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Isolation, Characterization and Structure of α-Lipoic Acid[1]

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Procedures are described for the isolation of crystalline α-lipoic acid from acid-hydrolyzed liver residue. This isolation represents a 300,000- to 600,000-fold concentration, with a 2.5% over-all recovery of activity in the form of crystalline α-lipoic acid. Data are presented which indicate that α-lipoic acid is the cyclic disulfide derived from 4,8-, 5,8- or 6,8-dimercapto-*n*-caprylic acid.

Previous studies have demonstrated that a variety of biological preparations contain a substance or substances which can replace acetate in its growth-promoting function for some lactic acid bacteria and are required for the oxidative decarboxylation of pyruvate.[2-4] The terms "acetate-replacing factor" and "pyruvate oxidation factor" were used to denote this biological activity, which has been shown to be exhibited by a group of chemically related catalytic agents.[5,6] Biological activity corresponding to that possessed by these factors has been shown to be exhibited by concentrates of "protogen,"[4] an essential growth factor for *Tetrahymena geleii*.[7]

These factors are released from materials of natural origin by the action of enzymes or by acid or base hydrolysis. Acid hydrolysis results in maximum release of activity in the form of only two of the active principles, which are acidic in nature. In a preliminary communication[8] it was announced that the less polar of the two acidic principles had been isolated in crystalline form and named α-lipoic acid. The more polar acid is referred to as β-lipoic acid. Subsequently, the isolation of Protogen-B, in the form of the crystalline S-benzylthiuronium salt, was reported.[9] The properties of Protogen-B are similar to those of β-lipoic acid.

Crystalline α-lipoic acid gives a positive qualitative test[10] for sulfur. A nitroprusside test for a thiol group is negative, but positive after treatment with sodium cyanide, suggesting a disulfide linkage. Elemental analyses and molecular weight determination by electrometric titration suggest the formula $C_8H_{14}O_2S_2$.

X-Ray diffraction powder data are given in Table I. α-Lipoic acid crystallizes from petroleum ether in the form of plates having positive optic sign, positive elongation and parallel extinction with a 2V value of 80–85°. The substance is optically active. Electrometric titration shows one acidic group, pK_a' 4.76. This value indicates an aliphatic carboxyl group separated by at least two methylene groups from any unsaturation or polar substitution. The shape of the titration curve shows that the formula given above is a molecular formula and not an empirical formula. Wave

(1) The isolation of crystalline α-lipoic acid was effected at the University of Texas (LJR) and in the Lilly Research Laboratories (GHFS) using a combination of procedures developed by the three coöperating groups and predominantly an assay procedure developed by one of the authors (ICG). Characterization and structural determination were carried out mainly by the other authors in the Lilly Research Laboratories.

(2) B. M. Guirard, E. E. Snell and R. J. Williams, *Arch. Biochem.*, **9**, 381 (1946).

(3) D. J. O'Kane and I. C. Gunsalus, *J. Bact.*, **54**, 20 (1947); **56**, 499 (1948).

(4) E. E. Snell and H. P. Broquist, *Arch. Biochem.*, **23**, 326 (1949).

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

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

(7) G. W. Kidder and V. C. Dewey, *Arch. Biochem.*, **20**, 433 (1949); E. L. R. Stokstad, C. E. Hoffman, M. A. Regan, D. Fordham and T. H. Jukes, *ibid.*, **20**, 75 (1949).

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

(9) R. L. Patterson, J. A. Brockman, Jr., F. P. Day, J. V. Pierce, M. E. Macchi, C. E. Hoffman, C. T. O. Fong, E. L. R. Stokstad and T. H. Jukes, THIS JOURNAL, **73**, 5919 (1951).

(10) F. Feigl, "Manual of Spot Tests," Academic Press, Inc., New York, N. Y., 1943, p. 163.

TABLE I

X-RAY POWDER DATA OF α-LIPOIC ACID

d	I/I_1	d	I/I_1	d	I/I_1
8.56	0.12	3.52	0.08	2.36	0.08
5.62	.04	3.27	.02	2.29	.08
5.30	.20	3.00	.16	2.23	.02
4.81	1.00	2.87	.02	2.20	.02
4.49	0.16	2.78	.02	2.12	.08
4.24	.04	2.73	.02	1.997	.02
4.01	.60	2.57	.08	1.888	.02
3.82	.12	2.46	.02	1.720	.02
3.67	.12				

lengths of infrared absorption bands in the rocksalt region are given in Table II with their assignments. The spectrum is similar to that of a simple aliphatic

TABLE II

INFRARED BANDS OF α-LIPOIC ACID

Wave length (μ)	Assignment
3.37	C–H
3–4 (broad)	Carboxyl O–H
5.68 (weak)	.....
5.84	Carboxyl C=O
6.90	$-CH_2-$
7.07	$-CH_2-\overset{\overset{O}{\parallel}}{C}-$
7.78	.....
8.0	C–OH
10.7	COOH (dimer)

carboxylic acid. The band at 5.8 μ is typical for an aliphatic carboxyl group. Infrared spectra obtained with calcium fluoride optics in the 3- to 4-micron region show the 3.37 μ band due to unsymmetrical methyl C–H stretching to be absent in α-lipoic acid but to be present in methyl-containing model compounds. In the ultraviolet region, α-lipoic acid shows no characteristic absorption down to 220 mμ.[11a]

Polarographic studies of α-lipoic acid showed that the sulfur is reducible at the dropping mercury electrode. According to Wawzonek[11b] the disulfide linkage is apparently the only sulfur grouping that can be reduced at the dropping mercury electrode.

The shape of the polarographic wave for the reduction of linear disulfides has been described by Kolthoff.[12] The shape of the reduction waves for two cyclic disulfides has been observed in this Laboratory to be different in that only one wave is present corresponding to the first reduction step observed in curves for linear disulfides. This step has been interpreted as due to the reduction of mercury out of a complex of mercury with the mercaptan formed by direct chemical reaction of the disulfide with the mercury at the electrode surface. α-Lipoic acid behaves in this respect like a cyclic disulfide.

(11a) NOTE ADDED IN PROOF.—M. Calvin and J. A. Barltrop, THIS JOURNAL, **74**, 6153 (1952), have presented the absorption spectrum of 6,8-thioctic acid,[14] which exhibits a maximum at approximately 333 mμ (estimated from figure) with ε approximately 170. The absorption spectrum of α-lipoic acid was determined at too low a concentration (0.2 mg. in 4 ml. ethanol) to detect this absorption. We have since confirmed Calvin and Barltrop's results with synthetic DL-α-lipoic acid.

(11b) S. Wawzonek, *Anal. Chem.*, **21**, 61 (1949).

(12) I. M. Kolthoff and C. Barnum, THIS JOURNAL, **63**, 520 (1941).

Desulfurization of α-lipoic acid by means of Raney nickel yielded a product identified as *n*-caprylic acid by comparison of the X-ray powder data of its silver salt with silver *n*-caprylate. One of the sulfur atoms is attached to the terminal carbon of the *n*-caprylic acid carbon skeleton since the infrared absorption data indicated the absence of a methyl group in the substance.

The conclusion drawn in an earlier note[13] that the polarographic behavior of α-lipoic acid resembled that of a six-membered disulfide ring rather than that of a five- or seven-membered disulfide ring was based on a quantitative comparison of a catalytic evolution of hydrogen in the polarography of dithiol forms. Two models each of dithiols corresponding to five-membered and six-membered rings and one dithiol corresponding to a seven-membered ring disulfide were the only models available. It is now felt that this evidence is inadequate as a basis for judging ring size. Systematic consideration will be given to this comparison in future work.

The data presented are consistent with the interpretation that α-lipoic acid is the cyclic disulfide derived from either 4,8-, 5,8- or 6,8- dimercapto-*n*-caprylic acid.[14]

Experimental

Assay Procedure.—A manometric assay[15] involving activation of pyruvate oxidation by resting *Streptococcus faecalis* cells, and by dried cell preparations,[16] has been used routinely during the course of this investigation. A growth assay[5] employing an acetate-free phosphate-buffered medium with *Streptococcus lactis* also has been used, particularly for preparation of bioautographs.

Activity is expressed, unless specified otherwise, in pyruvate oxidation factor units. One unit is equivalent to the manometric response produced by 1 mg. of Fleischmann Type 3 yeast extract.[15] Potency is expressed in units per mg. of solids.

Extraction of Acid-hydrolyzed Liver Residue with Benzene.—Procedures described previously for the acid hydrolysis of liver residue and subsequent extraction with benzene[17] were adapted to a larger scale. Two hundred and fifty pound batches of liver residue (from beef liver) were stirred at 121° for 4 hours with 210 gal. of 6 *N* sulfuric acid. The hydrolysate was filtered while hot through Hyflo Super-Cel by means of a filter-press and the insoluble material was washed with 40 gal. of hot water. The combined filtrates contained 45 to 90 × 10^6 units at a potency of 0.4 to 0.8 unit/mg. of liver residue employed.

The filtrates were stirred with two 10-gal. portions of benzene. The combined extracts contained 10 to 20 × 10^6 units at a potency of 100 to 200 units/mg. Further extraction of the hydrolysate removed a negligible amount of active material.

Purification of Benzene Extracts by Treatment with Sodium Bicarbonate.—The benzene extracts were evaporated *in vacuo* to a volume of 2 l. and extracted with a total of 600 ml. of 5% aqueous sodium bicarbonate solution. The aqueous extracts were acidified with 6 *N* sulfuric acid and extracted with a total of 250 ml. of benzene. This procedure resulted in an essentially quantitative recovery of ac-

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

(14) The synthesis of these three isomers and a comparison of their biological activities is reported in a recent communication (M. W. Bullock, J. A. Brockman, Jr., E. L. Patterson, J. V. Pierce and E. L. R. Stokstad, *ibid.*, **74**, 3455 (1952)). The isomer presumed to be 6,8-dithiooctanoic acid is by far the most active biologically of the three compounds.

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

(16) I. C. Gunsalus and G. H. F. Schnakenberg, unpublished data.

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

tive material at a potency of 750 to 1500 units/mg. The second benzene extract contained α- and β-lipoic acids in the approximate ratio of 2 to 1, as revealed by countercurrent distribution analysis.[6]

Esterification and Chromatography on Alumina.—To 10.92 g. of acidic material (second benzene extract) in 100 ml. of benzene was added slowly in the cold a solution of diazomethane in 150 ml. of benzene, prepared from 13.5 g. of N-nitrosomethylurea.[18] The reaction mixture was allowed to stand at room temperature for 1 hour and then evaporated *in vacuo*. The brown oil remaining was insoluble in Skellysolve B[19] but soluble in benzene and ethyl acetate. It was dissolved in 109 ml. of benzene and introduced onto a column (3 cm. diameter) prepared from a slurry of 250 g. of activated alumina[20] in benzene. The results are presented in Table III.

TABLE III

CHROMATOGRAM OF METHYL ESTERS ON ALUMINA

Fraction	Solvent	Total volume, ml.	Solids, g.	Potency, units/mg.	Total units
Acids			10.92	1907	20,800,000
Methyl esters				1800	19,700,000
1 + 2	Benzene	200		Inactive	
3 – 5	Benzene	150	2.80	4200	11,800,000
6 – 16	Benzene	550		Inactive	
17	EtAc	500	2.27	3040	6,900,000

The active fractions eluted from alumina columns with benzene (α-ester fractions) were rechromatographed on alumina. As an illustration of this procedure, a solution of 1.33 g. of an α-ester fraction in 7 ml. of Skellysolve B was introduced onto a column (1 cm. diameter) prepared from a slurry of 30 g. of alumina in Skellysolve B. The results are presented in Table IV. By esterification and the use of two alumina chromatograms, the α-lipoic acid present in the second benzene extract was recovered as its methyl ester in 64% yield at a potency of approximately 20,000 units/mg.

TABLE IV

CHROMATOGRAM OF α-ESTER FRACTION ON ALUMINA

Fraction	Solvent	Total volume, ml.	Solids, mg.	Potency, units/mg.	Total units
0			1330	4400	5,860,000
1 – 5	Skellysolve B	100	581	Inactive	
6 – 12	10% benzene[a]	140	211	20,400	4,300,000
13 + 14	10% benzene	40	38	6800	208,000
15	30% benzene	20			
16 – 18	30% benzene	60	56	Inactive	
19	Benzene	20			
20 + 21	Benzene	40	73	Inactive	
22	EtAc	20			
23	EtAc	40	38	7200	220,000

[a] In Skellysolve B.

Purification of Second Benzene Extract *via* Countercurrent Distribution.—A 12.0-g. sample of acidic material (1265 units/mg.) was distributed between 2-liter portions of benzene and 50% aqueous acetic acid in 10 separatory funnels according to the Craig method.[21] The benzene layers in separatory funnels 7 to 9 contained 5.0 g. of viscous oil possessing a potency of 2550 units/mg., of which > 90% represented α-lipoic acid, as revealed by countercurrent distribution analysis.[6] This material was esterified with diazomethane as described above. The results of a typical chromatogram of this type material on a 5 × 7-in., 200-g. alumina column are presented in Table V.

TABLE V

CHROMATOGRAM OF α-ESTER FROM DISTRIBUTION BETWEEN AQUEOUS ACETIC ACID AND BENZENE

Fraction	Solvent	Total volume, ml.	Solids, mg.	Potency, units/mg.	Total units
0			5000	2500	12,500,000
1–6	25% benzene[a]	1955		Inactive	
7–8	50% benzene	500			
9–16	50% benzene	1985	91	18,000 to >40,000	4,963,000
17–22	Benzene	1500	105	14,000 to 18,000	1,800,000
23–25	50% EtAc[b]	1000			1,812,000
26–27	EtAc	1000			

[a] In *n*-heptane obtained from Phillips Petroleum Co. [b] In benzene.

Chromatography on Florisil.—A solution of 135.6 mg. of a concentrate of methyl α-lipoate (27,000 units/mg.) from alumina chromatograms in 1 ml. of Skellysolve B was introduced onto a column (1 cm. diameter) prepared from a slurry of 13.6 g. of Florisil[22] in Skellysolve B. The results are presented in Table VI. This chromatogram yielded 88% of the methyl α-lipoate at a potency of 172,000 units/mg.

TABLE VI

CHROMATOGRAM OF METHYL α-LIPOATE CONCENTRATE ON FLORISIL

Fraction	Solvent	Total volume, ml.	Solids, mg.	Potency, units/mg.	Total units
0			135.6	27,000	3,660,000
1–5	10% benzene[a]	100	7.9	1,040	8,200
6–10	20% benzene	100	18.9	1,140	21,500
11–16	20% benzene	120	2.4	14,400	34,600
17	20% benzene	20	1.3	42,000	54,500
18–28	20% benzene	220	18.8	172,000	3,220,000
29	Benzene	20	14.2	17,600	250,000
30	Benzene	20	15.8	1,800	28,400
31	EtAc	20	33.6	6,000	201,600

[a] In Skellysolve B.

Saponification of Methyl α-Lipoate: Preparation of Crystalline α-Lipoic Acid.—The concentrates of methyl α-lipoate from Florisil columns were pale yellow oils. Attempts to obtain solid material from solutions of the oil in various solvents or by sublimation of the oil *in vacuo* were unsuccessful. The methyl ester was then saponified to obtain crystalline α-lipoic acid as illustrated by the following procedure. A suspension of 54 mg. of concentrate (160,000 units/mg.) in 10 ml. of 0.1 *N* potassium hydroxide was shaken for 6 hours at room temperature in an atmosphere of nitrogen. The saponification mixture was extracted with a total of 4.5 ml. of benzene. The unsaponified material consisted of a yellow oil, 17.9 mg. (780 units/mg.). The aqueous layer was acidified with 2 *N* sulfuric acid and extracted with a total of 8.2 ml. of benzene. The benzene extracts were evaporated *in vacuo*, leaving a yellow oil, which was extracted with a total of 1.5 ml. of warm Skellysolve B. The latter solution was cooled while scratching the inside of the container with a glass rod. Pale yellow platelets separated, 10 mg. (250,000 units/mg.)[23]; 29% recovery of active material. The mother liquor yielded 17.4 mg. of a yellow oil (125,000 units/mg.) from which further crystalline material could not be obtained. The potency of the crystalline α-lipoic acid was not increased by two recrystallizations from Skellysolve B.

(18) F. Arndt, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1946, p. 165.

(19) A *n*-hexane fraction, b.p. 60–68°, obtained from the Skelly Oil Co., Kansas City, Missouri.

(20) Merck aluminum oxide, Reagent Grade, is satisfactory. In most experiments pretreated Alcoa alumina (Aluminum Co. of America) was used. This alumina was stirred with 20% aqueous sodium cyanide, washed well with water and then stirred with 50% aqueous acetic acid, washed again with water, followed by activation at 175° for 48 hours.

(21) A. Weissberger, "Technique of Organic Chemistry," Vol. III, Interscience Publishers, Inc., New York, N. Y., 1950, p. 171.

(22) 60/100 mesh, obtained from the Floridin Co., Warren, Pa.

(23) Crystalline α-lipoic acid possesses a potency of approximately 15,000,000 acetate-replacing factor units/mg.[5]; *i.e.*, 1.7×10^{-6} μg./ml. of culture medium is capable of supporting half-maximal growth of *Streptococcus lactis* in the absence of acetate.

Examination of the crystalline material by means of bioautographs[5] and by countercurrent distribution analysis[6] revealed migration characteristics identical with those of the less polar principle present in acid-hydrolyzed liver residue.

Characterization of α-Lipoic Acid.—Crystalline α-lipoic acid melts at 47.5° (corrected) on a micro-stage. It is very soluble in benzene, ethyl acetate, methanol, less soluble in Skellysolve B and only sparingly soluble in water. The crystalline substance is optically active: $[\alpha]^{25}D$ +96.7° (1.88% in benzene).

Anal. Calcd. for $C_8H_{14}O_2S_2$: C, 46.57; H, 6.84; S, 31.08; mol. wt., 206. Found: C, 46.35, 46.41; H, 6.79, 6.58; S, 31.21; mol. wt. (electrometric titration), 217.

The X-ray diffraction data (Table I) were obtained using a powder camera, diameter 114.6 mm. and nickel filtered copper radiation, λ 1.5374 Å. Infrared absorption spectra (Table II) were obtained on carbon tetrachloride solutions, approximately 3% concentration in 0.1-mm. path and 0.05% in 10-mm. path microcells on a Beckman IR-2T spectrophotometer equipped with sodium chloride and calcium fluoride monochromators. Polarographic experiments were done manually with a Heyrovsky polarograph, E. H. Sargent model XII. The supporting electrolyte was 0.035 *M* potassium chloride and *M* perchloric acid, *p*H 1.27.

Desulfurization of α-Lipoic Acid.—A 3.1-mg. sample of α-lipoic acid was weighed into a small test-tube. To this was added 0.22 g. of ordinary Raney nickel and 1 ml. of 75% ethanol. The mixture was refluxed overnight and then filtered through a filter aid. The precipitate was washed four times with small quantities of dilute ammonium hydroxide solution and twice with concentrated ammonium hydroxide solution.

Air was bubbled through the filtrate for about three hours until no more ammonia was detected. The volume of filtrate was about 1 ml. The precipitate was removed by filtration and washed with two portions of water (0.2 and 0.1 ml.). These washings were combined with the filtrate and again passed through a filter. This filtrate was treated with 8 drops of 0.1 *N* silver nitrate solution. The silver salt was collected on a sintered glass filter and washed with several 0.1-ml. portions of water, ethanol and dry ether. The residue weighed about 2 mg.

Silver *n*-Caprylate.[24]—A mixture of 0.14 g. (0.001 mole) *n*-caprylic acid and 5 ml. of water was neutralized with 11 drops of 1:2 ammonium hydroxide solution. Excess ammonia was removed by bubbling air through the solution. The silver salt was precipitated by adding 10.5 ml. of 0.1 *N* silver nitrate solution. The precipitate was collected and washed with water until the filtrate gave a negative test for Ag^+. It was further washed with absolute alcohol and ether. The yield was 0.20 g.

Discussion

It is estimated that 10 tons of liver residue were processed during the course of developing and applying the procedures described. From this amount of liver residue approximately 30 mg. of crystalline α-lipoic acid was obtained, which sufficed to permit determination of the structural features of this catalytic agent. Because DL-α-lipoic acid is now available as a synthetic product,[25] improvements of the present isolation procedure have not been attempted.

Several factors contributed to the necessity of processing such a large amount of liver residue. Although the latter is one of the richest sources of α-lipoic acid, it contains only 1.6 to 3.2 μg./g. of this catalytic principle. The recovery of activity in the form of crystalline α-lipoic was only 2.5% according to the procedures described. Actually, these procedures are to be regarded as illustrating the individual steps rather than representing one continuous process. In practice, concentrates were often combined at each stage of the isolation. Many of the pooled concentrates did not result in isolation of crystalline α-lipoic acid. In this regard, attention is called to the fact that the second benzene extract consisted essentially of a mixture of organic acids, containing both α- and β-lipoic acids, the former in an amount of approximately 0.2 to 0.4%. The composition of this mixture of acids may have varied with different batches of liver residue, thus contributing to difficulties encountered in subsequent chromatographic steps. Furthermore, the low melting point of α-lipoic acid (47.5°) is thought to have caused difficulty in separating the principle in crystalline form from the acidic impurities present in saponified concentrates of methyl α-lipoate.

The discovery that α-lipoic acid possessed a disulfide linkage led to the identification of β-lipoic acid as an oxidized form of α-lipoic acid, probably a sulfoxide.[26] Procedures were then developed for interconverting these two principles,[27] which, if applied at an earlier stage, would have permitted a more efficient isolation to be achieved. It would seem that the presence of β-lipoic acid in acid-hydrolyzed liver residue is due to the oxidizing effect of hot sulfuric acid on α-lipoic acid. Use of a non-oxidizing hydrolytic agent may have avoided this interconversion.

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(24) F. W. Matthews, G. G. Warren and J. H. Mitchell, *Anal. Chem.*, **22**, 514 (1950).

(25) C. S. Hornberger, Jr., R. F. Heitmiller, I. C. Gunsalus, G. H. F. Schnakenberg and L. J. Reed, THIS JOURNAL, **74**, 2382 (1952); **75**, 1273 (1953).

(26) L. J. Reed, B. G. DeBusk, I. C. Gunsalus and G. H. F. Schnakenberg, *ibid.*, **73**, 5920 (1951).

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