[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TEXAS, AND THE CLAYTON FOUNDATION FOR RESEARCH, AND FROM THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF ILLINOIS]

Interrelationships of Lipoic Acids

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Treatment of α-lipoic acid with thiols, followed by oxidation with iodine, resulted in formation of a series of new compounds possessing biological activity corresponding to that of α-lipoic acid. These compounds appear to be mixed disulfides. The presence of such mixed disulfides in biological preparations is suggested. β-Lipoic acid is converted to α-lipoic acid by treatment with reduced glutathione, hydriodic acid or ethanol and hydrochloric acid. Procedures are described for the preparation of crystalline α-lipoic acid from concentrates of β-lipoic acid. α-Lipoic acid is converted to β-lipoic acid by treatment with hydrogen peroxide or aqueous potassium permanganate. These interconversions suggest that β-lipoic acid is an oxidized form of α-lipoic acid, probably a sulfoxide.

The existence in biological preparations of a group of chemically related substances which possess acetate-replacing and pyruvate oxidation factor activity has been demonstrated.[1,2] One of these catalytic agents, α-lipoic acid, has been isolated in crystalline form from acid-hydrolyzed liver residue.[3] α-Lipoic acid is a monocarboxylic acid containing a disulfide linkage and possessing the empirical formula $C_8H_{14}O_2S_2$.[3] The presence of this latter functional group suggested relationships of α-lipoic acid to other acetate-replacing and pyruvate oxidation factors present in biological preparations. Thus, interaction of naturally occurring thiols with α-lipoic acid might result in formation of mixed disulfides possessing biological activity corresponding to that of α-lipoic acid. Moreover, the occurrence in biological preparations of oxidized forms of α-lipoic acid and of the mixed disulfides, *i.e.*, sulfoxides, is suggested.

It is shown in Fig. 1 that treatment of α-lipoic acid with any of the indicated thiols, followed by oxidation to disulfides with iodine, resulted in formation of a series of new compounds possessing biological activity corresponding to that of α-lipoic acid. Controls in which the thiol compounds were first oxidized to the corresponding disulfides, then added to α-lipoic acid, allowed to stand, and chromatographed, gave the same results as with α-lipoic acid alone. Whereas α-lipoic acid is ninhydrin-negative, chromatograms of the reaction product of α-lipoic acid with β-mercaptoethylamine showed a ninhydrin-positive zone that corresponded exactly in outline and R_f value with the "extra" zone of growth revealed by the bioautograph (Fig. 1E). This behavior would be expected of a mixed disulfide containing a β-mercaptoethylamine residue as one component, since the latter fragment furnishes a ninhydrin-reactive amino group. Thus mixed disulfides are readily formed from α-lipoic acid, and these mixed disulfides retain the biological activity of α-lipoic acid. Since many thiols occur naturally (*e.g.*, cysteine, glutathione, homocysteine, cysteine peptides, proteins, etc.) in amounts far surpassing α-lipoic acid, similar mixed disulfides could be expected to occur in natural materials.

Acid hydrolysates of natural materials contain α-lipoic acid and a more polar acidic principle, β-lipoic acid.[1-3] These two substances can be distinguished and separated by countercurrent distribution[2] (Figs. 2 and 3) and by chromatography.[2-4] It has been observed that bioautographs prepared from crystalline α-lipoic acid always reveal two zones of growth (Fig. 1A). The lower zone corresponds in R_f value to that obtained with concentrates of β-lipoic acid (Fig. 1F). When chromatograms of crystalline α-lipoic acid are sectioned so as to separate the two active principles, which are then eluted and rechromatographed, the faster-moving principle (α-lipoic acid) again results in two zones of growth, whereas the slower-moving principle (β-lipoic acid) produces only the lower zone.

These observations suggest that β-lipoic acid is an oxidized form of α-lipoic acid, probably a sulfoxide. If such is the case, treatment of β-lipoic acid with reagents known to convert sulfoxides to sulfides should result in formation of α-lipoic acid. Figure 1G shows that treatment of a concentrate

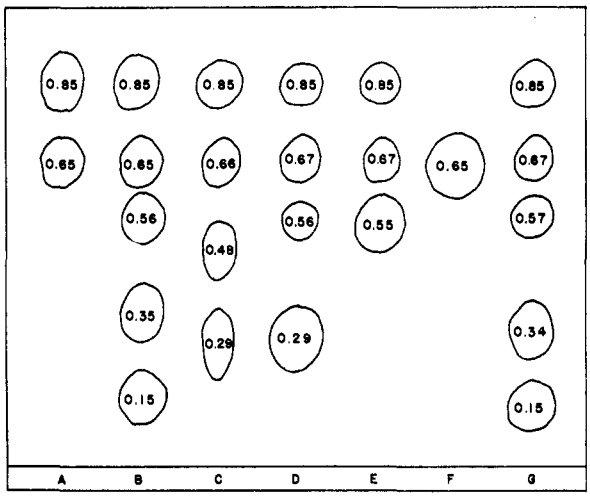

Fig. 1.—Diagram of bioautographs showing the formation of mixed disulfides from α-lipoic acid and thiol compounds. R_f values of zones of growth are given within the zone area: A, α-lipoic acid; B, α-lipoic acid + reduced glutathione; C, α-lipoic acid + cysteine; D, α-lipoic acid + thiomalic acid; E, α-lipoic acid + β-mercaptoethylamine; F, β-lipoic acid; G, β-lipoic acid + reduced glutathione. 2,6-Lutidine-water (65:35) used as the solvent system, with *Streptococcus lactis* as the test organism.[1] See Experimental section for treatment.

(1) L. J. Reed, B. G. DeBusk, P. M. Johnston and M. E. Getzendaner, *J. Biol. Chem.*, **192**, 851 (1951).

(2) I. C. Gunsalus, L. Struglia and D. J. O'Kane, *ibid.*, **194**, 859 (1952).

(3) L. J. Reed, I. C. Gunsalus, G. H. F. Schnakenberg, Q. F. Soper, H. E. Boaz, S. F. Kern and T. V. Parke, THIS JOURNAL, **75**, 1267 (1953).

(4) L. J. Reed, M. E. Getzendaner, B. G. DeBusk and P. M. Johnston, *J. Biol. Chem.*, **192**, 859 (1951).

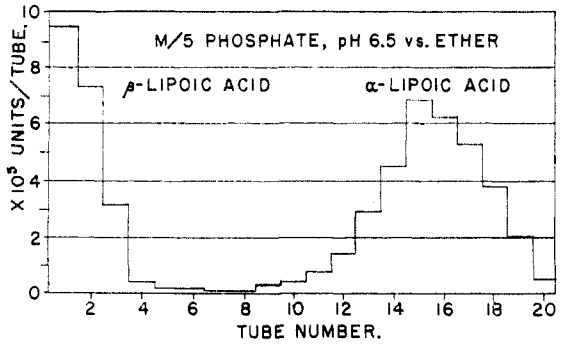

Fig. 2.—Separation of α- and β-lipoic acids by countercurrent distribution. *M*/5 phosphate buffer, *p*H 6.5, *vs.* diethyl ether, 50 ml. per phase, ether as mobile phase.

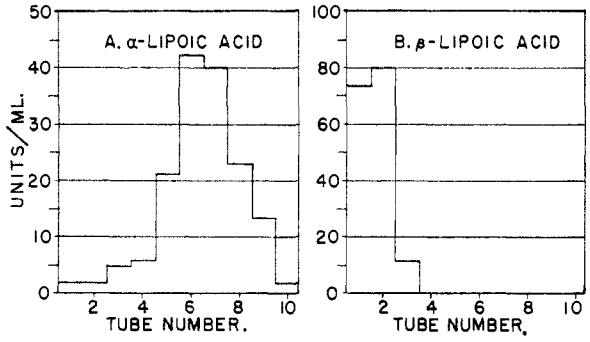

Fig. 3.—Analytical countercurrent distribution of α-lipoic acid (3A) and β-lipoic acid (3B) from the separation shown in Fig. 2.

of β-lipoic acid with reduced glutathione results in formation of α-lipoic acid as well as the same mixed disulfides resulting from treatment of α-lipoic acid with this thiol. Treatment of β-lipoic acid concentrates with hydriodic acid or with ethanol and hydrochloric acid at an elevated temperature produces α-lipoic acid, as revealed by bioautographs and countercurrent distribution analysis. These observations have been confirmed by isolation of crystalline α-lipoic acid from relatively crude concentrates of the methyl ester of β-lipoic acid after reduction of the latter with hydriodic acid as described below. As noted previously[3] β-lipoic acid constitutes approximately 35% of the total activity of benzene extracts of acid-hydrolyzed liver residue. It is possible to recover approximately 21% of this activity as crystalline α-lipoic acid.

α-Lipoic acid has been converted to β-lipoic acid by oxidation with hydrogen peroxide in glacial acetic acid and with aqueous potassium permanganate, as revealed by bioautographs and countercurrent distribution analysis. Highly purified preparations of β-lipoic acid have been obtained from oxidized concentrates of methyl α-lipoate. Although methyl α-lipoate is readily saponified to α-lipoic acid with dilute alkali,[3] the β-ester is converted to a biologically inactive product under these conditions. It may be that the $-S\overset{\uparrow}{\underset{O}{}}S-$ linkage, assumed to be present in β-lipoic acid, is hydrolyzed to a thiol group and a sulfinic acid group under alkaline conditions. β-Lipoic acid can be obtained from concentrates of the β-ester by acid hydrolysis. Preparations of the former have been obtained which possess a potency of 150,000 units/mg. (as compared to 250,000 units/mg. for crystalline α-lipoic acid), but attempts to crystallize such concentrates have been unsuccessful.[5]

Experimental

Preparation of Mixed Disulfides from α-Lipoic Acid and Thiols.—The method employed is essentially that of Brown and Snell.[6] A solution of 10 μg. of crystalline α-lipoic acid (20 μg. in the case of β-lipoic acid, employed as a concentrate, 125,000 units/mg.) and a 20-fold excess of the appropriate thiol in 0.5 ml. of water was allowed to stand at room temperature for 3 to 4 hours. The mixture was then oxidized with iodine to the disulfides, neutralized, and bioautographs of the reaction mixtures prepared as described previously,[1] employing 1–2 μl.[7] (equivalent to 0.01–0.02 μg. of crystalline α-lipoic acid) of each reaction mixture.

Separation of α- and β-Lipoic Acids by Countercurrent Distribution.—A benzene extract of acid-hydrolyzed liver residue[8] containing 3.1×10^6 units of active material was subjected to a 20-tube countercurrent distribution in a system consisting of *M*/5 potassium phosphate buffer, *p*H 6.5, and peroxide-free diethyl ether (50 ml. per phase, ether as mobile phase). After twenty transfers, the ether was removed by mild heating on a steam-cone and the tube contents assayed manometrically with *Streptococcus faecalis*.[8] As shown in Fig. 2, the active material was completely separated into two forms, a non-migrating fraction, *i.e.*, tubes 0 to 4, consisting of β-lipoic acid (2.1×10^6 units, 37%), and a migrating fraction, tubes 11 to 19, consisting of α-lipoic acid (3.5×10^6 units, 63%). The increase in total units as a result of countercurrent distribution occurs frequently at this stage of purification, and has been attributed to the removal of inhibitory substances.[2]

The contents of tubes 14 to 18 (250 ml.) were combined, acidified to *p*H 2.0 with hydrochloric acid, and extracted twice with an equal volume of peroxide-free ether. A sample of the combined ether extracts was subjected to a ten-tube countercurrent distribution between ether and phosphate buffer, *p*H 6.5. It is shown in Fig. 3A that all of the active material migrated as α-lipoic acid. The contents of tubes 1 to 3 were combined, acidified to *p*H 1.0 with concentrated hydrochloric acid and extracted three times with an equal volume of peroxide-free ether. A ten-tube countercurrent distribution at *p*H 6.5 showed that the active material, β-lipoic acid, was free of α-lipoic acid (Fig. 3B).

Conversion of β- to α-Lipoic Acid. (a).—A concentrate of β-lipoic acid (13,200 units) obtained as described above was autoclaved for one hour at 120° in the presence of 1 ml. of ethanol and 1 ml. of 8 *N* hydrochloric acid.[9] The reaction mixture was cooled, neutralized to *p*H 6.5 and subjected to a ten-tube countercurrent distribution at *p*H 6.5. Tubes 4 to 9 contained 11,000 units of α-lipoic acid and tubes 1 and 2 contained 500 units of β-lipoic acid. The total units recovered (11,500) amounted to 87% of the active material used for the experiment.

(b).—A β-ester fraction (7.85 g.) obtained from an alumina chromatogram[3] was adsorbed on Florisil (300 g.) and the column was washed with benzene until the eluate was colorless. The β-ester fraction (5.35 g.) was then eluted with ethyl acetate with essentially quantitative recovery of activity. To 2.43 g. of this fraction was added 30 ml. of 5 *N* potassium iodide and 17.2 ml. of 10 *N* hydrochloric

(5) It was reported subsequently (J. A. Brockman, Jr., E. L. R. Stokstad, E. L. Patterson, J. V. Pierce, M. Macchi and F. P. Day, THIS JOURNAL, **74**, 1868 (1952)) that Protogen-B, isolated from a water-insoluble fraction of liver in the form of a crystalline S-benzylthiuronium salt, is a sulfoxide. It would appear that β-lipoic acid and Protogen-B are identical.

(6) G. M. Brown and E. E. Snell, *Proc. Soc. Exp. Biol. Med.*, **77**, 13 (1951).

(7) Approximately 1000 times as much reaction product must be used to obtain a ninhydrin test as is used to prepare bioautographs.

(8) I. C. Gunsalus, M. I. Dolin and L. Struglia, *J. Biol. Chem.*, **194**, 849 (1952).

(9) M. Gazdar and S. Smiles, *J. Chem. Soc.*, **97**, 2248 (1910), describe the conversion of sulfoxides to sulfides by heating the former with alcoholic hydrogen chloride.

acid.[10] The mixture was shaken in an atmosphere of nitrogen for 10 minutes at room temperature and then extracted with a total of 40 ml. of benzene. The benzene extracts were washed consecutively with dilute sodium thiosulfate, 5% sodium bicarbonate, and water, and then evaporated *in vacuo*. The viscous oil remaining was diluted to 24.3 ml. with benzene and introduced onto a column prepared from a slurry of 150 g. of Florisil in benzene. The results are presented in Table I. The simple procedure described resulted in a 70-fold purification with approximately 40% recovery of active material. Saponification[3] of 31.1 mg. of α-ester (233,000 units/mg.) prepared in this manner resulted in 16.4 mg. of crystalline α-lipoic acid (250,000 units/mg.), m.p. 47.5°. The recovery of active material was 56%. The X-ray powder data of this sample of α-lipoic acid were identical with those of other samples isolated from acid-hydrolyzed liver residue.[3]

TABLE I

REDUCTION OF CRUDE β-ESTER FRACTION AND CHROMATOGRAM ON FLORISIL

Fraction	Solvent	Total volume, ml.	Solids, mg.	Potency, units/mg.	Total units
Crude β-ester			2430	3050	7,400,000
Reduced β-ester				2200	5,350,000
1-5	Benzene	300		Inactive	
6	Benzene	50	8.8	192,000	1,690,000
7	Benzene	50	5.7	226,000	1,290,000
8	Benzene	50	1.4	140,000	196,000
9	Benzene	50	0.1	181,000	18,100
10-20	Benzene	500		Inactive	
21	EtAc	140	1780	67	120,000

Conversion of α- to β-Lipoic Acid. (a).—A concentrate of α-lipoic acid (11,600 units), obtained by countercurrent distribution (Figs. 2 and 3A), in 5 ml. of *M*/5 phosphate buffer, *p*H 6.5, was treated with 0.05 ml. of 0.001 *N* potassium permanganate at room temperature for three minutes. The residual permanganate color was discharged with sodium thioglycolate, the *p*H readjusted to 6.5 with acetic acid, and the sample subjected to a ten-tube countercurrent distribution. Active material (11,500 units) was recovered in tubes one and two, accounting for a 97% conversion of α- to β-lipoic acid.

(b).—A solution of 200 mg. of an α-ester fraction, obtained from an alumina chromatogram,[3] and 0.035 ml. of 30% hydrogen peroxide in 1.4 ml. of glacial acetic acid was allowed to stand at room temperature for 3 hours. The acetic acid was removed *in vacuo*, the oily residue dissolved in 2 ml. of benzene and introduced onto a column (1-cm. diameter) prepared from a slurry of 20 g. of Florisil in benzene. The results are presented in Table II.

TABLE II

OXIDATION OF CRUDE α-ESTER FRACTION AND CHROMATOGRAM ON FLORISIL

Fraction	Solvent	Total volume, ml.	Solids, mg.	Potency, units/mg.	Total units
Crude α-ester			200	15,000	3,000,000
Oxidized α-ester				14,900	2,980,000
1	Benzene	50			600,000
2-3	Benzene	100		Inactive	
4-7	20% EtAc[a]	80		Inactive	
8-11	20% EtAc	80	9	151,500	1,360,000
12-14	20% EtAc	60		Inactive	
15	EtAc	50		Inactive	

[a] In benzene.

Stability of Methyl β-Lipoate to Alkaline and Acid Hydrolysis.—A concentrate of methyl β-lipoate (22,500 units), prepared by treating β-lipoic acid (Figs. 2 and 3B) with diazomethane,[3] was shaken in a glass-stoppered test-tube under nitrogen with 2 ml. of 0.2 *N* potassium hydroxide for one hour at room temperature. The contents of the tube were adjusted to *p*H 6.5 with dilute phosphoric acid and subjected to a ten-tube countercurrent distribution analysis. Tubes 1 and 2 contained 1300 units and tubes 4 to 9 contained 900 units. Thus, 90% of the methyl β-lipoate was inactivated by treatment with alkali.

To a concentrate of methyl β-lipoate, 22,500 units, was added 2 ml. of 1 *N* hydrochloric acid and the mixture was autoclaved at 120° for 25 minutes. The reaction mixture contained approximately 20,000 units of which approximately 80% consisted of β-lipoic acid and 20% of α-lipoic acid, as revealed by countercurrent distribution analysis.

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(10) These reagents have been used for the conversion of cystine disulfoxide to cystine (G. Toennies and T. F. Lavine, *J. Biol. Chem.*, **113**, 571 (1936)).

[CONTRIBUTION FROM THE LABORATORIES OF BACTERIOLOGY, UNIVERSITY OF ILLINOIS; THE LILLY RESEARCH LABORATORIES, ELI LILLY AND COMPANY; AND THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TEXAS]

Synthesis of DL-α-Lipoic Acid

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Treatment of 4-(α-tetrahydrofuryl)-butyric acid with thiourea and hydrobromic acid, followed by hydrolysis, produces dimercaptoöctanoic acid. Iodine oxidation of the dimercapto acid produces a cyclic disulfide. The properties of this cyclic disulfide are those to be expected for DL-α-lipoic acid, *i.e.*, biological activity one-half that of α-lipoic acid from natural material, no optical activity, elemental and infrared analysis identical with the natural material. Treatment of 4-(α-tetrahydrofuryl)-butyric acid with hydrobromic acid cleaves the ether linkage and forms a bromolactone. Treatment of the unstable bromolactone with thiourea and acid, followed by hydrolysis, gives an improved yield of biologically active dimercaptooctanoic acid. An observed rearrangement of the unstable bromolactone indicates that one of the isothiouronium groups may become attached to a carbon other than in the 5-position of the octanoic acid due to carbonium ion migration. The method of synthesis suggests that α-lipoic acid is the cyclic disulfide derived from either 4,8-, 5,8- or 6,8-dimercaptoöctanoic acid.

α-Lipoic acid, a catalytic agent for the oxidative decarboxylation of pyruvic acid, has been isolated from natural material,[1,2] characterized by physical data,[2,3] and its structure partially elucidated.[2,4] These studies indicate that α-lipoic acid is a cyclic disulfide obtained from 4,8-, 5,8- or 6,8-dimercaptooctanoic acid.[2,4] In a preliminary communica-

(1) L. J. Reed, B. G. DeBusk, I. C. Gunsalus and C. S. Hornberger, Jr., *Science*, **114**, 93 (1951).

(2) L. J. Reed, I. C. Gunsalus, G. H. F. Schnakenberg, Q. F. Soper, H. E. Boaz, S. F. Kern and T. V. Parke, THIS JOURNAL, **75**, 1267 (1953).

(3) L. J. Reed, Q. F. Soper, G. H. F. Schnakenberg, S. F. Kern, H. E. Boaz and I. C. Gunsalus, *ibid.*, **74**, 2383 (1952).

(4) L. J. Reed, B. G. DeBusk, C. S. Hornberger, Jr., and I. C. Gunsalus, *ibid.*, **75**, 1271 (1953).