Lipoic acid activation of the α-ketobutyrate oxidation system in cell-free extracts of *Streptococcus faecalis*

Cell suspensions and dried cell preparations of *Streptococcus faecalis*, strain 10Cl, harvested from a lipoic acid-deficient medium, oxidize pyruvate and α-ketobutyrate when lipoic acid is added to the system[1]. Until recently [2,3], however, attempts to activate cell-free extracts of the deficient cells with lipoic acid or its derivatives have been unsuccessful[4]. This report describes some of the conditions and components required for activation of the α-ketobutyrate oxidation system in cell-free extracts prepared from lipoic acid-deficient *S. faecalis* cells.

The cells were grown on a synthetic medium essentially as described by GUNSALUS *et al.*[5]. The cell paste (25 g) was suspended in sufficient 0.02 *M* potassium phosphate buffer (pH 7.0) to give a final volume of 50 ml. The suspension was subjected to sonic vibration for 45 minutes with a Raytheon 10KC oscillator and then centrifuged for 40 minutes at top speed of a Servall SS1 centrifuge at 4°. The cell-free extract contained approximately 80 mg of protein per ml. The rate of formation of propionyl phosphate, by dismutation of α-ketobutyrate, was used as the assay for the α-ketobutyrate oxidation system. The assay system (*cf.* [1,6]) contained 100 μM of potassium phosphate buffer (pH 7.0), 50 μM of potassium α-ketobutyrate, 0.2 μM of cocarboxylase, 0.1 μM of CoA, 0.23 μM of DPN, 6.4 μM of L-cysteine, 4 μM of $MgCl_2$, and extract, in a final volume of 1.0 ml. The reaction mixtures were incubated for 30 minutes at 30° in air and then assayed for propionyl phosphate by the hydroxamic acid method of LIPMANN AND TUTTLE[7].

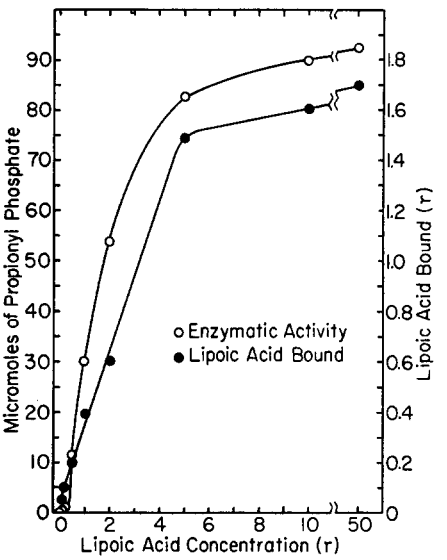

Fig. 1. Lipoic acid uptake and α-ketobutyrate dismutation activity of cell-free extract. 1 ml aliquots of extract (84 mg protein) and radioactive lipoic acid were incubated at 30° for 60 minutes and then dialyzed with agitation for 12 hours against three changes of 200 ml each of 0.02 *M* phosphate buffer (pH 7.0) at 4°. The dialyzed extracts were assayed for radioactivity and α-ketobutyrate dismutation activity. Results are based on 84 mg of protein. As a control, 5 γ of radioactive lipoic acid and 84 mg of crystalline egg albumin in 1 ml of 0.02 *M* phosphate buffer (pH 7.0) was dialyzed as described. 0.05 γ of radioactive lipoic acid remained in dialysis sack.

Preliminary incubation of the extract with lipoic acid was necessary for maximal α-ketobutyrate dismutation activity. With an excess of lipoic acid approximately 30 minutes of preincubation was required. That lipoic acid is taken up and retained in an enzymically active form during the preincubation was shown by experiments with DL-α-lipoic acid $^{35}S_2$[8]. Aliquots of the cell-free extract were incubated with increasing concentrations of radioactive lipoic acid and then dialyzed to remove free lipoic acid. Assay of the dialyzed preparations revealed a parallel increase in the amount of lipoic acid "bound" and the α-ketobutyrate dismutation activity, both reaching a maximum when approximately 1.5 γ of radioactive lipoic acid was "bound" per 84 mg of protein (Fig. 1). This amount of lipoic acid represents approximately 30% of that originally added.

Dialysis of the original extracts against 0.6% ethylenediaminetetraacetate in 0.02 *M* tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4), and then against 0.02 *M* Tris buffer pH 7.0), prior to incubation with radioactive lipoic acid, revealed that both magnesium ion and phosphate ion were required in the preincubation for "binding" of lipoic acid and enzymic activity. The optimum concentrations of magnesium and phosphate ions were approximately 0.002 *M* and 0.02 *M*, respectively. Magnesium ion could be replaced by manganous or cobaltous ions. Phosphate ion could not be replaced by either sulfate or chloride ions at a concentration of 0.02 *M*, but could be supplied equally well as the potassium or sodium salt.

Fractionation of the crude extracts with protamine sulfate yielded two enzyme fractions, one (Fraction PP) precipitated by protamine and eluted with 1 *M* phosphate buffer, pH 7.0, and the other (Fraction PS) remaining in the protamine supernatant. It was necessary to incubate both fractions together with lipoic acid for α-ketobutyrate dismutation activity (Table I). When extracts which had been incubated with lipoic acid were fractionated with protamine, Fraction PP was found to contain the complete α-ketobutyrate dismutation system. However, Fraction PS

TABLE I

COUPLING OF FRACTIONS FROM ACTIVATED AND UNACTIVATED EXTRACTS

Fractions in preincubation	*Fraction added at assay time*	*Propionyl phosphate formed*
PP	PS	0.3 μM
PS	PP	0.2
PP + PS	—	2.3
PP(Act.)*	—	2.5
PS(Act.)	PP	0.2
PP(Act.) + PS(Act.)	—	2.2
PP + PS(Act.)	—	1.8
PP and PS(Act.)**	—	0.3

Conditions: The protein content of the fractions (0.2 ml of each) was as follows: PP, 1.4mg; PS, 0.6 mg; PP(Act.), 1.8 mg; PS(Act.), 0.8 mg. The fractions were incubated for 90 minutes at 30° with 20 γ of lipoic acid, 4 μM of $MgCl_2$ and 0.2 μM of cocarboxylase, and then assayed for α-ketobutyrate dismutation activity (10 minute assay), without further addition of $MgCl_2$ or cocarboxylase, but with the indicated fractions added at time of assay.

* When this fraction was assayed without preincubation, the result was the same.
** Fractions were preincubated separately and combined at time of assay.

from the activated extract could be incubated with lipoic acid and Fraction PP from the unactivated extract to reconstruct the dismutation system. Thus far, attempts to detect a heat stable or dialyzable component which could replace Fraction PS have been unsuccessful.

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The utilization of acid soluble phosphorus in growing bacteria

In studying the kinetics of desoxyribose nucleic acid (DNA) or ribose nucleic acid (RNA) synthesis, by means of ^{32}P incorporation, it is essential to ascertain whether exchange of phosphorus takes place between these substances and other sources of phosphorus within, and without, the cell.

This problem has been studied recently by HERSHEY[1] by growing labelled *E. coli* cells in cold medium and looking for a redistribution of ^{32}P between the RNA and DNA fractions. HERSHEY concluded that the conservation of ^{32}P observed in both the RNA and the DNA indicated an absence of turnover or of exchange of phosphorus within these fractions.

We have confirmed HERSHEY's results using *Salmonella typhimurium*. These results in themselves, however, are not decisive. Similar results woud be obtained if both RNA and DNA exchanged phosphorus, through a common phosphorus pool, and if the exchange rates were proportional to the net rates of synthesis of these substances[1]. However, if such exchanges occurred, the total ^{32}P-activity of the trichloroacetic acid (TCA) soluble material, of which such a common phosphorus pool may be assumed to be a part, would remain more or less constant during growth and synthesis of new nucleic acid, the return of ^{32}P from the larger, insoluble fraction maintaining an almost constant total activity in the soluble fraction. The combination of a high rate of exchange *and* loss of activity from the soluble pool, whether to the medium of to other fractions than RNA and DNA, can be excluded, since we know that the ^{32}P of the nucleic acids is conserved over several generations of growth[1].

Accordingly, a culture of *Salmonella typhimurium* was diluted to a titer of $5 \cdot 10^5$ cells/ml in