme	Kidney enzyme
K_m	0.16 $\pm$ 0.05 (8)	0.30 $\pm$ 0.06 (10)
K_i with 5mM- P_i		
From slope	10.8 (8)	40.7 (6)
From intercept	—	38.5 (6)
K_i with 20mM- P_i		
From slope	12.5 (8)	56.6 (10)
From intercept	—	34.8 (10)

DISCUSSION

We feel confident in asserting that there cannot be more than a trace of glutamine synthetase in rat heart homogenates. It is impossible at this stage to be certain whether the absence of the enzyme is characteristic of all mammalian heart muscle, since the evidence is very conflicting. By direct measurement of enzyme activity, Trush (1963) found a capacity of about $5\mu\text{mol/h}$ per g in rabbit and cat heart homogenates. However, he also found about the same activity in rat heart homogenates. We have found, in agreement with Reiner & Hudson (1953), that the activity in high-speed supernatants may be 3–4 times that in homogenates, and Trush's (1963) value for rat heart would therefore be equivalent to $15\text{--}20\mu\text{mol/h}$ per g in our assay conditions. We could hardly have overlooked activity of this order, and indeed we consistently measured this amount of activity in supernatants from muscle homogenates. The results shown in Fig. 5 show that heart-muscle extracts do not contain an inhibitor that might mask glutamine synthetase activity. Wu (1963) failed to find activity not only in rat heart-muscle homogenates, but also in homogenates of skeletal muscle. Doell & Felts (1959) found that labelled glutamine was formed from [^{14}C]glutamate in isolated perfused rabbit heart, although J. Mowbray (personal communication) found that glutamine remained unlabelled in perfused rat heart. Ferdman, Silakova & Trush (1963) found [^{15}N]glutamine, and $^{15}\text{NH}_3$ of higher specific radioactivity, in rabbit hearts 1 h after intraperitoneal injection of [^{15}N]ammonium nitrate, but this is compatible with fairly rapid uptake of glutamine from plasma, as suggested by Ottaway (1969), together with diffusion of [^{15}N]ammonia from plasma to intracellular space. Finally, Keul, Doll, Stein, Singer & Reindell (1966) reported that the coronary arterio-venous difference in athletes in training is negative for glutamine and positive for glutamate, implying that glutamine synthesis is occurring in the tissue.

It is at least clear that if there is glutamine synthetase activity in mammalian heart, it is only a fraction of that occurring in avian heart. Activity as great as, or greater than, that found in kidney homogenates has been reported by Gothoskar *et al.* (1960a) for chicken heart, by Trush (1963) for pigeon heart, and by Wu (1963) for chicken, pigeon and goose heart homogenates. Gothoskar, Raina, Tate & Ramakrishnan (1960b) have pointed out that chick heart cells in tissue culture, unlike rat heart cells, do not require glutamine as a growth factor. It is reasonable to conclude that the glutamine found in rat heart has not been synthesized in that tissue, but almost certainly in the liver (Wu, 1964).

It follows that the rather high concentration of

glutamine in heart cannot be related to the detoxication of ammonia, or to the transport from heart to liver of amino N arising from the catabolism of amino acids. The results of Ottaway (1969) show that there is a steady hydrolysis of glutamine in rat heart, at least *in vitro*, and there is strong presumptive evidence that the ammonia so formed is removed by reductive synthesis of glutamate. This then undergoes transamination with oxaloacetate and pyruvate, so that amide N of glutamine continuously leaves the heart as the amino N of aspartate and alanine, chiefly the latter. The turnover-time of glutamine in rat heart is about 1 h; this relatively rapid turnover suggests that the maintenance of a constant concentration of glutamine in the heart is important, although the function of the amino acid remains unknown.

Table 2 shows that the maximal activity of the enzyme in skeletal muscle is about $20\mu\text{mol}$ of glutamylhydroxamate formed/h per g of tissue, which is equivalent to about $40\mu\text{mol}$ of glutamine/h per g (Table 1). It is possible that this is an underestimate, since Fig. 3(b) shows that the linear rates of hydroxamate formation over 30 min which we used for our estimates of activity in muscle were apparently much lower than the initial rates (during the first 2–5 min) with non-dialysed supernatants or homogenates. This was not so with kidney extracts, as shown in Fig. 3(a). The value of $40\mu\text{mol/h}$ per g may thus be a minimum estimate, but it is sufficiently high to conclude that the enzyme could be responsible for the formation of the glutamine of muscle. The actual rate of glutamine formation must chiefly depend on the concentration of NH_4^+ in the tissue, since the concentrations of glutamate and ATP *in vivo* are effectively saturating. The concentration of glutamate dehydrogenase in rat skeletal muscle is low (Wergedal & Harper, 1964); thus it seems likely that NH_4^+ formed intracellularly must be removed by synthesis of glutamine, unless it simply escapes by diffusion.

Our estimates of the glutamine synthetase activity of kidney extracts, with hydroxylamine as a substrate, were slightly higher than those of Reiner & Hudson (1953), who also used the supernatants from high-speed centrifugation of the homogenates. Both estimates are several times greater than those of Richterich & Goldstein (1958) and Wu (1963), both of whom used untreated homogenates. The values in Table 2 nevertheless underestimate the true maximal activity of the enzyme, since it can be shown (M. P. Gonzalez, personal communication) that if the ADP is continuously removed in the hydroxamate assay by the addition of phosphoenolpyruvate and pyruvate kinase, the rate of hydroxamate formation by kidney extracts increases threefold. We place more reliance on the results from the hydroxamate

assays, since Gonzalez has also shown that the amount of NADH that disappears in the NADH assay is less than the amount of hydroxamate that appears, suggesting that a side-reaction occurs, possibly the loss of some pyruvate by a transamination.

The chief importance of our kinetic results is the emphasis they throw on the difference between the muscle and kidney enzymes, but some subsidiary points are noteworthy. The inhibition by an excess of Mg^{2+} shown in Fig. 2 confirms that reported by Speck (1949b), Trush (1963) and Wu (1964). In conjunction with the strong inhibition of sheep brain glutamine synthetase by Ca^{2+} , even in the presence of equimolar concentrations of Mg^{2+} , reported by Elliott (1951), the evidence suggests that Mg^{2+} is bound to the enzyme and decreases its activity, as well as forming part of the active substrate $MgATP^{2-}$. A plausible explanation is that $MgATP^{2-}$ binds to the free enzyme, but the product, ADP, leaves the enzyme before the metal ion.

The Michaelis constants for ATP shown in Table 4, which were derived from studies with the NADH assay method, are about one-tenth of those reported by Speck (1949b), Elliott (1951) and Pamijans *et al.* (1962). The K_m values for ATP that can be derived from the results shown in Fig. 1 are approx. 1 mM, in agreement with the values of the earlier workers, and we conclude that the presence of traces of ADP in the hydroxamate assay markedly affects some of the rate constants of the enzyme. This effect is distinct from the product inhibition by ADP observed at higher concentrations of the latter (cf. Elliott, 1951). The difference between the K_m values for the muscle and kidney enzyme quoted in Table 4 is significant at the 1% level of probability.

The results of the extensive studies of Meister have been embodied in reaction diagrams (Meister, 1962, 1965, 1968) which imply a kind of Theorell-Chance mechanism (Cleland, 1963) for the enzyme. P_i is lost and ammonia (or hydroxylamine) is incorporated in a single step, and neither species forms part of a distinct intermediate enzyme complex. The steady-state rate of reaction for such a model, when the concentration of products other than P_i remains zero, is:

(1965). In our studies the enzyme was saturated with glutamate and hydroxylamine, and [ATP] was varied. Eqn. (4) can then be simplified by omitting all terms not containing [Glu] or [N], and it reduces to:

$$v = V'[ATP]/C_1 + [ATP](C_2 + C_3[P_i]) \quad (5)$$

where V' , C_1 , C_2 and C_3 are constants for given values of [Glu] and [N]. This equation predicts uncompetitive inhibition by phosphate, and this is what was found with the enzyme in kidney extracts. Thus in this instance our results are in agreement with the mechanism put forward by Meister. We do not regard it as serious that our results conflict with those of Pamijans, Krishnaswamy & Meister (quoted by Meister, 1962) since the inhibition is slight and could quite easily be overlooked. Alternatively, it is possible that Pamijans *et al.* used glutamate as the variable substrate: in this case eqn. (4) predicts that inorganic phosphate will not inhibit the reaction.

The results obtained with the muscle enzyme, on the other hand, cannot be accommodated within Meister's (1962) scheme. Competitive inhibition demands that the inhibitor reacts with the form of the enzyme that reacts with the substrate (Cleland, 1963), and this does not occur in the standard mechanism. Even the addition of a 'dead-end' enzyme- P_i complex to the scheme does not help, since it introduces $[P_i]^2$ terms into the denominator of the rate equation, whereas the values for K_i in Table 4 are notably independent of the phosphate concentration. 'Partial' inhibition mechanisms of the type discussed by Dixon & Webb (1964) give even more complex rate equations and can be excluded from consideration.

A mechanism that would explain our findings is one based on random release of ADP and P_i from the enzyme, as shown in Scheme 1. This mechanism is compatible with competitive inhibition of ATP by ADP, which has been shown for all the mammalian enzymes so far investigated, and which has been established for the chicken heart enzyme (which is known to be inhibited by P_i) by Gothoskar *et al.* (1960a). Completely random release of products

$$v = \frac{V[ATP][Glu][N]}{A + B[N] + C[ATP] + D[ATP][Glu] + E[ATP][N] + F[Glu][N] + G[ATP][Glu][N] + H[P_i] + J[ATP][P_i] + K[ATP][Glu][P_i]} \quad (4)$$

where [N] refers to ammonia or hydroxylamine, and A-K are complex constants. The order of addition of reactants is that specified by Meister

from the E-ADP- P_i complex shown in Scheme 1 leads to eqn. (6), in the absence of products other than phosphate, and if the enzyme is saturated

Scheme 1. Semi-random mechanism postulated to explain the inhibition of glutamine synthetase in muscle extracts by P_i . The rate equation for this mechanism eqn. (6) was computed according to Silvestri & Zahner (1967).

with glutamate and hydroxylamine, so that all terms not containing $[Glu]$ or $[N]$ may be omitted:

similar to the other mammalian enzymes so far described, and the muscle enzyme is therefore

$$v = \frac{K_1 K_3 K_5 K_7 K_{11} K_{15} (K_9 + K_{13}) [ATP] [Glu] [N] + K_1 K_3 K_5 K_7 K_{10} K_{13} K_{15} [ATP] [Glu] [N] [P_i]}{K_2 K_5 K_7 K_{11} K_{15} (K_9 + K_{13}) [N] + K_3 K_5 K_7 K_{11} K_{15} (K_9 + K_{13}) [Glu] [N] + K_1 K_5 K_7 K_{11} K_{15} (K_9 + K_{13}) [ATP] [N] + K_1 K_3 K_{11} K_{15} (K_6 + K_7) (K_9 + K_{13}) [ATP] [Glu] + K_1 K_3 K_5 \{K_{15} (K_7 K_9 + K_7 K_{11} + K_9 K_{11} + K_{11} K_{13}) + K_7 K_{11} K_{13}\} [ATP] [Glu] [N] + K_2 K_5 K_7 \{K_{11} K_{16} (K_9 + K_{13}) + K_{10} K_{13} K_{15}\} [N] [P_i] + K_3 K_5 K_7 \{K_{11} K_{16} (K_9 + K_{13}) + K_{10} K_{13} K_{15}\} [Glu] [N] [P_i] + K_1 K_5 K_7 K_{10} K_{13} K_{15} [ATP] [N] [P_i] + K_1 K_3 K_{10} K_{13} K_{15} (K_6 + K_7) [ATP] [Glu] [P_i] + K_1 K_3 K_5 K_{10} (K_7 K_{13} + K_7 K_{15} + K_{13} K_{15}) [ATP] [Glu] [N] [P_i] + K_2 K_5 K_7 K_{10} K_{13} K_{16} [N] [P_i]^2 + K_3 K_5 K_7 K_{10} K_{13} K_{16} [Glu] [N] [P_i]^2} \quad (6)$$

This equation still contains a $[P_i]^2$ term in the denominator as well as a $[P_i]$ term in the numerator, and is thus too complex to fit the data that we have assembled. If, however, it were to be assumed that P_i does not bind readily to the E-ADP complex, so that K_{10} is very small by comparison with the other rate constants, eqn. (6) would simplify to:

$$v = V[ATP]/[ATP] + K_a(1 + [P_i]/K_i) \quad (7)$$

which predicts competitive inhibition by phosphate. The mechanism suggested in Scheme 1 can be tested by systematic variation of other reactants and products, and this is now under investigation.

The difference in K_m for ATP, and in the type of inhibition by P_i , suggest strongly that the enzymes from muscle and kidney are not identical. Other evidence to support this conclusion is the strong inhibition of the muscle enzyme by mm-pentachlorophenol, whereas the kidney enzyme is only slightly affected, and the slight difference in the concentrations of ammonium sulphate that will precipitate the enzyme from the supernatant fractions. Some of these differences may be due to interactions between glutamine synthetase and tissue-specific proteins in the extracts, but others cannot be explained on these lines, and it appears that a genuine difference between the two enzymes exists. From the evidence provided by the phosphate-inhibition studies, we surmise that the kidney enzyme is

atypical. There are points of resemblance between its properties and those of the enzyme from chicken heart described by Gothoskar *et al.* (1960a), but the inhibition of the latter enzyme by phosphate was far more severe than that which we found. All the animal enzymes, including the muscle enzyme, so far described appear to differ considerably from the bacterial enzymes (Kingdon *et al.* 1968; Liess *et al.* 1968).

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