Biochem. J. (1970) **118**, 35–39
Printed in Great Britain

A Radiochemical Assay for Glutamine Synthetase, and Activity of the Enzyme in Rat Tissues

By PATRICIA LUND
Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford OX2 6HE, U.K.

(*Received* 30 *January* 1970)

1. A radiochemical assay for glutamine synthetase has been developed in which an ATP-regenerating system is incorporated to prevent accumulation of inhibitory amounts of ADP. It is particularly suitable for assay of the enzyme in crude tissue extracts containing high adenosine triphosphatase activity. 2. A survey of the distribution of the enzyme in tissues from normal male rats showed that activity is present in liver, brain cortex, kidney cortex, spleen, testis and retina. 3. The K_m of the enzyme for L-glutamate is approx. 1.5×10^{-2}M.

The methods generally used for the assay of glutamine synthetase [L-glutamate–ammonia ligase (ADP), EC 6.3.1.2] are modifications of those of Speck (1949) and Elliott (1951). The assay is based on the principle that hydroxylamine can replace ammonia, and that γ-glutamylhydroxamic acid formation can be readily measured colorimetrically. Although this method is adequate for enzyme preparations containing little or no adenosine triphosphatase activity, it was found to be unsatisfactory when applied to crude homogenates of rat tissues (other than liver) where glutamine synthetase activity compared with adenosine triphosphatase activity is relatively low, with the result that measured rates of glutamine synthetase are non-linear because of competitive inhibition by ADP (Elliott, 1951; Fry, 1955).

The need to develop a new assay for glutamine synthetase arose in connexion with a study of the control of the synthesis and degradation of glutamine. A special feature of the problem is the presence in the same cell of separate enzymes carrying out the two processes (glutamine synthetase and the 'glutaminases' respectively). Wu (1963*a*) reported that glutamine synthetase was present in rat liver, kidney, brain and testis, but absent from other rat tissues. A new survey seemed warranted when it was found that the enzyme may have been inhibited by ADP in most of the tissue extracts assayed under the conditions used by Wu (1963*a*). A radiochemical assay was therefore developed in which the inhibition by ADP was minimized by incorporating an ATP-regenerating system and using short incubation times. In addition, phosphate buffer was avoided to prevent activation of glutaminase. The main advantages of this method are that ammonia, the natural substrate, is used instead of hydroxylamine and that there is no interference from pigmented tissues, such as spleen, which give high tissue blanks in the colorimetric method.

EXPERIMENTAL

Rats. All enzyme assays were performed on fresh tissues from normal male rats of the Wistar strain.

Reagents. NAD^+, ATP, phosphoenolpyruvate (monopotassium salt), pyruvate kinase (approx. 150 international units/mg) and glutamate dehydrogenase in glycerol were obtained from the Boehringer (London) Corp., London W.5, U.K. L-Cycloserine was from Calbiochem, Los Angeles, Calif., U.S.A. L-[U-^{14}C]Glutamic acid (>225mCi/mmol; 50μCi/ml of aqueous solution containing 2% of ethanol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Pyruvate kinase was dialysed against 0.02M-tris–HCl buffer, pH7.4 (2×800ml), to remove NH_4^+ ions; the absence of ammonia was checked in the dialysed enzyme with Nessler's reagent. Complete removal of NH_4^+ ions is essential if the enzyme kinetics with regard to ammonia are to be studied.

Solutions of 0.5M-L-[U-^{14}C]glutamate were prepared as follows: 0.4ml of high-specific-radioactivity L-[U-^{14}C]-glutamic acid was mixed with 1ml of M-L-glutamate (pH7.4) and made up to 2ml with water. The specific radioactivity of the solution was determined as described below.

Thin-layer chromatography. Separation of glutamate and glutamine was carried out on MN-Polygram Cel300 (Macherey–Nagel and Co., Düren, Germany) supplied by Camlab Ltd., Cambridge, U.K. Chromatography was one-dimensional in a solvent system of chloroform–methanol–17% (w/w) ammonia (2:2:1, by vol.) (Bujard & Mauron, 1966).

Scintillator fluid. This contained 6–8g of 5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole (Butyl-PBD; CIBA Ltd., Duxford, Cambs., U.K.), 80g

of naphthalene (Thorn Electronics, Tolworth, Surrey, U.K.), 600ml of toluene and 400ml of 2-methoxyethanol.

Preparation of homogenates. Liver, kidney cortex, spleen, testis and lung were homogenized in a stainless-steel Potter–Elvehjem homogenizer (clearance 0.2mm), heart and skeletal muscle in a laboratory-model Silverson mixer–emulsifier (Silverson Machines Ltd., London S.E.1, U.K.), and brain cortex, retina (pooled from four animals), duodenal mucosa, pancreas and adrenals in small all-glass homogenizers. Homogenates were prepared from 1 part of fresh tissue and 4 parts of 0.9% NaCl and centrifuged at 200*g* to sediment fibrous particles. NaCl at this concentration was used because it had been reported to cause glutamine synthetase to become detached from the microsomes (Wu, 1963*b*). Later, in a survey of media used for homogenization of liver (water; 0.9% NaCl; 40mM-tris–HCl buffer, pH7.4, plus 1mM-EDTA; 40mM-tris–HCl buffer, pH7.4, plus 5mM-EDTA; 40mM-tris–HCl buffer, pH7.4, plus 5mM-cysteine; 0.25M-sucrose), the highest activity was found in extracts prepared in 40mM-tris–HCl buffer containing 1mM or 5mM-EDTA (approx. 25% increase over all other media tested).

Assay procedure. The incubation is in 3ml glass tubes containing 0.08ml of 0.5M-tris–HCl buffer, pH7.4, 0.02ml of 0.5M-$MgCl_2$, 0.01ml of M-NH_4Cl, 0.05ml of 0.2M-ATP, 0.075ml of 0.2M-phosphoenolpyruvate, 0.02ml of dialysed pyruvate kinase, 0.02ml of 0.2M-L-glutamine, 0.01ml of 0.1M-L-cycloserine, 0.075ml of 0.5M-L-[U-^{14}C]glutamate and 0.04ml of water. Pyruvate is produced from phosphoenolpyruvate in regenerating ATP, and with L-[^{14}C]glutamate and alanine aminotransferase present in the tissue extract forms α-oxo[^{14}C]glutarate, which overlaps with glutamine in the chromatography. This source of error is eliminated by including L-cycloserine, a powerful inhibitor of alanine aminotransferase (Otto, 1965), at a concentration of 2mM in the assay. The unlabelled glutamine pool is included to dilute the [^{14}C]glutamine formed and thus to minimize loss of label as a result of glutaminase activity. The added glutamine does not inhibit glutamine synthetase, and provides a marker in the subsequent chromatography.

The incubation mixture is equilibrated to 37°C before starting the reaction by adding 0.1ml of homogenate. At exactly 2, 4, 6 and 8min 0.1ml samples are withdrawn into 0.15ml of ethanol (95%) in small tubes kept in ice. The tubes are centrifuged in the cold, and separation of glutamate and glutamine is carried out by t.l.c. The following procedure is used: 5μl samples from each tube are spotted at approx. 1cm intervals along a line 1cm from the edge of a sheet (9cm×9cm) of MN-Polygram Cel 300. One-dimensional chromatography is carried out by standing the chromatogram in a small volume of the chloroform–methanol–ammonia solvent in a small glass chromatography tank with a lid. The chromatogram is removed from the tank when the solvent front is within 1cm of the top edge, air-dried and then sprayed thoroughly with 0.2% ninhydrin in butan-1-ol. After colour development at 100°C for 5min, the glutamine areas are cut out and the radioactivity is determined directly in a Beckman 200 liquid-scintillation counter in vials containing 10ml of scintillator fluid. Care is taken to standardize the technique so as not to introduce variations in the amounts of $^{14}CO_2$ lost in formation of the ninhydrin colour complex. There is no quenching of counts due to the blue colour. The radioactivity (c.p.m.) incorporated is corrected for a blank as described below, and activity is calculated from the known specific radioactivity of the stock 0.5M-L-[U-^{14}C]glutamate solution.

Determination of specific radioactivity of 0.5M-L-[U-^{14}C]-*glutamate solution.* The specific radioactivity of each new 0.5M-L-[U-^{14}C]glutamate solution must be determined. The c.p.m./ml is calculated from the radioactivity (c.p.m.) present in the glutamate area after chromatography of 5μl of the solution diluted to the same extent as in the enzyme assay (i.e. 0.075ml diluted to 1.25ml with 50% ethanol). This standard is run on the same chromatogram

Table 1. *Effectiveness of the ATP-regenerating system in preventing accumulation of ADP under the glutamine synthetase assay conditions*

All assays contained tris–HCl buffer, pH7.4, $MgCl_2$ and ATP at the same final concentration as used in the glutamine synthetase assay. The ATP-regenerating system (phosphoenolpyruvate and pyruvate kinase), where present, had also the same final concentration as in the glutamine synthetase assay. The tubes were equilibrated to 37°C, and the reaction was started by addition of 0.1ml of the fresh homogenates (1 part of tissue+4 parts of 0.9% NaCl). Incubation was for 6min. The reaction was stopped by addition of 0.1ml of 20% (v/v) $HClO_4$. After neutralization with KOH the supernatants were assayed for ADP by the method of Adam (1963). The initial ATP concentration, as determined by the method of Lamprecht & Trautschold (1963), was 18μmol/ml of incubation mixture.

	ADP formed (μmol/6min per ml of reaction mixture)	
	No ATP-regenerating system	+ATP-regenerating system
No homogenate	0.6	0.6
Liver	2.6	0.4
Brain cortex	4.8	1.1
Kidney cortex	5.2	0.8
Spleen	6.9	0.8
Testis	11.9	1.4
Thigh muscle	1.2	0.5
Duodenal mucosa	5.0	0.9
Pancreas	4.7	0.9

as the samples, and treated in the same way as described. At the same time 0.02 μmol of glutamine is run with the standard and the radioactivity of the glutamine area is measured to give the radioactivity (c.p.m.) in the blank that has to be subtracted from the radioactivity (c.p.m.) incorporated in the enzyme assays.

The L-glutamate content of the 0.5 M-L-[U-^{14}C]-glutamate solution is determined enzymically as described by Bernt & Bergmeyer (1963) after dilution of the stock solution to a calculated concentration of 1 μmol/ml. Several assays, containing amounts of glutamate between 0.025 and 0.1 μmol, are carried out for each new solution. ADP (0.1 μmol/cuvette) is included in the glutamate assay system to speed up completion of the reaction by activation of glutamate dehydrogenase (Tomkins, Yielding, Talal & Curran, 1963).

The specific radioactivity of the 0.5 M-[U-^{14}C]glutamate solution as determined was approx. 6×10^4 c.p.m./μmol.

RESULTS

Effectiveness of the ATP-regenerating system in preventing accumulation of ADP in the assay. The data in Table 1 show that ADP is rapidly formed from ATP by most of the tissue extracts when the ATP-regenerating system is omitted from the glutamine synthetase assay. Only liver and thigh-muscle extracts form relatively little ADP. After the 6 min incubation the [ADP]/[ATP] ratio for other tissues approaches, or is less than, the value of 0.3 reported by Elliott (1951) to cause a 50% inhibition of glutamine synthetase. In the presence of the ATP-regenerating system the proportion of ADP was decreased to approx. 10% of the added ATP.

Proportionality of activity to amount of homogenate in assay. Glutamine synthetase activity of liver homogenates was proportional to the amount of homogenate in the range 10–25 mg of tissue/assay. At 5 mg of tissue/assay activity was less reliably determined because the amount of ^{14}C incorporated into glutamine was low. Higher concentrations of tissue than 25 mg of tissue/assay were not tried. An arbitrary amount of tissue (20 mg/assay) was chosen for the survey of rat tissues so that activity, if present, would be detectable.

Calculation of glutamine synthetase activity. Activity is calculated from the plotted linear rate of radioactivity (c.p.m.) incorporated per unit of time, taking into account the specific radioactivity of the L-[U-^{14}C]glutamate solution used in the assay, the dilution of the incubation mixture on deproteinization and the amount of tissue in the assay. Activity is expressed as μmol of glutamine formed/min per g fresh wt. of tissue at 37°C. It is not necessary to determine the specific radioactivity of the glutamine formed. The assumptions are made that: (1) glutamate initially present in the homogenates is insignificant; (2) the proportion of added glutamate lost through side reactions is negligible during the 8 min incubation; (3) loss of labelled glutamine through glutaminase activity in the tissue extracts is minimized by the inclusion of the unlabelled pool of glutamine in the assay system.

Determination of K_m for L-glutamate. The K_m of glutamine synthetase for glutamate was determined in crude extracts because less than maximum rates of glutamine synthesis were reached at concentrations of glutamate below 50 mM, although the K_m of the purified sheep brain enzyme for L-glutamate is reported to be 2.5×10^{-3} M (Pamiljans, Krishnaswamy, Dumville & Meister, 1962).

Enzyme activity was assayed at different L-glutamate concentrations in supernatants from extracts after centrifugation at 9700g to remove some of the interfering glutaminase and adenosine triphosphatase activity associated with the mitochondria. The normal assay system was used with glutamate concentrations of 2–40 mM. For these experiments the incubation time was extended to 16 min and at the low glutamate concentrations 10 μl of the reaction mixture was spotted for chromatography. A K_m value of 1.5×10^{-2} M (average of three experiments; range 1.3×10^{-2}–1.9×10^{-2} M) was obtained for the liver enzyme, and 1.3×10^{-2} M (one experiment) for the brain enzyme. When the pool of unlabelled glutamine was omitted from the K_m determination in a brain-cortex extract, a value of 1.4×10^{-2} M was obtained with no apparent change in $V_{max.}$. The concentrations of other reactants in these experiments are at near-saturation. The K_m values for ammonia and ATP are 1.8×10^{-4} M and 2.3×10^{-3} M respectively (Pamiljans *et al.* 1962): the concentrations used in the assay are for both 20 mM. Wu (1963*a*) also found a relatively high apparent K_m for glutamate (1.1×10^{-2} M) in a crude liver extract, and Richterich van Baerle, Goldstein & Dearborn (1957) obtained a value of 5.5×10^{-2} M in a crude guinea-pig kidney extract. The discrepancy between these values and the value for the purified brain enzyme obtained by Pamiljans *et al.* (1962) cannot be explained.

Cysteine, which has been reported to activate both the purified enzyme and the enzyme in crude tissue extracts (Speck, 1947; Elliott, 1953; Wu, 1963*a*), had no effect on activity as measured by the radiochemical assay.

Effect of varying the NH_4^+ ion concentration in the glutamine synthetase assay. To check that the 20 mM-ammonium chloride used in the assay system was saturating a liver extract was assayed after addition of a range of concentrations of ammonium chloride. Again a 9700g supernatant was used to remove most of the interfering enzymes associated with the mitochondrial fraction. The unlabelled glutamine pool was omitted from the

assay to minimize endogenous ammonia production. The ATP-regenerating system and L-cycloserine were also omitted. The data in Fig. 1 show that activity is low and non-linear in the absence of added ammonium chloride, but is linear and not significantly affected by concentrations between 2 and 100mM-ammonium chloride. Determinations of the K_m for ammonia of glutamine synthetase in a crude extract are meaningless because of interference from endogenous ammonia production.

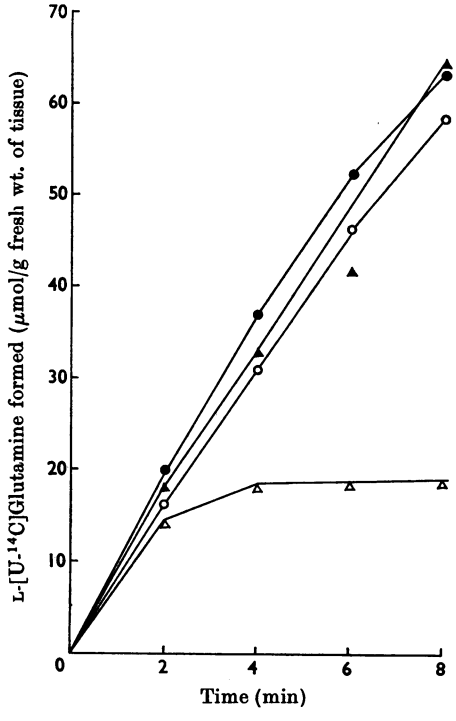

Fig. 1. Effect of NH_4^+ concentration on rate of glutamine synthetase activity in rat liver homogenate. The assay method was as described in the text, except that the ATP-regenerating system, the unlabelled glutamine pool and L-cycloserine were omitted. △, No added NH_4Cl; ●, 2mM-NH_4Cl; ▲, 20mM-NH_4Cl; ○, 100mM-NH_4Cl.

Glutamine synthetase activity in rat tissues. Results of the survey of the distribution of glutamine synthetase activity in tissues of normal fed male rats are given in Table 2. The relative activities are similar to those reported by Wu (1963*a*), although individual tissues have, in general, considerably higher activity. Spleen is now found to contain the enzyme. Formation of glutamine by retina was described by Krebs (1935), so it is not surprising to find the synthetase in this tissue.

DISCUSSION

Importance of the ATP-regenerating system in assays of glutamine synthetase in crude tissue extracts. The preliminary work in developing the radiochemical assay for glutamine synthetase served to draw attention to the shortcomings of previous assay methods when applied to crude homogenates of rat tissues other than liver. The main advantages of the radiochemical assay are: (1) it can be used when only very small amounts of tissue are available; (2) it can be used to study NH_4^+ as a reactant; (3) formation of glutamine, the natural end-product of the glutamine synthetase reaction, is measured, in contrast with other assays that depend on the formation of γ-glutamylhydroxamate. However, for routine assays, the ATP-regenerating system and the short incubation times can be incorporated into the colorimetric assay. This has been used to determine the activity in tissues of lower vertebrates and is far less time-

Table 2. *Glutamine synthetase activity in various rat tissues*

The assay system was as described in the text. The results refer to fed male rats. Values are means±S.E.M. with the numbers of observations in parentheses.

Tissue	L-Glutamine formed (μmol/min per g fresh wt. at 37°C)
Liver	8.8±0.70 (8)
Brain cortex	2.3±0.3 (5)
Kidney cortex	2.3±0.29 (8)
Spleen	2.5±0.16 (5)
Testis	2.4±0.2 (6)
Retina (pooled from three rats)	6.9 (1)
Thigh muscle	<0.5
Duodenal mucosa	<0.5
Pancreas	<0.5
Heart	<0.5
Adrenal gland	<0.5
Erythrocytes	<0.5
Lung	<0.5

consuming than the radiochemical assay (see Lund & Goldstein, 1969).

K_m for glutamate of glutamine synthetase. The implications of the finding that glutamine synthetase has a K_m for glutamate approx. 1.5×10^{-2} M should be considered. At normal physiological glutamate concentrations of 2.75 μmol/g (P. Lund, unpublished work) and 3.0 μmol/g (Brosnan, 1968) for liver and kidney respectively, there is likely to be little glutamine synthesis. Brain, having a glutamate content of 10.4 μmol/g (Brosnan, 1968), might be expected to synthesize glutamine provided that the other substrates, ATP and ammonia, are available. It is known that the brain synthesizes an appreciable amount of glutamine after an ammonia load (du Ruisseau, Greenstein, Winitz & Birnbaum, 1957; Berl, Takagaki, Clarke & Waelsch, 1962), but there is no information on glutamine synthesis under normal conditions. The ammonia contents of brain, liver and kidney are 0.34, 0.71 and 0.88 μmol/g respectively (Brosnan, 1968), and the data in Fig. 1 and the K_m for ammonia (1.8×10^{-4} M) determined by Pamiljans *et al.* (1962) indicate that the enzyme is saturated at low ammonia concentrations, so that synthesis could occur if a low continuous supply of ammonia were available. Liver and kidney are the tissues most likely to meet this requirement.

Non-equilibrium of glutamine synthetase. A consideration of the properties of glutamine synthetase shows that the reaction is not at equilibrium in any tissue *in vivo*. The equilibrium constant K of the enzyme is 1.2×10^3 at pH 7.0 and 37°C (Levintow & Meister, 1954), where

$$K = \frac{[\text{glutamine}][\text{orthophosphate}][\text{ADP}]}{[\text{glutamate}][\text{ATP}][\text{ammonia}]}$$

and the concentrations of reactants refer to the sum of all ionic species. When this value is corrected for the activity coefficient of ammonium glutamate and for the concentration of HPO_4^{2-} in the following equation (where brackets indicate concentrations and parentheses indicate thermodynamic activities):

$$K' = \frac{[\text{MgADP}^-][HPO_4^{2-}](\text{glutamine})}{[\text{MgATP}^{2-}](NH_4^+)(\text{glutamate}^-)}$$

K' becomes 4.42×10^2 (Benzinger, Hems, Burton & Kitzinger, 1959). Taking the liver as an example, the [glutamine]/[glutamate] ratio is approx. 1.5 and the tissue contents of ammonia and of HPO_4^{2-} are approx. 1 mM and 3 mM respectively; this means that for equilibrium to exist [ADP]/[ATP] must be about 10, which is not the case. In any event, such a ratio would be incompatible with activity because of the competitive inhibition by ADP (see Elliott, 1951). The range of values of the [ADP]/[ATP] ratio in whole liver is between 0.5 and 1.0, and the experiments of Klingenberg, Heldt & Pfaff (1969) indicate that the ratio in the cytoplasm is lower than in the mitochondria, and hence lower than the ratio found for the whole tissue. Thus compartmentation of some of the reactants is unlikely to be the explanation of the large deviation from equilibrium. Non-equilibrium in the glutamine synthetase system means that the tissue content of the reactants of the enzyme cannot be used to obtain information on the $[\text{ATP}]/[\text{ADP}][HPO_4^{2-}]$ ratio of the cytoplasm *in vivo*.

I am grateful to Sir Hans Krebs, F.R.S., for his helpful advice. This work was supported by a grant from the Medical Research Council and from the U.S. Public Health Service grant no. AM11748-01. The author is a member of the Medical Research Council External Staff.

REFERENCES

Adam, H. (1963). In *Methods of Enzymatic Analysis*, p. 573. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.

Benzinger, T., Hems, R., Burton, K. & Kitzinger, C. (1959). *Biochem. J.* **71**, 400.

Berl, S., Takagaki, G., Clarke, D. D. & Waelsch, H. (1962). *J. biol. Chem.* **237**, 2570.

Bernt, E. & Bergmeyer, H. U. (1963). In *Methods of Enzymatic Analysis*, p. 384. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.

Brosnan, J. T. (1968). D.Phil. Thesis: University of Oxford.

Bujard, E. & Mauron, J. (1966). *J. Chromat.* **21**, 19.

du Ruisseau, J. P., Greenstein, J. P., Winitz, M. & Birnbaum, S. M. (1957). *Archs Biochem. Biophys.* **68**, 161.

Elliott, W. H. (1951). *Biochem. J.* **49**, 106.

Elliott, W. H. (1953). *J. biol. Chem.* **201**, 661.

Fry, B. A. (1955). *Biochem. J.* **59**, 579.

Klingenberg, M., Heldt, H. W. & Pfaff, E. (1969). In *The Energy Level and Metabolic Control in Mitochondria*, p. 237. Ed. by Papa, S., Tager, J. M., Quagliariello, E. & Slater, E. C. Bari: Adriatica Editrice.

Krebs, H. A. (1935). *Biochem. J.* **29**, 1951.

Lamprecht, W. & Trautschold, I. (1963). In *Methods of Enzymatic Analysis*, p. 543. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.

Levintow, L. & Meister, A. (1954). *J. biol. Chem.* **209**, 265.

Lund, P. & Goldstein, L. (1969). *Comp. Biochem. Physiol.* **31**, 205.

Otto, K. (1965). *Hoppe-Seyler's Z. physiol. Chem.* **341**, 99.

Pamiljans, V., Krishnaswamy, P. R., Dumville, G. & Meister, A. (1962). *Biochemistry, Easton*, **1**, 153.

Richterich van Baerle, R., Goldstein, L. & Dearborn, E. H. (1957). *Enzymologia*, **18**, 327.

Speck, J. F. (1947). *J. biol. Chem.* **168**, 403.

Speck, J. F. (1949). *J. biol. Chem.* **179**, 1405.

Tomkins, G. M., Yielding, K. L., Talal, N. & Curran, J. F. (1963). *Cold Spring Harb. Symp. quant. Biol.* **28**, 461.

Wu, C. (1963*a*). *Comp. Biochem. Physiol.* **8**, 335.

Wu, C. (1963*b*). *Biochim. biophys. Acta*, **77**, 482.