ENZYME SYSTEMS IN THE MYCOBACTERIA

IX. THE REDUCTIVE ACETYLATION OF LIPOIC ACID*

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SUMMARY

The pyruvic dehydrogenase complex of the H37Ra strain of *Mycobacterium tuberculosis* catalyzes the reductive acetylation of lipoic acid. The reaction is inhibited by N-ethylmaleimide and not by arsenite. CoA does not participate in this reaction. A scheme is presented which attempts to unify these and other findings on the mechanism of the oxidative decarboxylation of pyruvate.

If the oxidative decarboxylation of [2-^{14}C]pyruvate is carried out in the absence of external oxidizing agents but in the presence of free acetaldehyde, the external acetaldehyde becomes labeled. This indicates that enzyme-bound acetaldehyde (or a compound which rearranges to acetaldehyde when it dissociates from the enzyme) is an intermediate in the oxidative decarboxylation of pyruvate.

INTRODUCTION

The participation of $lipS_2$ in the oxidative decarboxylation of pyruvate was demonstrated some years ago[3], however, its function was little understood. After the demonstration that "active acetyl" is the thiolester of acetate and CoA (see ref. 4) it was suggested by GUNSALUS[5] and by REED AND DEBUSK[6] that $lip(SH)_2$ serves in an analogous fashion. It was proposed that $lipS_2$ has the dual function of electron acceptor and acetyl acceptor. The product of this reaction, S-acetyl-lip(SH) could not be isolated. REED *et al.*[7] have investigated the role of both free and bound $lipS_2$ in the several reactions involved in pyruvate oxidation (see ref. 8 and 9 for recent reviews of this area of investigation).

We recently described the purification and properties of a soluble pyruvic dehydrogenase complex isolated from the H37Ra strain of *Mycobacterium tuberculosis*[10,11]. This enzyme complex carries out the reductive acetylation of $lipS_2$ by a mechanism similar to that first proposed by GUNSALUS[5] and by REED AND DEBUSK[6]. Enzyme-bound "acetaldehyde" is an intermediate in this reaction[5,12]. The reaction

Abbreviations: $lipS_2$ and $lip(SH)_2$, the oxidized and reduced forms of α-lipoic acid (1,2-dithiolane-3-valeric acid); S-acetyl-lip(SH), 6-S-acetyl-8-mercapto octanoic acid; Tris, tris(hydroxymethyl)amino methane; FAD, flavin adenine dinucleotide; DPN, diphosphopyridine nucleotide.

* For paper 8 in this series see[1]. A preliminary report of these findings has been published[2].

does not appear to involve CoA and, accordingly the mechanism differs from that suggested by SANADI *et al*[13] as applicable to the pyruvic and α-ketoglutaric dehydrogenases of mammalian muscle. While both the animal and the mycobacterial pyruvic dehydrogenases catalyze the oxidative decarboxylation of pyruvate and the formation of S-acetyl-CoA, there appear to be differences in the mechanisms of the reactions catalyzed by the two respective enzymes.

MATERIALS AND METHODS

The sources and preparation of enzymes and substrates, except as mentioned below, and analytical procedures are the same as previously described[10,11]. [2-^{14}C]pyruvate was obtained from Volk Radiochemical, Chicago ;(+)-α-lipS_2 was the gift of Dr. D. S. ACKER, duPont de Nemours and Co., and was also obtained by the resolution of commercial (±)-α-lipS_2 (see ref. 14). (±)-α-Lipoamide was the gift of Dr. D. R. SANADI and of Dr. A. F. WAGNER, Merck, Sharp and Dohme Research Laboratories.

The assay system for reductive acetylation contains, in a total volume of 0.25 ml, 50 μmoles of Tris buffer of pH 7.5, 4 μmoles of Li-pyruvate, 6 μmoles of (±)-α-lipS_2, 0.10 μmole of cocarboxylase, 2 μmoles of $MgCl_2$ and 0.2–2.0 mg of pyruvic dehydrogenase. The tubes are flushed with nitrogen, capped with a rubber stopper and incubated for 30 min at 30°. The tubes are opened and 0.25 ml of neutral, 2.5 *M* hydroxylamine is added. Incubation is continued for an additional 10 min at 22°. The reaction is stopped and the hydroxamic acid color is developed by the addition to the tube of 0.25 ml of 3 *N* HCl and 0.25 ml of 5 % $FeCl_3$. The slightly turbid mixture is thoroughly extracted with 0.5 ml of benzene. The resulting emulsion is clarified by centrifugation (20,000 × *g* for 5 min) and the absorbancy of the aqueous phase at 540 mμ is measured in the Beckman DU spectrophotometer. Under these conditions 1.0 μmole of acethydroxamic acid has a molar absorbancy index of $0.72 \cdot 10^3$ cm²/mole. One unit of enzyme activity is defined as that amount of enzyme which catalyzes the formation of 1.0 μmole of acethydroxamic acid under the conditions of the above assay (30 min, 30°, pH 7.5). Specific activity is expressed as units per mg of protein.

RESULTS

Characteristics of the assay system

The dependence of the rate of reductive acetylation on the concentration of substrates and enzyme and on pH and time is shown in Figs. 1 and 2. The highest specific activity routinely obtained ranges from 2 to 4. Although lipS_2 inhibits lipoic transacetylase[11] it does not inhibit the reductive acetylation system.

Formation of S-acetyl-lip(SH) from pyruvate

In an experiment, set up on twice the scale of the assay system, 0.76 μmole of an hydroxamic acid and 1.17 μmoles of –SH appeared. A sample of the reaction mixture, before addition of NH_2OH, was acidified with HCl and extracted three times with cold benzene. The pooled benzene extracts were evaporated to dryness and the residue was dissolved in methanol. The absorption spectrum of the product, corrected for the blank absorption (determined in control runs without enzyme or pyruvate) is shown in Fig. 3. About 0.51 μmole of S-acetyl-lip(SH) was recovered.

The discrepancy between the amounts of hydroxamic acid and free -SH groups formed is due to the presence of an S-acetyl-lip(SH) deacylase[11].

In a similar experiment, 20 volumes of 95 % ethanol were added to the tube after the reaction with NH_2OH was complete. The precipitate thus formed was

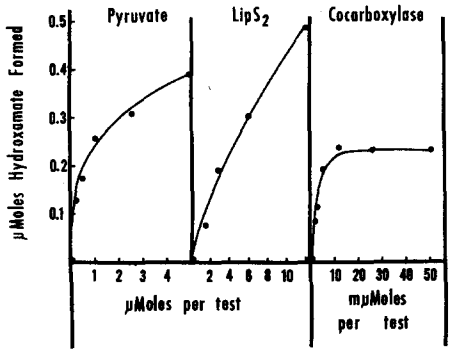


Fig. 1. Dependence of the rate of reductive acetylation on the concentrations of pyruvate, $lipS_2$ and cocarboxylase.

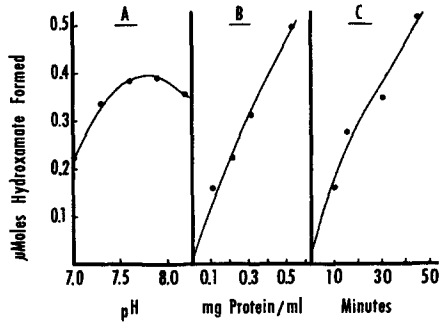


Fig. 2. Effect of pH, protein concentration and time on the reductive acetylation of $lipS_2$.

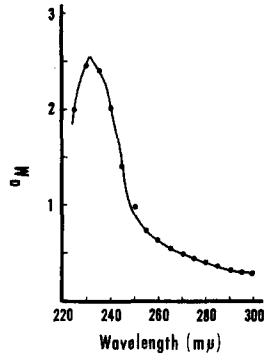


Fig. 3. Absorption spectrum of the product of the reductive acetylation of $lipS_2$.

removed by centrifugation. The alcohol phase was evaporated to dryness *in vacuo*. The residue was extracted several times with absolute ethanol and the combined ethanol extracts were concentrated *in vacuo*. Acethydroxamic acid was identified in this concentrate by ascending paper chromatography in two solvent systems (*n*-butanol–acetic acid–H_2O, (4:1:5, v/v) and *n*-amyl alcohol–acetic acid–H_2O, (4:1:5, v/v)[15]. The R_F's of the hydroxamate spots obtained by spraying the dried chromatograms with an acidified alcoholic solution of $FeCl_3$ (see ref. 16) were identical with those of known acethydroxamic acid.

(+)-α-lipoate as substrate of the pyruvic dehydrogenase

Attempts to show complete conversion of (+)-α-$lipS_2$ to S-acetyl-lip(SH) were unsuccessful due to the presence of S-acetyl-lipoate deacylase (eqn. 1).

$$\text{S-acetyl-lip(SH)} + H_2O \longrightarrow \text{acetate} + \text{lip(SH)}_2 \qquad (1)$$

A maximum of 70 % of the added (+)-α-$lipS_2$ was converted to the acetyl derivative.

That the acetylation was actually greater than this, is shown by the experiments described in Table I. (—)-α-lipS_2 was active in the reductive actylation system only to the extent that it was contaminated with (+)-α-lipS_2.

TABLE I

REDUCTIVE ACETYLATION OF (+)-α-lipS_2 AND THE ACTION OF S-ACETYL-LIPOATE DEACYLASE

The conditions are the same as those in the standard reductive acetylation assay system except that the (±)-α-lipoate was replaced with (+)-α-lipoate as shown below. Values reported have been corrected for the blanks observed in controls lacking lipS_2 or enzyme.

Expt. No.	*(+)-α-lipS_2 (μmoles)*	*Pyruvic dehydrogenase* (mg)*	*Products formed (μmoles)*		*Lipoate utilized*
			S-acetyl-lip(SH)	*Lip$(SH)_2$*	
1	1.5	0.23	0.26	0.14	0.40
2	1.5	0.45	0.66	0.46	1.12
3	1.5	0.60	0.76	0.49	1.25
4	0.90	0.45	0.34	0.23	0.57

* Specific activity 1.0.

Experiments in which (±)-α-lipoamide was used as substrate for reductive acetylation gave questionable results. A small amount of acetylated product was formed. The water insolubility of the amide made it necessary to use an ethanolic solution of the compound. Unfortunately ethanol proved to be highly inhibitory to the system. By contrast the pyruvic dehydrogenase of *Streptococcus faecalis* is unaffected by ethanol[9].

Attempts to isolate pure S-acetyl-lip(SH) formed by reductive acetylation of (+)-α-lipS_2 were unsuccessful. The product apparently polymerized and became insoluble during attempts to free it from residual (+)-α-lipS_2 which is highly inhibitory to the transacetylase. This frustrated our efforts to use pure S-acetyl-lip(SH) as an acetyl donor in coupled reactions.

Effect of potential inhibitors on the reductive acetylation reaction

Reductive acetylation of lipoate is not inhibited by $4 \cdot 10^{-2}$ *M* ethylenediaminetetraacetate or $1 \cdot 10^{-2}$ *M* arsenite. N-ethylmaleimide at $6 \cdot 10^{-3}$ *M* inhibits about 80 %.

Formation of acetoin

The pyruvic dehydrogenase of H37Ra can condense acetaldehyde (externally added) with "active acetaldehyde" (enzyme-bound) in the absence of lipoate. Under these conditions acetoin is formed. The requirements for acetoin formation are shown in Table II.

Demonstration of (E-acetaldehyde) as an intermediate in pyruvate oxidation

In these experiments the decarboxylation of pyruvate was carried out in the absence of an oxidizing agent (lipS_2) but in the presence of free acetyladehyde. [2-^{14}C]pyruvate was the substrate, [1-^{14}C]acetaldehyde was expected as the product. The reaction mixture was flushed with N_2 and incubated for 15 min at 38°. The reaction was stopped by the addition of $HClO_4$ and the acidified mixtures were held at 0° for 10 min before neutralization with KOH and centrifugation. A sample

of the neutral supernatant solution was analyzed for the presence of acetaldehyde by the diffusion method[10]. The sample (0.4 ml) was mixed with 50 μmoles of pH 7.0 phosphate buffer in the diffusion bulb and frozen in acetone–ice. The trapping tube

TABLE II

FORMATION OF ACETOIN FROM PYRUVATE

Each tube contained, in a total volume of 0.70 ml, 40 μmoles of phosphate buffer of pH 6.8, 5 μmoles of $MgCl_2$, 0.005 μmole of cocarboxylase, 4 μmoles of Li-pyruvate, 40 μmoles of acetaldehyde and 0.46 mg of pyruvic dehydrogenase of specific activity 1.7. The solutions were flushed with N_2, capped and incubated for 15 min at 38°. The reaction was stopped by the addition of 0.020 ml of $HClO_4$; the acid was neutralized with KOH and the residue removed by centrifugation. Acetoin was determined after conversion to diacetyl[16]. Values shown have been corrected for the blank obtained in a control run lacking acetaldehyde.

System	*Diacetyl formed (μg)*
Complete	12.3
Without pyruvate	5.1
Without cocarboxylase	1.3
Without $MgCl_2$	4.1
Without pyruvic dehydrogenase	0.69

contained 0.20 ml of 1.0% dinitrophenylhydrazine. Diffusion was carried out for 30 min at 38° under reduced pressure. After the diffusion assemblies were opened, 0.5 ml of 1.0% dinitrophenylhydrazine was added to each diffusion bulb. The hydrazones were formed during a 10 min incubation at room temperature. The hydrazones in the diffusion bulbs and trapping tubes were extracted with ethyl acetate. The dinitrophenylhydrazones were separated and identified by ascending paper chromatography. Three solvent systems were used: (a) *n*-butanol–ethanol–NH_4OH–H_2O (140:20:1.5:38), (b) *tert.*-amyl alcohol–ethanol–H_2O (100:20:80) and (c) ethanol–petroleum ether (80:20). The following standards, as the dinitrophenylhydrazones, were always chromatographed along with the experimental and control tube samples: [2-^{14}C]pyruvate, diacetyl, acetaldehyde and acetoin as well as dinitrophenylhydrazine itself. Samples and standards were applied to the origin as one inch streaks. After development of the chromatogram the papers were dried in air. The chromatogram developed by each streak was removed by cutting the paper into vertical strips; each strip was cut in half. The yellow spots on the first half were intensified and the characteristic colors of the dinitrophenylhydrazones were brought out by spraying the dried strip with a solution of 1% KOH in 95% ethanol. The second half of the streak was monitored for ^{14}C-containing areas by using a recording strip counter*.

Aliquot samples of the diffusion bulb and tube ethyl acetate solutions, from above, were plated on aluminum planchets and total ^{14}C contents were measured in a flow-gas counter. Results of a typical experiment are shown in Table III and Fig. 4. Control experiments showed that while negligible amounts of pyruvate diffuse from bulb to collecting tube under these conditions, essentially all the acetaldehyde diffuses.

These experiments can be summarized as follows. A strongly-labeled non-diffusible compound is formed non-enzymically. The R_F of its dinitrophenylhydrazone in all systems is identical with that of a non-radioactive dinitrophenylhydrazone which

* I wish to thank Dr. J. W. PORTER for making the radioisotope-detecting equipment available to me for these experiments.

TABLE III

EXCHANGE OF POOL ACETALDEHYDE WITH ENZYME-BOUND ACETALDEHYDE

Each tube contained, in a total volume of 0.60 ml; 30 μmoles of phosphate buffer of pH 6.9, 3 μmoles of $MgCl_2$, 0.003 μmole of cocarboxylase, 4 μmoles of Li-pyruvate, 2.0 μmoles of Na-[2-^{14}C] pyruvate (3.17 μC/μmole), 40 μmoles of acetaldehyde and 0.31 mg of pyruvic dehydrogenase of specific activity of 1.7.

System	^{14}C recovered		
	Non-diffusible (counts/min)	Diffusible	
		counts/min	Percent
Complete	$9.06 \cdot 10^5$	$24.8 \cdot 10^3$	2.7
Without enzyme	$8.76 \cdot 10^5$	$1.25 \cdot 10^3$	0.14
Without cocarboxylase	$9.60 \cdot 10^5$	$0.29 \cdot 10^3$	0.03
Without $MgCl_2$	$7.56 \cdot 10^5$	$9.51 \cdot 10^3$	1.3

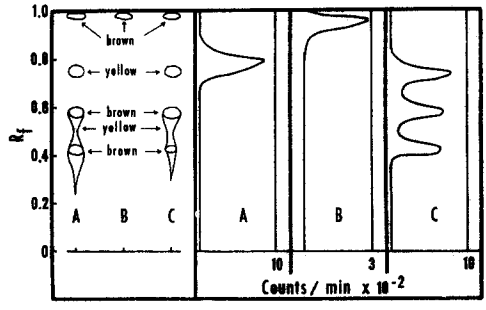


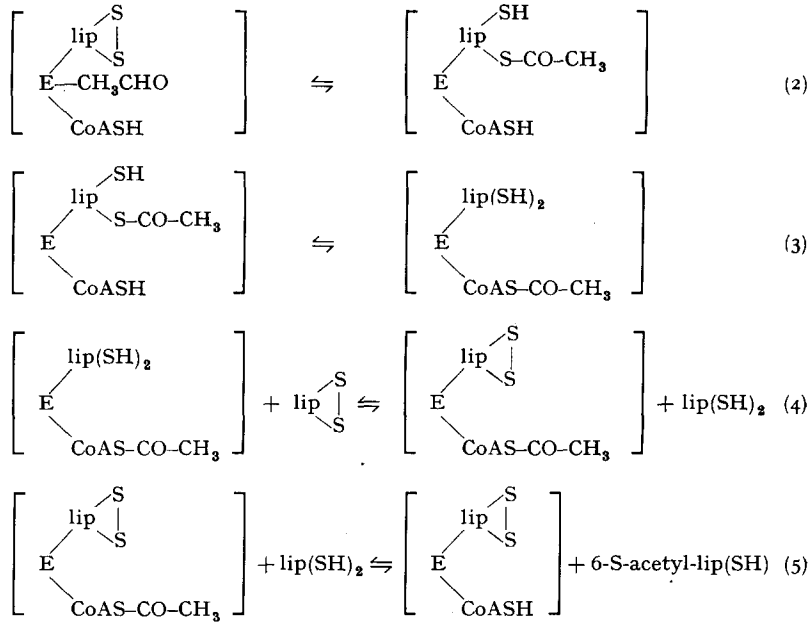
Fig. 4. Detection of [^{14}C]acetaldehyde by paper chromatography (left) and strip counting (right). Experiments as described in the text. A. 2,4-dinitrophenylhydrazones recovered from the nondiffusible residue of a control run (without enzyme). The single radioactive peak corresponds to that of [2-^{14}C]pyruvate. B. 2,4-dinitrophenylhydrazone of a diffusible compound recovered from the complete reaction system. C. 2,4-dinitrophenylhydrazones of the nondiffusible ^{14}C-containing compounds formed in the complete reaction system.

contaminates the dinitrophenylhydrazone of [2-^{14}C]pyruvate. Its R_F is less than that of the [2-^{14}C]pyruvate derivative. In the presence of the complete enzymic system a diffusible radioactive compound is formed which reacts with 2,4-dinitrophenylhydrazine. The R_F of its hydrazone is identical with that of the dinitrophenylhydrazone of acetaldehyde in three solvent systems. When the same experiment is carried out in absence of either enzyme or cocarboxylase this diffusible substance is not found. In the presence of carrier acetaldehyde the dinitrophenylhydrazone can be recrystallized (ethanol–water) to constant specific activity. Three recrystallizations are usually sufficient.

Despite the fact that free acetaldehyde exchanges with enzyme-bound "acetaldehyde" it was not possible to use acetaldehyde as an acetyl donor for lipoate.

Role of CoA in the reductive acetylation reaction

SANADI *et al.*[13] suggested that S-acetylCoA is an obligatory intermediate in the reductive acetylation of $lipS_2$. They proposed that the acetyl group of the initial enzyme-bound S-acetyl-lip(SH) is transferred to enzyme-bound CoA. The S-acetyl-CoA is then the substrate for the lipoic transacetylase which transfers the acetyl group to $lip(SH)_2$. This sequence is shown below. A similar sequence showing a requirement for added CoA was presented by REED *et al.*[7].



(2)

(3)

(4)

(5)

Our results do not support this proposal. (a) Treatment of the pyruvic dehydrogenase with Dowex-1 to remove CoA (see ref. 18) had no effect on the reductive acetylation reaction. (b) If acetyl CoA is formed in this reaction we should be able to transfer its acetyl group to phosphate by the addition of phosphotransacetylase and phosphate.

The acetyl group of acetyl P is readily hydrolyzed under conditions of temperature and pH which do not affect the acetyl group of S-acetyl-lip(SH). When this experiment, shown in Table IV, was carried out, all the hydroxamate formed was heat and acid-stable. In the absence of $lipS_2$ no hydroxamate was formed. Addition of free excess CoASH did not change this result. It would appear that S-acetyl-CoA is not an intermediate in the reductive acetylation reaction.

TABLE IV

EFFECT OF CoA ON THE REDUCTIVE ACETYLATION OF $lipS_2$

The standard reaction mixture with 0.31 mg of pyruvic dehydrogenase of specific activity 2.5, was used. Additions to the mixture are shown below.

Expt. No.	*Additions*	*Heat treatment*	*μmoles hydroxamate formed*
1	None	None	0.82
2	Phosphotransacetylase* + 5 μmoles Pi	None	0.65
3	None	100° for 3 min	0.64
4	Phosphotransacetylase + 5 μmoles Pi	100° for 3 min	0.69
5	0.05 μmole CoASH	None	0.72
6	Phosphotransacetylase + 5 μmoles Pi + 0.05 μmole CoASH	None	0.80
7	0.05 μmole CoASH	100° for 3 min	0.70
8	Phosphotransacetylase + 5 μmoles Pi + 0.05 μmole CoASH	100° for 3 min	0.64

* 0.89 μg of specific activity 8.4 (see ref. 18).

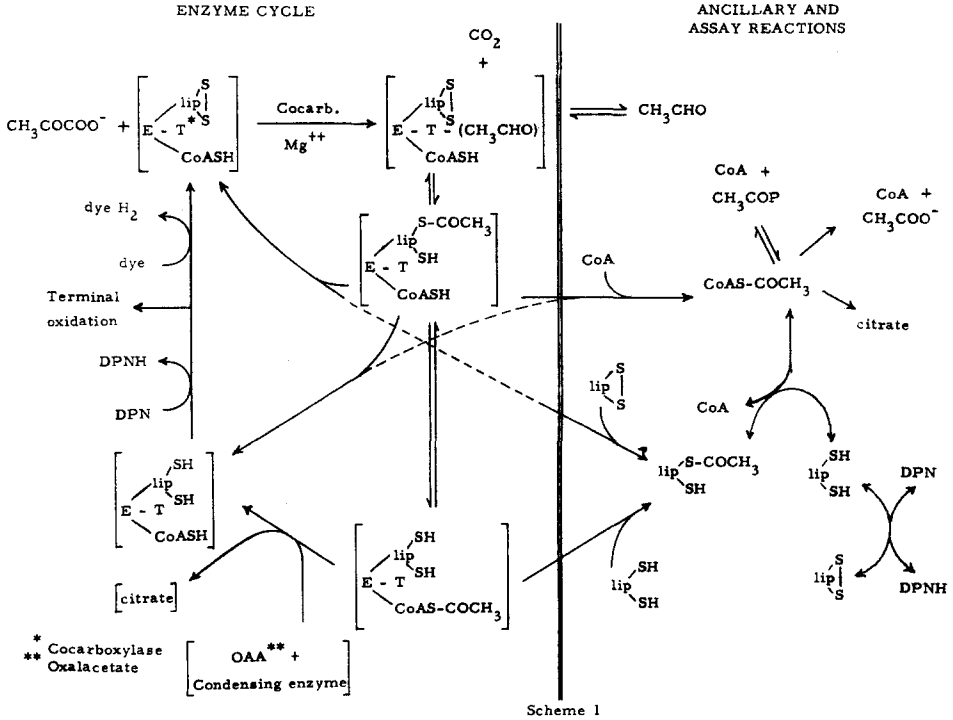
It should be pointed out, however, that preparations of this pyruvic dehydrogenase may contain a small amount of bound CoA. A typical dehydrogenase preparation contained 20 μg of pantothenic acid/g of soluble protein*.

DISCUSSION

The pyruvic dehydrogenase complex of H37Ra catalyzes the fundamental reaction characteristic of all pyruvic dehydrogenase complexes regardless of source, namely the oxidative decarboxylation of pyruvate to acetyl CoA. The product may be isolated as acetyl CoA, acetyl phosphate or acetate; the basic mechanism of the overall reaction appears to be the same whichever product is formed. The differences between the various dehydrogenase complexes may be ascribed, in part, to the isolation procedures and to the assay procedures. Thus the intermediation of $lipS_2$ in the overall reaction cannot be demonstrated if the assay system is based on reduction of indophenol or evolution of CO_2.

Previous attempts to demonstrate the reductive acetylation reaction have led to questionable results. SANADI *et al.*[13] suggested that this reaction, recently reported by GUNSALUS AND SMITH[9], is an artifact arising from reactions 2–5, above.

These several points of view and divergent results have been brought together and are shown in Scheme 1 which is presented as an overall unifying reaction sequence. Scheme 1 is obviously drawn from earlier suggestions[9,13,20] and is the result of many investigations, some of which have already been mentioned. The present investigation



Scheme 1

* I am grateful to the Wisconsin Alumni Research Foundation for this analysis.

provides information relative to the S → S acyl transfer step. (The reaction in which the acetyl group of enzyme-bound S-acetyl-lip(SH) is transferred to external $lipS_2$.)

REED *et al.*[7] showed that during the course of the oxidative decarboxylation of pyruvate by the apopyruvic dehydrogenase of *Escherichia coli* (a) there is no exchange between enzyme-bound $lipS_2$ and free $lip(S_2)$, $lip(SH)_2$, or oxidized and reduced lipoamides; (b) CoA is required for the overall reaction leading to the formation of acetyl phosphate; (c) interaction is possible between protein-bound and free $lip(S_2)$. In the absence of free CoA a very small (2 %) amount of hydroxylamine-reacting product was formed. This was assumed to be S-acetyl-lip(SH). These observations, taken with the results of SANGER[21], RYLE AND SANGER[22] and THOMAS AND REED[23], suggest a possible alternative to the mechanism suggested by REED *et al.*[7] and SANADI *et al.*[13] for the oxidative decarboxylation of pyruvate.

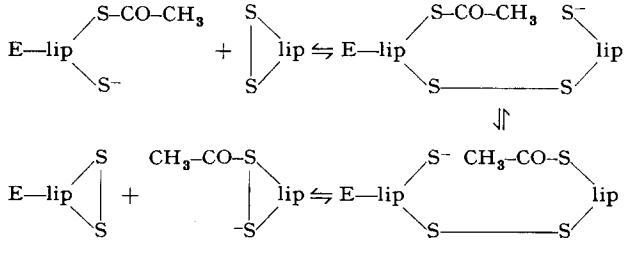
During an investigation by SANGER on the structure of insulin[21] it was shown that disulfide exchange reactions occur. This was confirmed in model systems[21,22]. Of particular interest was the finding that disulfide exchange reactions occur only slowly at neutral pH and that the reaction is strongly stimulated by the addition of thiols. The catalytic effect of thiols is reversibly inhibited by N-ethyl-maleimide. RYLE AND SANGER suggested the following mechanism for the thiol catalysis of disulfide exchange.

$$R^1SSR^1 + R^2S^- \rightleftharpoons R^1SSR^2 + R^1S^- \quad (6)$$

$$R^2S^- + R^1SSR^2 \rightleftharpoons R^2SSR^2 + R^1S^- \quad (7)$$

THOMAS AND REED[23] investigated the mechanism of the depolymerization of $polylipS_2$. Depolymerization at room temperature is dependent on the presence of OH^-. At pH 12 depolymerization is markedly stimulated by the presence of catalytic amounts of $lip(SH)_2$. *p*-Chloromercuribenzoate completely inhibits depolymerization; $lip(SH)_2$ reverses the inhibition. A mechanism similar to that proposed by RYLE AND SANGER was suggested by THOMAS AND REED to account for the catalytic effect of mercaptide ions on the depolymerization of $polylipS_2$.

This suggests that the pyruvic dehydrogenase complex of H37Ra may carry out a disulfide exchange reaction between bound S-acetyl-lip(SH) and free $lipS_2$. S-acetyl-lip(SH) can contribute the mercaptide ion to the reaction (Scheme 2). An S → S acetyl transfer is not uncommon in biological systems, *e.g.*, the transfers catalyzed by CoA-$lip(SH)_2$ transacetylases[11,13,24] and by the general CoA-thiol transacetylase of BRADY AND STADTMAN[25].



Scheme 2

The inhibition of reductive acetylation by N-ethyl-maleimide suggests the intermediation of a monothiol whereas the lack of inhibition by arsenite would preclude

the participation of a dithiol in the reaction. The effect of arsenite in suppressing the production of acetyl CoA by this same pyruvic dehydrogenase of H37Ra can be explained in terms of the binding of enzyme-bound lip$(SH)_2$ formed after transfer of the acetyl group to exogenous CoA. We have previously shown[10] that there is an absolute requirement for CoA for citrate formation by the pyruvic dehydrogenase of H37Ra supplemented with oxalacetate and condensing enzyme. This reaction is strongly inhibited by arsenite.

The picture of pyruvic oxidative decarboxylation by the enzyme of H37Ra which emerges from these studies differs only in detail from the schemes suggested earlier. It may, indeed, differ only in the binding strength between the several enzymes of the complex. Scheme I supplies a possible general explanation for observed results by segregating the enzyme-bound transfer and oxidation–reduction reactions from the artificial *in vitro* reactions. By extension of this scheme the results of most investigations can be explained in terms of siphoning off of various intermediates in the decarboxylation cycle into artificial assay systems.

The recent report of GRUBER AND WASSENAAR[26] on the inhibition of yeast carboxylase by acetaldehyde may provide an explanation for the inability of acetaldehyde to serve as an acetyl donor for lipoate in the pyruvic dehydrogenase system. SEARLS AND SANADI[27] and MASSEY[28] have independently shown that the FAD of pig heart ketoglutaric dehydrogenase is associated with the lipoyl dehydrogenase portion of the ketoglutaric dehydrogenase complex. MASSEY[29] has shown the identity of this lipoyl dehydrogenase with the STRAUB[30] diaphorase. MASSEY[28] has suggested that the ketoglutaric dehydrogenase goes through a cyclical reaction sequence similar to that proposed above using the pyruvic dehydrogenase complex as the model. Succinyl is thus generated and simultaneously transferred to E-lipS_2 and, in turn, is transferred from the enzyme-bound succinyl lipoate to CoA yielding succinyl CoA. The only difference of consequence between the cyclical mechanism suggested by MASSEY[28] and that suggested here is that MASSEY proposes that DPN is the physiological oxidant of the reduced flavin of lipoyl dehydrogenase. It appears reasonable to assume, however, that once the FAD of lipoyl dehydrogenase is reduced it is reoxidized *via* the cytochrome sequence rather than *via* DPN, FAD and then the cytochrome sequence[31].

ACKNOWLEDGEMENTS

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REFERENCES

1 D. S. GOLDMAN AND A. GELBARD, *Arch. Biochem. Biophys.*, 83 (1959) 360.
2 D. S. GOLDMAN, *Federation Proc.*, 17 (1958) 231, Abst. 909.
3 L. J. REED, B. G. DEBUSK, I. C. GUNSALUS AND C. S. HORNBERGER, JR., *Science*, 114 (1951) 93.

[4] F. LYNEN, E. REICHERT AND L. RUEFF, *Ann. Chem., Liebigs*, 574 (1951) 1.
[5] I. C. GUNSALUS, in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, Md., 1954, p. 545.
[6] L. J. REED AND B. G. DEBUSK, *J. Am. Chem. Soc.*, 75 (1953) 1261.
[7] L. J. REED, M. KOIKE, M. E. LEVITCH AND F. R. LEACH, *J. Biol. Chem.*, 232 (1958) 143.
[8] L. J. REED, *Advances in Enzymology*, 18 (1957) 319.
[9] I. C. GUNSALUS AND R. A. SMITH, *Proc. Intern. Symp. Enzyme Chem., (Tokyo and Kyoto)*, Maruzen, Tokyo (Japan), 1958, p. 77.
[10] D. S. GOLDMAN, *Biochim. Biophys. Acta*, 27 (1958) 513.
[11] D. S. GOLDMAN, *Biochim. Biophys. Acta*, 32 (1959) 80.
[12] T. P. SINGER AND J. PENSKY, *Biochim. Biophys. Acta*, 9 (1952) 316.
[13] D. R. SANADI, M. LANGLEY AND F. WHITE, *J. Biol. Chem.*, 234 (1959) 183.
[14] D. S. ACKER AND W. J. WAYNE, *J. Am. Chem. Soc.*, 79 (1957) 6483.
[15] A. R. THOMPSON, *Australian J. Sci. Research, Ser. B*, 4 (1951) 180.
[16] E. R. STADTMAN AND H. A. BARKER, *J. Biol. Chem.*, 184 (1950) 769.
[17] W. W. WESTERFELD, *J. Biol. Chem.*, 161 (1945) 495.
[18] E. R. STADTMAN, G. D. NOVELLI AND F. LIPMANN, *J. Biol. Chem.*, 191 (1951) 365.
[19] D. S. GOLDMAN, *Biochim. Biophys. Acta*, 28 (1958) 436.
[20] L. J. REED, *Proc. Intern. Symp. Enzyme Chem., (Tokyo and Kyoto)*, Maruzen, Tokyo (Japan), 1958, p. 71.
[21] F. SANGER, *Nature*, 171 (1953) 1025.
[22] A. P. RYLE AND F. SANGER, *Biochem. J.*, 60 (1955) 535.
[23] R. C. THOMAS AND L. J. REED, *J. Am. Chem. Soc.*, 78 (1956) 6148.
[24] I. C. GUNSALUS, L. S. BARTON AND W. GRUBER, *J. Am. Chem. Soc.*, 78 (1956) 1763.
[25] R. O. BRADY AND E. R. STADTMAN, *J. Biol. Chem.*, 211 (1954) 621.
[26] M. GRUBER AND J. S. WASSENAAR, *Biochim. Biophys. Acta*, 38 (1960) 355.
[27] R. L. SEARLS AND D. R. SANADI, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 697.
[28] V. MASSEY, *Biochim. Biophys. Acta*, 38 (1960) 447.
[29] V. MASSEY, *Biochim. Biophys. Acta*, 37 (1960) 314.
[30] F. B. STRAUB, *Biochem. J.*, 33 (1939) 787.
[31] D. S. GOLDMAN, *Advances in Tuberculosis Research*, in the press.