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3-HYDROXY-3-METHYLGLUTARYL COENZYME A LYASE FROM TISSUES OF THE OYSTER, *CRASSOSTREA VIRGINICA*, THE LITTLE SKATE, *RAJA ERINACEA* AND THE LAKE CHARR, *SALVELINUS NAMAYCUSH*: A SIMPLIFIED SPECTROPHOTOMETRIC ASSAY*

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Abstract—1. A continuous spectrophotometric assay for 3-hydroxy-3-methylglutaryl coenzyme A lyase has been developed which couples acetoacetate production to NADH oxidation with 3-hydroxybutyrate dehydrogenase.

2. The method is simpler than techniques involving citrate synthase and malate dehydrogenase, and does not require empirically determined stoichiometric correction factors.

4. Enzyme activity increases with pH over the physiological range, and is optimized at 5 mM $MgCl_2$ and low ionic strength.

5. The assay has been used successfully in tissues of oysters (*Crassostrea virginica*), little skates (*Raja erinacea*), and lake charr (*Salvelinus namaycush*).

INTRODUCTION

Although the roles of ketone bodies in the metabolism of mammals have been extensively investigated, such is not the case for many lower vertebrate and invertebrate groups. What data exist suggest that among vertebrates ketone bodies are quantitatively more important in taxa without serum albumin with which to transport free fatty acids (Zammit and Newsholme, 1979; Ballantyne and Moyes, 1987; Ballantyne *et al.*, 1987). One enzyme involved in the metabolism of ketone bodies is 3-hydroxy-3-methylglutaryl coenzyme A lyase (HMG CoA lyase; EC 4.1.3.4.). HMG CoA lyase is primarily of interest for its role in the major pathway in formation of ketone bodies in liver mitochondria (Lynen *et al.*, 1958; Dashti and Ontko, 1979). It also acts on an intermediate in the synthesis of cholesterol, and is involved in leucine degradation (Wanders *et al.*, 1988). HMG CoA lyase catalyses the reaction:

$$\text{HMG CoA} \xrightarrow{Mg^{2+}} \text{acetyl CoA} + \text{acetoacetate}$$

A variety of assays including the radiochemical methods proposed by Clinkenbeard *et al.* (1975) and Young and Berger (1981) have been used to assay this enzyme, however, spectrophotometric methods have predominated. Assays have been attempted using acetoacetate production as their basis, however, these are cumbersome "stop and kill" procedures requiring repeated sampling (Coon, 1962; Williamson *et al.*, 1968; Wanders *et al.*, 1988).

In an attempt to find a continuous method, Stegink and Coon (1968) developed a technique based on coupling production of acetyl CoA to a citrate synthase (CS)–malate dehydrogenase (MDH) system:

$$\text{acetyl CoA} + \text{oxaloacetate} \xrightarrow{CS} \text{citrate} + \text{CoA}$$

$$\text{malate} + H_2O + \text{NAD} \xrightarrow{MDH} \text{oxaloacetate} + \text{NADH}$$

This system has been modified by Hamprecht and Lynen (1970), Barth (1978), Dashti and Ontko (1979), and Kramer and Miziorko (1980). Equilibrium is first established in the MDH reaction. Added HMG CoA is converted to acetyl CoA by HMG CoA lyase. Condensation of the acetyl CoA with oxaloacetate forms citrate, pulling the MDH reaction from its equilibrium. While this is a continuous and therefore more convenient assay, calculation of HMG CoA lyase activity based on the perturbation of MDH from its equilibrium position requires the inclusion of an empirically determined stoichiometric factor to correct NADH reduction rate to give the true rate of acetyl CoA formation (Buckel and Eggerer, 1965; Pearson, 1965; Hardwick, 1968). As an alternative, the commercial availability of the enzyme 3-hydroxybutyrate dehydrogenase (BHB dh) in sufficiently purity should allow acetoacetate production to be coupled to NADH oxidation directly in a continuous spectrophotometric assay.

$$\text{acetoacetate} + \text{NADH} \xrightarrow{\text{BHB dh}} \text{3-hydroxybutyrate} + NAD^+ + H^+$$

In this paper, the CS/MDH coupled assay of Barth (1978) whose validity is based on previous

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comparison to the acetoacetate production assay of Williamson *et al.* (1968) is compared with the proposed assay for HMG CoA lyase. The new method is optimized in a teleost, *Salvelinus namaycush*, and applied to an elasmobranch, *Raja erinacea*, and an invertebrate mollusc, *Crassostrea virginica*.

MATERIALS AND METHODS

Experimental animals

Lake charr, *Salvelinus namaycush* (Walbaum) were maintained in freshwater at 10°C and fed a commercial salmonid diet (84G, Martin Feed Mills Ltd., Elmira, Ontario) *ad libitum*. Little skates, *Raja erinacea*, were collected by trawl near St. Andrews, New Brunswick and maintained at 30 parts per thousand (ppt) salinity at 10°C and fed a diet of minced herring, *ad libitum*. Oysters, *Crassostrea virginica*, were obtained from Malpeque Bay, Prince Edward Island through a local seafood supplier and maintained at 10°C at 30 ppt salinity, without feeding.

Enzyme extraction

Lake charr were killed with a blow to the head and samples of red muscle and liver tissue removed. Skates, killed by cervical incision, yielded samples of brain, heart, kidney, liver and red muscle for analysis. Adductor muscle, gill, hepatopancreas and mantle tissues were taken from oysters. All tissues were quickly blotted dry, weighed and immersed in ice-cold 50 mM imidazole buffer pH 8.1 with 0.1% Triton X-100. Tissues were homogenized using a Polytron PT10 unit for 3 bursts of 10 seconds each. Adductor muscle, mantle, hepatopancreas and gill tissue of the oyster were homogenized in an identical manner. Homogenates were centrifuged at 18,200 *g* for 20 min and the resulting supernatant used directly in enzyme assays.

Reagents and enzyme assays

All chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri) with the exception of $MgCl_2$ obtained from Fisher Scientific. Citrate synthase and malate dehydrogenase used in assays were porcine heart enzymes (Sigma C3260 and M7383 respectively). Two preparations of 3-hydroxybutyrate dehydrogenase (BHB dh) were used: a purified lyophilized enzyme from *Pseudomonas lemoignei* (Sigma H5132, 50 units/mg protein) and a crystalline suspension from *Rhodopseudomonas spheroides* (Sigma H6126, 7.6 units/mg protein).

Maximal enzyme activities were determined using a Varian DMS 100 UV-Visible spectrophotometer equipped with thermostatted cell changer maintained at 10°(±1°)C with a Haake D8 circulating temperature bath. Reaction rate was determined by monitoring increase or decrease of NADH at 340 nm. Activity is expressed in units, where one unit equals one μmol substrate converted to product per min. All assays were conducted in 50 mM imidazole buffer, pH 8.1. The reaction was monitored initially following addition of homogenate to determine activity due to endogenous electron transport chain enzymes or NADH dehydrogenase (usually 15–30% of the HMG CoA lyase activity), then 0.2 mM DL-3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) was added. The difference in activity before and after HMG CoA addition was taken to be the true HMG CoA lyase activity.

CS/MDH-based assay. Reactions were conducted in a 1 ml volume with substrate and enzyme concentrations as follows: 2.5 mM L-cysteine, 0.66 mM L-malate, 5 mM $MgCl_2$, 2 mM NAD, 55 units MDH and 4.4 units CS (Barth, 1978).

BHB dh-based assay. Reactions proceeded in a 1 ml volume with 10 mM $MgCl_2$, 0.2 mM NADH and 1 unit 3-hydroxybutyrate dehydrogenase.

Enzyme activities are expressed in units per gram wet weight where one unit is equal to 1 μmol substrate converted per minute.

Assay comparisons

Lake charr tissues were selected for detailed examination. CS/MDH- and BHB dh-based assays were compared using duplicate assays over a range of homogenate additions for both muscle and liver tissue. To minimize the effects of time-dependent decay of activity, the order in which homogenate concentrations were assayed was randomized. For each assay, resulting activities were regressed against wet weight added tissue by least-squares methods to facilitate comparison. The CS/MDH-based assay was corrected for MDH equilibrium effects by multiplying by the stoichiometric factor 1.33 (Pearson, 1965; Hardwick, 1968; Barth, 1978).

Optimization of assay conditions

For the BHB dh assay in lake charr muscle tissue, a profile of activity was prepared over the physiological range of pH from 7.0 to 8.5. Because Mg^{2+} is a required cation in the reaction, the effect of varying magnesium concentration was tested to ensure saturating levels, with $MgCl_2$ of between 0 and 20 mM added. The effects of ionic strength on the activity of muscle enzyme were investigated by varying concentrations of KCl between 0 to 0.1 M. Change in absorbance without HMG CoA addition may be due to NADH dehydrogenase activity. Because such dehydrogenases may be sensitive to rotenone (Dashti and Ontko, 1979) additions of 1.0 μM rotenone in ethanol were made. The stabilization of enzyme sulfhydryl groups using 2.5 mM cysteine was also tested.

Species comparison

For each tissue of each organism, BHB dh-based assays were performed, as described.

RESULTS

Assay comparisons

Figure 1 shows the linearity of both assays with respect to homogenate addition in red muscle ($r^2 = 0.97$ for BHB dh, $r^2 = 0.99$ for CS/MDH). In each assay, preincubation slopes typically represented 15–30% of slopes following HMG CoA addition. The BHB dh assay gave consistently higher activity per unit tissue addition than did the CS/MDH assay. Due to excessive turbidity in concentrated liver samples, HMG CoA lyase activity shown in Fig. 2 was not a linear function of amount of tissue added beyond 10 mg/ml for either assay. In muscle tissue both assays demonstrated linearity for up to one hr with 20 mg muscle tissue/ml under optimal assay conditions.

Optimization of assay conditions

No differences were found in activity between assays conducted with either Sigma BHB dh preparation tested.

Activity of HMG CoA lyase increased continuously with pH increase over the range pH 7.15 to 8.35, as shown in Fig. 3.

With magnesium concentration up to 5–8 mM $MgCl_2$, the activity of the enzyme increased (Fig. 4). Concentrations above 8 mM did not result in significantly higher activity.

KCl concentrations also affected enzyme activity. Figure 5 demonstrates that as KCl was increased activity decreased to approximately 50% relative

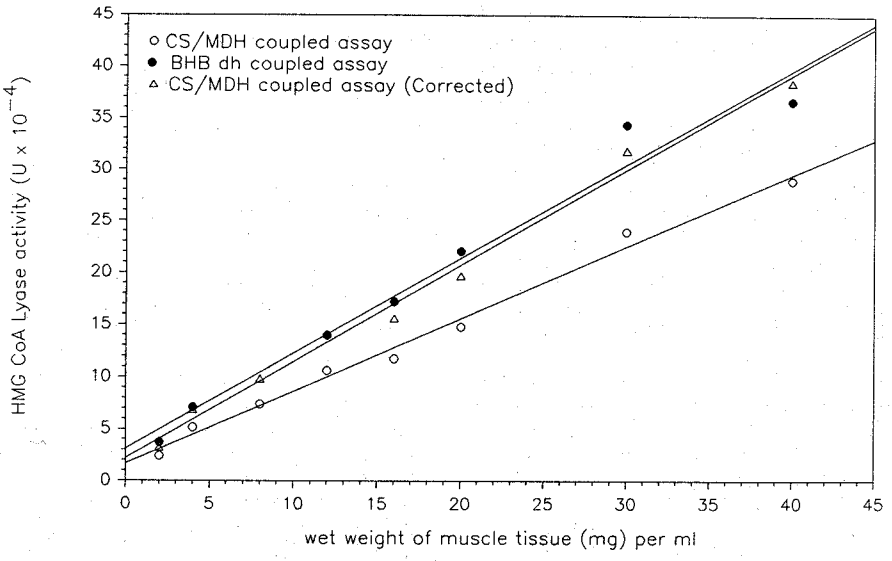

Fig. 1. Comparison of CS/MDH and BHBdh coupled assays of HMG CoA lyase activity versus added lake charr red muscle tissue. Assay conditions were as described in Materials and Methods.

activity at 0.5 M. Increases in KCl beyond 0.5 M did not produce further significant decreases.

Rotenone addition did not consistently decrease preincubation activity. In all cases, corrected enzyme activities were no different than those measured without rotenone.

Cysteine additions had no significant effect on enzyme activities in any case for the BHB dh assay.

Species comparisons

Mean activities and standard errors of HMG CoA lyase for each tissue of each species are given in Table 1. Values for laboratory rat and rabbit liver obtained from the literature are included for comparison. Both charr and skates had very similar levels in liver and red muscle with liver activity being approximately 10-fold higher in each species. Oyster tissue activities were far lower than vertebrate tissues tested. Adductor muscle activity was highest, followed by hepatopancreas, mantle and gill.

DISCUSSION

The BHB dh assay for HMG CoA lyase presented here offers important advantages over previous methods. It is much simplified from the CS/MDH-based methods, requiring only a single coupling enzyme and eliminates the multiple sampling needed for the Williamson *et al.* (1968) technique. The problems of the effects of MDH equilibria whereby initial NADH concentrations bias calculated factors are eliminated.

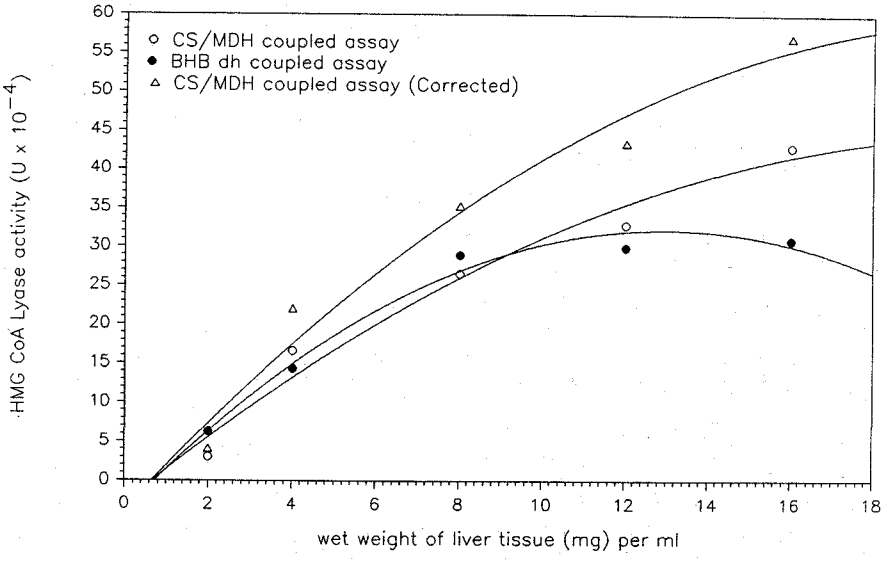

Fig. 2. Comparison of CS/MDH and BHBdh coupled assays of HMG CoA lyase activity versus added lake charr liver tissue. Assay conditions were as described in Materials and Methods.

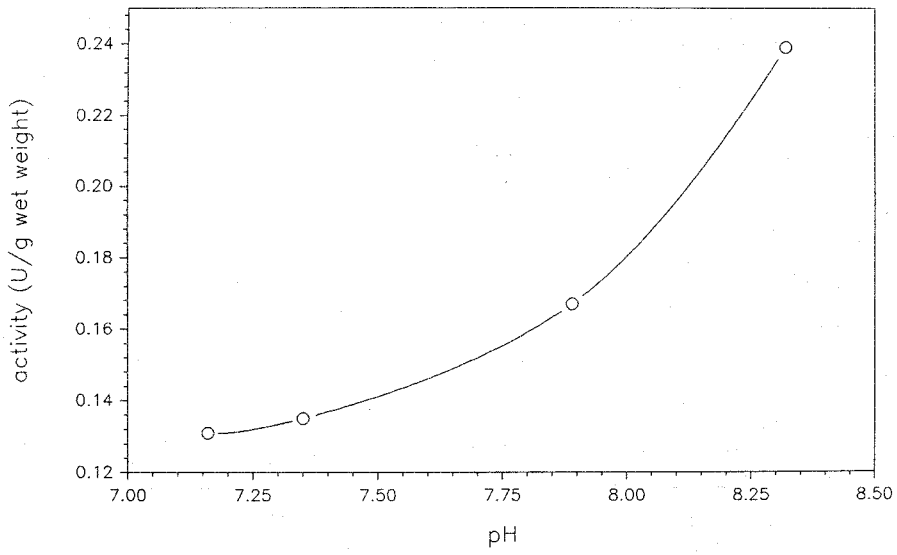

Fig. 3. Effect of pH on BHB coupled assay of HMG CoA lyase in lake charr red muscle tissue. Assay conditions were as described in Materials and Methods.

Response of activity to pH over the range tested closely matches that found by Stegink and Coon (1968) for the bovine enzyme and Kramer and Miziorko (1980) for the avian enzyme. Optimal activity is probably found between 8.9 and 9.5, however, Stegink and Coon (1968) recommended assays be performed at 8.1 to reduce non-enzymatic acetylation and auto-oxidation of sulfhydryl groups. The absorbance changes noted prior to HMG CoA addition may be attributable to these sources. This agrees with our finding that rotenone and cysteine failed to eliminate preincubation absorbance changes.

An apparent K_m of 1.0×10^{-3} M for $MgCl_2$ has been reported by Stegink and Coon (1968). Using the present assay, in lake charr, magnesium concentrations greater than 5 mM saturate the enzyme. Lowest levels of activity (approximately 50% of maximal activity) resulted from omission of $MgCl_2$.

The reduction in activity HMG CoA lyase by increasing KCl concentration may be due to the effects of increasing ionic strength. Optimal assay conditions for lake charr should maintain ionic strength as low as possible.

Values for HMG CoA lysase activity in lake charr liver closely correspond to those measured by

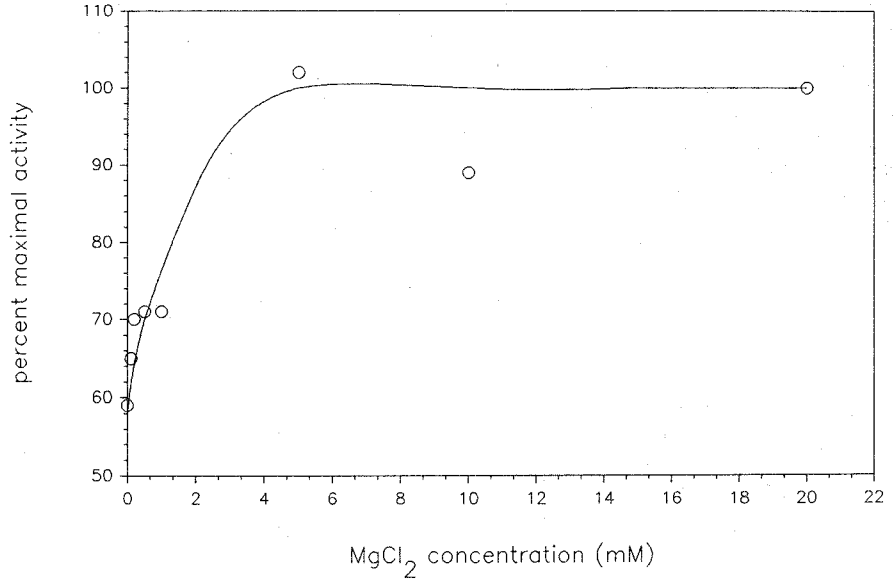

Fig. 4. Effect of $MgCl_2$ concentration on BHB coupled assay of HMG CoA lyase in lake charr red muscle tissue. Enzyme activity is expressed as a percentage of that obtained under standard conditions with 20 mM added $MgCl_2$. Assay conditions were as described in Materials and Methods.

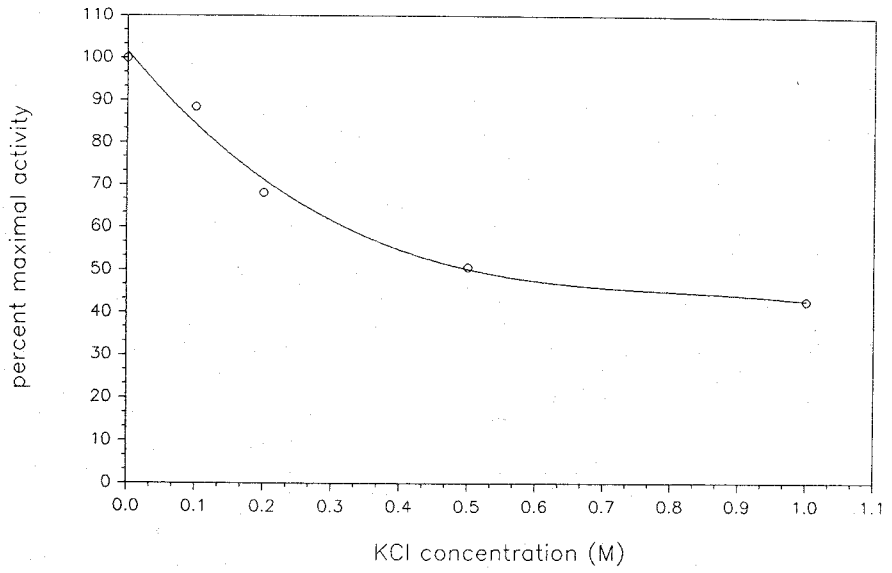

Fig. 5. Effect of KCl concentration on BHB coupled assay of HMG CoA lyase in lake charr red muscle tissue. Enzyme activity is expressed as a percentage of that obtained under standard conditions with no added KCl. Assay conditions were as described in Materials and Methods.

Zammit *et al.* (1979), who found 1.3 U/g wet weight in liver tissue of *Salmo gairdneri*. Activities obtained for *Raja clavata* liver of 0.71 U/g wet weight (Zammit *et al.*, 1979) are comparable to this study's findings, as well.

Comparing values obtained in this study to mammalian values, it is necessary to correct for both a 15°C assay temperature difference and a radically different metabolic rate. Vertebrate ectotherms generally have a 10-fold lower metabolic rate than do homeotherms such as mammals (Hochachka and Somero, 1984), and for invertebrate molluscs this is almost certainly lower. Taking these factors into account, the enzyme activities obtained are relatively high compared to vertebrates. HMG CoA lyase also appears to play an important role in non-hepatic tissues, suggesting that ketogenesis is not confined to the livers of these organisms. This supports an earlier contention (Ballantyne and Moyes, 1987; Ballantyne *et al.*, 1987; Ballantyne and Chamberlin, 1988) that intertissue transport of fatty acids may be limited in osmoconforming organisms due to lack of carrier proteins. Ketone bodies, consequently, may provide a more soluble transport form of lipid carbon. The ease with which the BHB dh-based assay can be applied, and its success in a variety of organisms will facilitate future comparative investigation of these metabolic pathways.

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Table 1. Maximal activity of HMG CoA lyase in tissues of oyster, skate, lake charr, laboratory rat and rabbit. Assays for oyster, skate and lake charr were performed at 10°C as described in Materials and Methods

Animal	Tissue	HMG CoA lyase activity (units/gram wet weight) Mean	S.E.	*n*
Oyster	adductor	0.045	0.009	6
(*Crassostrea virginica*)	gill	0.025	0.008	6
	hepatopancreas	0.039	0.007	6
	mantle	0.028	0.009	6
Skate	brain	0.052	0.006	6
(*Raja erinacea*)	heart	0.243	0.134	6
	kidney	0.744	0.134	6
	liver	1.553	0.272	6
	red muscle	0.149	0.030	6
Lake charr	liver	1.495	0.028	3
(*Salvelinus namaycush*)	red muscle	0.148	0.051	3
Laboratory rat*	liver	8.8	1.0	11
Rabbit†	liver	2.8	0.2	7

*Data from Williamson *et al.* (1968).
†Data from Beis (1985).

REFERENCES

Ballantyne J. S. and Moyes C. D. (1987) Osmotic effects on fatty acid, pyruvate and ketone body oxidation in oyster gill mitochondria. *Physiol. Zool.* **60,** 713–721.

Ballantyne J. S., Moon T. W. and Moyes C. D. (1987) Compatible and counteracting solutes and the evolution of ion and osmoregulation in fishes. *Can. J. Zool.* **65,** 1883–1888.

Ballantyne J. S. and Chamberlin M. E. (1988) Adaptation and evolution of mitochondria: Osmotic and ionic considerations. *Can. J. Zool.* **66,** 1028–1035.

Barth C. A. (1978) Compartmentalization of ketone body synthesis in rat liver. In *Biochemical and Clinical Aspects of Ketone Body Metabolism* (Edited by Soling H. and Seufert C.), pp. 50–58, Georg Thieme, Stuttgart.

Beis A. (1985) Activities of enzymes of ketone body metabolism in liver and muscle tissues of suckling rabbits. *Comp. Biochem. Physiol.* **81B,** 245–249.

Buckel W. and Eggerer H. (1965) Zur optischen bestimmung von citrat-synthase und von acetyl-coenzym A. *Biochem. Z.* **343,** 29–32.

Clinkenbeard K. D., Reed W. D., Mooney R. A. and Lane M. D. (1975) Intracellular localization of the 3-hydroxy-3-methylglutaryl coenzyme A cycle enzymes in liver. *J. biol. Chem.* **250,** 3108–3116.

Coon M. J. (1962) β-hydroxy-β-methylglutaryl CoA cleavage enzyme from heart. *Meths Enzymol.* **5,** 900–903.

Dashti N. and Ontko J. A. (1979) Rate limiting function of 3-hydroxy-3-methylglutaryl-coenzyme-A synthase in ketogenesis. *Biochem. Med.* **22,** 365–374.

Hamprecht B. and Lynen F. (1970) Verfahren zur bestimmung der 3-hydroxy-3-methylglutaryl coenzym A-reduktase-aktivität in rattenleber. *Eur. J. Biochem.* **14,** 323–336.

Hardwick D. C. (1968) Continuous coupled assay for acetyl-coenzyme A production: Correction of observed rates. *Biochem. J.* **110,** 747–748.

Hochachka P. W. and Somero G. N. (1984) *Biochemical Adaptation*, Princeton University Press, New Jersey.

Kramer P. R. and Miziorko H. M. (1980) Purification and characterization of avian liver 3-hydroxy-3-methylglutaryl coenzyme A lyase. *J. biol. Chem.* **255,** 11023–11028.

Lynen F., Henning U., Bublitz C., Sörbo B. and Kröplin-Rueff L. (1958) Der chemische mechanismus der acetassigsäurebildung in der leber. *Biochem. Z.* **330,** 269–295.

Pearson D. J. (1965) A source of error in the assay of acetyl-coenzyme A. *Biochem. J.* **95,** 23c–24c.

Stegink L. D. and Coon M. J. (1968) Stereospecificity and other properties of highly purified β-hydroxy-β-methylglutaryl coenzyme A cleavage enzyme from bovine liver. *J. biol. Chem.* **243,** 5272–5279.

Stegink L. D. and Coon M. J. (1970) β-hydroxy-β-methylglutaryl-CoA cleavage enzyme (bovine liver). *Meths Enzymol.* **17A,** 823–829.

Wanders R. J. A., Schutgens R. B. H. and Zoeters P. M. H. (1988) 3-Hydroxy-3-methylglutaryl-CoA lyase in human skin fibroblasts: study of its properties and deficient activity in 3-hydroxy-3-methylglutaric aciduria patients using a simple spectrophotometric method. *Clin. chim. Acta* **171,** 95–102.

Williamson D. H., Bates M. W. and Krebs H. A. (1968) Activity and intracellular distribution of enzymes of ketone body metabolism in rat liver. *Biochem. J.* **108,** 353–361.

Young N. L. and Berger B. (1981) Assay of S-3-hydroxy-3-methylglutaryl CoA reductase. *Meths Enzymol.* **71,** 498–509.

Zammit V. A. and Newsholme E. A. (1979) Activities of enzymes of fat and ketone-body metabolism and effects of starvation on blood concentrations of glucose and fat fuels in teleost and elasmobranch fish. *Biochem. J.* **184,** 313–322.

Zammit V. A., Beis A. and Newsholme E. A. (1979) The role of oxo-acid CoA transferase in the regulation of ketogenesis in the liver. *FEBS Lett.* **103,** 212–215.