ENZYME SYSTEMS IN THE MYCOBACTERIA

VI. FURTHER STUDIES ON THE PYRUVIC DEHYDROGENASE SYSTEM

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SUMMARY

1. The pyruvic dehydrogenase complex of the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis* has been resolved into two fractions.

2. The first fraction, precipitating at high ammonium sulfate concentrations, contains a lipoic dehydrogenase. This enzyme catalyzes the oxidation of $lip(SH)_2$ by DPN; the reverse reaction has not been demonstrated. The lipoic dehydrogenase has been purified at least twenty-fold over the crude cell-free extract. DL-α-lipoamide is reduced by DPNH and the lipoic dehydrogenase. E_0' for the $lip(SH)_2 \rightleftharpoons lipS_2$ couple at 22° and pH 6.0 was shown to be —0.23 V.

3. The second fraction, precipitating at low ammonium sulfate concentrations, contains, in addition to the pyruvic dehydrogenase, a lipoic transacetylase. This enzyme catalyzes the reversible transfer of an acetyl group from acetyl CoA to $lip(SH)_2$. The enzyme has been purified at least fifteen-fold over the crude cell-free extract.

4. Associated with the pyruvic dehydrogenase–lipoic transacetylase complex is a lipoic deacylase which hydrolytically cleaves S-acetyl-lip(SH) to acetate and $lip(SH)_2$.

5. The possible existence of a soluble pyruvic dehydrogenase complex of enzymes in cell-free extracts of H37Ra and the presence of another form of lipoic acid are discussed.

INTRODUCTION

The oxidative decarboxylation of pyruvate by cell-free extracts of the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis* was described in previous publications from this laboratory[1,2]. With either oxygen or dyes (ferricyanide or 2,6-dichlorophenol-indophenol) as electron acceptors the pyruvic dehydrogenase system of H37Ra catalyzes the formation of acetate from pyruvate. When CoA and DPN are present the pyruvic dehydrogenase system catalyzes the formation of acetyl CoA from pyruvate.

The following abbreviations will be used: CoA (acetylation coenzyme), DPN and DPNH (oxidized and reduced forms of diphosphopyridine nucleotide), TPN and TPNH (oxidized and reduced forms of triphosphopyridine nucleotide), $lipS_2$ and $lip(SH)_2$ (oxidized and reduced forms of α-lipoic acid), DPT (thiamine pyrophosphate), tris (tris(hydroxymethyl)aminomethane buffer).

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Pyruvic dehydrogenase systems have been isolated in several laboratories from several strains of bacteria and from animal tissues. The histories and characteristics of these enzyme systems have been recently reviewed[3,4,5]. The pyruvic dehydrogenase systems of *Escherichia coli*[3,6] and *Streptococcus faecalis*[3] were each resolved into two fractions, neither of which alone can carry out the formation of acetyl CoA from pyruvate. One fraction apparently contains the enzymes necessary for the oxidative decarboxylation of pyruvate to acetyl CoA, a reaction in which lipoic acid is required as indicated in reaction (1).

$$\text{pyruvate} + \text{CoA} + \text{lip}S_2 \xrightarrow{\text{DPT, Mg}^{++}} \text{acetyl CoA} + \text{lip}(SH)_2 + CO_2 \qquad (1)$$

The second fraction contains a lipoic dehydrogenase[7] which catalyzes the oxidation of $\text{lip}(SH)_2$ according to reaction (2).

$$\text{lip}(SH)_2 + \text{DPN}^+ \rightleftharpoons \text{lip}S_2 + \text{DPNH} + H^+ \qquad (2)$$

The overall reaction obtained when the system is reconstituted is the sum of reactions (1) and (2).

$$\text{pyruvate} + \text{CoA} + \text{DPN}^+ \longrightarrow \text{acetyl CoA} + \text{DPNH} + H^+ + CO_2 \qquad (3)$$

Although reaction (1) has been recognized for some time to represent the sum of several intermediate steps, the nature of these intermediate reactions has remained obscure. The evidence that lipoic acid plays a key role in the oxidative decarboxylation of α-keto acids led REED[8] and GUNSALUS[3] to propose, independently, the following mechanism for this decarboxylation. The decarboxylation of pyruvate (reaction (4)) is catalyzed by the pyruvic dehydrogenase in the form of a DPT-Enzyme complex.

$$\text{pyruvate} + \text{(DPT-Enz)} \xrightarrow{\text{Mg}^{++}} \text{(“acetaldehyde”-DPT-Enz)} + CO_2 \qquad (4)$$

The hypothetical “acetaldehyde” intermediate is oxidized to “acetyl”, $\text{lip}S_2$ being the oxidizing agent in this step (reaction (5)).

$$\text{(“acetaldehyde”-DPT-Enz)} + \text{lip}S_2 \longrightarrow \text{S-acetyl-lip(SH)} + \text{(DPT-Enz)} \qquad (5)$$

The acetyl group is then transferred to CoA. This transfer is catalyzed by a lipoic transacetylase[9] according to reaction (6).

$$\text{S-acetyl-lip(SH)} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{lip}(SH)_2 \qquad (6)$$

The sum of these reactions is reaction (1).

The present report describes the resolution of the pyruvic dehydrogenase system of H37Ra into two fractions and the reconstitution of dehydrogenase activity by recombination of the two fractions. One fraction is shown to contain, in addition to the pyruvic dehydrogenase, a lipoic transacetylase. The second fraction contains a lipoic dehydrogenase. The latter two enzymes have been purified and their characteristics studied.

METHODS AND MATERIALS

The preparation and assay of the soluble pyruvic dehydrogenase system of H37Ra, the analytical methods used and the sources of reagents have been previously described[1,2]. In the early stages of this work Dr. LESTER REED furnished generous

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amounts of DL-α-lipoic acid. DL-lip(SH)$_2$ was prepared by borohydride reduction of lipS_2[9]. Free sulfhydryl groups were determined by ferricyanide reduction[10].

RESULTS

A. Resolution and reconstitution of the pyruvic dehydrogenase system

The results of a typical fractionation of the pyruvic dehydrogenase system of H37Ra are shown in Table I. The activity of the system at each of the three stages of the fractionation is given in terms of both the indophenol and the DPN assay. Based on the former assay the enzyme has been purified approximately 60 times; in terms of the latter assay there has been essentially no purification. It is apparent that the additional enzymic components required for the reaction in which DPN is the electron acceptor either have been removed from the pyruvic dehydrogenase or have been inactivated.

TABLE I

RESOLUTION OF THE PYRUVIC DEHYDROGENASE SYSTEM

Purification step	*Pyruvic dehydrogenase activity*			
	Indophenol assay		*DPN assay*	
	*Specific activity**	*Total units*	*Specific activity***	*Total units*
Crude extract	*ca.* 5			
1st $(NH_4)_2SO_4$	19	52,300	26.4	72
2nd $(NH_4)_2SO_4$	50	47,600	18.4	18
Gel eluate	274	19,700	33.0	2.4

* ΔD_{600}/mg protein/10 min × 1000.
** Δmμmoles DPNH/mg protein/5 min.

TABLE II

RECONSTITUTION OF THE PYRUVIC DEHYDROGENASE SYSTEM

Enzyme fraction added	*Δ mμmoles DPNH/5 min*
1. Pyruvic dehydrogenase* (0.228 mg; specific activity = 274)	8.69
2. Protein fraction A** (0.298 mg)	6.43
3. Protein fraction B (0.226 mg)	0.0
4. Protein fraction C (0.210 mg)	0.0
1 + 2	16.4
1 + 3	24.0
1 + 4	29.6

* Gel eluate, as described in Table I.
** A crude cell-free extract of H37Ra was fractionated with ammonium sulfate into 3 fractions whose salt saturation limits are as follows: A (0.25–0.40), B (0.40–0.60) and C (0.60–0.85).

The data of Table II demonstrate that resolution of activities has actually been accomplished. Reconstitution of the system is achieved by the combination of the fraction containing the pyruvic dehydrogenase with the fraction of the cell-free extract which precipitates between the ammonium sulfate saturation limits of 0.60–0.85. One of the components of this latter fraction is a lipoic dehydrogenase [reaction (2)],

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the properties of which enzyme are described below. The distribution of lipoic dehydrogenase activity among the several fractions of the cell-free extract is shown in Table III. Fig. 1 shows how the rate of DPN reduction is affected by the amount of lipoic dehydrogenase added to the resolved pyruvic dehydrogenase.

The fraction in which the pyruvic dehydrogenase is localized also contains a lipoic transacetylase [reaction (6)]. The component study which establishes the lipoic transacetylase reaction is summarized in Table IV. Acetyl CoA is generated from acetyl-P and CoA in the presence of phosphotransacetylase. The crude lipoic transacetylase system is completely dependent on the presence of acetyl-P and lip$(SH)_2$ and partially dependent on the presence of CoA. More purified preparations of the lipoic transacetylase show a complete requirement for CoA. The purification and description of the lipoic transacetylase are presented below.

TABLE III

LIPOIC DEHYDROGENASE ACTIVITY IN FRACTIONS OBTAINED BY AMMONIUM SULFATE FRACTIONATION OF A CELL-FREE EXTRACT OF H37Ra

Ammonium sulfate fraction	*Specific activity of the lipoic dehydrogenase**
Pyruvic dehydrogenase** (specific activity = 274)	0.119
Protein fraction A***	0.075
Protein fraction B	0.130
Protein fraction C	0.305

* μmoles of lip$(SH)_2$ oxidized/mg protein/h at 22°. See below for the lipoic dehydrogenase assay system.

** Gel eluate, as described in Table I.

*** Ammonium sulfate fractions as described in Table II.

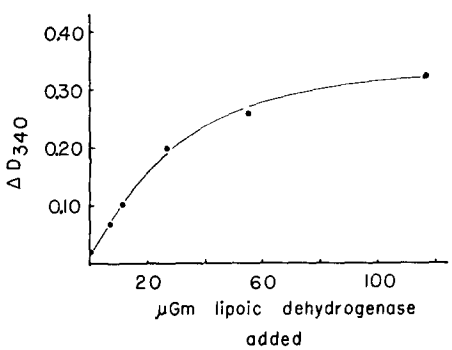

Fig. 1. Reconstruction of the DPN-pyruvic dehydrogenase system. Each cuvette contained, in μmoles: tris buffer of pH 7.0 (40), L-cysteine (10), $MgCl_2$ (5), CoA (0.15), DPT (0.025), Li-pyruvate (10), DPN (1), pyruvic dehydrogenase (0.08 mg) and lipoic dehydrogenase as indicated. The final volume was 1.0 ml; incubation was carried out for 4 min at 22°.

Effect of inhibitors on the reconstituted pyruvic dehydrogenase system

The reconstituted pyruvic dehydrogenase system [reaction (3)] is not inhibited by arsenate, versene or *p*-chloromercuribenzoate at concentrations of 1.0, 1.0 and 0.03 μmoles/ml, respectively. Reaction (3) is inhibited by arsenite. The inhibition is

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complete at $1 \cdot 10^{-5}$ *M* arsenite and about 30 percent at $1 \cdot 10^{-7}$ *M* arsenite. These observations are similar to those of PETERS[12] and GUNSALUS[13] who demonstrated the sensitivity of pyruvic oxidation to trivalent arsenicals. This observation lead PETERS *et al.* to suggest that a dithiol was involved as a coenzyme in the oxidation of pyruvate.

TABLE IV

DEMONSTRATION OF LIPOIC TRANSACETYLASE ACTIVITY IN H37Ra EXTRACT

Description	*μmoles heat-stable hydroxamate formed*
Complete system	0.62
less phosphotransacetylase	0.65
less acetyl-P	0
less CoA	0.15
less lip$(SH)_2$	0

The complete system contained, in μmoles: Tris buffer of pH 7.5 (80), DL-lip$(SH)_2$ (3), CoA (0.3), acetyl-P (8), phosphotransacetylase (0.92 units), and crude cell-free extract of H37Ra (1.14 mg). The final volume was 0.9 ml. The reaction mixtures were incubated for 10 min at 38°. The reaction was stopped and acetyl-P destroyed by the addition of 0.1 ml of 1.0 *N* HCl followed by boiling the acidified mixtures for 3 min. Heat-stable acetyl-groups were measured by the hydroxamic acid method[11].

B. Purification and properties of the lipoic dehydrogenase

Lipoic dehydrogenase assay

The assay system for the lipoic dehydrogenase contains, in μmoles: phosphate buffer of pH 6.0 (60), DL-lip$(SH)_2$ (5), DPN (1.0) and lipoic dehydrogenase (0.01 to 1.0 mg). The final volume is 1.0 ml and the reaction is carried out at 22°. The reduction of DPN is followed for 5 minutes: the rate of DPNH formation is linear over this period provided that no more than 0.02 μmoles of DPNH are produced. A unit of lipoic dehydrogenase activity is defined as that amount of enzyme which catalyzes, under the above conditions, the reduction of 1.0 μmoles of DPN per minute. Specific activity is defined as units of enzymic activity per mg of protein.

Purification of lipoic dehydrogenase

All steps are carried out at 0 to 2°. The crude cell-free extract of H37Ra[14] is fractionated at pH 7.5 with saturated ammonium sulfate. The fraction precipitating below 0.60 saturation is removed by centrifugation and is discarded. The concentration of ammonium sulfate in the supernatant solution is raised to 0.85 saturation by addition of the solid salt. The precipitate (AS-1) is removed by centrifugation at 5,000 × g for 1.5 h and is dissolved in and dialyzed against 0.02 *M* tris buffer of pH 7.5.

Sufficient 1.0 *M* acetic acid is added to the dialyzed solution of AS-1 to adjust the pH to 5.3. The clear solution is fractionated at this pH with saturated ammonium sulfate. The fraction precipitating between the saturation limits of 0.53 and 0.59 (AS-2) is removed by centrifugation and is dissolved in and dialyzed against 0.02 *M* tris buffer of pH 7.5.

The dialyzed solution of AS-2 is next treated with calcium phosphate gel. For each mg of protein in the dialyzed AS-2 solution, 2 mg of gel are added. The gel is

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recovered by centrifugation; the supernatant solution is discarded. The gel is washed three times with one-half its original volume of a 1.5 % solution of ammonium sulfate of pH 5.4. These washes are discarded. The lipoic dehydrogenase is eluted by washing the gel 4 times with one-half its original volume of a 5 % solution of ammonium sulfate of pH 5.4. These washes are pooled. The lipoic dehydrogenase is precipitated by adding solid ammonium sulfate to the gel eluated to about 0.9 saturation. The mixture is stirred for three hours and then centrifuged at 20,000 × g for 30 min. The residue (AS-3) is dissolved in 0.05 *M* tris buffer of pH 7.0.

Results of a typical purification of the lipoic dehydrogenase by this procedure are shown in Table V.

TABLE V

PURIFICATION OF THE LIPOIC DEHYDROGENASE OF H37Ra

Fraction	*Volume* ml	*Protein*			*Activity*		
		mg per ml	*Total mg*	*Percent recovered*	*Specific activity*	*Total units*	*Percent recovered*
Crude extract	2,720	16.3	45,330	100			
AS-1	240	20.1	4,800	10.6	0.0066	31	100
AS-2	16.5	26.4	462	1.0	0.033	15	50
AS-3	6.1	12.0	73	0.16	0.13	9.5	31

Characteristics of the assay system

Fig. 2 and 3 show lipoic dehydrogenase activity as a function of pH and of the concentrations of lip$(SH)_2$, DPN and enzyme, respectively.

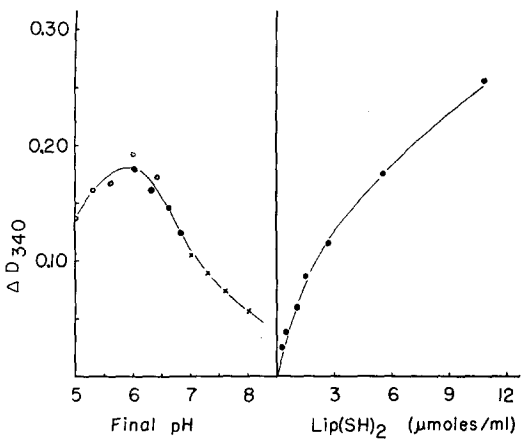

Fig. 2. Lipoic dehydrogenase activity as a function of pH and lip$(SH)_2$ concentration. Assay conditions as described in the text; 0.070 mg of lipoic dehydrogenase of specific activity of 0.093. The reaction was allowed to proceed for 5 min at 22°. O—O, citrate phosphate buffer, 50 μmoles per ml; ●—●, phosphate buffer, 60 μmoles per ml; ×—×, tris buffer 60 μmoles per ml.

Equilibrium of the lipoic dehydrogenase reaction

GUNSALUS[3,7] originally reported the apparent irreversibility of reaction (2). This observation was not confirmed by SANADI AND SEARLS[15] in their investigation of the

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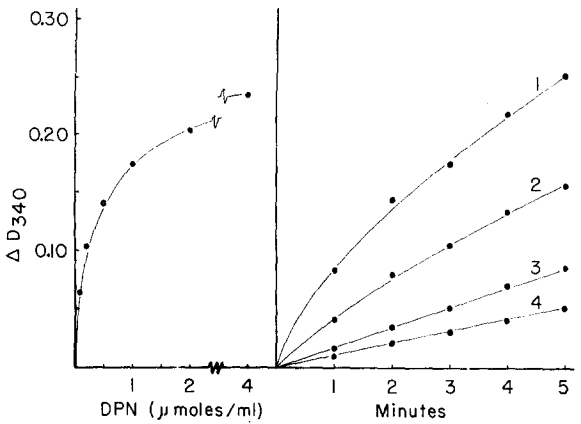

Fig. 3. Lipoic dehydrogenase activity as a function of DPN and enzyme concentrations. Assay conditions as described in the text. Lipoic dehydrogenase of specific activity 0.093 was used. The reaction was allowed to proceed for 5 min. Protein concentrations, in μg/ml: curve 1 (117), curve 2 (58.5), curve 3 (29.3) and curve 4 (11.7).

DPN-linked lipoic dehydrogenase of the pig heart α-ketoglutaric oxidase system. The results of the present study are in agreement with those of GUNSALUS and in disagreement with those of SANADI AND SEARLS. The lipoic dehydrogenase of H37Ra catalyzes the reduction of DPN by $lip(SH)_2$ but not the reverse reaction under the conditions of the lipoic dehydrogenase assay.

The above observations are of particular interest since reaction (2) reaches a definite equilibrium (Fig. 4, Curve 1). That the change in optical density followed is actually due to DPNH formation is shown in Fig. 4 (Curve 2). In this experiment the DPNH formed from reaction (2) is oxidized by the addition of oxalacetate:

$$\text{oxalacetate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{L-malate} + \text{DPN}^+ \qquad (7)$$

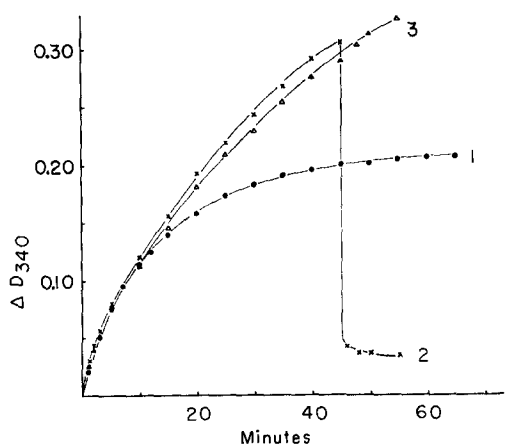

Fig. 4. Equilibrium studies of the lipoic dehydrogenase reaction. Curve 1: The cuvette contained, in μmoles: phosphate buffer of pH 6.0 (60), DL-$lip(SH)_2$ (2.70), DPN (0.20) and lipoic dehydrogenase of specific activity 0.093 (0.117 mg). The final volume was 1.0 ml. Curves 2 and 3: Conditions the same as Curve 1 except that DPN was increased to 1.0 μmole. After 45 minutes 2.0 μmoles of oxalacetate were added to cuvette 2.

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Preparations of the lipoic dehydrogenase of H37Ra contain sufficient malic dehydrogenase to carry out this reaction in the absence of added malic dehydrogenase.

The equilibrium value for reaction (2) was determined; the method and results are shown in Table VI. The uncertainty of the equilibrium value results from the slow oxidation of both –SH and DPNH during the incubation period. We have shown that about 8% of the available –SH groups disappear during the 90 min incubation. Taking the E_0' for the DPNH $\rightleftharpoons$ DPN couple as —0.320 V at pH 7.0[16] or —0.292 V at pH 6.0 and 22°, then E_0' for the lip(SH)$_2$ $\rightleftharpoons$ lipS_2 couple at 22° and pH 6.0, based on the equilibrium constant, is —0.23 V. This value is considerably more positive than that reported by REED[17] for the lipothiamide couple (—0.41 V)and by GUNSALUS[3] for the lipoic couple. SANADI AND SEARLS[14] calculated the E_0' for the lipoamide$_{red}$ $\rightleftharpoons$ lipoamide$_{ox}$ couple as —0.30 V at pH 7.1 and 23°. Polarographic determinations of the lip(SH)$_2$ $\rightleftharpoons$ lipS_2 couple by KE[18] gave a value of —0.30 V at pH 7.0 and 30°.

TABLE VI

DETERMINATION OF THE APPARENT EQUILIBRIUM CONSTANT FOR THE LIPOIC DEHYDROGENASE REACTION

Each cuvette contained 60 μmoles of phosphate buffer of pH 6.0 and 0.023 units of lipoic dehydrogenase. Lip(SH)$_2$ and DPN were added as indicated. The final volume was 1.0 ml; the reaction was carried out at 22° until no further change in optical density at 340 mμ was noted (50 to 90 min).

$$K_{eq} = \frac{(\text{lip}S_2)\ (\text{DPNH})\ (\text{H}^+)}{(\text{lip}(SH_2)\ (\text{DPN}^+)}$$

Expt. No.	*Final concentrations (moles/l)*					K_{eq}
	Lip(SH)$_2$	*DPN*	*LipS_2*	*DPNH*	*H+*	
1	$1.68 \cdot 10^{-3}$	$6.08 \cdot 10^{-5}$	$2.12 \cdot 10^{-5}$	$2.12 \cdot 10^{-5}$	$1.0 \cdot 10^{-6}$	$4.2 \cdot 10^{-9}$
2	$0.99 \cdot 10^{-3}$	$1.34 \cdot 10^{-4}$	$2.99 \cdot 10^{-5}$	$2.99 \cdot 10^{-5}$	$1.0 \cdot 10^{-6}$	$6.7 \cdot 10^{-9}$
3	$0.638 \cdot 10^{-3}$	$2.86 \cdot 10^{-4}$	$4.16 \cdot 10^{-5}$	$4.16 \cdot 10^{-5}$	$1.0 \cdot 10^{-6}$	$9.4 \cdot 10^{-9}$
4	$2.67 \cdot 10^{-3}$	$1.31 \cdot 10^{-4}$	$3.33 \cdot 10^{-5}$	$3.33 \cdot 10^{-5}$	$1.0 \cdot 10^{-6}$	$3.1 \cdot 10^{-9}$
5	$2.64 \cdot 10^{-3}$	$3.48 \cdot 10^{-4}$	$6.21 \cdot 10^{-5}$	$6.21 \cdot 10^{-5}$	$1.0 \cdot 10^{-6}$	$4.2 \cdot 10^{-9}$
					Average	$5.5 \cdot 10^{-9}$

The value at 22° and pH 6.0 for the lip(SH)$_2$ $\rightleftharpoons$ lipS_2 couple is uncertain to the extent that the assumptions of the values $\Delta E_0'/\Delta$pH and $\Delta E_0'/\Delta T$ for DPN are uncertain. These latter values are not available and have been estimated assuming that both these curves can be extrapolated to pH 6.0 and 22°, respectively. We may conclude, however, that at pH 6.0 and 22° the lip(SH)$_2$ $\rightleftharpoons$ lipS_2 couple is about 60 mV more positive than the DPNH $\rightleftharpoons$ DPN couple. The apparent irreversibility of reaction (2) may be referable to any of several observations. First, as is discussed below, lipS_2 inhibits the lipoic transacetylase. It is possible that lipS_2 also inhibits the dehydrogenase, perhaps by remaining bound to the enzyme. Second, the rate of the reverse reaction may be many times slower than the rate of the forward reaction. The present state of purity of the dehydrogenase is not sufficiently high to allow a direct test of this possibility since very large amounts of protein would have to be added to the reaction mixture.

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Reduction of lipoamide by lipoic dehydrogenase

Oxidized lipoamide* is reduced by DPNH in the presence of the lipoic dehydrogenase of H37Ra. These results are in marked contrast to the inactivity of $lipS_2$ in an identical system. The stoichiometry of this reduction is shown in Table VII. The K_s for the reduction of lipoamide by DPNH has not yet been accurately determined. A preliminary calculation shows the value to be on the order of $1 \cdot 10^{-5}$ M/L. SANADI AND SEARLS[15] recently reported that the lipoic dehydrogenase associated with the α-ketoglutaric oxidase complex of pig heart muscle catalyzes the reduction of lipoamide by DPNH. The data presented here confirm their results.

TABLE VII

REDUCTION OF DL-α-LIPOAMIDE BY DPNH CATALYZED BY THE LIPOIC DEHYDROGENASE OF H37Ra

Each cuvette contained, in μmoles: phosphate buffer of pH 6.0 (40), DPNH (0.10), DL-α-lipoamide (No 1, 0.15; No 2, 0.30) and lipoic dehydrogenase (0.016 unit). The final volume was 1.0 ml; the reaction was carried out at 22°, for 85 min. DPNH disappearance was calculated from the change in optical density at 340 mμ; free –SH groups by the ferri-ferrocyanide method corrected for the DPNH present in the sample removed for analysis. The values shown are corrected for a blank reaction containing no lipoamide.

Experiment No.	*Δ μmoles DPNH*	*μmoles –SH produced*
1	0.0219	0.0316
2	0.0718	0.0889

K_s *values for the lipoic dehydrogenase system*

K_s values for DPN and $lip(SH)_2$ were determined from the data shown in Figs. 1b and 2a. For DPN this value is $2.5 \cdot 10^{-4}$ M and for $lip(SH)_2$ it is $5.3 \cdot 10^{-3}$ M. This latter value is somewhat lower than that reported by SANADI AND SEARLS[15].

Coenzyme specificity of the lipoic dehydrogenase

In all reactions catalyzed by the lipoic dehydrogenase of H37Ra DPN or DPNH cannot be replaced by TPN or TPNH.

Presence of alcohol dehydrogenase in lipoic dehydrogenase preparations

CUTULO[19] reported recently the isolation of a DPN-linked lipoic dehydrogenase from yeast. This enzyme was active as a general thiol dehydrogenase. A re-evaluation of this enzyme by MACLEOD *et al.*[20] showed that the enzyme isolated was actually alcohol dehydrogenase. The substrate was a diffusible compound present in the undialyzed yeast enzyme preparations; the thiols changed the equilibrium constant of the alcohol dehydrogenase and were not substrates for the dehydrogenase. We have accordingly investigated the lipoic dehydrogenase of H37Ra for alcohol dehydrogenase activity. Under the conditions of the lipoic dehydrogenase assay, there was no evidence of alcohol dehydrogenase activity in our lipoic dehydrogenase preparation.

* The generous gift of Dr. ARTHUR WAGNER of the Research Laboratories, Merck Sharp and Dohme, Rahway, N. J.

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C. Purification and properties of the lipoic transactylase

Lipoic transacetylase assay

The lipoic transacetylase assay system is composed of two linked reactions catalyzed, respectively, by phosphotransacetylase (reaction 8) and lipoic transacetylase (reaction 9).

$$\text{acetyl-P} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + P_i \quad (8)$$

$$\text{acetyl CoA} + \text{lip(SH)}_2 \rightleftharpoons \text{S-acetyl-lip(SH)} + \text{CoA} \quad (9)$$

$$\text{acetyl P} + \text{lip(SH)}_2 \rightleftharpoons \text{S-acetyl-lip(SH)} + P_i \quad (10)$$

The assay system contains, in μmoles: tris buffer of pH 7.5 (80), acetyl-P (6.0), CoA (0.3), DL-lip(SH)$_2$ (5.0)*, phosphotransacetylase of *E. coli*[21] (0.06 unit) and lipoic transacetylase (0.5 to 2.0 mg). The final volume is 0.90 ml. Incubation is carried out at 30° for 10 min at which time the reaction is stopped by the addition of 0.10 ml 1.0 *N* HCl. Residual acetyl-P is destroyed by boiling the acidified mixture for 4 min. S-acetyl-lip(SH), which is stable under these conditions[10], is determined by the hydroxamic acid method of LIPMANN AND TUTTLE[11]. One unit of lipoic transacetylase is defined as that amount of enzyme which catalyzes the formation of 1.0 μmoles of S-acetyl-lip(SH) under the above conditions. Specific activity is defined as units of enzymic activity per mg of protein.

Purification of the lipoic transacetylase

Lipoic transacetylase activity is always concentrated in those fractions enriched in pyruvic dehydrogenase activity. Accordingly, the purification procedure is essentially identical to that already described for the pyruvic dehydrogenase[1]. The only changes in the procedure are in the dialysis steps. The lipoic transacetylase is rapidly and irreversibly inactivated by dialysis; this inactivation is markedly reduced if dialysis is carried out in a medium containing 0.05 *M* tris buffer of pH 7.5, 0.001 *M* $MgCl_2$ and 0.002 *M* cysteine. Results of a typical fractionation for the lipoic transacetylase are given in Table VIII. The anomolous increase in total units recovered will be discussed below.

TABLE VIII

PURIFICATION OF THE LIPOIC TRANSACETYLASE OF H37Ra

Fraction No.	*Volume ml*	*Protein*			*Activity*		
		mg per ml	*Total mg*	*Percent recovered*	*Specific activity*	*Total units*	*Percent recovered*
Crude extract	915	17.0	15,550	100			
AS-1	76	46.0	3,496	22	0.042	103	100
AS-2	27	59.1	1,596	10	0.093	148	144
0.05 *M* Gel El	30	14.8	437	0.28	0.69	437	426
0.10 *M* Gel El	50	5.16	258	0.17	0.60	154	150
Combined gel eluates			695	0.45		591	576

* Solutions of DL-lip(SH)$_2$ are prepared by suspending a weighed amount of DL-lip(SH)$_2$ in 0.01 *M* ethylenediaminetetraacetate; sufficient KOH is added to neutralize the free acid and the solution is diluted to final volume with water. Solutions of DL-lip(SH)$_2$ were prepared fresh daily.

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Characteristics of the assay system

The assay system is quite insensitive to changes in pH. Essentially identical rates of formation of S-acetyl-lip(SH) are obtained over the pH range of 7.0 to 8.2 in the standard assay system. The effect of variations in the concentrations of $lip(SH)_2$ and CoA, time and lipoic transacetylase are shown in Figs. 5 and 6. Phosphotransacetylase, which is always associated with the lipoic transacetylase-pyruvic dehydrogenase complex of H37Ra[2], is always in excess. Elimination of the *E. coli* phosphotransacetylase has little effect on the assay system. The purified lipoic transacetylase has an absolute requirement for $lip(SH)_2$, acetyl-P and CoA.

Extent of formation of S-acetyl-lip(SH)

Fig. 7 shows the results of an experiment in which the formation of S-acetyl-lip(SH) was followed for 40 minutes. The lipoic transacetylase fraction (2 mg) had a

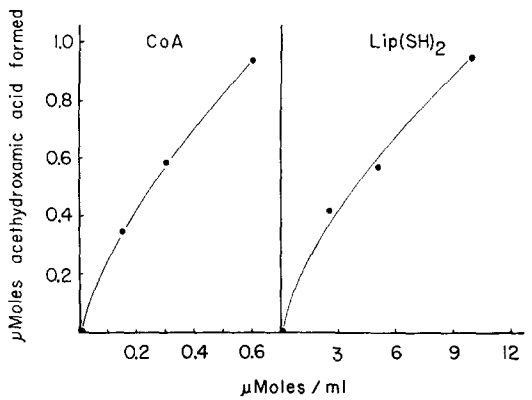

Fig. 5. Lipoic transacetylase activity as a function of the concentrations of CoA and $lip(SH)_2$ Lipoic transacetylase (1.59 mg) of specific activity 0.63 was used. Assay conditions as described in the text.

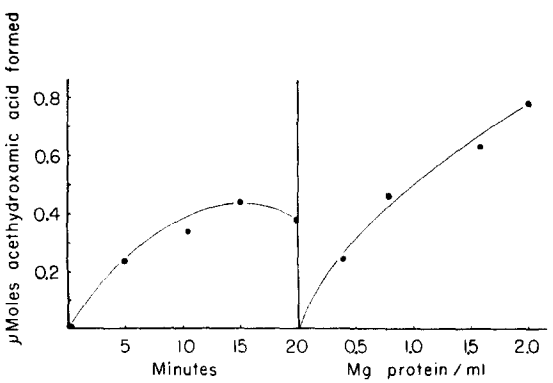

Fig. 6. Lipoic transacetylase activity as a function of time and enzyme concentration. Lipoic transacetylase (3.96 mg) of specific activity 0.63 was used in the first series.

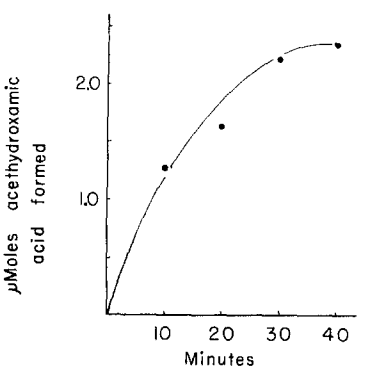

Fig. 7. Formation of S-acetyl-lip(SH) as a function of time. Each cuvette contained, in µmoles: tris buffer of pH 7.5 (80), DL-$lip(SH)_2$ (5), CoA (0.5), acetyl-P (6), phosphotransacetylase of specific activity 25 (2.2 µgm) and lipoic transacetylase. The final volume was 0.90 ml; incubation temperature was 30°. Acetyl P was destroyed and acethydroxamic acid determined as described in the text. Values shown for formation of hydroxamic acid have been corrected for no-enzyme blanks.

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specific activity of 0.64. After 40 minutes one-half of the added DL-lip(SH)$_2$ had been acetylated. This is in agreement with the work of GUNSALUS *et al.*[9] who showed that only L-lip(SH)$_2$ is acetylated.

Lipoic transacetylase has been used to prepare substantial amounts of S-acetyl-lip(SH). In a typical experiment the reaction mixture contained, in μmoles, tris buffer of pH 7.5 (150), DL-lip(SH)$_2$ (40), CoA (0.75), acetyl-P (30), phosphotransacetylase (0.18 unit) and lipoic transacetylase (4.8 units). The final volume was 2.40 ml. The reaction mixture was incubated under nitrogen for 75 minutes at 38°; 0.25 ml of 1.0 *N* HCl was added and the acidified mixture was boiled for 3 minutes. A sample was removed and assayed for acid-stable hydroxamic acid; 21.6 μmoles were found. S-acetyl-lip(SH) and unreacted lip(SH)$_2$ were removed from the acidified mixture by repeated extractions at 0° with benzene. The benzene extracts were combined and evaporated to dryness. A yellow solid was recovered which was brought into solution in 15 % ethanol with sufficient KOH to bring the pH to about 6.5. Assay of this solution for hydroxamic acid showed 17 μmoles S-acetyl-lip(SH) to be present.

Chromatography of acethydroxamic acid

The conversion of the acid-labile acetyl- of acetyl-phosphate to the acid-stable acetyl- of acetyl-lip(SH) was shown by chromatography of the hydroxamic acid. A lipoic transacetylase assay system was prepared as described above. Control tubes were prepared containing a) no lipoic transacetylase and b) no acetylphosphate. Incubation was carried out for 60 min at 30°. Sufficient HCl was added to bring the pH to 1 and the reaction mixtures were boiled. After neutralization, the mixtures were reacted with NH_2OH. The hydroxamic acids were extracted and chromatographed on paper[22] in two different solvent systems. A spot corresponding to acethydroxamic acid was obtained only from the chromatographed complete reaction system.

Absorption spectrum of S-acetyl-lip(SH)

The heat-stable acetylated lipoic acid formed in the above experiments shows a strong absorption in the ultra-violet region (Fig. 8). Absorption at about 235 mμ is characteristic of acetylated thiols[23].

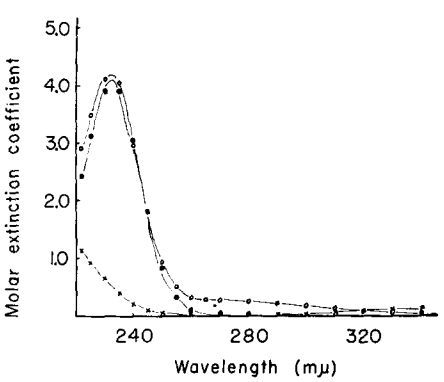

Fig. 8. Absorption spectrum of S-acetyl-lip(SH). O—O, S-acetyl-lip(SH) formed enzymatically, ●—●, 8-S-acetyl-lip(SH) formed by the reaction of acetic anhydride with DL-lip(SH)$_2$. ×—×. DL-lipS$_2$.

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S-acetyl-lip(SH) deacylase

It is apparent from the data of Table VIII that more units of lipoic transacetylase activity are recovered than were present in the crude cell-free extract. This apparent increase in total transacetylase activity is actually caused by the partial removal, during the purification of the transacetylase, of an enzyme which deacylates S-acetyl-lip(SH).

$$\text{S-acetyl-lip(SH)} + H_2O \longrightarrow \text{lip(SH)}_2 + \text{acetate} \qquad (11)$$

The action of this enzyme was shown by the following experiment. A reaction mixture containing four times the quantities normally present in the standard assay system was prepared. This mixture was incubated for 20 min at 30°, acidified with HCl and boiled for 3 min. The boiled mixture was chilled in ice, neutralized with KOH and centrifuged. Analysis of the neutralized mixture showed that it contained 1.76 μmoles of S-acetyl-lip(SH) per ml. Samples containing 1.41 μmoles of S-acetyl-lip(SH), were incubated for 10 min at 30° with 0, 1.38 and 2.30 mg, respectively, of a crude acetyl-lipoic deacylase preparation (corresponding to the AS-1 fraction of the transacetylase) and 0, 0.056 and 0.086 μmoles of S-acetyl-lip(SH), respectively, disappeared from the incubation mixtures. The deacylase does not hydrolyze acetyl CoA or S-acetyl-glutathione.

General thioltransacetylase activity

BRADY AND STADTMAN[23] isolated from pigeon liver a series of thioltransacetylases which catalyze the general reaction:

$$\text{S-acetyl-CoA} + \text{R-SH} \rightleftharpoons \text{S-acetyl-R} + \text{CoA-SH} \qquad (12)$$

A series of sulfhydryl compounds were reported to be active as acetyl acceptors.

If reduced glutathione is used as acetyl acceptor in place of lip(SH)_2 in the H37Ra lipoic transacetylase assay system a small but measurable amount of S-acetyl-glutathione is formed. The rate of formation of S-acetyl-glutathione is about 10 % of the rate of formation of S-acetyl-lip(SH) under equivalent conditions. This is in agreement with the data of BRADY AND STADTMAN. The relationships between the several lipoic transacetylases and the thioltransacetylases is not yet clear. The thioltransacetylase of BRADY AND STADTMAN, after considerable purification, reacts with several thiols as acceptors. Neither the lipoic transacetylase of *E. coli* nor that of H37Ra has been sufficiently purified to permit a critical study of thiol acceptors.

Inhibition of the lipoic transacetylase reaction by $lipS_2$

In experiments designed to show a possible stimulatory effect of lipS_2 on the DPN-pyruvic dehydrogenase system (reaction 3), DPNH formation was consistently inhibited when lipS_2 was added to the system. These anomolous results were clarified by a study of the effect of lipS_2 on the lipoic transacetylase system. The inhibition of lipoic transacetylase by lipS_2 is shown in Table IX. These results explain our inability 1) to demonstrate the utilization of S-acetyl-lip(SH) as an acetyl donor for the enzymic formation of acetyl CoA (reaction 6), and 2) to show the reversal of the lipoic dehydrogenase reaction by coupling the dehydrogenase to lipoic- and phospho-transacetylases. In both these systems the concentration of lipS_2 added either as such or as a contaminant of S-acetyl-lip(SH) preparations is sufficient to completely inhibit the lipoic transacetylase.

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TABLE IX

INHIBITION BY $lipS_2$ OF THE LIPOIC TRANSACETYLASE REACTION

Conditions were those of the standard lipoic transacetylase assay

Experiment No.	*μmoles* DL-*lip*S_2 *added*	*μmoles S-acetyl-lip(SH) formed*
1	0	0.51
2	1.0	0.31
3	2.5	0.16

DISCUSSION

DPN-linked lipoic dehydrogenases have been isolated from extracts of *E. coli*[3,7] and other microorganisms[24]. Previous work by DOLIN AND GUNSALUS[25] and by KORKES *et al.*[6] showed that the pyruvic dehydrogenase systems of both *E. coli* and *Streptococcus faecalis* can be resolved into two enzymic components, A and B. HAGER AND GUNSALUS[7] subsequently demonstrated that the effectiveness of fraction B in reactivating the resolved pyruvic dehydrogenase system is proportional to the content of lipoic dehydrogenase in this fraction. Of particular interest is that all investigators who have studied the lipoic dehydrogenase reaction have noted that the enzyme requires extremely high concentrations of $lip(SH)_2$ for maximal activity. A possible explanation for these observations is that in the isolated dehydrogenase reaction the coenzyme form of α-lipoic acid is tightly bound to the dehydrogenase. The free acid may be visualized as either exchanging with or being reduced by the tightly bound form; thus a high concentration of the free coenzyme might be required for maximum rates of hydrogen transfer.

The possibility must also be considered that neither free lipoic acid itself nor a firmly bound form of lipoic acid is the actual coenzyme of the pyruvic dehydrogenase. This possibility is supported by the activity of lipoamide in the lipoic dehydrogenase system. This activity, originally reported by SANADI AND SEARLS[15] has been confirmed by GUNSALUS[24], by REED[26] and in the present report. Lipoamide may be structurally closer to the true coenzyme form than lipoic acid itself.

REED[26] has recently presented evidence for lipoic acid activation by adenosinetriphosphate. Lipoyl-adenylate is more active than either $lipS_2$ or lipoamide in a pyruvate dismutation system. Lipoyl-adenylate would presumably be bound into the complex of enzymes which comprises the pyruvic dehydrogenase system and the lipoate moiety would undergo reductive acetylation, deacetylation and oxidation. If this hypothesis is correct then free lipoic acid may be considered as either binding very poorly to the resolved lipoic dehydrogenase or exchanging very slowly with the bound lipoyl-adenylate.

In relating the activities of the isolated lipoic dehydrogenase and lipoic transacetylase to the metabolic activities of the intact cell certain factors must constantly be borne in mind. The experimental conditions necessary for the demonstration of these enzymic activities have no relation to physiological reality; the *in vivo* concentration of lipoic acid is vanishingly small. It must be remembered that the conditions for the isolation of lipoic acid from cells are quite harsh[27]. The instability of lipoic acid and its derivatives is well known[28]. The possibility exists that the isolated

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coenzyme, α-lipoic acid, is a relatively stable fragment or transformation product of the true coenzyme of α-keto-acid oxidation.

A consideration of these very low concentrations of lipoic acid in cells leads to the conclusion that, *in vivo*, this coenzyme probably does not exist in a free form but is bound firmly to its apo-enzymes[26]. It therefore becomes necessary to study the pyruvic dehydrogenase and the acetyl transfer reactions as part of a complex of enzymic activities and not as isolated reactions.

The pyruvic dehydrogenase system of H37Ra appears to be part of an enzyme complex which catalyzes several reactions involving pyruvate. Our evidence for the presence of such a complex is that a group of related activities fractionates together through a series of purification steps[1]. We have not yet succeeded in separating the several enzymes one from another although three activities (lipoic transacetylase, phosphotransacetylase and alanine dehydrogenase[29]) may be eliminated by differential inactivation. Once the cell-free extract has been fractionated with ammonium sulfate into three major fractions[1] no significant dissociation of enzymic activities from the proposed complex can be detected during the purification sequence. The following enzymes have been shown to be present in this complex: pyruvic dehydrogenase, lipoic transacetylase, phosphotransacetylase, acetyl-lipoic deacylase, lipoic dehydrogenase and alanine dehydrogenase. The lipoic and alanine dehydrogenases exist in both a bound and a free form. The free form of each has been extensively purified. SCHWEET *et al.*[30] purified the pyruvic dehydrogenase of pigeon breast muscle. The purified enzyme has a molecular weight of about four million, considerably higher than the molecular weight of other pyridinoproteins. As the pyruvic dehydrogenase is purified it becomes enriched in bound lipoic acid[31]. Isolation of a high molecular weight enzyme complex containing a small amount of tightly bound α-keto-acid oxidation coenzyme lends support for the hypothesis that the pyruvic dehydrogenase is part of an enzyme complex and would also account for the low *in vivo* concentration of α-lipoic acid.

While the presence of a soluble pyruvic dehydrogenase complex in extracts of H37Ra is an attractive hypothesis the evidence in support of such an hypothesis is insufficient to warrant drawing definite conclusions.

ADDENDUM IN PROOF

Further evidence in support of the hypothesis that α-lipoic acid may not be the physiologically active form of this coenzyme has been recently presented by MASSEY[32]. In his study of the lipoic dehydrogenase activity of pig heart diaphorase, MASSEY showed that DL-α-lipoamide is approximately 350 times more active than DL-α-lipoic acid as substrate for this coupled reaction system.

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Fe++-DEPENDENT ALKALINE PHOSPHATASE OF YEAST

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SUMMARY

The common occurence of a ferrous iron-dependent alkaline phosphatase in crystalline preparations of yeast alcohol dehydrogenase is reported. Some of the properties of the enzyme together with a method for separating it from ethanol dehydrogenase are described.

INTRODUCTION

Brewer's yeast contains a specialized phosphatase that cleaves *p*-nitrophenyl phosphate to equimolar amounts of orthophosphate and *p*-nitrophenol. This activity on

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