Enzymes of the β-hydroxy-β-methylglutaryl-coenzyme A cycle in *Rhodopseudomonas spheroides*

The operation of the β-hydroxy-β-methylglutaryl-CoA cycle[1] depends upon enzymes that synthesize and cleave hydroxymethylglutaryl-CoA. These are the condensing enzyme[2] (1) and the cleavage enzyme[3] (2).

$$\text{Acetyl-CoA} + \text{acetoacetyl-CoA} \rightarrow \text{hydroxymethylglutaryl-CoA} + \text{CoA} \quad (1)$$

$$\text{Hydroxymethylglutaryl-CoA} \rightarrow \text{acetoacetate} + \text{acetyl-CoA} \quad (2)$$

The two component enzymes are present in animal and yeast preparations and the cycle has been shown to function in the formation of free acetoacetate by liver[1,4,5]. Hydroxymethylglutaryl-CoA may be reduced to mevalonic acid by the TPNH-specific hydroxymethylglutaryl-CoA reductase[6,7] and it is therefore a precursor of a wide range of poly-isoprenoid compounds. Previous work[8] has shown the presence of the hydroxymethylglutaryl-CoA-cleavage enzyme in extracts of *Rhodopseudomonas spheroides*, but had failed to show the synthesis of hydroxymethylglutaryl-CoA. This communication describes the demonstration of the condensing enzyme in ultrasonic extracts of *R. spheroides* by measurement of decrease in extinction due to acetoacetyl-CoA, and by incorporation of [1-^{14}C]acetyl-CoA into hydroxymethylglutaryl-CoA.

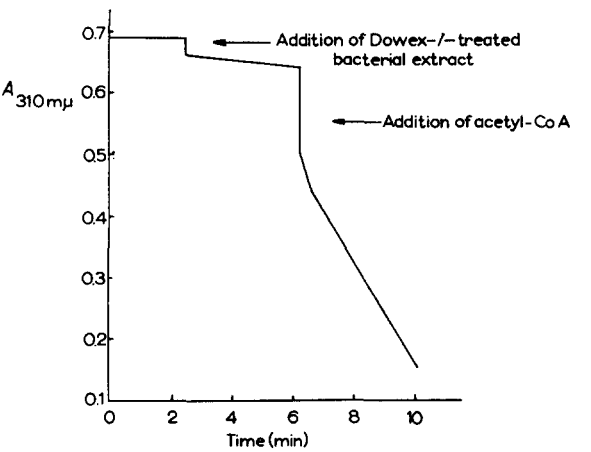

Fig. 1. In a microcuvette (d = 1 cm) were placed Tris (pH 8.5), 75 μmoles; $MgCl_2$, 5 μmoles; acetoacetyl-CoA, 0.03 μmole. The absorption, at 310 mμ, relative to a blank lacking acetoacetyl-CoA was observed. Bacterial extract (20 μg protein) and acetyl-CoA (0.03 μmole) were added as indicated.

R. spheroides (N.C.I.B. 8253) was grown either photosynthetically under anaerobic conditions in the light or in the dark under conditions of high aeration. Under the latter conditions the culture contained only slight amounts of bacteriochlorophyll and carotenoids. In either case the medium (malate, glutamate, salts, and B-group vitamins), the growth conditions and the preparation of cell-free extracts by ultrasonic treatment were as previously described[9,10]. Acetyl-CoA, acetoacetyl-CoA and hydroxymethylglutaryl-hydroxamate were prepared by methods described previously[8].

Hydroxymethylglutaryl-CoA-condensing enzyme was estimated by measuring the decrease in absorption at 310 mμ due to acetoacetyl-CoA on addition of acetyl-CoA[1,11]. The high level of activity of β-ketothiolase in extracts of this organism necessitated the removal of free CoA from the bacterial extract by passing a portion

of the extract (15 mg protein) down a column (7.5 × 1.5 cm) of Dowex-1-XB (100–200 mesh, chloride form) at 0°. Acetyl-CoA was purified by paper chromatography[12].

Fig. 1 shows that the decrease in extinction due to acetoacetyl-CoA is markedly increased on the addition of purified acetyl-CoA. As the hydroxymethylglutaryl-CoA-condensing enzyme acts it releases an equimolar amount of hydroxymethylglutaryl-CoA and CoA and the latter thus becomes available for cleaving the remaining acetoacetyl-CoA by action of β-ketothiolase. A certain amount of CoA may also be released by deacylation of acetyl-CoA. For these reasons an accurate assay of hydroxymethylglutaryl-CoA-condensing enzyme in crude preparations was not possible, but extracts of organisms grown either photosynthetically or aerobically had approximately the same activity. The presence of hydroxymethylglutaryl-CoA-condensing enzyme was confirmed by the incorporation of [1-^{14}C]acetyl-CoA into hydroxymethylglutaryl-CoA which was identified as the hydroxamate. [1-^{14}C]Acetate, CoA and ATP were used as a source of labelled acetyl-CoA, utilising the acetate thiokinase known to be present in this organism.

The hydroxamates were isolated by the method of LYNEN[1] and separated by paper chromatography, their position on the paper being found by spraying with

TABLE I

INCORPORATION OF [1-^{14}C]ACETYL-CoA INTO HYDROXYMETHYLGLUTARYL-CoA BY EXTRACT OF *R. spheroides*

The reaction mixture contained in a total volume of 5.0 ml: Tris (pH 8.5), 500 μmoles; $MgCl_2$, 10 μmoles; CoA, 0.5 μmole; ATP, 50 μmoles; acetate (sodium salt), 500 μmoles (containing 25 μC [1-^{14}C]acetate); acetoacetyl-CoA, 4.5 μmoles; cysteine, 50 μmoles; Dowex-1-treated bacterial extract (photosynthetically grown), 10 mg protein. The mixture was incubated at 34° for 1 h, after which 5 ml 2 *M* NH_2OH (pH 7.0) were added and it was stood at room temperature for 40 min. A control containing bacterial extract that had been heated at 100° for 15 min was treated in a similar manner. To both control and the experimental tubes were added approx. 10 μmoles of carrier hydroxymethylglutaryl-hydroxamate, prepared by reacting hydroxymethylglutaryl-anhydride with NH_2OH. The hydroxamates were isolated and separated by paper chromatography[1] prior to determining their radioactivity.

	Radioactivity present at R_F 0.43 after correction for background count (counts/min)
Control	60
Experimental	3250

5% $FeCl_3$ and 0.1 *N* alcoholic HCl. The chromatograms from both the control and the experimental system had only one, rather streaked, spot (purplish-brown) with an R_F of 0.43, corresponding to that of the carrier hydroxymethylglutaryl-hydroxamate[1]. There was significant incorporation of labelled acetate into hydroxymethylglutaryl-CoA, measured as the hydroxamate, in the experimental system described (Table I). The only region of the chromatogram containing significant amounts of radioactivity was in this area; there was no accumulation of labelled acetyl-CoA. The small amount of radioactivity present on the chromatogram of the control evidently represented slight carry-over of [^{14}C]acetate into the hydroxamate preparation.

With the demonstration of the hydroxymethylglutaryl-CoA-condensing enzyme all the enzymes necessary for the synthesis of hydroxymethylglutaryl-CoA from acetate have been shown in extracts of *R. spheroides*. These bacteria synthesize large

amounts of carotenoids and phytol, and presumably hydroxymethylglutaryl-CoA is a precursor of these isoprenoid compounds. The role of the hydroxymethylglutaryl-CoA-cleavage enzyme is not clear; there is no evidence that it is required for the synthesis of acetoacetate, which is formed by CoA transferase from acetoacetyl-CoA in extracts of *R. spheroides*[8].

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[1] F. LYNEN, U. HENNING, C. BUBLITZ, B. SÖRBO AND L. KRÖPLIN-RUEFF, *Biochem. Z.*, 330 (1958) 269.
[2] H. RUDNEY AND J. J. FERGUSON, *J. Am. Chem. Soc.*, 79 (1957) 5580.
[3] B. K. BACHHAWAT, W. G. ROBINSON AND M. J. COON, *J. Biol. Chem.*, 216 (1955) 727.
[4] F. J. R. HIRD AND R. H. SYMONS, *Biochem. J.*, 76 (1960) 25P.
[5] I. C. CALDWELL AND G. I. DRUMMOND, *Biochem. Biophys. Research Communs.*, 4 (1961) 127.
[6] I. F. DURR, H. RUDNEY AND J. J. FERGUSON, *Federation Proc.*, 18 (1959) 219.
[7] F. LYNEN, B. W. AGRANOFF, H. EGGERER, U. HENNING AND E. M. MÖSLEIN, *Angew. Chem.*, 71 (1959) 657.
[8] N. G. CARR AND J. LASCELLES, *Biochem. J.*, 80 (1961) 70.
[9] J. LASCELLES, *Biochem. J.*, 62 (1956) 78.
[10] J. LASCELLES, *Biochem J.*, 72 (1959) 508.
[11] H. RUDNEY AND J. J. FERGUSON, *J. Biol. Chem.*, 234 (1959) 1076.
[12] E. R. STADTMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, Inc., New York, 1959, p. 931.

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Incorporation of inorganic phosphate into alkaline phosphatase from *Escherichia coli*

Inorganic phosphate has been shown to be incorporated into calf-intestinal alkaline phosphatase, since [^{32}P]SerP could be isolated from acid hydrolysates of highly purified enzyme preparations that had been incubated with $^{32}P_i$[1,2]. Evidence was obtained that $^{32}P_i$ is incorporated at the active site of the enzyme[2,3]. As a highly purified preparation of alkaline phosphatase from *Escherichia coli*[4] is now available, similar experiments have been performed with this enzyme. It was found that $^{32}P_i$ is incorporated into the bacterial phosphatase in essentially the same way as into the calf-intestinal enzyme. This reaction has also been studied by SCHWARTZ AND LIPMANN[5].

The enzyme was purchased from Worthington Biochemical Corp. (Lot no. 6111, chromatographically purified). Before use, it was dialyzed against 0.002 *M* Tris–acetic acid (pH 8.0). The purity of the enzyme was tested by chromatography on a DEAE-cellulose column, as described in the legend to Fig. 1. The fractions were analyzed for protein by measuring the ultraviolet absorbancy/cm at 280 mμ, and for enzyme activity by a phenyl phosphate method[6]. The chromatogram, shown in Fig. 1, suggests a high purity of the enzyme used.

In all the experiments, 0.7 mg of enzyme, as estimated by a modified Folin