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Lipoic acid and diabetes—Part III: Metabolic role of acetyl dihydrolipoic acid

S. S. WAGH, V. M. GANDHI, C. V. NATRAJ and K. K. G. MENON
Hindustan Lever Research Centre, Andheri (East), Bombay 400099, India

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Abstract. Rat liver lipoyl transacetylase catalyzes the formation of acetyl dihydrolipoic acid from acetyl coenzyme A and dihydrolipoic acid. In an earlier paper the formation of acetyl dihydrolipoic from pyruvate and dihydrolipoic acid catalyzed by pyruvate dehydrogenase has been reported. Acetyl dihydrolipoic acid is a substrate for citrate synthase, acetyl coenzyme A carboxylase and fatty acid synthetase. The V_{max}. for citrate synthase with acetyl dihydrolipoic acid was identical to acetyl coenzyme A (approximately 1 µmol citrate formed/min/mg protein) while the apparent K_m was approximately 4 times higher with acetyl dihydrolipoic acid as the substrate. This may be due to the fact that synthetic acetyl dihydrolipoic acid is a mixture of 4 possible isomers and only one of them may be the substrate for the enzymatic reaction. While dihydrolipoic acid can replace coenzyme A in the activation of succinate catalyzed by succinyl coenzyme A synthetase, the transfer of coenzyme A between succinate and acetoacetyl dihydrolipoic acid catalyzed by succinyl coenzyme A: 3 oxo-acid coenzyme A transferase does not occur.

Keywords. Lipoic acid; acetyl coenzyme A; acetyl dihydrolipoic acid; citrate synthase.

Introduction

In an earlier study (Natraj *et al.,* 1984) we have shown that lipoic acid administration in alloxan diabetic rats alleviated many of the biochemical abnormalities in diabetes. Major impairments of carbohydrate, fat and protein metabolisms occur due to reduced uptake and utilization of glucose in diabetes. Adequate intracellular concentrations of glucose are met through its supply from liver and kidney where it is synthesised from precursors such as alanine, lactate, etc. resulting in increased gluconeogenic rates (Hers and Hue, 1983). The energy requirements in the liver and kidney are primarily met through fatty acid oxidation resulting in elevated levels of acetyl coenzyme A (CoA), acetoacetate and ß-hydroxy butyrate (Randle, 1966). Higher levels of acetyl CoA in diabetes are believed to be responsible for the inhibition of pyruvate dehydrogenase (EC 1.2.4.1) (Kerbey *et al.,* 1976) and activation of pyruvate carboxylase (EC 6.4.1.1) (Williamson *et al.,* 1969; Ruderman *et al.,* 1976; Utter *et al.,* 1964). From this point of view, reduction in acetyl CoA levels would therefore be expected to have a beneficial effect in diabetes.

Abbreviations used: CoA, Coenzyme A; DTO, dihydrolipoic acid; DTNB, 5, 5 dithiobis (2-nitrobenzoic acid); BSA, bovine serum albumin; DTT, dithiothreitol; NAD, nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; TLC, thin layer chromatography.

Like CoA, dihydrolipoic acid (DTO) possesses a sulphydryl group and can form thiol esters. In the present study we have shown that acetyl DTO was formed from acetyl CoA and DTO thus leading to a net reduction in acetyl CoA levels. Furthermore, acetyl DTO replaced acetyl CoA in acetyl CoA carboxylase (EC 6.4.1.2) and fatty acid synthetase catalyzed reactions which are key to fatty acid biosynthesis. Similarly, DTO was found to replace CoA in succinyl CoA synthetase (EC 6.2.1.4) but not in succinyl CoA: 3 oxo-acid CoA transferase (EC 2.8.3.5). Thus thiol esters of DTO performed some of the functions of CoA thioesters and not others. The implications of this selective role of DTO in bringing about the changes in diabetes have been discussed.

Materials and methods

D,L-Lipoic acid was purchased from British Drug House, England. [^{14}C]-Bicarbonate, and [1_^{14}C] -acetic anhydride were purchased from Bhabha Atomic Research Centre, Bombay. Bovine serum albumin (BSA), CoA, dithiothreitol (DTT), 5,5 dithiobis (2-nitrobenzoic acid) (DTNB), malonyl CoA, nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), oxalacetate and sodium pyruvate were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. All other reagents were ofanalytical grade. The rats used were an inbred Haffkine Wistar strain maintained on Hindustan Lever pellet feed.

Acetyl DTO was prepared according to Gunsalus *et al.* (1956). Radioactively labelled acetyl CoA and acetyl DTO were prepared according to the procedure of Simon and Shemin (1953). For malonyl DTO the procedure described for malonyl CoA by Trams and Brady (1960) was modified as follows. Thiophenyl malonate was made from malonic acid and thiophenol, and reacted with DTO in bicarbonate buffer (0·1 M, pH 8·5). Chloroform extract of the acidified reaction mixture showed 4 spots on thin layer chromatography (TLC) in chloroform: acetone: formic acid (9:1:0·1). The spot with an R_f of 0·5 was identified as malonyl DTO by the hydroxamate method (Lipmann and Tuttle, 1945). The other bands corresponded to mixed malonate esters with thiophenol and DTO.

Acylation of DTO catalyzed by lipoyl transacetylase

The procedure described by Brady and Stadman (1954) for isolation and purification of pigeon liver lipoyl transacetylase was adapted to rat liver. The enzyme was partially purified (upto 75 % ammonium sulphate saturation step). The assay mixture in a final volume of 1·5 ml contained 100 μmol of Tris buffer pH 7·5,0·5 μmol of acetyl CoA and 10 μmol of DTO. The reaction was started by adding the enzyme (approximately 500 μg protein) and incubated at 25°C for 30 min and terminated by adding 0·2 ml of chilled 6 N HCl and extracted with benzene. The organic layer was washed with water, dried over sodium sulphate and the solvent removed under a stream of nitrogen gas. Acetyl DTO was estimated according to Lipmann and Tuttle (1945).

Citrate synthase and acetyl DTO

Citrate synthase was isolated from normal rat liver mitochondria and partially purified

as described by Shepherd and Garland (1969) with the following modifications. The mitochondrial pellet from 60 g of liver was sonicated (20 KHz for 8 min) in 80 ml of 0·1M potassium phosphate buffer, pH 7·4 and the homogenate was subjected to ammonium sulphate fractionation. The precipitate obtained at 50–75 % saturation was dissolved in 10 ml of 0·1M potassium phosphate buffer, pH 7·4 and dialysed against 3 litres of distilled water. The dialysate was loaded onto a DEAE-sephacel column (2× 15 cm) and was successively washed with 1 litre each of 2 mM and 8 mM potassium phosphate buffer, pH 7·4. The column was eluted with 18 mM potassium phosphate buffer, pH 7·4 and fractions of 250 ml were collected. The 3rd and 4th fractions had all the enzyme activity and was precipitated by 50–75 % ammonium sulphate saturation, dissolved in 10 ml of buffer and dialysed against 2 litre distilled water for 24 h. The dialyzed extract was used as the source of the enzyme.

The condensation of acetyl CoA or acetyl DTO with oxalacetate results in the release of free CoA or DTO which was measured spectrophotometrically using DTNB. The assay mixture in a final volume of 2 ml contained: 200 μmol of Tris buffer, pH 8·0, 25–200 nmol of acetyl CoA or 20–150 nmol of acetyl DTO, 0·2 μmol of DTNB and 100 μl ofenzyme (approximately 20 μg protein). The reference cuvette contained all the ingredients except oxalacetate. The reaction was monitored initially for 3 min for absorbance increase at 412 nm and the assay was started by adding 0·5 μmol oxalacetate in the sample cuvette only. The change in absorbance at 412 nm was monitored.

The product of the reaction was identified by estimating the citrate formed according to Stern (1957).

Carboxylation of acetyl DTO to malonyl DTO catalyzed by acetyl CoA carboxylase

Acetyl CoA carboxylase was partially purified from normal rat livers (up to 25 % ammonium sulphate saturation step) according to the procedure of Inoue and Lowenstein (1975). The enzyme was assayed by estimating the amount of $NaH^{14}CO_3$ incorporated into malonate. The assay mixture, in a final volume of 2 ml contained: 200μmol Tris HCl buffer, pH 7·5,2·5 μmol DTT, 0·8 μmol acetyl CoA or acetyl DTO, 40 μmol of $NaH^{14}CO_3$ ($4·1 \times 10^6$ CPM), 10 μmol of ATP, 40 μmol of citrate, 40 μmol of NaCl and 1 mg BSA. The reaction was started by adding an aliquot of the enzyme (approx. 100 μg protein) and incubated at 37°C for 15 min. The reaction was stopped by adding 1 ml of 6 N HCl and evaporating the mixture to dryness in a hot water bath under a stream of nitrogen. The residual radioactivity was counted in a scintillation spectrometer (Packard). The control for this experiment contained all the ingredients except ATP and the radioactivity incorporated was estimated and the value was subtracted from the experimental values.

In a separate experiment, 0·8 μmol of CoA was added along with acetyl DTO and the assay performed as described above.

The product of the reaction was identified by extracting the acidified reaction mixture with chloroform and the solvent removed under a stream of nitrogen gas. The residue was separated on TLC using hexane: ether: acetic acid (80:20:1). The spots corresponding to acetyl DTO and malonyl DTO were eluted and estimated by conversion to hydroxamate according to the method of Lipmann and Tuttle (1945).

Acetyl DTO as a substrate for fatty acid synthetase

Fatty acid synthetase was isolated and partially purified upto the DEAE cellulose chromatography step (Stoops *et al.,* 1979) and was assayed by coupling with acetyl CoA carboxylase and measuring the rate of oxidation of NADPH. The assay mixture in a final volume of 1 ml contained: 40 μmol of Tris HCl buffer, pH 7·5, 8 μmol of $MgCl_2$, 8 μmol of sodium citrate, 3 μmol of DTT, 20 μmol of sodium bicarbonate, 0·6 mg BSA, 0·1 μmol acetyl CoA or acetyl DTO, 0·1 μmol of NADPH, acetyl CoA carboxylase (approximately 100 μg protein), and fatty acid synthetase (approximately 100 μg protein). The assay mixture was taken in the sample and reference cuvettes in a double beam spectrophotometer (Shimadzu, UV 240) and the reaction was started by adding 3 μmol of ATP to the sample cuvette only and the absorbance change at 340 nm followed spectrophotometrically. This procedure would correct for NADPH oxidase that may be present in the enzyme sample which would otherwise have given higher values for enzyme activity.

The product of the above assay was identified by substituting acetyl DTO with [l-^{14}C]-acetyl DTO. The reaction mixture was incubated for 15 min at 37°C and acidified with 1 ml of 6 N HCl. The products were extracted into chloroform and the solvent was evaporated under a stream of nitrogen gas. The fatty acids were separated by TLC using hexane:ether:acetic acid (80:20:1). The radioactivity in the spot corresponding to palmitate was determined.

Activation of succinate catalyzed by succinyl CoA synthetase

The enzyme was isolated from rat heart according to the method described by Murakami and Nishimura (1974). The enzyme was assayed by measuring the increase in absorbance at 235 nm for the succinyl thioester formed.

The assay mixture in a final volume of 1 ml contained: 50 μmol of Tris succinate buffer, pH 7·4, 10 μmol of $MgCl_2$, 0·1 μmol of thiol (CoA or DTO) and 200 μg of enzyme protein. The reaction was started by adding the enzyme and the increase in absorbance at 235 nm with time was monitored spectrophotometrically (Sungman, 1969).

Noninvolvement of succinyl DTO in succinyl CoA: 3 oxo-acid CoA transferase

The enzyme was isolated from rat hearts and purified by ammonium sulphate fractionation (35–65 % saturation), acetone precipitation (57 % saturation) and heat and acid treatment (Stern *et al.,* 1956). The enzyme was concentrated by ammonium sulphate precipitation (72% saturation) and dialysed. The dialysate was further purified by DEAE-sephacel column chromatography (Hersh and Jencks, 1967). The purified enzyme was free of thiolase activity.

The enzyme activity was determined by measuring the decrease in the 310 nm absorbance of acetoacetate thioester with time. The assay mixture in a final volume of 1 ml contained: 70 μmol of Tris-HCl buffer, pH 8·1, 50 μmol of succinate, 0·2 μmol of acetoacetyl thioester and 50 μg of enzyme protein. The hydrolysis of acetoacetyl thioester was followed for 2-3 min and the reaction was started by the addition of succinate. One unit of enzyme activity is defined as an absorbance change of 0·01/min at 310 nm which is equivalent to $2{\cdot}5 \times 10^{-3}$ μmol of acetoacetyl CoA or acetoacetyl DTO.

Results

Formation of acetyl DTO

The assay of lipoyl transacetylase described by Brady and Stadman (1954) used a system generating acetyl CoA from acetyl phosphate and CoA. Formation of acetyl CoA was monitored by taking aliquots of the assay mixture and estimating the thioester content by the hydroxamate assay. In order to demonstrate that excess acetyl CoA in the liver can be transacylated to acetyl DTO, the former was used in the present study as the acyl donor. The enzyme activity was measured by separating acetyl DTO from acetyl CoA by making use of their differential solubility in benzene and estimating the thioester by conversion to hydroxamate. The results showed that rat liver lipoyl transacetylase catalyzed the conversion of acetyl CoA to acetyl DTO at 90 nmol/ min/mg protein in the presence of excess DTO.

Acetyl DTO as a substrate for citrate synthase

Crude citrate synthase contains an active hydrolase which hydrolyses acetyl DTO at appreciable rates. Purified citrate synthase was virtually free of any hydrolase activity as evidenced by negligible DTO formation in the absence of any oxalacetate. The results of citrate synthase assay are shown in figure 1. In the presence of acetyl DTO it was found that there was a rapid release of free -SH groups as measured by the reaction with DTNB . The K_m value for acetyl CoA was estimated to be 14 μM. With acetyl DTO as the substrate, the V was identical to that ofacetyl CoA but the K_m value was found to be 62 μM which was approximately 4 times larger than that for acetyl CoA. The product of the reaction was identified as citrate by the colorimetric method described by Stern (1957).

Carboxylation of acetyl DTO catalyzed by acetyl CoA carboxylase

The results demonstrated that acetyl DTO served as a substrate for acetyl CoA carboxylase and was converted to malonyl DTO. Specific activity of the enzyme with acetyl DTO as a substrate (44·7 nmol ofHCO_3^- fixed/min/mg protein) was nearly 60 % of that with acetyl CoA (74·9 nmol of HCO_3^- fixed/min/mg protein). The velocities observed with and without added CoA were similar suggesting that acetyl DTO was not converted first to acetyl CoA and then to malonyl DTO. This indicates that acetyl CoA is unlikely to be the intermediate for the carboxylation of acetyl DTO.

Acetyl DTO as a substrate for fatty acid synthetase

Fatty acid synthetase catalyzes the successive condensation of 7 malonyl CoA molecules with acetyl CoA to produce palmitate. In the present study malonyl-CoA or malonyl-DTO was produced *in situ* from acetyl CoA or acetyl DTO catalyzed by acetyl CoA carboxylase. This reaction was coupled to fatty acid synthetase and the overall rate was monitored through oxidation of NADPH. In a separate assay, [l-^{14}C] -acetyl CoA and [l-^{14}C]-acetyl DTO were used as substrates and the radioactivity incorporated into palmitate was quantified.

From table 1 it can be seen that acetyl DTO was converted to palmitate with the

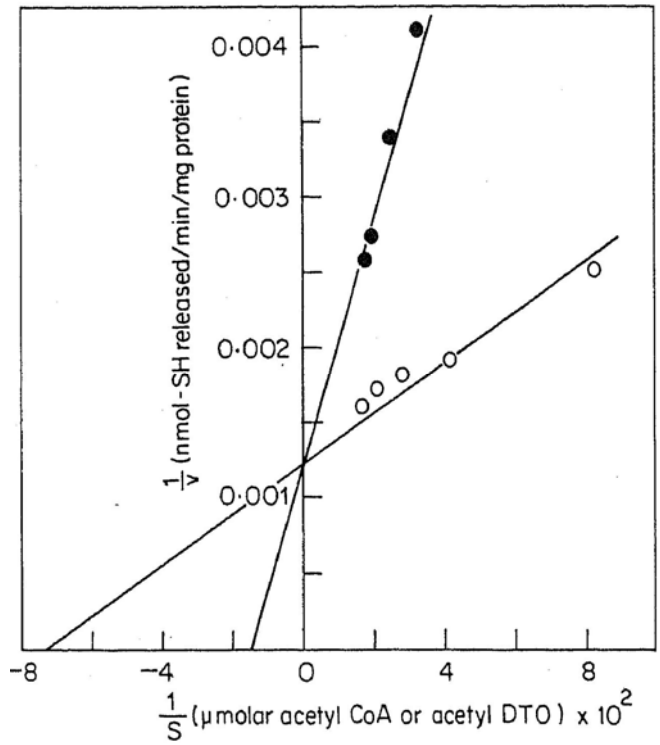

Figure 1. Effect of varying concentrations of acetyl CoA (O) and acetyl DTO (●) on citrate synthase activity. The assay mixture in a volume of 2 ml contained: 200μmolTris buffer, pH 8·0, 0·5 μmol oxalacetate, 25–200 nmol of acetyl CoA or 20–150nmol of acetyl DTO and 0·2μmolDTNB. The reaction was started by adding an aliquot of citrate synthase (approximately 20 μg protein) and recording the absorbance change at 412 nm. The results analysed by Lineweaver-Burk plot.

Table 1. Acetyl DTO as a precursor for palmitate.

Substrate	Activity	
	nmol NADPH oxidized/ min/mg/protein	nmol [^{14}C]-acetyl CoA/DTO incorporated/min/mg/protein
Acetyl CoA	75·0 (5·6)	43·2 (5·4)
Acetyl DTO	37·0 (2·7)	22·2 (2·7)

Fatty acid synthetase was isolated from normal rat livers and the activity estimated by coupling with acetyl CoA carboxylase and determining the rate of oxidation of NADPH (nmol/min/mg protein) or, the radioactivity incorporated from [1-^{14}C]-acetyl CoA or acetyl DTO into palmitate (nmol/min/mg protein). The numbers in parentheses are the nmol of palmitate produced/min/mg protein.

relative rate approximately 50% of that obtained with acetyl CoA both by the spectrophotometric and radioactivity assays. After correcting for losses during recovery ofpalmitate in the radioactive assay, the rate of palmitate formation (numbers in parentheses in table 1) was identical in both assays.

Succinyl CoA synthetase

Succinyl CoA synthetase catalyzes the activation of dicarboxylic acids with CoA in the presence of GTP. As observed with citrate synthase and acetyl CoA carboxylase, DTO substituted for CoA, although the enzyme activity with DTO as the substrate was approximately 40% of that with CoA (10 and 4 nmol of thioester formed/min/mg protein with CoA and DTO respectively).

Succinyl DTO does not participate in succinyl CoA: 3 oxo-acid CoA transferase

The enzyme isolated from rat heart was purified to eliminate the risk of any thiolase contamination which would otherwise have interfered with the assay. The results of the thiol transfer from acetoacetyl CoA or acetoacetyl DTO to succinate are shown in table 2. The enzyme catalyzed the transfer of CoA between acetoacetyl CoA and succinate but not between acetoacetyl DTO and succinate indicating that the enzyme had a high degree of specificity for the thiol moiety.

Table 2. Transfer of DTO catalyzed by succinyl CoA: 3 oxo-acid CoA transferase.

	Activity	
	Substrate converted	
Substrate	A_{310}/min/mg protein	μmol/min/mg protein
Acetoacetyl CoA	70·6	0·176
Acetoacetyl DTO	ND	ND

ND, Not detectable.

The enzyme was isolated from normal rat heart and purified to remove any contaminating thiolase activity. The enzyme was assayed by following the decrease in 310 nm absorbance.

Discussion

In an earlier study (Gandhi *et al.,* 1985) the mode of action of lipoic acid in diabetes was shown to be through its effects on certain enzymes of fatty acid oxidation and gluconeogenesis, which were slowed down, thus accounting for a reduction in ketosis and gluconeogenesis. It was shown that DTO replaced CoA effectively in the oxidative decarboxylation of pyruvate catalyzed by pyruvate dehydrogenase to form acetyl DTO. DTO was found to activate long chain fatty acids as DTO thioesters catalysed by fatty acyl CoA synthetase (EC 6.2.1.3). Two important differences between CoA and DTO observed were that (i) acetoacetyl DTO was a poor substrate for thiolase (EC 2.3.1.9)

while acetoacetyl thioesters of pantetheine and N-acetyl ß-mercaptoethylamine have been reported to be good substrates (Stern and Drummond, 1961) and (ii) acetyl DTO did not activate pyruvate carboxylase to the same extent as acetyl CoA. It appeared therefore that DTO had a specific role and was not a nonspecific sulphydryl compound, whose only resemblance to CoA was in its thiol group(s); such a specific role is most likely to be in fatty acid biosynthesis. We have presented some evidence to substantiate this hypothesis.

In the present study, we have examined the origin and the metabolic fate of acetyl DTO. Lipoyl transacetylase has been isolated from rat liver and shown to catalyze the formation of acetyl DTO from acetyl CoA and DTO. Acetyl DTO has been shown to be a substrate for citrate synthase, acetyl CoA carboxylase and fatty acid synthetase, 3 enzymes which catalyze crucial steps in the synthesis of palmitate. Thus DTO by substituting for CoA in these reactions may be expected to support fatty acid biosynthesis under the conditions where CoA availability may become limiting as has been suggested in diabetes (Sauer and Erfle, 1966), or even in normal conditions as an alternate pathway for fatty acid biosynthesis.

The role of DTO in relation to the metabolic fate of acetoacetate has also been investigated. DTO has been shown to replace CoA in the activation of succinate catalyzed by succinyl CoA synthetase. However, the DTO moiety in acetoacetyl DTO is not transferred to succinate in the reaction catalyzed by succinyl CoA: 3 oxo-acid CoA transferase. It must be mentioned that Moore and Jencks (1982) have shown that CoA transfer from acetoacetyl CoA to succinate is highly specific for the nonreacting portions ofthe CoA molecule; neither N-acetyl ß-mercaptoethylamine nor pantotheine can replace CoA in this reaction.

An important difference between acetyl CoA and acetyl DTO in all these reactions is that, for a given concentration ofacetyl CoA/acetyl DTO, the enzymatic reaction in the presence of acetyl CoA was faster than that with acetyl DTO. This is indicated from studies with citrate synthase which showed that the *V*max for the two substrates was similar, but the K_m for acetyl DTO was approximately four times of that for CoA. It must be pointed out that synthetic acetyl DTO is a mixture of4 possible isomers: (+) 8 - S-acetyl, (—)8-S-acetyl, (+)6-S-acetyl and (—)6-S-acetyl dihydrolipoic acid. It is probable that only one of these is the substrate with the result that the actual concentration of the active compound is considerably lower than the total (one fourth?). Support for this interpretation also stems from observations by Gunsalus *et al.* (1956) who showed that the product of the enzymatic acylation of exogenous DTO by acetyl CoA catalyzed by extracts of *Escherichia coli* was the 6-S-acetyl derivative. Studies by O'Connor *et al.* (1982) with *E. coli* and bovine kidney pyruvate dehydrogenase showed that the principal product was the 8-S-acetyl derivative. It is probable that this specificity may also be found in the other enzyme reactions we have examined. At present the chemical identity of the acetyl derivative is not known and work is in progress to clarify this issue.

These investigations clearly demonstrate the need to re-examine the role of lipoic acid in intermediary metabolism. While its function as a coenzyme in the enzyme complexes that catalyze the oxidative decarboxylation of á-keto acids is well established, the findings from this and the previous study (Gandhi *et al.,* 1985) open up the possibility that DTO may substitute for CoA in other enzyme reactions in

intermediary metabolism. The question that remains to be answered is whether infact DTO is of physiological importance in some of these metabolic functions under normal physiological conditions in the animal and if so, do DTO thioesters actually participate in fatty acid biosynthesis? Answers to this question form the theme of the experiments in progress in our laboratory.

References

Brady, R. O. and Stadman, E. R. (1954) *J. Biol. Chem.,* **211,** 621.

Gandhi, V. M., Wagh, S. S., Natraj, C. V. and Menon, K. K. G. (1985) *J. Biosci.,* **9,** 117.

Gunsalus, I. C., Barton, L. S. and Gruber, W. (1956) *J. Am. Chem. Soc.,* **78,** 1763.

Hers, H. G. and Hue, L. (1983) *Ann. Rev. Biochem.,* **52,** 617.

Hersh, L. B. and Jencks, W. P. (1967) *J. Biol. Chem.,* **242,** 3468.

Inoue, H. and Lowenstein, J. M. (1975) *MethodsEnzymol.,* **35,** 3.

Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. and Denton, R. M. (1976) *Biochem. J.,* **154,** 327.

Lipmann, F. and Tuttle, L. C. (1945) *J. Biol. Chem.,* **159,** 21.

Moore, S. A. and Jencks, W. P. (1982) *J. Biol. Chem.,* **257,** 10893.

Murakami, Y. and Nishimura, J. S. (1974) *Biochim. Biophys. Acta,* **336,** 252.

Natraj, C. V., Gandhi, V. M. and Menon, K. K. G. (1984) *J. Biosci.,* **6,** 37.

O'Connor, T. P., Riche, T. E. and Paukstelis, J. V. (1982) *J. Biol. Chem.,* **257,** 3110.

Randle, P. J. (1966) *Symp. Med. Hoechst,* **12,** 97.

Ruderman, N. B., Aoki, T. T. and Cahill, G. F. Jr., (1976) in *Gluconeogenesis—Regulation in Mammalian Species* (eds R. W. Hanson and M. A. Mehlman) (New York: Academic Press) p. 515.

Sauer, F. and Erfle, J. D. (1966) *J. Biol. Chem.,* **241,** 30.

Shepherd, D. and Garland, P. B. (1969) *Methods Enzymol.,* **13,** 11.

Simon, E. J. and Shemin, D. (1953) *J. Am. Chem. Soc.,* **75,** 2520.

Stern, J. R. (1957) *Methods Enzymol.,* **3,** 426.

Stern, J. R., Coon, M. J., Delcampillo, A. and Schneider, N. C. (1956) *J. Biol. Chem.,* **251,** 15.

Stern, J. R. and Drummond, C. I. (1961) *J. Biol. Chem.,* **236,** 2892.

Stoops, J. K., Ross, P., Arslanian, M. J., Aune, K. C. and Wakil, S. J. (1979) *J. Biol. Chem.,* **254,** 7418.

Sungman, C. (1969) *Methods Enzymol.,* **13,** 62.

Trams, E. G. and Brady, R. O. (1960) *J. Am. Chem. Soc.,* **82,** 2972.

Utter, M. F., Keech, D. B. and Scrutton, M. C. (1964) *Adv. Enzyme Regul.,* **2,** 49.

Williamson, J. R., Browning, E. T. and Scholz, R. (1969) *J. Biol. Chem.,* **244,** 4607.