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

procedure[7], was incubated with 40 mμmoles of $^{32}P_i$ ($1.86 \cdot 10^6$ counts/min/mμmole) at 0° in a final volume of 0.4 ml, as previously described for the calf-intestinal enzyme[2]. The enzyme was inactivated by addition of 0.1 ml of 2 *N* HCl and precipitated by

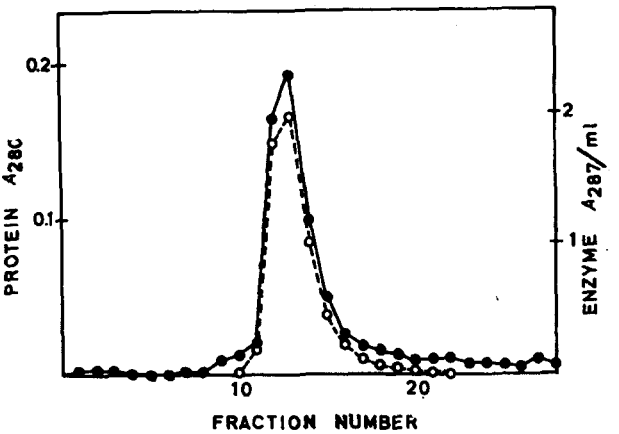

Fig. 1. Chromatography of 5 mg of enzyme on DEAE-cellulose. Column dimensions: 12 × 70 mm. Gradient elution started with 150 ml of 0.01 *M* Tris–acetic acid (pH 7.5), in the closed mixing chamber and 0.20 *M* Tris-acetic acid (pH 7.5) + 0.1 *M* sodium acetate in the reservoir flask. Fraction volume: 4–5 ml. ●—●, absorbancy at 280 mμ; O—O, enzyme activity.

2.5 ml of acetone. After washing with 2 ml of acetone, containing 1% of 2 *N* HCl, the precipitates were dried *in vacuo* at room temperature and hydrolyzed with 2 *N* HCl at 100° for 20 h. [^{32}P]SerP was isolated from the dried hydrolysates by chromatography on Dowex 50W-X8, and further identified by rechromatography on Dowex 1-X10, paper electrophoresis and paper chromatography[2].

In one series of experiments, enzyme was incubated with $^{32}P_i$ for 10 min at pH 4.0, 5.0, 6.0 and 7.6 and, in addition, for 15 sec, 1 and 2 min at pH 5. The total radioactivity of the [^{32}P]SerP fractions from the Dowex 50W columns was determined, and used as an estimate of the amount of phosphorus incorporated. The results are given in Table I. It is seen that the incorporation was greatest at pH 4 and decreased with rising pH, being very small at pH 7.6. At pH 5, $^{32}P_i$ was incorporated more slowly than into the calf-intestinal enzyme[2,3]. Assuming the enzyme to be pure, with a molecular weight of 80000 (see ref. 4), and that 25% of the protein-bound phosphorus was isolated as [^{32}P]SerP (see ref. 2), the incorporation at pH 4 was 0.6 mole of phosphate per mole of enzyme:

$$\frac{1 \cdot 2.44 \cdot 10^6 \cdot 80000}{0.25 \cdot 1.86 \cdot 10^6 \cdot 0.7 \cdot 10^6} = 0.6 \text{ mole}$$

Although this figure is highly approximate, it indicates that $^{32}P_i$ may be incorporated in stoichiometric amounts.

In order to substantiate the assumption that the $^{32}P_i$ was incorporated at the active site of the enzyme, some experiments were performed at pH 5 in the presence of glucose 6-phosphate, sodium arsenate or EDTA. It is reasonable to assume that glucose 6-phosphate and sodium arsenate are bound to the active site of the enzyme by virtue of their function as substrate and competitive inhibitor, respectively. EDTA has been shown to inhibit the enzyme by binding to a metal at the active site[4]. Enzyme was therefore incubated for 15 sec with $^{32}P_i$ + 40 mμmoles of glucose

TABLE I

$^{32}P_i$ INCORPORATION AT DIFFERENT pH VALUES AND INCUBATION TIMES

0.7 mg of enzyme incubated with 40 mμmoles of $^{32}P_i$ ($1.86 \cdot 10^6$ counts/min/mμmole) at 0°.

pH	*Incubation time (min)*	*[^{32}P]SerP isolated (counts/min × 10^{-6})*
4	10	2.44
5	0.25	0.65
5	1	1.45
5	2	1.72
5	10	1.97
6	10	0.86
7.6	10	0.09

6-phosphate, or for 10 min with $^{32}P_i$ + 40 mμmoles of sodium arsenate. In other experiments, EDTA was added to the enzyme to a final concentration of 0.01 *M*. After 2 h in a water bath at 25°, the enzyme was incubated for 10 min with $^{32}P_i$ at 0° in the usual way. Enzyme was also incubated for 10 min with $^{32}P_i$ at 0° before the addition of EDTA. After 2 h at 25°, the incubation mixture was cooled to 0° and inactivated. In addition, enzyme was incubated with $^{32}P_i$ alone (without EDTA), but otherwise treated as the two former incubation mixtures. It is seen from Table II that glucose 6-phosphate, arsenate and EDTA inhibited the incorporation reaction, indicating that the $^{32}P_i$ was incorporated at the active site of the enzyme. When EDTA was added after the $^{32}P_i$, most of the incorporated phosphate was released. The control experiments without EDTA ruled out the possibility that the lack of incorporation was due to inactivation of the enzyme.

In other control experiments, enzyme inactivated by heat or acid was incubated with $^{32}P_i$. The enzyme was either inactivated at 0° with 0.1 ml of 2 *N* HCl for 5 min, or heated in a boiling-water bath for 5 min before the addition of $^{32}P_i$. In these cases, only traces of [^{32}P]SerP could be isolated ($0.001 \cdot 10^6$ counts/min or less).

TABLE II

INHIBITION OF $^{32}P_i$ INCORPORATION AT pH 5 BY GLUCOSE 6-PHOSPHATE, SODIUM ARSENATE AND EDTA

0.7 mg of enzyme incubated at pH 5 and 0° with 40 mμmoles of $^{32}P_i$ ($1.86 \cdot 10^6$ counts/min/mμmole) in the presence of 40 mμmoles of glucose 6-phosphate, 40 mμmoles of sodium arsenate or EDTA, to a final concentration of 0.01 *M*. Controls incubated with $^{32}P_i$ alone. Inactivation by acid at 0° in all cases.

Enzyme incubated with:	*[^{32}P]SerP isolated (counts/min × 10^{-6})*
$^{32}P_i$ for 15 sec (same experiment as in Table I)	0.65
$^{32}P_i$ + glucose 6-phosphate for 15 sec	0.06
$^{32}P_i$ for 10 min (same experiment as in Table I)	1.97
$^{32}P_i$ + sodium arsenate for 10 min	0.86
$^{32}P_i$ for 10 min at 0° after preincubation with EDTA for 2 h at 25°	0.01
$^{32}P_i$ for 10 min at 0° after preincubation without EDTA for 2 h at 25°	1.86
$^{32}P_i$ for 10 min at 0° followed by EDTA for 2 h at 25°	0.14
$^{32}P_i$ for 10 min at 0° and then for 2 h at 25°	2.10

The results show that the $^{32}P_i$ incorporation into the alkaline phosphatase from *Escherichia coli* was highly similar to that into the calf-intestinal enzyme. This supports the conclusion that the $^{32}P_i$ incorporation is related to the mechanism of action of alkaline phosphatases.

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[1] L. ENGSTRÖM, *Acta Soc. Med. Upsaliensis*, 64 (1959) 214.
[2] L. ENGSTRÖM, *Biochim. Biophys. Acta*, 52 (1961) 49.
[3] L. ENGSTRÖM, *Biochim. Biophys. Acta*, 54 (1961) 179.
[4] A. GAREN AND C. LEVINTHAL, *Biochim. Biophys. Acta*, 38 (1960) 470.
[5] J. H. SCHWARTZ AND F. LIPMANN, personal communication.
[6] L. ENGSTRÖM, *Biochim. Biophys. Acta*, 52 (1961) 36.
[7] M. EGGSTEIN AND F. H. KREUTZ, *Klin. Wochenschr.*, 33 (1955) 879.

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Failure of ultraviolet-irradiated *Escherichia coli* to produce a cross-reacting protein

Irradiation with ultraviolet light inhibits the ability of cultures of *Escherichia coli* to synthesize active β-galactosidase. This synthesis decreases with increasing radiation dosage at a considerably greater rate than does total protein synthesis (Fig. 1). For example, an irradiation which was sufficient to reduce enzyme synthesis by 80% only reduced protein synthesis by 20%. Alkaline phosphatase, aspartate transcarbamylase, tryptophanase and D-serine deaminase exhibit similar ultraviolet sensitivities[1]. If these enzymes are to be regarded as typical cellular proteins, one wonders why their synthesis should be particularly sensitive to radiation.

One possible explanation is that irradiated bacteria synthesize proteins which are closely related to the enzymes, but are devoid of catalytic activity because of some small structural change. The observation that about half of the radiation-induced β-galactosidase-negative mutants are capable of synthesizing a cross-reacting material which will precipitate antibody to β-galactosidase makes this hypothesis particularly attractive[2]. Alternatively, the irradiated bacteria may simply synthesize a reduced amount of normal enzyme and either no abnormal protein, or some additional protein which, although derived from the enzyme, is devoid of any immunologically detectible structural similarity.

Immunological tests were performed on material produced immediately after irradiation in order to determine if the irradiated organisms now synthesize a cross-reacting material in the place of active enzyme, thus testing the first hypothesis. The test employed detects cross-reacting material by its ability to prevent combination and precipitation of antibody and active enzyme. If enzyme remaining in the supernatant after precipitation is plotted against enzyme input, the point at which residual