A Difference in the Incorporation of ^{14}C into Hippurate Glycine from DL-[2- ^{14}C]-Glutamate and DL-[5- ^{14}C]-Glutamate in Guinea Pigs

By KATHLEEN V. ROWSELL and TAHSIN A. J. ALI

Department of Medical Biochemistry, University of Manchester, Manchester M13 9PT, U.K.

and EDWARD V. ROWSELL

Department of Biochemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD, U.K.

(Received 17 July 1975)

The specific radioactivity of urinary hippurate glycine was determined after injecting guinea pigs with benzoate and either DL-[2- ^{14}C]-glutamate or DL-[5- ^{14}C]-glutamate. The isotope dilution factor for the formation of [^{14}C]glycine was significantly greater (30%) with C-2 labelled glutamate. With either form of labelled glutamate the hippurate glycine was largely carboxyl-group labelled. The observations suggest a route for the incorporation of glutamate carbon into glycine that involves C-5 but not C-2. A hypothesis for glycine biosynthesis from L-glutamate is advanced, consistent with these findings, that includes conversion of L-glutamate to 4-hydroxy-2-oxoglutarate, the scission of the latter to glyoxylate and pyruvate, and the formation of glycine by transamination.

Leuthardt (1941) investigated the formation of glycine in guinea-pig liver by incubating slices in the presence of benzoate and measuring the accumulation of hippurate. The most noteworthy finding was an increase in hippurate formation reproducibly observed on the addition of L-glutamine, almost as much as with the addition of glycine itself. L-Glutamate was much less effective, but this does not exclude the possibility that L-glutamine was active after amide hydrolysis. There is a permeability barrier towards L-glutamate at the liver cell membrane (see e.g. Hems *et al.*, 1968), but L-glutamine penetrates easily and once inside the cells is readily hydrolysed to L-glutamate (Schwerin *et al.*, 1950). Leuthardt (1941) suggested a mechanism for the direct provision of the whole glycine molecule from L-glutamine or from L-glutamate, namely hydroxylation at C-3 and then scission between the C-2 and C-3 atoms.

However, after injecting rats or guinea pigs with DL-[1,2- ^{13}C]-glutamate and benzoate, Shemin (1949) isolated urinary hippurate labelled only in the glycine carboxyl group position. Thus he excluded Leuthardt's (1941) hypothesis (according to which glycine would have been labelled in both carbon atoms) and sugges-

ted that L-glutamate carbon was incorporated into glycine through a metabolic pathway stemming from some member of the citric acid cycle beyond the stage of 2-oxoglutarate decarboxylation, probably via L-serine. A route that would now find general favour is the reaction sequence(s) of Scheme 1. The weight of evidence supports route (a) rather than (b) for serine biosynthesis via the glycolytic pathway (see e.g. Feld & Sallach, 1973), but by either route L-glutamate labelled in the C-1 and C-2 positions would form glycine labelled only in the carboxyl group.

But a problem remains. If L-glutamine (via hydrolysis to L-glutamate) required so many metabolic steps for its carbon contribution to glycine biosynthesis, it seems improbable that it would be almost as effective as glycine itself in promoting hippurate formation as Leuthardt (1941) found. A much shorter route than either shown in Scheme 1 would appear to be involved. Moreover, Evered & Wass (1966) reported ^{14}C incorporation into hippurate glycine after incubating guinea-pig liver slices with benzoate and L-[U- ^{14}C]-glutamine and the dilution factor for the specific radioactivity (glut-

-
- (a) L-Glutamate $\rightarrow$ 2-oxoglutarate $\rightarrow$ succinate $\rightarrow$ fumarate $\rightarrow$ malate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ 2-phosphoglycerate $\rightarrow$ 3-phosphoglycerate $\rightarrow$ 3-phosphohydroxypyruvate $\rightarrow$ 3-phospho-L-serine $\rightarrow$ L-serine $\rightarrow$ glycine
(b) L-Glutamate $\rightarrow$ 3-phosphoglycerate $\rightarrow$ glycerate $\rightarrow$ hydroxypyruvate $\rightarrow$ L-serine $\rightarrow$ glycine
2-phosphoglycerate

Scheme 1. Routes for the conversion of L-glutamate into glycine via the citric acid cycle, the glycolytic pathway and L-serine biosynthesis

amine as against glycine) was not high, about 8 (D. F. Evered, personal communication).

The hypothesis underlying the present investigation is outlined in Scheme 2(a). Following Leuthardt (1941) we suggest that enzymic hydroxylation of L-glutamate (or a closely related metabolite) may occur, but in the C-4 position. Apart from this one speculative step, Scheme 2(a) comprises well-established metabolic reactions. The conversion of 4-hydroxy-L-glutamate via 4-hydroxy-2-oxoglutarate to pyruvate and glyoxylate is the terminal sequence of reactions in the degradation of L-hydroxyproline (see, e.g., Adams, 1970); the enzymes responsible are present in liver (see e.g. Goldstone & Adams, 1962; Maitra & Dekker, 1964). The final conversion to glycine could be catalysed by L-alanine-glyoxylate aminotransferase which is also predominantly a liver enzyme (Rowsell *et al.*, 1972).

The experimental objective was to determine the isotope dilution factor [(sp. radioactivity of L-[^{14}C]glutamate injected)/(specific radioactivity for [^{14}C]glycine excreted as hippurate)] after injecting guinea pigs with benzoate, carrier L-glutamate and either DL-[2- ^{14}C]glutamate or DL-[5- ^{14}C]glutamate. If the only significant pathway for carbon transfer from L-glutamate into glycine was that shown in Scheme 1, we would expect the same isotope dilution factor for both C-2 and C-5-labelled L-glutamate. With the oxidative decarboxylation of 2-oxo[^{14}C]glutarate derived from either form of labelled L-glutamate the succinate produced would carry ^{14}C randomly distributed between its carboxyl groups and there would be the same degree of ^{14}C incorporation into the glycine eventually formed. But if the hypothetical pathway in Scheme 2(a) was in operation to a significant extent, it would be reasonable to anticipate some difference in the isotope dilution factor.

Experimental

Radiochemicals

DL-[2- ^{14}C]Glutamic acid (3.16 mCi/mmol) was obtained from Philips-Duphar, Petten, Holland; DL-[5- ^{14}C]glutamic acid (11.4 mCi/mmol) was from Schwarz-Mann, Orangeburg, N.Y., U.S.A., and [1- ^{14}C]glycine (56 mCi/mmol) and [2- ^{14}C]glycine (51 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.

Radioactivity counting procedure

The scintillation fluid was a mixture of 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene dissolved in 1 litre of sulphur-free toluene (A.R. grade). An Intertechnique Model SL 40 liquid-scintillation spectrometer was used (Intertechnique Ltd., Portslade, Sussex, U.K.). Small samples of aqueous solutions (<50 μl) were counted for radioactivity by mixing them with 4 ml

of methanol (A.R. grade) before adding 14 ml of scintillation fluid. When it was necessary to employ a larger sample, this was applied to a strip of chromatography paper (2 cm $\times$ 7.5 cm) and dried in a stream of cold air; 4 ml of methanol and 14 ml of scintillation fluid were then added. Each determination was made in duplicate. The same method of counting for radioactivity on paper was used with ^{14}C -labelled glycine derived from urinary hippurate, after chromatographic separation. The background count rate was obtained with a blank vial containing the same volume of methanol and scintillation fluid (and, where appropriate, a paper strip), recording at least 1000 total counts. ^{14}C samples always showed at least ten times the background radioactivity and were counted long enough to accumulate at least 10000 total counts. The radioactivity recorded (d.p.m.) was corrected for quenching and for background.

Purification of ^{14}C -labelled amino acids

A neutral pH mixture of about 50 μCi of commercially obtained ^{14}C -labelled amino acid with 10 μmol of the corresponding unlabelled carrier was applied to a column (5 cm $\times$ 0.6 cm) of Amberlite 1R-120 resin (H^+ form). After washing well with deionized water, the amino acid was eluted with 30 ml of aq. 2 M- NH_3 . The eluate was evaporated to dryness under decreased pressure at 50°C. The residue was dissolved in water (about 2.0 ml) and further fractionated by descending paper chromatography for 24 h on Whatman No. 1 paper with phenol saturated with water. Several marker amino acids were run separately on the same paper to be located by the ninhydrin reaction. After normal drying, papers were washed in dry acetone to remove any traces of phenol. The distribution of radioactivity was mapped by radioautography: a few days' exposure with Standard Medical X-ray film SD-54 (Kodak Ltd., Hemel Hempstead, Herts., U.K.). On each occasion the bulk of the radioactivity had run as a band in the expected position. This band was cut out and eluted with about 25 ml of water and radioactivity was determined by liquid-scintillation counting. A sample (about 0.5 μCi) was then subjected to the same procedure to assess radiochemical purity, eluting into 5.0 ml of water. Recoveries as single spots with the correct R_F values were as follows: DL-[2- ^{14}C]glutamate, 98%; DL-[5- ^{14}C]glutamate, 99%; [1- ^{14}C]glycine, 99%; [2- ^{14}C]glycine, 99%. The purified labelled amino acids were stored in the 'deep freeze.'

No systematic attempt was made to confirm the ^{14}C distribution patterns in the labelled glutamate samples commercially obtained. However, purified samples were analysed for 1- ^{14}C and $^{14}\text{CO}_2$ recoveries (as compared with total d.p.m.); these were about 0.1 and 0.7% respectively for the assumed DL-[2- ^{14}C]glutamate and DL-[5- ^{14}C]glutamate.

Injection of guinea pigs and urine collection

Guinea pigs (Duncan Hartley strain) were maintained on Oxoid laboratory-animal diet SG 1 (Oxoid Ltd., London, SE1 9HF, U.K.). They were starved 24h before being injected intraperitoneally with a solution (2.0ml/100g body wt.) that was 0.175 M with respect both to sodium benzoate and to sodium L-glutamate and which contained purified DL-[2-¹⁴C]glutamate or DL-[5-¹⁴C]glutamate (0.4–0.8 μ Ci). Each guinea pig was then left in a metabolic cage (Metabowl; Jencons Scientific Ltd., Hemel Hempstead, Herts., U.K.) for 4h, with free access to water but not to food. The urine was collected and the volume noted.

Chemical and radioactivity analysis of urinary hippurate glycine

After acidification to pH 1 with 1 M-HCl the urine was extracted with 75 ml of anaesthetic ether for 12h in a microextractor which had been made with its mixing chamber as an ascending coil with nodules on the inner surface, a device to retard the ether in its passage through the urine and so maximize the extraction rate. In other respects the extractor design was as recommended by Wayman & Wright (1945, see Fig. 2). With a urine volume greater than 15 ml, extractions were made separately on half-volume samples. The ether extract was evaporated to dryness and the weight recorded. A weighed portion (the bulk) was hydrolysed by refluxing for 6h with 2 M-HCl (1 ml/mg). The HCl extract was evaporated to dryness under decreased pressure at 50°C and the residue was re-extracted several times with water and re-evaporated to dryness to remove traces of HCl. After a final extraction in a small volume of water the solution was neutralized with NaOH and made up to a known volume (about 3 ml). Samples of the hydrolysate, 2.5 and 5.0 μ l in duplicate, accompanied by duplicate glycine standards (10 μ l of a 0.01 M solution) were subjected to descending chromatography for 24h on Whatman No. 1 paper with phenol saturated with water. The glycine spots produced by the hydrolysate were then measured colorimetrically after reaction with ninhydrin following the procedure of Kay *et al.* (1956). The method was tested with 10.0 ml of the normal urine of guinea pigs and with 10.0 ml of the same urine containing added sodium hippurate (60 mg). The glycine recovery for the added hippurate was 97.5%.

The same chromatographic procedure was used before ¹⁴C estimations, with duplicate 100 and 200 μ l samples of the hydrolysate. Radioactivity in the glycine position was located by radioautography (2–3 weeks exposure) and these areas were cut out for immediate counting (as described above). Often the applied samples had diffused such that the radioactivity occupied a relatively large area of paper (of the order 7 cm $\times$ 5 cm); in such cases the excised area was cut into two strips for separate counting.

Duplicates agreed within 5% for both chemical and ¹⁴C measurements. There was linearity between the amount of hydrolysate chromatographed and the corrected count rate (d.p.m.) observed. Colorimetric measurements were accepted only when they were in the region of proportionality with the amount of hydrolysate taken for analysis.

Estimation of C-1 label in ¹⁴C-labelled amino acids

The procedure was based on the observations of Van Slyke *et al.* (1941). A sample of ¹⁴C-labelled amino acid, 25–50 μ mol of glycine or glutamate, was mixed with 2.5–5.0 ml of 5% (w/v) (0.28 M) ninhydrin in 0.1 M-HCl in a 200 ml Kjeldahl flask [(ninhydrin)/(amino acid) molar ratio >15]. The flask was continuously flushed out with a slow stream of N₂ and the mixture was boiled gently for 1 h. With refluxing in the neck of the flask there was little decrease in the volume of the reaction mixture. The emergent gas mixture was passed, in sequence, through 100 ml of concentrated H₂SO₄, an empty trap, 100 ml of methanol and finally through two scintillation vials, in series, each containing 4 ml of 1 M-Hyaminate hydroxide in methanol as a CO₂ trap. For counting, the Hyamine vials were removed and 14 ml of scintillation fluid was added to each, in the process washing Hyamine adherent to the inlet tube into the vial.

The ¹⁴C-labelled amino acid was introduced either in solution (not >0.5 ml) or on paper (not >15 cm²) cut into small pieces, and mixtures with ninhydrin were kept on ice until the whole apparatus was assembled with the N₂ flow in operation. CO₂ absorption in the hyamine was facilitated by using drawn-out inlet tubes so that the gas was delivered as a stream of fine bubbles. A methanol wash was included to diminish the loss of methanol from the hyamine solution in the absorption vials which would decrease its volume and increase its viscosity.

The trapping efficiency was tested by liberating CO₂ in the reaction vessel at room temperature (20°C) from a mixture of 0.1 ml of 2 M-HCl and 10 μ l of 0.05 M-NaH¹⁴CO₃ (duplicate counts 857 and 865 d.p.m.). The total recovery in the hyamine vials was 887 d.p.m. (i.e. close to 100%). In this, and in all experiments with ninhydrin, radioactivity accumulated in the second vial was <1% of that of the first vial. The whole technique was checked with samples of purified [¹⁴C]glycine (30 μ mol, about 10⁶ d.p.m.). With glycine introduced either in solution or on paper, isotope recoveries as ¹⁴CO₂ with [1-¹⁴C]glycine were >93% and <0.1% with [2-¹⁴C]glycine. Labelled formaldehyde from [2-¹⁴C]glycine was apparently not carried over into the hyamine.

Calculations

The carrier for injected DL-[¹⁴C]glutamate was sodium L-glutamate and in calculating specific radio-

activities (d.p.m./ μ mol) for injected glutamate, only counts associated with L-[14 C]glutamate have been considered (i.e. half the total counts); these values have been used for the measurement of isotope dilution factors i.e., (d.p.m. per μ mol of glutamate injected)/(d.p.m. per μ mol of urinary hippurate glycine excreted). We have assumed that the metabolism of D-glutamate would be negligible; at least with the rat this appears to be the case (Wilson & Koepe, 1961).

Unless otherwise stated, Student's *t* test was employed in assessing the significance of a difference between mean values.

Results

Incorporation of radioactivity into urinary hippurate glycine from DL-[2- 14 C] or DL-[5- 14 C]glutamate

The conditions and findings for the whole series of experiments are summarized in Table 1. The mean isotope dilution factor for C-2-labelled glutamate (49.1) was 30% higher than for C-5 labelled-glutamate (37.9), and both by Student's *t* test and by the Mann-Whitney *U* test (as described by Roscoe, 1969) the difference was significant ($P < 0.01$). It appeared that

the sex of the animals is not a relevant factor; for example, in experiments either with DL-[2- 14 C]-glutamate or with DL-[5- 14 C]glutamate there was no significant difference in the mean isotope dilution factor for males and females ($P > 0.2$). There was no significant difference in the percentage of injected benzoate recovered as hippurate glycine ($P > 0.2$) for C-2- as against C-5-labelled glutamate.

In some experiments, a sample of an aqueous solution of the evaporated residue from the ether extract of urine was chromatographed before hydrolysis. The whole length of the developed chromatogram was then quite free of radioactivity, as measured by radioautography. Hippuric acid runs close to the solvent front in phenol saturated with water (as revealed under u.v. light), and presumably the radioactivity had been totally eluted from the paper. After paper chromatography with hydrolysed samples, except for a trace of colour development in response to ninhydrin and a trace of radioactivity, both in the glutamate position, the only ninhydrin spots and the only radioactive areas were in the glycine position, well separated from glutamate. (The appearance of glutamate under these conditions may be an indication of a slight degree of benzoate conjunction with L-

Table 1. *Incorporation of radioactivity into urinary hippurate glycine after injection of guinea pigs with benzoate and DL-[2- 14 C]glutamate or DL-[5- 14 C]glutamate*

For details see the Experimental section. Isotope dilution factors were calculated as (specific radioactivity of injected L-[14 C]glutamate)/(specific radioactivity of urinary hippurate glycine). Mean values $\pm$ S.D. or range (in parentheses) are given.

Position of 14 C label in glutamate	No. of expts.	Body wt. (g)	Sp. radioactivity of injected L-glutamate (d.p.m./ μ mol)	Benzoate recovery as hippurate glycine in urine (%)	Sp. radioactivity of hippurate glycine in urine (d.p.m./ μ mol)	Isotope dilution factor
C-2	10	433 (380-650)	950 (485-1367)	42.1 $\pm$ 11.0	19.9 (9.1-29.0)	49.1 $\pm$ 8.6
C-5	11	493 (360-640)	821 (391-1203)	37.9 $\pm$ 5.8	22.2 (9.4-34.8)	37.9 $\pm$ 3.7

Table 2. *Carboxyl labelling in hippurate glycine excreted by guinea pigs after injection with DL-[2- 14 C] or DL-[5- 14 C] glutamate*

Samples (200 μ L) of hydrolysed urinary hippurate from separate experiments described in Table 1 were chromatographed. The radioactive glycine areas were located and cut out for direct reaction with ninhydrin. The liberated 14 CO₂ was collected and counted for radioactivity. For further details see the Experimental section.

Source of hippurate glycine label	Glycine separated by paper chromatography		14 CO ₂ recovered (d.p.m.)	Recovery of total glycine radioactivity as 14 CO ₂ (%)
	(μ mol)	(d.p.m.)		
DL-[2- 14 C]Glutamate				
(a)	27.5	681	632	93
(b)	40.7	695	572	82.5
DL-[5- 14 C]Glutamate				
(a)	46.7	437	505	116
(b)	30.0	611	611	100

Scheme 2. Hypothetical route for the conversion of L-glutamate into glycine

The numbering (subscripts in parentheses) of the C atoms in each compound refers to the C atoms of the glutamate from which they are originally derived. With (c) some randomization of ¹⁴C distribution would be expected (via malate and fumarate) and serine would also be labelled in the C-3 position.

glutamine.) These observations gave some assurance that the procedure adopted for the chemical and radioactivity analysis of urinary hippurate glycine was adequately specific.

Measurement of the C-1 label in urinary hippurate [¹⁴C]glycine

After chemical and radioactivity measurements the urinary hippurate glycine derived from some experi-

ments with DL-[2-¹⁴C]glutamate or DL-[5-¹⁴C]-glutamate was investigated with respect to ¹⁴C distribution. In all experiments (with ninhydrin treatment) there was a high recovery of glycine total counts as ¹⁴CO₂ (Table 2). Evidently the ¹⁴C-glycine was labelled principally in the carboxyl position.

Discussion

Considered separately, the values for isotope dilution measured in the present study tell us little. Each is the result of many interrelated factors such as the rates of transfer of carbon from glutamate to glycine, the dilution of label by intermediates along these pathways, the size of various glycine pools and their rates of formation, and so on. In particular it is obvious that this precludes any quantitative measurement of the rate of glycine biosynthesis from glutamate and, in any case, administration of loading concentrations of benzoate and glutamate in our experiments might well affect the normal rate.

However, the reproducible difference in the isotope dilution factor observed for the two forms of labelled glutamate (with [2-¹⁴C]glutamate it was about 30% greater than with [5-¹⁴C]glutamate, $P < 0.01$) is noteworthy. Apparently at least part of the C-5 carbon of glutamate was transferred metabolically into glycine more efficiently than glutamate C-2 carbon. Whatever the explanation in detail, it would appear that there is a pathway for the transfer of carbon from the C-5 position into glycine which is different from that followed by carbon from the C-2 position.

A speculation which accords with this general conclusion has been outlined (see the introduction and Scheme 2). Thus [1-¹⁴C]glycine could be formed from [5-¹⁴C]glutamate by a relatively short route: either via 2-oxoglutarate, 4-hydroxy-2-oxoglutarate and glyoxylate, or via 4-hydroxyglutamate, 4-hydroxy-2-oxoglutarate and glyoxylate (Scheme 2a). The same route could be joined eventually by ¹⁴C from the C-2 position of glutamate: after hydroxylation and the scission of 4-hydroxy-2-oxo[2-¹⁴C]glutarate, unlabelled glyoxylate and [2-¹⁴C]pyruvate would be liberated; by oxidative decarboxylation the pyruvate would become [1-¹⁴C]acetyl-CoA; with entry into and metabolism along the citric acid cycle 2-oxo-[5-¹⁴C]glutarate would be formed and thence [1-¹⁴C]glyoxylate and [1-¹⁴C]glycine (Scheme 2b). An alternative route from [2-¹⁴C]pyruvate is shown in Scheme 2(c), in this case leading to [2-¹⁴C]glycine formation. By either route from [2-¹⁴C]glutamate the isotope dilution would be greater than with the route from [5-¹⁴C]glutamate.

With the operation of the route generally believed to be responsible for the biosynthesis of glycine (i.e. via glycolysis intermediates and serine, see Scheme 1), we would expect the same degree of isotope dilution

with C-2- or C-5-labelled glutamate. It would seem from our observations that this cannot be the only pathway from glutamate. A simultaneous operation of the pathway via serine and the hypothetical pathway via glyoxylate is a possibility, and to the extent that glycine biosynthesis from glutamate occurred by the former (common) pathway, the difference in isotope dilution factors for C-2- and C-5 labelled glutamate would be narrowed.

There is some suggestion in the results shown in Table 2 that with [2-¹⁴C]glutamate as precursor, the C-2 position of glycine might be labelled to a small extent and this would occur by the route shown in Scheme 2(c). The label was largely in the glycine carboxyl group, however, whether derived from [2-¹⁴C]- or [5-¹⁴C]-glutamate and this would be so via any of the pathways shown in Schemes 1, 2(a) or 2(b). Conceivably the extent of labelling of the glycine carboxyl group might be affected by exchange reactions with CO₂ (labelled or unlabelled) under the influence of the glycine-cleavage-enzyme complex (Sato *et al.* 1969). It would be difficult, however, to explain the lower isotope dilution observed with [5-¹⁴C]glutamate in terms of a more efficient carbon transfer into glycine via CO₂. There is no obvious route by which CO₂ is more readily liberated from the C-5 position of glutamate than from the C-2 position; moreover, observations at least with rat liver have shown no difference in the rate of ¹⁴CO₂ formation for DL-[5-¹⁴C] and DL-[2-¹⁴C]glutamate (D'Adamo & Haft, 1965).

Not all the possible metabolic pathways for ¹⁴C disposal following the injection of C-2- or C-5-labelled glutamate have been considered here; e.g. oxaloacetate formed from glutamate may enter the citric acid cycle, or glutamate may first be decarboxylated in the brain to form 4-amino-*n*-butyric acid etc. By no established metabolic route, however, does it seem possible (noting the various possible metabolic derivatives of [2-¹⁴C]- and [5-¹⁴C]-glutamate) to account for the difference in the isotope dilution factor for [¹⁴C]glycine formation. Our findings in fact point to the possibility of a novel pathway for glycine biosynthesis which involves carbon derived from the C-5 position of glutamate but not from the C-2 position. The experimental approach used in the present study precluded any prospect of obtaining direct evidence for the specific suggestion of a pathway via 4-hydroxy-2-oxoglutarate and glyoxylate, but our observations are consistent with that suggestion and encourage further investigation.

References

- Adams, E. (1970) *Int. Rev. Connect. Tissue Res.* **5**, 1-91
- D'Adamo, A. F. & Haft, D. E. (1965) *J. Biol. Chem.* **240**, 613-617
- Evered, D. F. & Wass, M. (1966) *Biochem. J.* **98**, 30P-31P

- Feld, R. D. & Sallach, H. J. (1973) *Arch. Biochem. Biophys.* **159**, 757–766
- Goldstone, A. & Adams, E. (1962) *J. Biol. Chem.* **237**, 3476–3485
- Hems, R., Stubbs, M. & Krebs, H. A. (1968) *Biochem. J.* **107**, 807–815
- Kay, R. E., Harris, D. C. & Entenman, C. (1956) *Arch. Biochem. Biophys.* **63**, 14–25
- Leuthardt, F. (1941) *Hoppe-Seyler's Z. Physiol. Chem.* **270**, 113–124
- Maitra, U. & Dekker, E. E. (1964) *J. Biol. Chem.* **239**, 1485–1491
- Roscoe, J. T. (1969) *Fundamental Research Statistics for the Behavioural Sciences*, 175–180, Holt, Rinehart and Winston, New York
- Rowell, E. V., Snell, K., Carnie, J. A. & Rowell, K. V. (1972) *Biochem. J.* **127**, 155–165
- Sato, T., Kochi, H., Sato, N. & Kikuchi, G. (1969) *J. Biochem. (Tokyo)* **65**, 77–83
- Schwerin, P., Bessman, S. P. & Waelsch, H. (1950) *J. Biol. Chem.* **184**, 37–44
- Shemin, D. (1949) *Cold Spring Harbor Symp. Quant. Biol.* **14**, 161–167
- Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941) *J. Biol. Chem.* **141**, 627–669
- Wayman, M. & Wright, G. F. (1945) *Ind. Eng. Chem. Anal. Ed.* **17**, 55–56
- Wilson, W. E. & Koeppe, R. E. (1961) *J. Biol. Chem.* **236**, 365–369