Differential Effects of 2-Oxo Acids on Pyruvate Utilization and Fatty Acid Synthesis in Rat Brain

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1. The effects of 2-oxo-4-methylpentanoate, 2-oxo-3-methylbutanoate and 2-oxo-3-methylpentanoate on the activity of pyruvate dehydrogenase (EC 1.2.4.1), citrate synthase (EC 4.1.3.7), acetyl-CoA carboxylase, (EC 6.4.1.2) and fatty acid synthetase derived from the brains of 14-day-old rats were investigated. 2. The pyruvate dehydrogenase enzyme activity was competitively inhibited by 2-oxo-3-methylbutanoate with respect to pyruvate with a K_i of 2.04 mM but was unaffected by 2-oxo-4-methylpentanoate or 2-oxo-3-methylpentanoate. 3. The citrate synthase activity was inhibited competitively (with respect to acetyl-CoA) by 2-oxo-4-methylpentanoate ($K_i \sim 7.2$ mM) and 2-oxo-3-methylbutanoate ($K_i \sim 14.9$ mM) but not by 2-oxo-3-methylpentanoate. 4. The acetyl-CoA carboxylase activity was not inhibited significantly by any of the 2-oxo acids investigated. 5. The fatty acid synthetase activity was competitively inhibited (with respect to acetyl-CoA) by 2-oxo-4-methylpentanoate ($K_i \sim 930 \mu\text{M}$) and 2-oxo-3-methylpentanoate ($K_i \sim 3.45$ mM) but not by 2-oxo-3-methylbutanoate. 6. Preliminary experiments indicate that 2-oxo-4-methylpentanoate and 2-oxo-3-phenylpropionate (phenylpyruvate) significantly inhibit the ability of intact brain mitochondria from 14-day-old rats to oxidize pyruvate. 7. The results are discussed with reference to phenylketonuria and maple-syrup-urine disease. A biochemical mechanism is proposed to explain the characteristics of these diseases.

The branched-chain amino acids L-leucine, L-isoleucine and L-valine are essential nutrients for higher mammals. Normally their metabolism is via a non-specific transaminase (Taylor & Jenkins, 1966), giving rise to 2-oxo-4-methylpentanoate, 2-oxo-3-methylpentanoate and 2-oxo-3-methylbutanoate respectively. These oxo acids are then oxidatively decarboxylated to give the CoA ester of the corresponding aliphatic acids, 3-methylbutanoyl-CoA, 2-methylbutanoyl-CoA and 3-methylpropionyl-CoA. It has been suggested that one enzyme is responsible for decarboxylating both 2-oxo-4-methylpentanoate and 2-oxo-3-methylpentanoate and a separate enzyme for 2-oxo-3-methylbutanoate (Bowden & Connelly, 1968; Connelly *et al.*, 1968; Johnson & Connelly, 1972). Further, tissue studies indicate that the branched-chain 2-oxo acid dehydrogenases are mainly located in the liver and kidney (Wohlhueter & Harper, 1970) and are predominantly mitochondrial in localization (Wohlhueter & Harper, 1970; Johnson & Connelly, 1972). Interest in the metabolism of the branched-chain amino acids has increased since the clinical description of maple-syrup-urine disease (or branched-chain ketonuria) by Menkes *et al.* (1954) in young children. This condition appears to be another example of a genetically linked inborn error of amino acid metabolism, and is charac-

terized by high urine and plasma concentrations of the branched-chain amino acids leucine, isoleucine and valine and their respective 2-oxo acids (Westall *et al.*, 1957; Dancis *et al.*, 1960; Dent & Westall, 1961). On the basis of these data and other investigations (Dancis *et al.*, 1963) it was proposed that the enzyme defect in maple-syrup-urine disease was the genetically linked absence of the enzyme responsible for oxidative decarboxylation of the branched-chain 2-oxo acids. The work of Bowden & Connelly (1968) and Connelly *et al.* (1968), indicating the existence of separate enzymes for the oxidative decarboxylation of 2-oxo-4-methylpentanoate or 2-oxo-3-methylpentanoate and 2-oxo-3-methylbutanoate, has led to the suggestion that it is the first of these two enzymes that is genetically absent but that the second is strongly inhibited by the accumulation of 2-oxo-4-methylpentanoate consequent on the absence of the first.

Clinically and pathologically, individuals suffering from maple-syrup-urine disease show many symptoms similar to those suffering from phenylketonuria, in particular the impaired synthesis and deposition of myelin. We have described the inhibition of rat brain citrate synthase (EC 4.1.3.7) and fatty acid synthetase by phenylpyruvate (2-oxo-3-phenylpropionate) and have suggested this as a possible mechanism for the impaired myelin forma-

tion in phenylketonuria (Land & Clark, 1973a,c). In view of the clinical similarities between maple-syrup-urine disease and phenylketonuria, and the chemical similarities between the metabolites responsible for these effects (2-oxo acids), we have investigated the effects of 2-oxo-4-methylpentanoate, 2-oxo-3-methylpentanoate and 2-oxo-3-methylbutanoate on several enzymes involved in fatty acid synthesis in rat brain, namely pyruvate dehydrogenase (EC 1.2.4.1), citrate synthase (EC 4.1.3.7), acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthetase. As in the previous work (Land & Clark, 1973a,b,c) these enzymes were derived from the brains of 14-day-old rats, since it is at this time in the brain development that myelin deposition is at its maximum (Davison & Dobbing, 1968).

Methods

Materials and animals

Chemicals. L-Leucine, DL-isoleucine, DL-valine and L-phenylalanine were obtained as the free acids and 2-oxo-4-methylpentanoate, 2-oxo-3-methylpentanoate (racemic mixture), 2-oxo-3-methylbutanoate and 2-oxo-3-phenylpropionate (phenylpyruvate) as sodium salts from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other compounds and reagents were of the purest grade available and were made up in double glass-distilled water.

Animals. Unweaned rats of either sex (13–16 day-old) of Wistar strain were used in all experiments. Each mitochondrial or supernatant preparation was derived from a single litter, culled to ten pups. Mitochondria were prepared by the method of Clark & Nicklas (1970) and the supernatant fraction was obtained essentially by the method of Saggerson & Greenbaum (1970) as described previously (Land & Clark, 1973c).

Enzyme assays

These were carried out essentially by methods described previously (Land & Clark, 1973c).

Citrate synthase. This was measured in frozen and thawed (three times) rat brain mitochondrial preparations by measurement of the appearance of free CoA from acetyl-CoA. The reaction was carried out at 25°C and contained as a routine 65 nmol of acetyl-CoA, 130 nmol of potassium oxaloacetate, 130 nmol of Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)], 130 nmol of Tris-HCl, pH 8.0, +0.08% Triton X-100 in a final volume of 1.3 ml. Approx. 20 µg of mitochondrial protein was used for each assay.

Acetyl-CoA carboxylase. This assay was carried out at 37°C by measuring the incorporation of $\text{KH}^{14}\text{CO}_3$ into acid-soluble material in the presence

of acetyl-CoA. The reaction mixture consisted of 40 µmol of triethanolamine buffer, pH 6.8, 80 µmol of potassium citrate, 20 µmol of MgCl_2 , 8 µmol of MnCl_2 , 30 µmol of $\text{KH}^{14}\text{CO}_3$ (2 µCi), 10 µmol of ATP, 1 µmol of dithiothreitol, 0.2 µmol of acetyl-CoA and 2 mg of bovine plasma albumin in a final volume of 1 ml. Approx. 1–1.5 mg of supernatant protein was used for each run and the assay was carried out for 30 min.

Fatty acid synthetase. This enzyme activity was measured by following NADPH oxidation on the addition of malonyl-CoA to a supernatant fraction from rat brain. The reaction mixture contained 100 µmol of potassium phosphate, pH 6.5, 0.5 µmol of NADPH, 50 nmol of acetyl-CoA and 5 µmol of dithiothreitol in a final volume of 1 ml. Approx. 2 mg of supernatant protein was used for each run and the reaction was initiated by the addition of 75 nmol of malonyl-CoA. The results are expressed as nmol of malonyl-CoA incorporated/min per mg of supernatant protein, assuming 2 mol of NADPH oxidized/mol of malonyl-CoA incorporated (Lynen, 1969).

Pyruvate dehydrogenase. Pyruvate dehydrogenase was measured in rat brain mitochondrial preparations at 340 nm essentially by the method of Reed & Willms (1966). The reaction mixture contained in a final volume of 1 ml (final concns.): 100 mM-Tris-HCl, pH 8.0, 5 mM-NAD⁺, 1 µM-rotenone, 10 mM-oxamate, 2 mM-thiamin pyrophosphate, 1 mM-CoA, 5 mM-Mg²⁺, 1 mM-dithiothreitol, 5 mM-pyruvate and approx. 0.3 mg of mitochondrial protein. The reaction was started by the addition of pyruvate and the initial velocity was taken as the rate of NADH formation during the first 30 s of linear increase in absorbance. Pyruvate dehydrogenase activity measured by this technique was 97% of the activity of pyruvate dehydrogenase as measured by an alternative assay coupling the formation of acetyl-CoA to an azo dye by pigeon liver arylamine acetyl transferase (Land & Clark, 1973b).

Mitochondrial respiration experiments

Mitochondrial respiration was measured polarographically at 28°C by using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The incubation medium contained (final concns.): 100 mM-KCl, 75 mM-mannitol, 25 mM-sucrose, 10 mM-phosphate-Tris, 10 mM-Tris-HCl, 0.05 mM-EDTA. The final pH was 7.4. To this rat brain mitochondria were added (approx. 1–2 mg of protein/ml) followed by 2.5 mM-malate and 5 mM-pyruvate (final concns.). State 3 (Chance & Williams, 1956) was induced by the addition of 250 µM-ADP. The amino or oxo acid under investigation was added at the same time as pyruvate. Protein was measured by the method of Lowry *et al.* (1951).

Table 1. *Effect of 2-oxo acids on enzymes from young rat brain*

Enzyme preparations of pyruvate dehydrogenase, acetyl-CoA carboxylase and fatty acid synthetase were prepared from brains of 14-day-old rats, and of citrate synthase from brains of 13-day-old rats. In all cases the final concentration of the inhibitor was 2mM and those of substrates were as detailed below. Control activities were: pyruvate dehydrogenase, 14.2 nmol of NADH formed/min per mg of mitochondrial protein in the presence of 100 μ M-pyruvate; citrate synthase, 206 nmol of acetyl-CoA incorporated/min per mg of mitochondrial protein in the presence of 15 μ M-acetyl-CoA; acetyl-CoA carboxylase, 0.157 nmol of H^{14}CO_3 incorporated/min per mg of supernatant protein in the presence of 33.5 μ M-acetyl-CoA; fatty acid synthetase, 0.96 nmol of malonyl-CoA incorporated/min per mg of supernatant protein in the presence of 60 μ M-acetyl-CoA.

2-Oxo acid added (2mM)	Enzyme activity (% of control)			
	Pyruvate dehydrogenase	Citrate synthase	Acetyl-CoA carboxylase	Fatty acid synthetase
2-Oxo-4-methylpentanoate	99	83	93	69
2-Oxo-3-methylbutanoate	77	88	93	89
2-Oxo-3-methylpentanoate	99	95	92	82
2-Oxo-3-phenylpropionate (phenylpyruvate)	92	72	91	62

Results and Discussion

Preliminary experiments were carried out to see which, if any, of the 2-oxo acids were inhibitors of the enzymes studied, by measuring the activity of the enzymes from brains of 13–14-day-old rats in the presence of 2mM (final concentration) of the 2-oxo acid. This concentration of 2-oxo acid is of the same order as that found physiologically in maple-syrup-urine disease (Synderman *et al.*, 1964). The results are given in Table 1 and include the effects of phenylpyruvate for comparison. For pyruvate dehydrogenase, citrate synthase and acetyl-CoA carboxylase, the substrates (i.e. pyruvate, acetyl-CoA) are non-saturating, but with fatty acid synthetase the acetyl-CoA concentration is approaching saturation. Pyruvate dehydrogenase under these conditions was markedly inhibited by 2-oxo-3-methylbutanoate (77% of control activity) but not by 2-oxo-4-methylpentanoate, 2-oxo-3-methylpentanoate or phenylpyruvate (cf. Land & Clark, 1973*b*). Citrate synthase, however, was inhibited by 2-oxo-4-methylpentanoate (to 83% of control) and to a lesser extent by 2-oxo-3-methylbutanoate (to 88% of control), whereas phenylpyruvate inhibited markedly (to 72% of control; cf. Land & Clark, 1973*c*). Acetyl-CoA carboxylase was not inhibited to any extent under these conditions by any of the 2-oxo acids tested, whereas the fatty acid synthetase was inhibited strongly by 2-oxo-4-methylpentanoate (to 69% of control) and phenylpyruvate (to 62% of control; cf. Land & Clark, 1973*c*). Some inhibition was also observed of the fatty acid synthetase by 2-oxo-3-methylpentanoate to 82% of control but very little by 2-oxo-3-methylbutanoate.

Further studies were carried out on the enzymes from the brains of 14-day-old rats in those cases where extensive inhibition was apparent. In all

cases the data were analysed on a Honeywell DDP 516 computer by using a program derived from the original algorithm of Cleland (1963), which incorporated a convergence sub-routine (available on request) to yield the enzyme parameters and inhibitor constants. These have been tabulated in Table 2. 2-Oxo-3-methylbutanoate acted as a classical competitive inhibitor with respect to pyruvate on the pyruvate dehydrogenase with a K_i of 2.04mM. Other experiments showed that the pyruvate dehydrogenase from adult (50-day-old) rat brain was also competitively inhibited by 2-oxo-3-methylbutanoate with a K_i of 1.9mM (J. B. Clark & J. M. Land, unpublished work). This is in agreement with the report of Kanazaki *et al.* (1969) who observed that pig heart pyruvate dehydrogenase was competitively inhibited by 2-oxo-3-methylbutanoate with a K_i of 3.7mM, but contrasts with the statement by Blass & Lewis (1973) that none of the 2-oxo acids produced in maple-syrup-urine disease and phenylketonuria effectively inhibit the isolated brain pyruvate dehydrogenase, even at concentrations one or two orders of magnitude higher than those found in these diseases. However, Blass & Lewis (1973) do not appear to have included 2-oxo-3-methylbutanoate in their reported studies.

It was also found that the citrate synthase from brains of 14-day-old rats was competitively inhibited with respect to acetyl-CoA by 2-oxo-4-methylpentanoate (K_i 7.2mM) and 2-oxo-3-methylbutanoate (K_i 14.9mM) (Table 2). The fatty acid synthetase was markedly inhibited by 2-oxo-4-methylpentanoate (K_i 930 μ M) but less so by 2-oxo-3-methylpentanoate (K_i 3.45mM), both these compounds acting as competitive inhibitors with respect to acetyl-CoA. Thus of the inhibitions reported in this paper the most effective are the inhibition of the fatty acid synthetase by 2-oxo-4-methylpentanoate (K_i 930 μ M)

Table 2. Summary of kinetic data of pyruvate dehydrogenase, citrate synthase and fatty acid synthetase from young rat brains

All enzymes are derived from brains of 14-day-old rats. They were prepared and assayed as outlined in the Methods section. The results are best-fit values derived from computer analysis and are expressed as the mean $\pm$ S.D. The units of activity for pyruvate dehydrogenase were nmol of NADH formed/min per mg of mitochondrial protein; for citrate synthase, nmol of acetyl-CoA incorporated/min per mg of mitochondrial protein; for fatty acid synthetase, nmol of malonyl-CoA incorporated/min per mg of supernatant protein (assuming 2 mol of NADPH oxidized/mol of malonyl-CoA incorporated).

Enzyme	$V_{\max}$ (nmol/min per mg of protein)	K_m (μ M)	K_i (mM)		
			2-Oxo-4- methylpentanoate	2-Oxo-3- methylbutanoate	2-Oxo-3- methylpentanoate
Pyruvate dehydrogenase	22.2 $\pm$ 0.4	53.1 $\pm$ 4.7 (pyruvate)	—	2.04 $\pm$ 0.03	—
Citrate synthase	1221 $\pm$ 28	22.0 $\pm$ 1.2 (acetyl-CoA)	7.2 $\pm$ 0.04	14.0 $\pm$ 0.9	—
Fatty acid synthetase	0.79 $\pm$ 0.03	7.6 $\pm$ 1.1 (acetyl-CoA)	0.93 $\pm$ 0.08	—	3.45 $\pm$ 0.30

Table 3. Effect of amino acids and their analogue 2-oxo acids on respiration of rat brain mitochondria

Mitochondria were prepared from brains of 16-day-old rats by the method of Clark & Nicklas (1970). Respiration was followed polarographically at 28°C in a medium containing (final concns.): 100 mM-KCl, 75 mM-mannitol, 25 mM-sucrose, 10 mM-phosphate-Tris, 10 mM-Tris-HCl, 0.05 mM-EDTA, pH 7.4. To this were added mitochondria (1.45 mg of protein/ml), 2.5 mM-malate and 5 mM-pyruvate. Each amino or oxo acid was added to a final concentration of 2 mM and state 3 respiration was induced by adding 250 μ M-ADP.

Addition (final concn. 2 mM)	State 3 (+ADP) respiration		State 4 (–ADP) respiration	
	(ng-atoms of O/ min per mg of protein)	(% of control)	(ng-atoms of O/ min per mg of protein)	(% of control)
None	89	100	30	100
DL-Valine	85	95	25.5	86
L-Leucine	91	102	29.5	99
DL-Isoleucine	82	92	29	97
L-Phenylalanine	89	100	30	100
2-Oxo-3-methylbutanoate	93	104	37.5	126
2-Oxo-4-methylpentanoate	60	67	33	111
2-Oxo-3-methylpentanoate	79	88	33	111
2-Oxo-3-phenylpropionate	39	43	28	94

and of the pyruvate dehydrogenase by 2-oxo-3-methylbutanoate (K_i 2.04 mM). Whether such inhibitions actually occur in maple-syrup-urine disease will depend on the concentrations of these 2-oxo acids present in such cases. As with phenylketonuria, such data are limited and variable. The values range from 800 μ M [total brain-tissue concentration of 2-oxo acids (Dreyfus & Prensky, 1967)] to 2–4 mM [total plasma concentration (Synderman *et al.*, 1964)] although concentrations as high as 10 mM have been reported (Patrick, 1961). It would appear therefore that the fatty acid synthetase could be extensively inhibited in maple-syrup-urine disease and although the inhibition of the pyruvate dehydrogenase would be less, it might well be sufficient to have far-reaching consequences. Such inhibitions might explain the defective myelination and neurological disorders

that are clinically characteristic of patients suffering from maple-syrup-urine disease (cf. phenylketonuria) (Dancis & Levitz, 1972). Further, the observations of Silberberg (1969) that myelination in newborn rat cerebellar cultures was markedly inhibited by 2-oxo-4-methylpentanoate (1–2 mM concentration) but not by 2-oxo-3-methylbutanoate or 2-oxo-3-methylpentanoate, are entirely consistent with our results indicating that 2-oxo-4-methylpentanoate is a competitive inhibitor of the fatty acid synthetase with a K_i (930 μ M) well within the reported concentrations in maple-syrup-urine disease.

There have been, however, a number of reports indicating that in brain slices or homogenates 2-oxo-4-methylpentanoate markedly inhibits [14 C]pyruvate decarboxylation (Dreyfus & Prensky, 1967; Bowden *et al.*, 1970, 1971; Patel *et al.*, 1973). To

resolve the apparent paradox that 2-oxo-3-methylpentanoate inhibits pyruvate decarboxylation in homogenates but not with the isolated pyruvate dehydrogenase, some preliminary experiments were carried out with functionally intact brain mitochondria (Table 3). Of the amino acids and 2-oxo acids tested, only 2-oxo-4-methylpentanoate and phenylpyruvate exhibited any marked effects. Both of these compounds caused marked inhibition (67% of control for 2-oxo-4-methylpentanoate and 43% of control for phenylpyruvate) of the state 3 (+ADP) respiration rate of brain mitochondria from young animals oxidizing pyruvate. However, the state 4 (−ADP) respiration was not affected (Table 3). Similar results were also observed with brain mitochondria from adult rats (J. B. Clark & J. M. Land, unpublished work). The exact mechanism of the inhibition of pyruvate oxidation by rat brain mitochondria by 2-oxo-4-methylpentanoate and phenylpyruvate remains to be elucidated but those results suggest that these compounds in some way modify the availability of pyruvate to the pyruvate dehydrogenase in the mitochondria. These observations would also explain the apparent differences in results obtained with slices and homogenates as compared with the isolated pyruvate dehydrogenase enzyme.

Thus in both phenylketonuria and maple-syrup-urine disease, phenylpyruvate and 2-oxo-4-methylpentanoate accumulate and they are potent competitive inhibitors of the brain fatty acid synthetase, and possibly other enzyme reactions involving acetyl-group transfer (Land & Clark, 1973c; the present paper). In addition and possibly more important, both these metabolites markedly inhibit the ability of brain mitochondria to oxidize pyruvate and malate whereas they do not inhibit the pyruvate dehydrogenase complex itself. Further investigations of the permeability properties of brain mitochondria to 2-oxo acids may help elucidate the mechanism of this inhibition.

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References

- Blass, J. P. & Lewis, C. A. (1973) *Biochem. J.* **131**, 31–37
- Bowden, J. A. & Connelly, J. L. (1968) *J. Biol. Chem.* **243**, 3526–3531
- Bowden, J. A., Brestel, E. P., Cope, W. T., McArthur, C. L., Westfall, D. N. & Fried, M. (1970) *Biochem. Med.* **4**, 69–76
- Bowden, J. A., McArthur, C. L. & Fried, M. (1971) *Biochem. Med.* **5**, 101–108
- Chance, B. & Williams, G. R. (1956) *Advan. Enzymol. Relat. Subj. Biochem.* **17**, 65–134
- Clark, J. B. & Nicklas, W. J. (1970) *J. Biol. Chem.* **245**, 4724–4731
- Cleland, W. W. (1963) *Nature (London)* **198**, 463–465
- Connelly, J. L., Danner, D. J. & Bowden, J. A. (1968) *J. Biol. Chem.* **243**, 1198–1203
- Dancis, J. & Levitz, M. (1972) in *Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S., eds.), 3rd edn., pp. 426–439, McGraw Hill, New York
- Dancis, J., Levitz, M. & Westall, R. G. (1960) *Pediatrics* **25**, 72–79
- Dancis, J., Hutzler, J. & Lewitz, M. (1963) *Pediatrics* **32**, 234
- Davison, A. N. & Dobbing, J. (1968) in *Applied Neurochemistry* (Davison, A. N. & Dobbing, J., eds.), pp. 253–286, Blackwell, Oxford
- Dent, C. E. & Westall, R. G. (1961) *Arch. Dis. Childhood* **36**, 259–268
- Dreyfus, P. D. & Prensky, A. L. (1967) *Nature (London)* **214**, 276
- Johnson, W. A. & Connelly, J. L. (1972) *Biochemistry* **11**, 1967–1973
- Kanazaki, T., Hayakawa, T., Hamada, M., Fukuyoshi, Y. & Koike, M. (1969) *J. Biol. Chem.* **244**, 1183–1187
- Land, J. M. & Clark, J. B. (1973a) *Biochem. Soc. Trans.* **1**, 463–466
- Land, J. M. & Clark, J. B. (1973b) *Biochem. J.* **134**, 539–544
- Land, J. M. & Clark, J. B. (1973c) *Biochem. J.* **134**, 545–555
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lynen, F. (1969) *Methods Enzymol.* **14**, 17–33
- Menkes, J. M., Hurst, P. L. & Craig, J. M. (1954) *Pediatrics* **14**, 462–466
- Patel, M. S., Auerbach, V. H., Grover, W. D. & Wilbur, D. O. (1973) *J. Neurochem.* **20**, 1793–1796
- Patrick, A. D. (1961) *Arch. Dis. Childhood* **36**, 269
- Reed, L. J. & Willms, C. R. (1966) *Methods Enzymol.* **9**, 247–265
- Saggerson, E. D. & Greenbaum, A. L. (1970) *Biochem. J.* **119**, 221–242
- Silberberg, D. H. (1969) *J. Neurochem.* **16**, 1141–1146
- Synderman, S. E., Morton, P. M., Roitman, E. & Holt, L. E. (1964) *Pediatrics* **34**, 454–472
- Taylor, R. T. & Jenkins, W. T. (1966) *J. Biol. Chem.* **241**, 4396–4405
- Westall, R. G., Dancis, J. & Miller, S. (1957) *Amer. J. Dis. Child.* **94**, 571–572
- Wohlhueter, R. M. & Harper, A. E. (1970) *J. Biol. Chem.* **245**, 2391–2401