STUDIES ON THE NATURE AND REACTIONS OF PROTEIN-BOUND LIPOIC ACID

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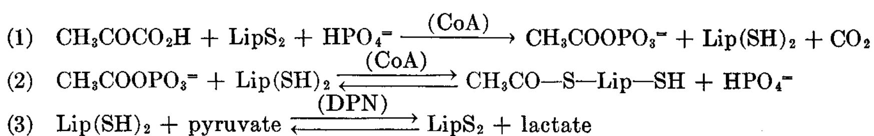
Studies on the activation of an apopyruvate dehydrogenation system obtained from extracts of lipoic acid-deficient *Streptococcus faecalis* cells indicated that in its functional form lipoic acid is bound to protein in covalent linkage through its carboxyl group, *i.e.* as "lipoyl enzyme" (1). Further support for this proposal is furnished by the present finding that a partially purified enzyme, "lipoyl-X hydrolase," obtained from *S. faecalis* extracts, liberated lipoic acid from the protein-bound form present in the *Escherichia coli* (Crookes strain) pyruvate dehydrogenation system, thereby inactivating the latter system. Reactivation required the same components and conditions as were found necessary to activate the *S. faecalis* apopyruvate dehydrogenation system. A preliminary communication of this work has been reported elsewhere (2).

The availability of a method of releasing lipoic acid from the protein-bound form and of reactivating the apoenzyme has enabled us to study the mechanism of certain enzymatic reactions which have been carried out with free lipoic acid or structurally related compounds. Gunsalus and his collaborators reported (3–6) that *E. coli* Fraction A, in the presence of CoA[1] and phosphotransacetylase, catalyzed Reactions 1 and 2, and that Fraction B, in the presence of DPN and lactic dehydrogenase, catalyzed Reaction 3.



(1) $CH_3COCO_2H + LipS_2 + HPO_4^{=} \xrightarrow{(CoA)} CH_3COOPO_3^{=} + Lip(SH)_2 + CO_2$

(2) $CH_3COOPO_3^{=} + Lip(SH)_2 \underset{}{\overset{(CoA)}{\rightleftharpoons}} CH_3CO{-}S{-}Lip{-}SH + HPO_4^{=}$

(3) $Lip(SH)_2 + \text{pyruvate} \overset{(DPN)}{\rightleftharpoons} LipS_2 + \text{lactate}$

The mechanism of Reaction 1 is uncertain. It has been established (3, 6) that Reaction 2 involves a coupling of Reactions 4 and 5 and that Reaction 3 involves a coupling of Reactions 6 and 7.

[1] The following abbreviations are used: adenosine triphosphate, ATP; coenzyme A, CoA and CoA-SH; thiamine pyrophosphate, TPP; diphosphopyridine nucleotide, DPN; lipoic acid, $lipS_2$; dihydrolipoic acid, $lip(SH)_2$.

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(4) $CH_3COOPO_3^{=} + HS—CoA \rightleftarrows CH_3CO—S—CoA$
$+ HPO_4^{=}$ (phosphotransacetylase)

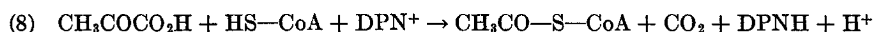
(5) $CH_3CO—S—CoA + Lip(SH)_2 \rightleftarrows CH_3CO—S—Lip—SH$
$+ HS—CoA$ (dihydrolipoic transacetylase)

(6) $Lip(SH)_2 + DPN^+ \rightleftarrows LipS_2 + DPNH + H^+$ (dihydrolipoic dehydrogenase)

(7) $DPNH + H^+ +$ pyruvate $\rightleftarrows DPN^+ +$ lactate (lactic dehydrogenase)

Reaction 6, with free lipoic acid or lipoamide as substrate, is catalyzed also by a purified mammalian α-ketoglutarate dehydrogenation complex (7).

E. coli Fraction A plus Fraction B catalyze Reaction 8 (3), and the α-ketoglutarate dehydrogenation complex catalyzes a similar reaction with α-ketoglutarate as substrate (8).



(8) $CH_3COCO_2H + HS—CoA + DPN^+ \rightarrow CH_3CO—S—CoA + CO_2 + DPNH + H^+$

Since Fraction A and the α-ketoglutarate dehydrogenation complex contain protein-bound lipoic acid (5, 9), which presumably functions catalytically in Reaction 8, it has not been possible to decide whether the reactions which occur with free lipoic acid, dihydrolipoic acid, or the corresponding amides involve (*a*) exchange between free and protein-bound lipoic acid; (*b*) coupling between free and protein-bound lipoic acid; or (*c*) reaction of the appropriate enzyme with free material. A basis for deciding which of these mechanisms is applicable to Reactions 1, 5, and 6 is provided in the present communication.

Materials and Methods

Pertinent information concerning preparation of enzyme fractions, source of materials, and assay procedures has been provided in an accompanying communication (1). Methyl *dl*-lipoate and methyl *dl*-dihydrolipoate were prepared as described by Gunsalus *et al.* (10). *dl*-7,9-Dithiolnonanoic acid was prepared by sodium borohydride reduction (10) of *dl*-1,2-dithiolane-3-caproic acid (11). The product was a colorless liquid; iodine equivalents, 110 (calculated, 111).

Synthesis of Lipoamides—*dl*-Lipoamide, *dl*-*N*,*N*-diethyllipoamide, and *dl*-lipoanilide were synthesized[2] in 12 to 16 per cent yield from *dl*-lipoic acid, through *dl*-lipoyl chloride, by a method similar to that reported by Wagner *et al.* (12) for the preparation of *dl*-lipoamide. *dl*-Lipoamide was obtained as yellow crystals from benzene-Skellysolve B (*n*-hexane); m.p. 129–130° (uncorrected); $\lambda_{max}^{95 \text{ per cent ethanol}}$ 332 mμ (ε 143).

$C_8H_{15}NOS_2$ (205.33).	Calculated.	C 46.79, H 7.36, N 6.82
	Found.	" 47.07, " 7.03, " 6.47

[2] Thomas, R. C., and Reed, L. J., unpublished results.

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dl-N,N-Diethyllipoamide was obtained as a yellow oil; n_D^{25} 1.5347°; $\lambda_{max}^{95 \text{ per cent ethanol}}$ 332 mμ (ϵ 143).

$C_{12}H_{23}NOS_2$ (261.44).	Calculated.	C 55.13, H 8.87, N 5.36
	Found.	" 54.86, " 8.68, " 5.09

dl-Lipoanilide was obtained as yellow crystals from benzene-Skellysolve B; m.p. 72–73° (uncorrected). The ultraviolet absorption spectrum of this compound exhibited a plateau in the 290 to 330 mμ region.

$C_{14}H_{19}NOS_2$ (281.43).	Calculated.	C 59.75, H 6.81, N 4.98
	Found.	" 60.15, " 6.93, " 4.84

dl-p-Carbethoxylipoanilide was prepared from *dl*-lipoic acid via *dl*-lipoic anhydride (1). To a stirred solution of 1.0 gm. of *dl*-lipoic acid in 5 ml. of benzene was added 0.5 gm. of *N,N'*-dicyclohexylcarbodiimide. After standing at room temperature for 1 hour, the mixture was filtered, and to the filtrate was added 0.825 gm. of ethyl *p*-aminobenzoate. The solution was allowed to stand overnight at room temperature and then was heated for 2 hours at 55–65°. The reaction mixture was washed successively with 1 N HCl, water, 5 per cent $NaHCO_3$, and finally with water. The organic layer was dried and then evaporated *in vacuo*. The residue was crystallized from benzene-Skellysolve B to yield 781 mg. (45 per cent) of yellow crystals; m.p. 83–84° (uncorrected); $\lambda_{max}^{CH_3OH}$ 332 mμ (ϵ 150).

$C_{17}H_{23}NO_3S_2$ (353.49).	Calculated.	C 57.76, H 6.56, N 3.96
	Found.	" 58.25, " 6.78, " 4.50

An improved synthesis of *dl*-lipoamide was devised, based on the mixed carbonic-carboxylic anhydride method (13). To a stirred solution of 1.03 gm. (5 mmoles) of *dl*-lipoic acid and 0.51 gm. (5 mmoles) of triethylamine in 10 ml. of tetrahydrofuran at −5° was added dropwise a solution of 0.68 gm. (5 mmoles) of isobutyl chloroformate (Eastman Kodak) in 2 ml. of tetrahydrofuran. After 10 minutes at this temperature, 10 ml. of cold tetrahydrofuran saturated with anhydrous ammonia were added, and anhydrous ammonia was bubbled into the reaction mixture for approximately 10 minutes. The mixture was allowed to warm to room temperature and then the solvent was removed *in vacuo*. The residue was extracted with a total of 30 ml. of warm chloroform. The extract was washed successively with 10 ml. portions of 1 N HCl, water, 5 per cent $KHCO_3$, and finally water. The organic layer was dried over anhydrous sodium sulfate and the solvent was removed *in vacuo*. The yellow solid was recrystallized from 4.5 ml. of 95 per cent ethanol to yield 822 mg. (82 per cent) of yellow plates; m.p. 130–131° (uncorrected). A mixture of the product and an authentic sample of *dl*-lipoamide melted at 130–131° (uncorrected).

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dl-Dihydrolipoamide was prepared by reduction of *dl*-lipoamide with sodium borohydride. A suspension of 200 mg. of *dl*-lipoamide in 4 ml. of methanol and 1 ml. of water was cooled to 0° and stirred while a cold solution of 200 mg. of sodium borohydride in 1 ml. of water was added. The reaction mixture was stirred until it became clear and colorless (approximately 45 minutes). The solution was acidified with dilute hydrochloric acid and extracted with chloroform. The chloroform extract was dried and evaporated *in vacuo*. The residue was crystallized from benzene-Skellysolve B (2.5:1) to yield 172 mg. (83 per cent) of white plates; m.p. 66–67° (uncorrected); iodine equivalents, 102.4 (calculated 103.7).

$C_8H_{17}NOS_2$ (207.36). Calculated. C 46.34, H 8.27, N 6.76
Found. " 46.81, " 8.22, " 7.24

Assay Procedures

p-Carbethoxylipoanilide-Hydrolyzing Enzyme—The reaction mixture contained 40 γ of *p*-carbethoxylipoanilide, enzyme, and 0.02 M potassium phosphate buffer (pH 7.0) in a total volume of 1.0 ml. After 1 hour at 30° the reaction was stopped by the addition of 1 ml. of 15 per cent trichloroacetic acid. The mixture was diluted to 5 ml., centrifuged, and diazotizable amine was determined by the Bratton-Marshall procedure (14). 1 unit corresponds to the production of 1 γ of ethyl *p*-aminobenzoate per hour. The response was linear for 1 to 6 units.

Lipoyl-X Hydrolase—The reaction mixture contained 4 units of *E. coli* pyruvate dehydrogenation system (1) (specific activity 40 to 50), enzyme, and 0.02 M potassium phosphate buffer (pH 7.0) in a total volume of 0.25 ml. The mixture was incubated for 1 hour at 30° and then assayed for pyruvate dismutation activity as described previously (1). 1 unit of lipoyl-X hydrolase is defined as that amount of enzyme which produced a loss of 1 unit of pyruvate dismutation activity under these conditions. The response was reasonably linear for 0.5 to 3.5 units. Pyruvate dismutation activity is expressed as micromoles of acetyl phosphate produced in 30 minutes, unless specified otherwise.

The assay system for Reaction 1 was essentially the same as that described by Chin and Gunsalus (4, 5). Dihydrolipoic transacetylase and dihydrolipoic dehydrogenase activities were determined according to the procedures of Hager and Gunsalus (15, 3). Acetyl phosphate and thio ester were determined by the hydroxamic acid procedure of Lipmann and Tuttle (16), and sulfhydryl by the method of Boyer (17) or Hager (15).

Results

Activity of Lipoamides with Enzyme Preparations from S. faecalis—During the course of studies with cell-free extracts of lipoic acid-deficient *S. faecalis*,

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several amides of lipoic acid were synthesized for testing as antagonists of lipoic acid. These derivatives did not inhibit "activation" of the cell-free

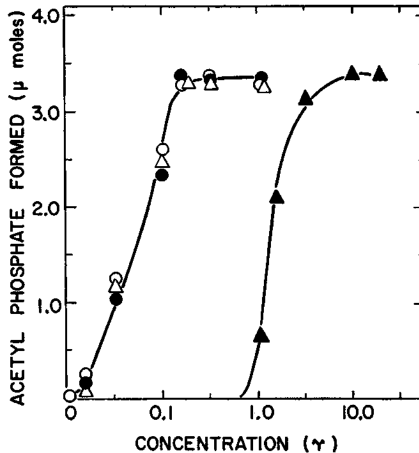

FIG. 1. Activation of cell-free *S. faecalis* extract by amides of lipoic acid. The reaction mixtures contained extract (4.3 mg. of protein), 0.8 μmole of $MgSO_4$, 0.04 μmole of TPP, 4 μmoles of potassium phosphate buffer (pH 7.0), and varying amounts of lipoic acid, ○, lipoamide, ●, lipoanilide, △, or *N*,*N*-diethyllipoamide, ▲, in a final volume of 0.2 ml. The mixtures were incubated for 1 hour at 30° and then assayed for pyruvate dismutation activity.

TABLE I

Activity of Amides with S. faecalis Enzyme Fractions

Compound	Preincubation mixture	Acetyl phosphate formed
		μmoles
Lipoic acid	Complete	4.5
	No ATP	0.4
Lipoamide	Complete	4.5
	No ATP	0.4
Lipoanilide	Complete	4.5
	No ATP	0.5

The complete system contained *S. faecalis* Fraction PP-1 (360 γ of protein), Fraction PS-2A (225 γ of protein), Fraction PS-2B (115 γ of protein), and 0.005 μmole of *dl*-lipoic acid or the amides. Other components and conditions were as described previously (1).

extract by lipoic acid. On the contrary, these amides were found to be capable of replacing lipoic acid (Fig. 1). The amides also replaced lipoic acid when partially purified preparations of the apopyruvate dehydrogenation system (Fraction PP-1) and the lipoic acid-activating system (Fractions PS-2A and PS-2B) were employed (Table I). It is significant that

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ATP was required with the amides as well as with lipoic acid to produce an active pyruvate dehydrogenation system. This observation suggested that hydrolysis of the amides occurred during incubation with the cell-free extract or the partially purified enzyme preparations, since the ATP requirement was shown previously (1) to be associated with activation of the carboxyl group of lipoic acid. To test this hypothesis, as well as to obtain a potentially useful substrate to follow enzyme purification, *p*-carbethoxylipoanilide was synthesized. It was observed that a diazotizable

TABLE II

Release of Lipoic Acid from E. coli Pyruvate Dehydrogenation System

Sample	Acetyl phosphate formed	Lipoic acid content
	μmoles per mg. protein	*γ per mg. protein*
E. coli preparation	43	0.41
Incubation mixture	3*	0.05*
Dialysate		0.45*

2 ml. of *E. coli* pyruvate dehydrogenation system (1) (4.2 mg. of protein) and 2 ml. of gel-treated *S. faecalis* Fraction PS-1 (25 mg. of protein) were incubated for 1 hour at 30°. An aliquot (0.1 ml.) of the incubation mixture was assayed for pyruvate dismutation activity and the remainder was dialyzed for 12 hours with stirring against 36 ml. of distilled water. A mixture of 2 ml. of the *E. coli* preparation and 2 ml. of 0.02 M potassium phosphate buffer (pH 7.0) served as a control, and was treated in the same manner. An equal volume of 4 N HCl was added to each of the dialyzed enzyme preparations and the resulting mixtures were autoclaved for 1 hour at 15 pounds. The hydrolysates were adjusted to pH 7.0 with dilute KOH and aliquots were assayed for lipoic acid by the manometric assay of Gunsalus *et al.* (18). The dialysate was not autoclaved with acid prior to assay.

* These results are based on the amount of *E. coli* preparation employed. The *S. faecalis* preparation did not exhibit a detectable amount of pyruvate dismutation activity; nor did it contain a detectable amount of lipoic acid.

amine, presumably ethyl *p*-aminobenzoate, was produced when *p*-carbethoxylipoanilide was incubated with the *S. faecalis* extract. A survey of the *S. faecalis* fractions obtained previously (1) revealed that approximately 70 per cent of the hydrolytic activity of the extract was present in Fraction PS-1. It should be mentioned that this fraction, as usually obtained, exhibited little, if any, activity in the lipoic acid-activating system assay (1).

Release of Lipoic Acid from Pyruvate Dehydrogenation Systems—When a partially purified *E. coli* pyruvate dehydrogenation system was incubated for 1 hour at 30° with *S. faecalis* Fraction PS-1, the activity of the former system was decreased markedly (Table II). Shorter periods of incubation resulted in less inactivation. The incubation mixture was dialyzed, and

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the dialyzed preparation, the dialysate, and a dialyzed sample of the *E. coli* pyruvate dehydrogenation system were assayed for lipoic acid. The enzyme preparations were autoclaved with dilute acid before assay to release lipoic acid from the protein-bound form. The assay results (Table II) indicate that incubation of the *E. coli* preparation with Fraction PS-1 released approximately 88 per cent of the protein-bound lipoic acid in a dialyzable form. The fact that slightly more lipoic acid activity was found in the dialysate than in the original *E. coli* preparation is probably due to partial destruction of lipoic acid during acid hydrolysis of the enzyme preparations (12, 19).

A 10 ml. portion of the dialysate was reduced in volume to 1 ml. by lyophilization. Bioautographs of this sample, prepared as described previously (20), showed only zones of growth at R_F values corresponding to α- and β-lipoic acids (21).

Additional evidence that Fraction PS-1 releases lipoic acid from the protein-bound form was furnished by the observation that this fraction released radioactive lipoic acid from a pyruvate dehydrogenation system (Fraction PP-1*) prepared from an *S. faecalis* extract which had been preincubated with radioactive lipoic acid (1). Thus, incubation of 1 ml. of Fraction PP-1* (11 mg. of protein, specific activity 6.4, 0.032 γ of radioactive lipoic acid per mg. of protein) with 0.3 ml. of Fraction PS-1 (0.5 to 0.7 ammonium sulfate fraction, 2.7 mg. of protein, specific activity 21) for 1 hour at 30° resulted in essentially complete inactivation of the former preparation and released approximately 88 per cent of the radioactivity in a dialyzable form, which was identified as lipoic acid by means of radioautographs (22).

Reactivation of E. coli and S. faecalis Apopyruvate Dehydrogenation Systems—If inactivation of the pyruvate dehydrogenation systems by *S. faecalis* Fraction PS-1 were due simply to release of lipoic acid from the protein-bound form, it would be anticipated that the inactive preparations could be reactivated in the same manner as was found necessary to activate the apopyruvate dehydrogenation system obtained from cell-free extracts of lipoic acid-deficient *S. faecalis* (1). That such is the case is indicated by the data in Table III. It was found that the inactive *E. coli* and *S. faecalis* preparations could be reactivated by incubation with lipoic acid and ATP (or synthetic lipoyl adenylate) and the lipoic acid-activating system from either *E. coli* or *S. faecalis*. It should be mentioned that a minimal amount of lipoyl-X hydrolase was used to inactivate the pyruvate dehydrogenation systems, and an excess of lipoic acid, ATP, and the lipoic acid-activating system was used to reactivate the inactive preparations. When an excess of lipoyl-X hydrolase was employed, it was necessary to separate this enzyme from the apopyruvate dehydro-

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genation system in order to obtain complete reactivation of the latter system. This separation was accomplished, in the case of the *E. coli* preparation, by adjusting the pH of the incubation mixture to 4.5 to 5.0. The apopyruvate dehydrogenation system, but not lipoyl-X hydrolase, precipitated at this pH.

Further Purification of Lipoyl-X Hydrolase[3]—Further purification of Fraction PS-1 resulted in a separation of the activities associated with

TABLE III

Reactivation of Apopyruvate Dehydrogenation Systems

Preincubation mixtures	Acetyl phosphate formed	
	E. coli	*S. faecalis*
	μmoles	*μmoles*
Complete	1.6	2.2
No lipoic acid	0	0.1
" ATP	0.2	0.1
" lipoic acid-activating system	0	0
Lipoyl adenylate instead of lipoic acid and ATP	1.8	2.0
Control*	1.8	2.0

The pyruvate dehydrogenation systems were inactivated as described in the text, and then dialyzed with stirring for 12 hours against 1 liter of 0.02 M potassium phosphate buffer (pH 7.0). The complete system contained 0.02 μmole of *dl*-lipoic acid and 0.02 μmole of ATP, or 0.04 μmole of lipoyl adenylate, gel-treated *E. coli* Fraction PS′-1 (The results were essentially the same when *S. faecalis* Fraction PS-2 was used as the source of the lipoic acid-activating sytem.) (1) (240 γ of protein), and inactivated *E. coli* pyruvate dehydrogenation system (equivalent to 42 γ of control) or inactivated *S. faecalis* pyruvate dehydrogenation system (equivalent to 425 γ of control). Other components and conditions were as described previously (1).

* Preparations of the pyruvate dehydrogenation systems were incubated for 1 hour with 0.02 M phosphate buffer (pH 7.0) instead of Fraction PS-1, and then dialyzed as described above.

hydrolysis of *p*-carbethoxylipoanilide and "lipoyl-X" (Table IV). A representative fractionation was carried out (at 4°) as follows: 10 ml. of Fraction PS-1 were adjusted to pH 6.0 with 0.1 N hydrochloric acid and then stirred for 30 minutes with aged (1 year) calcium phosphate gel (23) (pH 6.0, 1 mg., dry weight, per mg. of protein). The gel was collected by

[3] The presence of the lipoic acid-activating system in *S. faecalis* extract and Fraction PS presented technical difficulties in measuring the lipoyl-X hydrolase activity of these two preparations by the assay procedure described under "Materials and methods." Consequently, figures are not available for the activity of these preparations.

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centrifugation and discarded. The supernatant fluid was fractionated with a saturated solution of ammonium sulfate adjusted to pH 7.0 (Beckman glass electrode) with concentrated ammonium hydroxide. The precipitate obtained between 0.5 and 0.7 saturation was dissolved in 3.5 ml. of 0.02 M potassium phosphate buffer (pH 7.0) and the solution was dialyzed with stirring for 4 hours against the same buffer.

It was observed subsequently that freshly prepared calcium phosphate gel adsorbed an appreciable amount of lipoyl-X hydrolase at a gel to protein ratio of 1:1. The active protein could be eluted with 0.1 M potassium phosphate buffer, pH 6.5. The enzyme which hydrolyzed the synthetic substrate, *p*-carbethoxylipoanilide, could be eluted with 0.1 M phosphate buffer, pH 7.4. This enzyme has not been investigated further.

Table IV

Purification of Lipoyl-X Hydrolase

Fraction	Volume	Protein	Activity		Recovery	
			Procedure A	Procedure B	Procedure A	Procedure B
	ml.	*mg.*	*units per mg. protein*	*units per mg. protein*	*per cent*	*per cent*
PS-1	10	230	3.9	3.5	100	100
Gel supernatant	10.5	115	3.9	6.4	50	92
0.5-0.7 $(NH_4)_2SO_4$	3.9	35	1.4	21.0	5	91

Procedure A = *p*-carbethoxylipoanilide-hydrolyzing enzyme assay; Procedure B = lipoyl-X hydrolase assay.

Lack of Exchange of Protein-Bound Lipoic Acid with Free Material in Model Reactions—A direct test of mechanism (*a*), *i.e.* exchange between protein-bound lipoic acid and free material, was performed with a preparation of the pyruvate dehydrogenation system (Fraction PP-1*) from *S. faecalis*, which contained protein-bound radioactive lipoic acid (1). Reactions 1, 2, and 3 were carried out in the presence of this preparation, with unlabeled lipoic acid, lipoamide, dihydrolipoic acid, or dihydrolipoamide. Aliquots of the reaction mixtures were assayed to determine the amount of free material utilized in the reactions, the remainder of each reaction mixture was dialyzed, and the radioactivity of the dialyzed preparations was measured. The results obtained (Table V) indicate that there was essentially no exchange between the protein-bound radioactive lipoic acid and the free, unlabeled lipoic acid, dihydrolipoic acid, or the corresponding amides during the course of Reactions 1, 2, and 3, even though the amount of free material utilized was approximately 5000 to 17,000 times the amount of radioactive lipoic acid bound to protein.

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Participation of Protein-Bound Lipoic Acid in Model Reactions—Evidence pertaining to mechanisms (*b*) and (*c*) was obtained by measuring the enzymatic activities, as exhibited in Reactions 1, 2, and 3, of pyruvate and apopyruvate dehydrogenation systems prepared from *S. faecalis* and *E. coli*. It was found (Table VI and Table VII, Experiment A) that

TABLE V

Lack of Exchange of Protein-Bound Lipoic Acid in Model Reactions

Assay system	Free material reacting		Bound lipoic acid remaining	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
	μmoles per mg. protein	*μmoles per mg. protein*	*γ per mg. protein*	*γ per mg. protein*
Reaction 1	1.2	1.5	0.031	0.030
" 2	0.7	0.8	0.031	0.031
" 3	2.6*	2.6*	0.031	0.031
Control			0.031	

The assay systems were similar to those described by Gunsalus and collaborators ("Materials and methods"). Each system contained 0.7 ml. of *S. faecalis* Fraction PP-1* (1) (7.7 mg. of protein; specific activity 6.4; 0.032 γ of protein-bound radioactive lipoic acid per mg. of protein) in a final volume of 2.0 ml. In Experiment 1, 20 μmoles of unlabeled potassium *dl*-lipoate (Reaction 1) or potassium *dl*-dihydrolipoate (Reactions 2 and 3) were employed and in Experiment 2, 20 μmoles of the corresponding amides (see footnote 4). Phosphotransacetylase was not added to the assay systems for Reactions 1 and 2 since Fraction PP-1* contained this enzyme (13 units per mg.). The reaction mixtures were incubated for 30 minutes (Reaction 3) or 1 hour (Reactions 1 and 2) and 0.5 ml. aliquots were removed for determination of the products formed. In Reaction 1, acetyl phosphate and dithiol were measured and were equivalent; in Reaction 2, heat-stable thio ester was measured, and in Reaction 3, the amount of dithiol oxidized. The remainder of each incubation mixture was dialyzed with agitation for 6 hours against three changes of 150 ml. each of 0.02 M potassium phosphate buffer (pH 7.0) and the radioactivity of 0.1 ml. aliquots of the dialyzed preparations was measured. The control tube contained 0.7 ml. of Fraction PP-1* and 1.3 ml. of 0.02 M phosphate buffer (pH 7.0). It was incubated for 1 hour at 30° and the contents were dialyzed as described above.

* These figures do not represent maximal rates since the amount of Fraction PP-1* employed (to facilitate measurement of radioactivity in the dialyzed reaction mixtures) furnished a large excess of dihydrolipoic dehydrogenase.

Reaction 1, but not Reactions 2 and 3, required protein-bound lipoic acid. Since previous data (Table V) indicated that protein-bound lipoic acid did not exchange with free lipoic acid during the course of Reaction 1, the present finding suggests that Reaction 1 involves mechanism (*b*), *i.e.* coupling between free and protein-bound lipoic acid. The data also in-

[4] In this and subsequent experiments, solutions of the amides in 95 per cent ethanol were employed at levels up to 0.05 ml. per ml. of reaction mixture. Control experiments showed that this amount of ethanol did not affect the results.

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dicate that Reactions 2 and 3 involve mechanism (*c*), *i.e.* reaction of the appropriate enzyme with free dihydrolipoic acid.

The data in Table VII, Experiment B, and in Table V, show that lipoamide and dihydrolipoamide can replace lipoic acid and dihydrolipoic acid, respectively, as substrates in the model reactions. With the amides, as well as the acids, protein-bound lipoic acid was required for Reaction 1, but not for Reactions 2 and 3. In additional experiments with the *E. coli* pyruvate dehydrogenation system it was observed that approximately 12 per cent of the hydroxylamine-reactive material produced in Reaction 1 with lipoamide as substrate was heat-stable and benzene-soluble, suggesting that it was a thio ester, presumably *S*-acetyl dihydrolipoamide. It was observed also that only 2.5 per cent as much hydroxylamine-reactive material was produced in the absence of exogenous CoA as was produced in the presence of CoA. Attempts to identify this small amount of material as a thio ester gave inconclusive results.

TABLE VI

Enzymatic Activities of Apopyruvate and Pyruvate Dehydrogenation Systems from S. faecalis

System	Activity measured				
	Pyruvate dismutation	Reaction 1		Reaction 2	Reaction 3
	Ac-P	Ac-P	SH/2	Thio ester	SH/2 oxidized
Unactivated	0	0	0	1.3	174
Activated	60	3.2	3.3	1.7	174

The results are expressed as micromoles of product formed per hour per mg. of protein. The enzymatic activities were determined as described under "Materials and methods." The amounts of *S. faecalis* apopyruvate dehydrogenation system (Fraction PP-1, specific activity 30 (1)) employed in the assays were as follows: pyruvate dismutation, 60 γ; Reaction 1, 600 γ; Reaction 2, 600 γ; Reaction 3, 60 γ. Fraction PP-1 was activated as described previously (1).

It should be noted (Table VII, Experiment B) that more than one-half of the *dl*-lipoamide or dihydrolipoamide was utilized in Reactions 1 and 3 but not in Reaction 2 (3.8, 3.2, and 2.3 of 5 μmoles, respectively). Other experiments with an excess of the *E. coli* pyruvate dehydrogenation system showed that 5 μmoles of *dl*-lipoamide or dihydrolipoamide were utilized completely in Reactions 1 and 3, but not more than 2.5 of 5 μmoles were utilized in Reaction 2. The significance of these results with regard to the mechanism of Reaction 1 will be discussed later. The data also confirm the finding of Gunsalus and collaborators (3, 6) that *E. coli* dihydrolipoic transacetylase, but not the dihydrolipoic dehydrogenase, exhibits optical

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specificity. The dihydrolipoic dehydrogenase from *S. faecalis* also does not exhibit optical specificity (*cf.* Table V and Gunsalus *et al.* (10)).

The data in Table VII support and extend the observation of Sanadi and Searls (7) that the dihydrolipoic dehydrogenase reaction (Reaction 6),

TABLE VII

Enzymatic Activities of Pyruvate and Apopyruvate Dehydrogenation Systems from E. coli

System	Activity measured				
	Pyruvate dismutation	Reaction 1		Reaction 2	Reaction 3
	Ac-P	Ac-P	SH/2	Thio ester	SH/2 oxidized
Experiment A					
Untreated	76	2.9	3.2	2.4	124
Inactivated	0	0.6	0.5	2.4	110
Reactivated	76	3.3	3.8	2.5	110
Experiment B					
Untreated		6.0	6.0	3.7	305
Inactivated		0.5	0.6	3.6	298
Reactivated		5.6	5.6	3.7	305

The results are expressed as micromoles of product formed per hour per mg. of protein. The amounts of *E. coli* pyruvate dehydrogenation system (specific activity 38 (1)) employed in the assays were as follows: pyruvate dismutation, 63 γ; Reaction 1, 630 γ; Reaction 2, 630 γ; Reaction 3, Experiment A, 42 γ; Experiment B, 21 γ. The assay conditions were the same as in Table VI, except that in Experiment A 5 μmoles of potassium *dl*-lipoate or *dl*-dihydrolipoate were employed, and in Experiment B 5 μmoles of *dl*-lipoamide or *dl*-dihydrolipoamide. Also, phosphotransacetylase was not added to the assay systems for Reactions 1 and 2 since the *E. coli* preparation contained this enzyme (66 units per mg.). The *E. coli* preparation was inactivated, dialyzed, and reactivated as described in Table III. The dihydrolipoic dehydrogenase activities of the inactivated and reactivated preparations were corrected for a blank activity exhibited by the lipoyl-X hydrolase preparation.

catalyzed by a mammalian α-ketoglutarate dehydrogenation complex, proceeded at a faster rate with free lipoamide as substrate than with free lipoic acid. Thus, the rate of Reaction 3, which is the sum of Reactions 6 and 7, was approximately 2.5 times as fast with dihydrolipoamide as with dihydrolipoic acid. Reactions 1 and 2 also proceeded at faster rates with the amides than with the corresponding acids. In a separate experiment with the *E. coli* pyruvate dehydrogenation system, it was observed that the rate of Reaction 1 with methyl lipoate as substrate was

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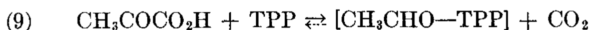
approximately twice that obtained with lipoic acid. The rates of Reactions 2 and 3 with methyl dihydrolipoate were approximately the same as with dihydrolipoic acid. It is also significant that the lipoic acid homologue, *dl*-1,2-dithiolane-3-caproic acid, produced no "activation" of the *S. faecalis* apopyruvate dehydrogenation system (1) at levels up to 10 γ, but was utilizable as substrate in Reaction 1. The reduced form of the homologue, *dl*-7,9-dithiolnonanoic acid, was utilizable in Reactions 2 and 3. The rates obtained with the homologues in the three reactions under assay conditions similar to those of Table VII were, respectively, approximately 70, 135, and 75 per cent of the rates obtained with lipoic acid and dihydrolipoic acid.

DISCUSSION

Before the present investigation Seaman and Naschke (24) reported that treatment of purified mammalian pyruvate and α-ketoglutarate dehydrogenation complexes with both alumina and an enzyme fraction from pigeon liver removed lipoic acid from the complexes and was accompanied by inactivation of the preparations. Activity was restored by adding a catalytic amount of lipoic acid to the treated preparations. It was suggested that the pigeon liver fraction catalyzed a reversible release of lipoic acid from the protein-bound form and that the liberated lipoic acid was adsorbed on the alumina. Our results indicate that lipoyl-X hydrolase cleaves a covalent bond linking the lipoyl moiety to protein, and that an energy-requiring reaction involving a different enzyme system is necessary to reform this bond. It is possible that lipoyl-X hydrolase, the lipoic acid-activating system, and ATP were present in the crude pigeon liver fraction.

Since the over-all pyruvate dehydrogenation reaction (Reaction 8) involves protein-bound lipoic acid, it would appear that intermediate reactions obtained with free lipoic acid or structurally related compounds can represent at best model reactions and not physiologically occurring reactions (25). The proposal of Gunsalus (5) that Reaction 1 involves a reductive acylation of free lipoic acid, followed by acyl transfer to CoA and then to phosphate, is difficult to reconcile with the present finding that protein-bound lipoic acid is required in the over-all reaction. Furthermore, the proposed reaction sequence is not consistent with the observation that both enantiomorphs of lipoamide can be utilized in Reaction 1, in view of the fact that dihydrolipoic transacetylase exhibits optical specificity. A reaction sequence which appears to be consistent with the data may be represented by Reactions 9 to 12 and the phosphotransacetylase reaction (Reaction 4).



$$(9) \quad CH_3COCO_2H + TPP \rightleftarrows [CH_3CHO\text{—}TPP] + CO_2$$

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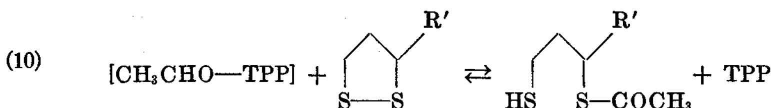
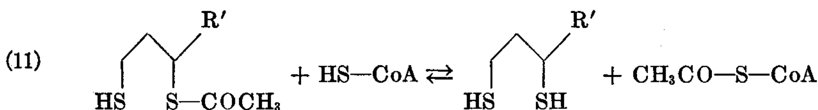
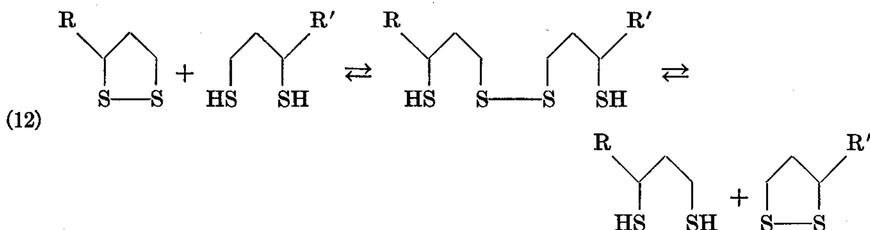
(10)



(11)



(12)



R = $—(CH_2)_4CO_2H$, $—(CH_2)_4CONH_2$, etc. R′ = $—(CH_2)_4CO$—enzyme

Reactions 9 to 11 were postulated previously (3) as intermediate steps in pyruvate dehydrogenation (Reaction 8). Reaction 12 represents a "disulfide interchange" reaction (26, 27) between protein-bound dihydrolipoic acid and free lipoic acid (or lipoamide, etc.). The formation of a small amount of free thio ester, presumably *S*-acetyl dihydrolipoamide, during the course of Reaction 1 with lipoamide as substrate may be attributed to interaction of acetyl CoA (produced in Reaction 11) and free dihydrolipoamide (produced in Reaction 12), catalyzed by dihydrolipoic transacetylase. The report (5) that a small amount of free thio ester, assumed to be *S*-acetyl dihydrolipoic acid, is formed in Reaction 1 in the absence of exogenous CoA appears to be in conflict with the reaction sequence proposed above. It is possible that the enzyme preparation (*E. coli* Fraction A) contained a small amount of CoA, and that the thio ester was formed as suggested above. An alternative possibility is that a reaction similar to Reaction 12 occurs between protein-bound *S*-acetyl dihydrolipoic acid and free lipoic acid. The latter reaction presumably would have to involve an acetyl transfer as well as a "disulfide interchange." In any event, it is apparent that further study of pyruvate oxidation in the presence of free disulfide (5, 28) is in order.

The finding that the dihydrolipoic transacetylase and dehydrogenase reactions, as obtained with free dihydrolipoic acid or structurally related dithiols, do not involve protein-bound lipoic acid indicates that these two enzymes can react directly with the free dithiols. The natural substrates for these enzymes are presumably protein-bound *S*-acetyl dihydrolipoic acid and dihydrolipoic acid, respectively. As mentioned previously, it is not yet possible to specify the nature of the group or the enzyme to which

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the lipoyl moiety is attached. The observation that lipoamide and dihydrolipoamide are more effective substrates in the model reactions than lipoic acid and dihydrolipoic acid or the corresponding methyl esters suggests that the lipoyl moiety may be attached to a basic group. From the evidence available it does not appear that the lipoyl moiety is attached to either dihydrolipoic transacetylase or dihydrolipoic dehydrogenase, but rather to an enzyme which catalyzes one of the earlier steps in pyruvate dehydrogenation, perhaps Reaction 10.

SUMMARY

1. An enzyme preparation from lipoic acid-deficient *Streptococcus faecalis* is described which released lipoic acid from the protein-bound form present in pyruvate dehydrogenation systems prepared from *Escherichia coli* and *S. faecalis*, yielding apopyruvate dehydrogenation systems.

2. Reactivation of the apopyruvate dehydrogenation systems required incubation of the preparations with lipoic acid and adenosine triphosphate (or synthetic lipoyl adenylate) and the lipoic acid-activating system from either *S. faecalis* or *E. coli*.

3. The formation of acetyl phosphate, CO_2, and free dihydrolipoic acid or structurally related dithiols from pyruvate and the corresponding free disulfides is mediated through protein-bound lipoic acid, but does not involve an exchange of protein-bound lipoic acid and free material. It is suggested that a "disulfide interchange" reaction between free disulfide and protein-bound dihydrolipoic acid is involved.

4. Evidence is presented that the dihydrolipoic transacetylase and dihydrolipoic dehydrogenase reactions, as obtained with free dihydrolipoic acid or structurally related dithiols, are not mediated through protein-bound lipoic acid. It appears that these enzymes react directly with the free dithiols.

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