BBA 75575

FURTHER STUDIES OF THE TRANSPORT OF AMINO ACIDS IN RAT LIVER SLICES

J. C. CRAWHALL AND M. G. DAVIS

Division of Clinical Biochemistry, Royal Victoria Hospital, McGill University Clinic, Montreal (Canada) and Professorial Medical Unit, St. Bartholomews Hospital, London (Great Britain)

(Received July 23rd, 1970)

(Revised manuscript received October 5th, 1970)

SUMMARY

Further studies of amino acid transport by the rat liver slice have shown that the transport of α-aminoisobutyric acid is inhibited by glycine as well as dinitrophenol, Na^+-free medium, and iodoacetate. Glycine itself is actively transported by the rat liver slice, although some metabolism also takes place. Cystine is transported by a single transport system, although reduction to cysteine occurs intracellularly and to some extent in the medium also. Cysteine is transported faster than cystine and to greater concentration gradients. Kinetic studies showed that cystine was transported by a single system that was inhibited by glycine but not by α-aminoisobutyric acid. Two transport systems were involved in cysteine transport, each inhibited to a certain extent by α-aminoisobutyric acid and glycine. Lysine and valine both exist at a higher concentration intracellularly than in the plasma *in vivo* but no intracellular gradients were obtained after *in vitro* incubations. It is suggested that the intracellular gradients for these amino acids are maintained by protein catabolism.

INTRODUCTION

Some *in vivo* studies in the cat[1] and in the rat[2] have shown concentrations of some amino acids to be higher in the liver than in plasma. The uptake of α-aminoisobutyric acid by rat liver *in vivo* has been shown to be increased by insulin but this effect was not observed in adrenalectomised animals[3]. Perfusion studies with isolated rat liver have shown that an amino acid concentration gradient can be maintained between the tissue and the perfusate *in vitro*[4,5]. The transport of amino acids by the liver slice has recently been investigated by several workers. It was shown that alanine uptake was inhibited by ethanol in the rat liver slice[6] and glutamic acid did not appear to enter the liver slice by an active transport system[7]. CRAWHALL AND SEGAL[8] were unable to demonstrate an active transport system for certain synthetic sugars but α-aminoisobutyric acid was taken up by liver slices to give a concentration gradient. This was confirmed in a separate investigation by TEWS AND HARPER[9] who also showed that dinitrophenol and ouabain were partial inhibitors of the system. The system was also saturable with α-aminoisobutyric acid and inhibited by anaero-

biosis. Liver slices have also been used to study the incorporation of amino acids into protein[10–12].

In this paper we report further studies on α-aminoisobutyric acid uptake by rat liver slices and the effect of Na^+-free medium, inhibition by iodoacetic acid and competitive inhibition by glycine. The transport of glycine itself has been studied and a comparison made between rates of transport and metabolism for that amino acid. The transport of cystine and cysteine were also studied and kinetic constants for their transport were determined. Lysine and valine were maintained at higher concentrations in the tissue than in the plasma *in vivo* but *in vitro* no radioactive gradient was obtained.

MATERIALS AND METHODS

Chemicals

α-Amino[1-^{14}C]isobutyric acid, uniformly labeled [$^{14}C_2$]glycine, lysine and valine, [^{35}S]cystine, and [*carboxy*-^{14}C]inulin were obtained from the Radiochemical Centre (Amersham, England). Further supplies of [*carboxy*-^{14}C]inulin were obtained from Calbiochem (Los Angeles, Calif.) and New England Nuclear Corp. (Boston, Mass.). Purity of the radiochemical amino acids was checked by high-voltage electrophoresis. Unlabeled L-amino acids were obtained from Hopkins and Williams Ltd. (Chadwell Heath, England). *N*-Ethylmaleimide and dithiothreitol were obtained from Calbiochem (Los Angeles, Calif.) and 2,4-dinitrophenol from British Drug Houses Ltd. (Poole, England).

Preparation of tissue

Male Sprague–Dawley rats, aged 6 weeks and weighing approx. 150 g, were used. They were fed on rat chow (Dixons Feeds 41B, Ware, England) and starved for 24 h before the experiment when they were killed by stunning followed by decapitation. The liver was removed and immersed in ice-cold 0.9% NaCl, and 0.5-mm slices were cut on a Stadie-Riggs microtome. Slices were kept chilled in oxygenated Krebs–Ringer bicarbonate buffer[13]. Three rats were normally used in each experiment.

Incubation procedure

A slice of liver from each rat, was placed in 25-ml conical flasks, containing 0.5 μC radioactive amino acid in 2 ml of Krebs–Ringer bicarbonate buffer. Each slice weighed approx. 20 mg. Incubations were carried out in triplicate in an atmosphere of O_2–CO_2 (95:5, by vol.), unless otherwise indicated. The flasks were gased for 30 sec, sealed with rubber turnover closures (Suba-seal Works, Barnsley, England), and incubated in a metabolic shaker (Gallenkamp) at 37°. For anaerobic experiments, the tissue was preincubated in an atmosphere of nitrogen–CO_2 (95:5, by vol.) before addition of the radioactive amino acid. At the end of the incubation period the slices were removed, dipped in 0.9% NaCl, blotted, and weighed on a torsion balance (Roller Smith Precision Balances, Federal Pacific Electric Co. N.J., U.S.A.).

The amino acids were extracted by one of two procedures. In one the slices were placed in 2 ml water in conical centrifuge tubes and heated at 100° in a water bath for 6 min. In the other the slices were added to 2 ml of 5% trichloroacetic acid and homogenised in a tissue grinder (Kontex Glass Co., Vineland, U.S.A.). The pre-

cipitate was removed by centrifugation and the trichloroacetic acid extracted by ether (3 × 2 ml). For experiments in Na^+-free medium, NaCl and $NaHCO_3$ in the Krebs–Ringer bicarbonate buffer were replaced with Tris[14].

Determination of tissue water

After incubation the slices were blotted, weighed, dried overnight in a crucible at 110°, and reweighed. The difference between the weights of the tissue before and after drying gave the total tissue water, which was expressed as a percentage of the wet tissue weight.

Determination of extracellular space

[*carboxy*-^{14}C]Inulin, obtained from three different commercial sources, was added to the incubation medium. Incubations were carried out for various periods of time, corresponding to the incubation periods used for the various amino acids. The calculation of extracellular space was carried as described by ROSENBERG *et al.*[15].

Identification of metabolites

Portions of the incubation medium and tissue extracts (10 μl) were submitted to high-voltage electrophoresis (High-Voltage Electrophoresis Unit, Model 2, Miles Hivolt Ltd, Shoreham, Sussex, England), using Whatman 3 MM paper and 6.8% formic acid solution as a buffer. Electrophoresis was carried out at room temperature, and at 4 kV for 30 min. The paper was then dried and cut into strips, which in turn were cut into segments (approx. 10 segments, each 2 cm long). These were immersed in 10 ml of a toluene-based phosphor (2,5-bis-2-(5-*tert.*-butylbenzoxazolyl)-thiophene, Ciba, Duxford, Cambridge, England) and the radioactivity counted in an automatic liquid scintillation counter (Nuclear Chicago). The areas of radioactivity were identified by comparison with known marker substances.

Determination of isotope distribution ratios

Portions of the incubation medium and tissue extracts (0.2 ml) were placed in liquid scintillation vials, and ethanol (2.8 ml) and the toluene-based phosphor (7 ml, 2,5-bis-2-(5-*tert.*-butylbenzoxazolyl)-thiophene) were added. The radioactive isotope distribution ratios were calculated by the method of ROSENBERG *et al.*[16], substituting the values for tissue water and extracellular fluid determined for liver slices.

Determination of the rate of transport of cysteine and the determination of the cystine–cysteine oxidoreduction state

Rat liver slices were incubated in buffer containing [^{35}S]cystine to which dithiothreitol had been added (final concentration 2 or 4 mM depending on the cysteine concentration and duration of incubation). After incubation the extent of cystine reduction was determined by homogenising the tissue in a solution of *N*-ethylmaleimide (1.0 ml, 20 mM in 0.1 M phosphate buffer (pH 7.4)). This reacts instantly with all free thiol groups and, hence, prevents any reoxidation to the disulfide. Trichloroacetic acid (10%, 1 ml) was added. The precipitated protein was removed by centrifugation and the trichloroacetic acid removed by ether extraction. Further details of this procedure have been described by CRAWHALL AND SEGAL[17] and by CRAWHALL

AND DAVIS[18]. The cysteine–*N*-ethylmaleimide derivative was separated from cystine by high-voltage electrophoresis, and the extent of reduction determined by counting the appropriate radioactive sections of the high-voltage electrophoresis strip.

RESULTS

Determinations of tissue water and extracellular space

Results of 12 determinations of total tissue water gave a value of 68.1 ± 3.4% (S.D.) comparable to previously reported values[8,9,19]. The results did not vary with the duration of incubation. The results of extracellular space determinations showed that the final equilibration of inulin with the extracellular fluid did not occur until 30 min of incubation and these results were similar using [^{14}C]inulin from three different manufacturers. The mean equilibrium value of 34.3% is comparable to the value of 35% reported by CAHILL *et al.*[20] but is higher than the mean value of 27% reported by several other workers[7,9,17]. The inulin space remained the same when liver slices were incubated in Na^+-free medium, but under anaerobic conditions the inulin space became equal to the total tissue water space.

Inhibitors and kinetics of α-aminoisobutyric acid transport

This study of inhibitors of α-aminoisobutyric acid transport showed that dinitrophenol even at a concentration of 0.5 mM was only a partial inhibitor. Iodoacetic acid (0.5 mM) and anaerobiosis were more effective inhibitors, the most effective being Na^+ deficiency when Na^+-free incubation medium was used (Fig. 1).

The rate of transport of α-aminoisobutyric acid was measured at different concentrations, from 0.2 to 2 mM for incubations of 60 min at 37°. The results were expressed graphically in a double reciprocal plot which showed that α-aminoisobutyric acid was transported by a single system over this concentration range, with a K_m of 1.25 mM and v_{max} of 0.022 μmole/g wet weight of tissue per min. Similar transport experiments carried out with α-amino[^{14}C]isobutyric acid in the presence of glycine

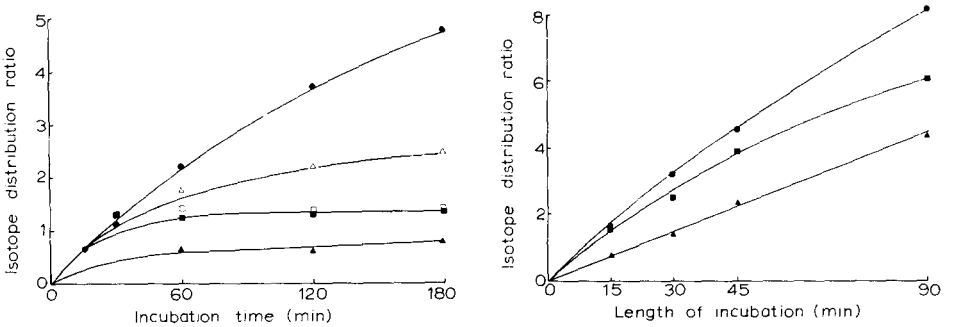

Fig. 1. Isotope distribution ratio obtained by incubation of rat liver slices in Krebs–Ringer bicarbonate buffer at 37° containing α-amino[^{14}C]isobutyric acid (0.2 mM). ●—●, control (no inhibitors); △—△, incubation with dinitrophenol (0.5 mM); □—□, incubation with iodoacetic acid (0.5 mM); ■—■, anaerobic incubation; ▲—▲, incubation in Na^+-free medium.

Fig. 2. Isotope distribution ratio after incubation of rat liver slices in Krebs–Ringer bicarbonate buffer containing [2-^{14}C]glycine. ●—●, 0.05 mM glycine; ■—■, 0.2 mM glycine; ▲—▲, isotope distribution ratio determined as free glycine after separation of glycine from metabolites by high-voltage electrophoresis. (Glycine concentration in medium, 0.05 mM.)

(20 mM) indicated that glycine was an inhibitor of α-amino-isobutyric acid transport and obeyed the kinetics of competitive inhibition in that it increased the K_m to 2.5 mM without affecting v_{max} (ref. 21).

Transport and metabolism of glycine

Initial experiments on the transport of [^{14}C]glycine by liver slices showed that a rapid uptake of radioactivity occurred (Fig. 2). The intracellular radioactivity was analysed by high-voltage electrophoresis and it was found that 70% of the radioactivity in the medium and 50% in the tissue remained as glycine during incubation periods of up to 90 min. The amount of glycine remaining in the tissue after each period of incubation was then calculated and it was shown that a considerable gradient for glycine was still achieved (lower graph Fig. 2).

Auto- and hetero-exchange of glycine and α-aminoisobutyric acid

Amino acids which share the same transport system may demonstrate the phenomenon of exchange diffusion, whereby the efflux of one preloaded amino acid accelerates the uptake of the same amino acid carrying a radioactive label (auto-exchange) or of a different radioactive amino acid sharing the same transport system (hetero-exchange). This possibility was investigated, using the technique reported by SCHWARTZMAN *et al.*[22]. Rat liver slices were incubated in either glycine (20 mM) or α-aminoisobutyric acid (20 mM) for 30 min. At the end of this incubation the slices were removed and placed in new medium containing either of the [^{14}C]amino acids at a concentration of 0.01 mM for α-aminoisobutyric acid or 0.02 mM for glycine. The rate of uptake of these amino acids was measured for increasing periods of time, from 2 min through to 60 min. No significant difference was observed between these results and paired normal controls, despite the fact that glycine was a competitive inhibitor of α-aminoisobutyric acid transport and, hence might be expected to share the same transport system.

Cystine and cysteine transport

The technique for studying cysteine transport was similar to that described by CRAWHALL AND SEGAL[23] for kidney cortex slices, and was based on the use of dithiothreitol in the incubation medium to keep cysteine in the reduced state during aerobic incubation. Cystine transport was investigated by the method described by CRAWHALL AND DAVIS[18] for the rat everted jejunal sac. The results (Fig. 3) indicated that cysteine was transported faster and for a longer period than cystine and gave concentration gradients greater than that observed for cystine. Anaerobic incubation completely stopped the formation of a concentration gradient by both these amino acids. Increasing the concentration of cysteine and cystine to 0.4 and 0.2 mM, respectively, decreased the initial rates of uptake of both these amino acids, indicating that saturation of the transport mechanisms was occurring.

The extent of cystine reduction and metabolism in the tissue homogenate was determined as decribed in MATERIALS AND METHODS. When cystine was incubated with liver slices under aerobic conditions, cystine was found to be mainly (75%) in the reduced form intracellularly (*cf.* ref. 17).

Intracellular reduction to cysteine was decreased to 44% in anaerobic incubations compared with aerobic incubations, but the formation of other metabolites

increased considerably during the incubation period. The electrophoretic properties of the principal metabolite corresponded to taurine. There was some reduction of cystine to cysteine in the incubating medium under both aerobic and anaerobic conditions. This phenomenon was observed to a lesser extent with incubations using everted gut sacs[18] and the explanation proposed was that cysteine efflux occurs from the tissue into the medium and then undergoes a thiol–disulfide exchange with [^{35}S]-cystine in the medium with the liberation of [^{35}S]cysteine. Cysteine appeared to be transported faster than cystine and the true rate of transport of cystine might in fact be less than that observed because of the contribution of the transport of [^{35}S]-cysteine derived from [^{35}S]cystine in the medium.

Experiments to measure directly the rate of cysteine transport by rat liver slices were carried out with dithiothreitol added to the medium. Cysteine remained in the reduced state in both the tissue and medium for incubation periods of up to 2 h.

The possibility of dithiothreitol directly affecting other amino acid transport systems in the rat liver slice was investigated by incubating liver slices in medium containing either [^{14}C]glycine (0.2 mM) or α-amino[^{14}C]isobutyric acid (0.2 mM) for 60 min and in which paired flasks either did or did not contain dithiothreitol (4 mM). The uptake of glycine and α-aminoisobutyric acid was decreased in the presence of dithiothreitol by 19 and 16%, respectively. Using kidney cortex slices, CRAWHALL AND SEGAL[23] showed that dithiothreitol did not significantly affect the rate of uptake of α-aminoisobutyric acid and some other amino acids.

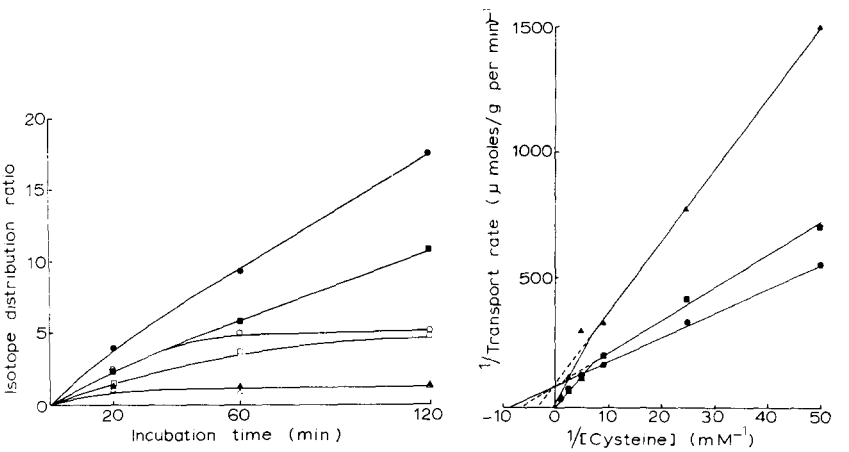

Fig. 3. Isotope distribution ratio obtained after incubation of rat liver slices in Krebs–Ringer bicarbonate buffer at 37° containing either [^{35}S]cystine or cysteine. ●—●, [^{35}S]cystine (0.01 mM) with dithothreitol (2 mM) giving cysteine concentration of 0.02 mM; ■—■, [^{35}S]cystine (0.2 mM) with dithiothreitol (2 mM) giving cystine concentration of 0.4 mM; ▲—▲, [^{35}S]cystine (0.2 mM) with dithiothreitol (2 mM), anaerobic; ○—○, 0.01 mM [^{35}S]cystine; □—□, 0.2 mM [^{35}S]cystine; △—△, 0.2 mM [^{35}S]cystine, anaerobic.

Fig. 4. Rate of transport of [^{35}S]cystine by rat liver slices incubated in Krebs–Ringer bicarbonate buffer containing [^{35}S]cystine at different concentrations and dithiothreitol. Incubation time 30 min. Plotted as a double reciprocal plot. ●—●, incubation with different concentrations of [^{35}S]cystine with dithiothreitol (2 mM); ■—■, incubation with different concentrations of [^{35}S]cystine with dithiothreitol (2 mM) in presence of α-aminoisobutyric acid (20 mM); ▲—▲, incubation with different concentrations of [^{35}S]cystine with dithiothreitol (2 mM) in presence of glycine (20 mM). The transport rates for the four lower concentrations of cysteine are mean values of nine separate determinations which were in good agreement with each other. The results of the high concentrations were the mean of triplicate determinations.

Transport kinetics of cystine and cysteine in the rat liver were studied by carrying out the incubations for a fixed period of time (30 min) with various concentrations of substrate. The possibility that α-aminoisobutyric acid and glycine inhibited transport was investigated by adding these amino acids to the incubation medium (20 mM). The results were expressed as a double reciprocal plot of rate of transport (corrected for diffusion) against the reciprocal of the concentration of cystine or cysteine in the medium. For cystine this gave a straight line indicating a single saturable transport system having a K_m of 0.56 mM and v_{max} 0.020 μmole cystine per g wet weight of tissue per min. Glycine (20 mM) inhibited cystine transport by 60% with an increase of K_m to 1.1 mM. α-Aminoisobutyric acid did not affect the rate of transport of cystine except at the lowest concentration (0.01 mM) where a slight decrease was observed.

Results for cysteine showed the presence of two transport systems (Fig. 4). One system was observed at low concentrations of cysteine (0.2 mM or less) and had a high affinity for cysteine (K_m = 0.11 mM) and a low capacity (v_{max} = 0.012 μmole cysteine per g wet weight of tissue per min); it was inhibited strongly by glycine and weakly by α-aminoisobutyric acid. Inhibition appeared to be competitive in both cases. The other transport system was for cysteine concentrations of 0.2 mM and above. This was a high-capacity, low-affinity system (v_{max} = 0.14 μmole cysteine per g wet weight of tissue per min and K_m approx. 4 mM), inhibited competitively by glycine but not by α-aminoisobutyric acid. Cysteine transport had previously been shown to be inhibited by α-aminoisobutyric acid in Ehrlich ascites cells[24].

Transport of lysine and valine

Incubation of rat liver slices in a medium containing L-[^{14}C]lysine (0.2 mM) showed that the amino acid rapidly entered the tissue and equilibrium was reached after 30 min but no concentration gradient was achieved. Amino acid analyses showed that valine also existed at a higher concentration intracellularly in liver slices than in the plasma, but incubation of the slices in medium containing L-[^{14}C]valine (0.2 mM) again did not give rise to a concentration gradient *in vitro*. Analysis of the intracellular radioactivity by high-voltage electrophoresis after *in vitro* incubation with both amino acids showed that the radioactivity corresponded principally to the anticipated amino acid.

DISCUSSION

Previous studies in the transport of amino acids by rat liver slices[8,9] were restricted to the non-metabolised amino acid, α-aminoisobutyric acid which is concentrated in the slice by an energy-dependent, Na^+-dependent mechanism. The present studies showed that α-aminoisobutyric acid transport was also inhibited by the –SH reacting substance, iodoacetic acid. Incubation under anaerobic conditions also inhibited the uptake of this amino acid but the permeability of the tissue was affected by this procedure as shown by the increase of the inulin space. Glycine was shown to be a competitive inhibitor of α-aminoisobutyric acid transport, a phenomenon which has been observed in other tissues such as the fetal and adult calvaria of the rat[25]. More definite kinetic studies of glycine transport were rendered difficult by the extent of metabolism of glycine in the liver slice but the evidence shown in Fig. 2

indicated that a true intracellular gradient for glycine uptake by the slice readily occurred. This would necessarily underestimate the true rate of transport because much of the radioactivity within the tissue must have entered as glycine before conversion to other metabolites

Two other amino acids, cystine and cysteine were also shown to be taken up from the incubating medium by the liver slice to form an intracellular concentration gradient. The kinetic analysis of this transport process is relatively uncomplicated in the case of cysteine, where the intracellular and extracellular forms are the same and two separate transport systems were observed with different affinity constants for cysteine. This phenomenon has previously been observed for cysteine in human kidney cortex slices[26] but not in rat kidney cortex slices[27]. The kinetics of cystine transport is more difficult to analyse because of its rapid reduction to cysteine in various tissues. Glycine was apparently an inhibitor of both cysteine and cystine transport. The latter result was unexpected because glycine had not previously been shown to be an inhibitor of the cystine–basic amino acid transport system but it was also shown that under these experimental conditions, some of the cystine in the medium was reduced to cysteine and it is possible that glycine was inhibiting the transport of this amino acid which was transported faster than cystine if glycine was not present.

These experiments also showed that although the liver slice was able to form a concentration gradient for several amino acids, it was apparently unable to do so for lysine and valine. It has been shown that various amino acids, including lysine and valine exist at a higher concentration in the intracellular fluid of the rat liver *in vivo* than in the plasma[4,28] and it is known that protein degradation can make a significant contribution to the concentrations of free amino acids in the liver[4,29]. Our own preliminary investigations have suggested that *in vivo* lysine may be concentrated in rat liver by incorporation into protein followed by intracellular release during protein catabolism[30]. This possibility has been discussed in relation to the release of amino acids from perfused rat liver[31]. Further experiments are being carried out to try to define the circumstances under which some amino acids appear to be readily transported into the liver slice against a concentration gradient whilst others do not and to extend our studies with other naturally occurring amino acids.

ACKNOWLEDGEMENTS

These studies were carried out in partial fulfilment of the requirements for a Ph.D. of the University of London by M.G.D. (submitted September 1969) and supported in part by the Canadian Medical Research Council Grant No. Ma-3331.

REFERENCES

1 H. H. TALLAN, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 927.
2 H. N. CHRISTENSEN, A. J. ASPEN AND E. G. RICE, *J. Biol. Chem.*, 220 (1956) 287.
3 R. B. SANDERS AND T. R. RIGGS, *Endocrinology*, 80 (1967) 29.
4 H. SCHIMASSEK AND W. GEROK, *Biochem. Z.*, 343 (1965) 407.
5 J. W. CHAMBERS, R. H. GEORG AND A. D. BASS, *Mol. Pharmacol.*, 1 (1965) 66.
6 N. FREINKEL, A. K. COHEN, R. A. ARKY AND A. E. FOSTER, *J. Clin. Endocrinol.*, 25 (1965) 76.
7 R. HEMS, M. STUBBS AND H. A. KREBS, *Biochem. J.*, 107 (1968) 807.
8 J. C. CRAWHALL AND S. SEGAL, *Biochim. Biophys. Acta*, 163 (1968) 163.

9 J. K. TEWS AND A. E. HARPER, *Biochim. Biophys. Acta*, 183 (1969) 601.
10 J. B. MELCHIOR AND H. TARVER, *Arch. Biochem. Biophys.*, 12 (1947) 301.
11 B. M. HANKING AND S. ROBERTS, *Biochim. Biophys. Acta*, 104 (1965) 427.
12 J. I. CLIFFORD AND K. R. REES, *Biochem. J.*, 102 (1967) 65.
13 H. A. KREBS AND K. HENSELEIT, *Z. Physiol. Chem.*, 210 (1932) 33.
14 M. FOX, S. THIER, L. E. ROSENBERG AND S. SEGAL, *Biochim. Biophys. Acta*, 79 (1964) 107.
15 L. E. ROSENBERG, S. J. DOWNING AND S. SEGAL, *Am. J. Physiol.*, 202 (1962) 800.
16 L. E. ROSENBERG, A. BLAIR AND S. SEGAL, *Biochim. Biophys. Acta*, 54 (1961) 479.
17 J. C. CRAWHALL AND S. SEGAL, *Biochem. J.*, 105 (1967) 891.
18 J. C. CRAWHALL AND M. G. DAVIS, *Biochem. J.*, 112 (1969) 571.
19 K. D. HECKMANN AND D. S. PARSONS, *Biochim. Biophys. Acta*, 36 (1959) 203.
20 G. F. CAHILL, J. ASHMORE, A. SCOTT EARLE AND S. ZOTTU, *Am. J. Physiol.*, 192 (1958) 491.
21 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
22 L. SCHWARTZMAN, A. BLAIR AND S. SEGAL, *Biochim. Biophys. Acta*, 135 (1967) 120.
23 J. C. CRAWHALL AND S. SEGAL, *Biochim. Biophys. Acta*, 121 (1966) 215.
24 H. N. CRISTENSEN, M. LIANG AND E. C. ARCHER, *J. Biol. Chem.*, 242 (1967) 5237.
25 G. A. M. FINERMAN AND L. E. ROSENBERG, *J. Biol. Chem.*, 241 (1966) 1487.
26 S. SEGAL AND J. C. CRAWHALL, *Biochem. Med.*, 1 (1967) 141.
27 S. SEGAL AND J. C. CRAWHALL, *Proc. Natl. Acad. Sci. U.S.*, 59 (1968) 231.
28 H. N. CHRISTENSEN AND A. M. CULLEN, *Biochim. Biophys. Acta*, 150 (1968) 237.
29 J. C. GAN AND H. JEFFAY, *Biochim. Biophys. Acta*, 148 (1967) 448.
30 J. C. CRAWHALL AND M. G. DAVIS, *Clin. Sci.*, 37 (1969) 565.
31 L. E. MALLETTE, J. H. EXTON AND C. R. PARK, *J. Biol. Chem.*, 244 (1969) 5724.