STUDIES ON REPORTED α-LIPOIC ACID INHIBITIONS*

By D. E. Bottrill† and J. T. Wiskich‡

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Summary

Pure lipoic acid was found to be without effect on the electron-transport systems of beef heart mitochondria, cauliflower bud mitochondria, and beetroot mitochondria. The activity of purified malate and lactate dehydrogenases was also unaffected.

Solutions of lipoate, when subjected to photolysis, potently inhibited malate and NADH oxidation by isolated mitochondria. The effects were found not to be secondary to changes in mitochondrial structure. Purified enzymes responded similarly. The responses could not be duplicated or prevented by a number of similar compounds but could be prevented by bovine serum albumin.

The effective product of photolysis was not identified but it is suggested that one product is a dimer of lipoic acid having two sulphydryl groups and linked by a disulphide bond. The effects may be due to a disulphide–sulphydryl interchange between the photolytes and the enzyme systems.

I. Introduction

Lipoic acid (6,8-dithio-n-octanoic acid) has been shown to take part in those reactions of the Krebs cycle involving decarboxylation of α-keto acids (Gunsalus 1956; Liebecq 1956; Covello 1957; Reed 1957, 1960; Koike and Reed 1960; Massey 1960). From an investigation on the metabolism of isolated plant mitochondria, Wiskich and Morton (1960) reported that added oxidized α-lipoic acid inhibited the oxidation of NADH and of substrates with NAD-linked dehydrogenases. The oxidation of succinate was not affected under these conditions. It was also reported that oxidized α-lipoic acid inhibited oxidative phosphorylation and stimulated the oxidation of reduced cytochrome *c*. The results are relevant to those of Goldman (1959) and of Koike and Reed (1960) who observed that lipoate acetyltransferase was inhibited by slight contaminations of lipoic acid and to those of Koike, Shah, and Reed (1960) who observed that free lipoic acid inhibited hydrogen transfer between NADH and bound lipoate. The above reactions are possible sites of the inhibitions

* This work was carried out under the direction of the late Professor R. K. Morton in the Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, in the Plant Physiology Unit, CSIRO, and in the Botany Department, University of Sydney.

† Present address: 8 Brook Av., Glen Osmond, S.A. 5064.

‡ Present address: Botany Department, University of Adelaide, S.A. 5000.

reported by Wiskich and Morton (1960) with α-oxoglutarate as substrate but not with NADH or malate as substrates. Henderson and Eakin (1960) also observed a non-specific inhibition of a range of enzymes by cyclic disulphides and closely related compounds, in particular, an NAD-dependent malate dehydrogenase which was especially sensitive. Ziegler, Green, and Doeg (1959) also reported a lipoic acid-sensitive diaphorase activity in a lipid–flavin–protein complex isolated from beef heart mitochondria.

The effect of lipoic acid on many of the above enzyme systems has been re-examined and this paper reports evidence that these effects are not due to lipoic acid but to products of lipoic acid formed in response to temperature or light treatments.

II. Materials and Methods

(*a*) *Enzyme Assays*

Mitochondria were prepared from red beetroot tissue (*Beta vulgaris* L.) by the method of Wiskich, Morton, and Robertson (1960) and from cauliflower buds (*Brassica oleracea* L.) by the method of Laties (1953). Oxidative phosphorylation and electron-transport-chain studies were performed as described by Wiskich, Morton, and Robertson (1960). Electron-transport particles were prepared by the procedure of Crane, Glenn, and Green (1956). Enzymes of the NADH oxidation system were assayed according to Minakami, Ringler, and Singer (1960). Malate dehydrogenase, prepared by the procedure of Siegel and Englard (1960), was assayed using malate plus NAD^+ (Wolfe and Nielands 1956) and oxaloacetate plus NADH (Ochoa 1957) as substrate combinations. The purified enzyme had a K_m at pH 10·0 of $8{\cdot}48 \times 10^{-5}$M, in close agreement with that reported by Wolfe and Nielands (1956). Rabbit skeletal muscle lactate dehydrogenase (Sigma) was assayed by the method of Colowick and Kaplan (1957).

(*b*) *Photolysis of Lipoic Acid*

Solutions of lipoic acid were irradiated with daylight or with light from a xenon arc lamp which passed through EEL filters. The products of photolysis were subjected to the following tests: nitroprusside test for sulphydryl groups; methylene blue test (Johnson and Nishita 1952) and lead acetate papers for hydrogen sulphide; and chromatography.

(*c*) *Titration of Sulphydryl Groups*

A combination of the methods described by Grunert and Phillips (1951), MacDonnell, Silva, and Feeney (1951), and Tsao and Bailey (1953) was used. To 0·1 ml of lipoic acid (approx. 0·3 μmole) were added 1 ml of 0·45M sodium carbonate, 1 ml of 1·5 mM *p*-chloromercuribenzoate (as measured spectrophotometrically at 234 nm—Boyer 1954), and 0·5 ml of 40 mM sodium nitroprusside. The excess *p*-chloromercuribenzoate was titrated with 0·5 mM cysteine monohydrochloride monohydrate (Merck).

(*d*) *Chemicals*

NAD^+, NADH, and ATP were obtained from P.L. Biochemicals. Cytochrome *c* was extracted from beef heart and purified on Amberlite CG–50 ion-exchange resin.

Lipoic acid, obtained from Nutritional Biochemical Corp., Cleveland, and Sigma Chemical Co., St. Louis, was purified by recrystallization from n-cyclohexane under a red photographic safety lamp (Thomas and Reed 1956). All solutions were stored in the dark at 4°C. The purity of the preparations was determined by spectrophotometry (using an extinction coefficient of 157 at 330 nm—Reed and Nui 1955), titration of carboxyl groups, melting point determinations, chromatography using n-butanol saturated with 0·5M NH_4OH (Patterson *et al.* 1951; Reed and Debusk 1954), and nitroprusside tests for sulphydryl groups.

III. Results

(a) *Studies on Enzyme Systems*

In contrast to the results reported by Wiskich and Morton (1960) recrystallized lipoic acid had no effect on oxidative phosphorylation of isolated cauliflower mitochondria (Table 1). Likewise, the enzyme systems of the electron-transfer chain were

Table 1

EFFECT OF PURE LIPOIC ACID ON THE OXIDATIVE PHOSPHORYLATION OF CAULIFLOWER MITOCHONDRIA

Expt. No.	Solvent	Substrate	Lipoic Acid (mM)	$Q^{N}_{O_2}$*	P/O†	P/N‡
1	Ethanol	Succinate	0	78·9	1·27	7·07
	Ethanol	Succinate	0·5	72·2	1·24	8·10
2	Na_2CO_3	Succinate	0	95·4	1·67	14·3
	Na_2CO_3	Succinate	0·5	93·5	1·54	13·0
3	Na_2CO_3	Malate	0	46·5	2·85	11·95
	Na_2CO_3	Malate	0·5	34·2	2·72	8·17

* No. of microlitres of oxygen respired per 1 mg mitochondrial protein.

† No. of μmoles of orthophosphate esterified per 1 μl oxygen respired.

‡ No. of μmoles of orthophosphate esterified per 1 mg mitochondrial protein.

Table 2

EFFECT OF LIPOIC ACID ON NADH OXIDATION BY ISOLATED CAULIFLOWER AND BEETROOT MITOCHONDRIA

Expt. No.	Enzyme System	Concn. of Lipoic Acid Added (mM)	Rate ($10^3\Delta E$/min)
1*	Cauliflower:	Control	63·0
	NADH oxidation	0·25	59·0
		0·25¶	4·7
2†	Beetroot:	Control	66·0
	NADH oxidation	0·13	66·0
		0·13¶	11·0
3‡	Beetroot:	Control	231
	Reduced NAD–cyto-	0·13	227
	chrome *c* oxidase	0·13¶	117
4‡	Beetroot:	Control	0·800
	Cytochrome *c*	0·13	0·808
	oxidase	0·13¶	1·940

* NADH oxidation measured spectrophotometrically. Lipoic acid dissolved in ethanol; 40 μg mitochondrial nitrogen per reaction.

† NADH oxidation measured as in experiment 1; lipoic acid dissolved in Na_2CO_3; 54 μg mitochondrial nitrogen per reaction.

‡ Cytochrome *c* reduction and oxidation was measured spectrophotometrically. Rates of reduction and oxidation are given as the first-order velocity constant (Smith 1955). 37 μg mitochondrial nitrogen per reaction.

¶ Lipoic acid light-treated at room temperature.

not affected by recrystallized lipoic acid but were affected by samples of recrystallized lipoic acid that had received light treatment (Table 2). The enzyme system causing NADH oxidation was sensitive to added KCN, amytal, and antimycin A and there was no lipoyl dehydrogenase activity; therefore, NADH was regarded as being oxidized via the electron-transfer chain. In each case the response to light-treated lipoic acid is similar to that reported by Wiskich and Morton (1960) for pure lipoic acid. These responses were observed in mitochondria from a number of sources but the magnitude of the reponses was not constant (Table 3).

TABLE 3

EFFECTS OF LIGHT-TREATED LIPOIC ACID ON OXIDATION RATES OF ENZYME SYSTEMS IN MITOCHONDRIA ISOLATED FROM OTHER PLANT TISSUES

Lipoic acid concentration 0·1 mM

Enzyme System	Oxidation Rate (as % of control)			
	Silver Beet Petiole	Cauliflower Bud	Pea Cotyledons	Wheat Root
NADH oxidation	39	61	76	67
Reduced NAD: cytochrome *c* oxidase	44	53	83	86
Cytochrome *c* oxidase	270	379	307	200

It was observed that light-treated lipoic acid could cause mitochondrial swelling (Fig. 1), which was reversible with ATP and magnesium. However, this effect on

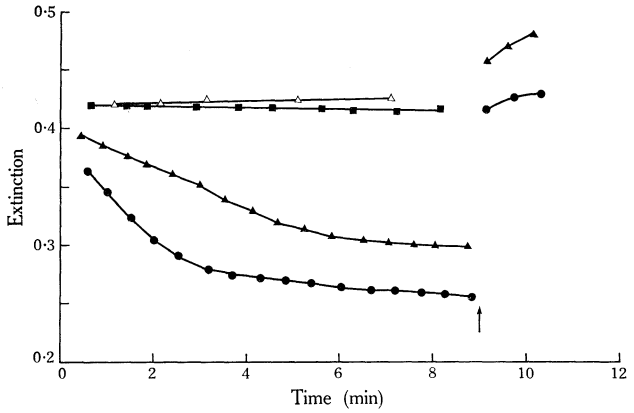


Fig. 1.—Swelling of beetroot mitochondria in 0·4M sucrose containing 0·28M Tris–0·31M acetic acid buffer, pH 7·2. ■ Plus 13 mM NADH. ▲ Plus 5 mM lipoic acid. ● Plus 5 mM lipoic acid and 13 mM NADH. △ Plus 5 mM lipoic acid, 13 mM NADH, and 0·17% of bovine serum albumin. The lipoic acid had received a light treatment. The arrow indicates the addition of 3 mM $MgCl_2$ and 5 mM ATP.

mitochondrial structure could not be the primary action of light-treated lipoic acid since the above responses could be demonstrated in mitochondria swollen by hypo-

tonicity, mitochondrial fragments (Table 4), and mitochondria under conditions where swelling could not occur, as in the presence of ATP and magnesium (Table 5).

TABLE 4

EFFECTS OF LIGHT-TREATED LIPOIC ACID ON OXIDATION RATES OF ENZYME SYSTEMS IN MITOCHONDRIAL FRAGMENTS PREPARED WITH DEOXYCHOLATE

Beetroot mitochondria were homogenized with 0·3% deoxycholate and centrifuged at 20,000 *g* for 10 min to remove an inactive fraction. Rates are expressed as changes in extinction per hour per milligram mitochondrial nitrogen

Enzyme System	Control Oxidation Rate	Rate with Lipoic Acid (0·1 mM) Present	Rate with Lipoic Acid as % of Control Rate
Reduced NAD: cytochrome *c* oxidase	9·2	4·0	44
Cytochrome *c* oxidase	50	83	166

TABLE 5

EFFECT OF LIGHT-TREATED LIPOIC ACID ON "CONTRACTED" BEETROOT MITOCHONDRIA

"Contracted" mitochondria treated with 5 mM ATP and 3 mM $MgCl_2$. Lipoic acid concentration 0·1 mM. Rates are expressed as changes in extinction per hour per milligram mitochondrial nitrogen

Enzyme System	"Normal" Rates			"Contracted" Rates		
	Control	With Lipoic Acid	As % of Control	Control	With Lipoic Acid	As % of Control
NADH oxidation	150	13	9	35	13	37
Cytochrome *c* oxidase	1224	2893	236	1471	2180	148

The effective substance was tightly bound to the mitochondria because particles pre-incubated with light-treated lipoic acid and washed by centrifugation still showed the effects (Table 6).

TABLE 6

EFFECT OF PRETREATMENT WITH LIGHT-TREATED LIPOIC ACID ON BEETROOT MITOCHONDRIAL ACTIVITY

The beetroot mitochondria were incubated in 0·4M sucrose with and without 5 mM lipoic acid at 0°C for 20 min, collected, and washed by centrifuging. Rates are expressed as changes in extinction per hour per milligram of mitochondrial nitrogen

Enzyme System	Control	Pretreated Rate	Rate as % of Control
NADH oxidation	106	37	35
Reduced NAD: cytochrome *c* oxidase	306	199	65
Cytochrome *c* oxidase	493	853	173

These results led us to examine the effect of lipoic acid on purified enzymes. Henderson and Eakin (1960) reported that lipoic acid inhibited malate and lactate dehydrogenases but these enzymes failed to respond to added recrystallized lipoic acid (Table 7). Malate dehydrogenase was inhibited by light-treated lipoic acid while lactate dehydrogenase was only inhibited by lipoic acid solutions stored in the dark at room temperature. Inhibitory products are therefore formed from lipoic acid under the influence of both light and temperature.

TABLE 7

EFFECTS OF LIPOIC ACID ON ACTIVITY OF MALATE AND LACTATE DEHYDROGENASES

Enzyme	Substrate	Lipoic Acid Concn. (mM)	Rate ($10^3\Delta E$/min)
Malate dehydrogenase from beef heart	Malate+NAD^+	Nil	16·8
		0·5*	19·5
	Oxaloacetate +NADH	Nil	32·1
		0·5*	29·3
Malate dehydrogenase from beetroot mitochondria	Malate+NAD^+	Nil	620
		0·13*	674
		0·13†	735
		0·13‡	520
Lactate dehydrogenase	Pyruvate+NADH	Nil	1090
		0·13*	996
		0·13†	251
		0·13‡	930

* Kept dark and refrigerated. † Kept dark at room temperature.

‡ Light-treated at room temperature.

The effects caused by light-treated lipoic acid could not be duplicated by cysteine, cystine, oxidized or reduced glutathione, 2,3-dimercaptopropanol, or short-chain fatty acids. Furthermore, none of these compounds nor EDTA, arsenite, or ascorbate protected the mitochondria from the effects of light-treated lipoic acid. Only bovine serum albumin proved to be an effective protecting agent, presumably because it bound the products of photolysis of lipoic acid solutions.

It was observed that fresh solutions of commercially available lipoic acid may or may not be inhibitory to enzymes of the NADH oxidation system and required varying periods of pretreatment to develop inhibitory characteristics. Inhibitory effects of lipoic acid solutions subjected to short-term light treatment increased when solutions were stored overnight in the dark at 4°C. Prolonged light treatment could abolish the stimulatory effect on cytochrome *c* oxidase.

(*b*) *Chemistry of Lipoic Acid*

The lipoic acid samples used were found to be about 80% pure by the assay procedures used. The melting point was found to be 59–59·5°C and the pK_a was 4·90–4·96. Chromatography resulted in only one detectable spot. The spectrum of purified lipoic acid (Fig. 2) remained constant if the solutions were kept in the dark and

refrigerated. This constancy was taken to indicate stability. There was no reduction in the extinction caused by the dithiolane ring on addition of cyanide as was reported by Calvin (1954). However, if the solutions were exposed to light and ambient temperatures spectral changes occurred (Figs. 2 and 3). The extinction decreased at 330 nm

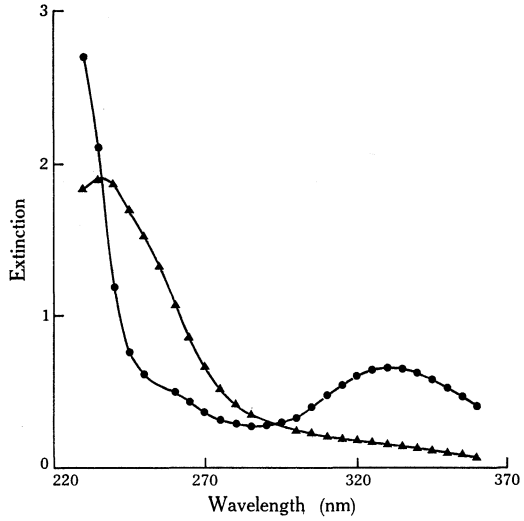


Fig. 2.—Absorption spectra of a sample of pure lipoic acid before (●) and after (▲) 14 days photolysis.

and increased at 250 nm (Fig. 3). The changes in the spectrum remained constant if solutions were stored in the dark after photolysis. Using the decrease in extinction at 330 nm as a measure of change and a series of EEL filters it appeared that the

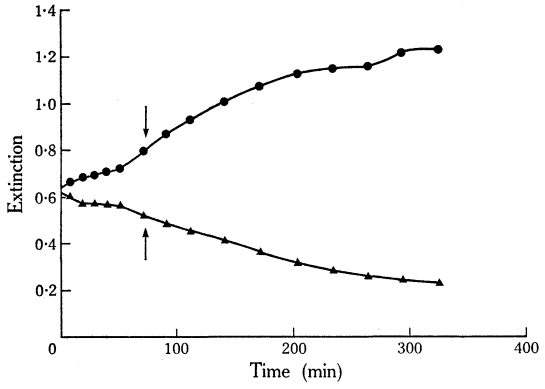


Fig. 3.—Spectral changes of an ethanolic solution of lipoic acid during photolysis. ● Change at 250 nm. ▲ Change at 330 nm. The arrows indicate removal of EEL filter No. 621, thus increasing light intensity threefold.

maximum rate of change occurred in the ultraviolet region of the spectrum. Spectral changes occurred with water, ethanol, or benzene as solvent and eventually a characteristic "sulphydryl" odour developed.

A positive nitroprusside test was obtained with light-treated ethanolic solutions of lipoic acid but no sulphides could be detected by the methylene blue test or by lead acetate papers with photolysis at low ambient temperatures. However, at higher ambient temperatures photolysis did produce detectable sulphides. Titration of the

sulphydryl groups showed that one group was present per molecule of lipoic acid. Chromatography of the light-treated lipoic acid indicated one spot (other than lipoic acid itself) when the chromatogram was developed with either bromophenol blue or nitroprusside, indicating that both the carboxylic and sulphydryl groups were still on the one molecule.

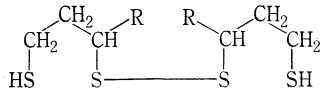
The product of light-treated lipoic acid was unlike the product formed in alkaline solutions since it failed to yield lipoic acid when subjected to the depolymerization procedure of Reed and Nui (1955).

IV. Discussion

Pure lipoic acid had no effect on any of the enzymes or enzyme systems examined (Tables 1, 2, and 7). Minakami (personal communication) also found that pure lipoic acid was without effect on NADH oxidation. However, light-treated lipoic acid caused an inhibition of most enzymes and enzyme systems tested (Tables 2–7; Fig. 1). Solutions of lipoic acid stored in the dark at room temperature inhibited lactate dehydrogenase while the light-treated sample of lipoic acid was without effect (Table 7). These results indicate that at least two types of reaction proceed in lipoic acid solutions stored at room temperature, one requiring light pretreatment and the other occurring entirely in the dark. It is the products of these reactions which alter the rates of enzyme reaction. A similar conclusion was drawn by Searls and Sanadi (1961) who suggested that the variability observed in experiments with lipoic acid may be related to its ease of polymerization.

Polymerization of lipoic acid is known to take place in alkaline solutions (Reed and Nui 1955) but the products of photolysis of lipoic acid are different in that they are not depolymerized by the same procedures as are used for depolymerizing the polymer formed in alkaline solutions. Similarly it is unlikely that the products formed in ethanolic solutions in the dark are similar to those formed in alkaline solutions since such solutions are acidic. Barltrop, Hayes, and Calvin (1956) suggested that under certain conditions the disulphide bond of lipoic acid cleaves with the formation of a sulphydryl and a sulphenic acid moiety. Such a reaction is possible since Benesch and Benesch (1958) have reported the formation of sulphenic acid and sulphydryl groups from activated disulphides under acid conditions. The opening of the five-membered ring in light treatment of lipoic acid is indicated by the decrease in extinction at 330 nm (Reed 1960) and the formation of equimolar sulphydryl moieties.

In most cases sulphenic acid moieties are highly unstable (Reid 1958; Cecil and McPhee 1959) and can react together to form a disulphide bond. The spectrum of light-treated lipoic acid has an inflection at 252 nm (Fig. 2) indicating the presence of linear disulphide bonds (Reed 1960). This evidence, together with that from co-chromatography of the sulphydryl and carboxylic groups, would suggest the following structure for the product of light-treated lipoic acid:



where R = $-(CH_2)_4COOH$. A solution of the proposed dimer would be expected to undergo sulphydryl–disulphide interchange, with the formation of a trimer and

dihydrolipoic acid. Dihydrolipoic acid is unstable in acid conditions, liberating hydrogen sulphide (Covello and de Vena 1960). Hydrogen sulphide was liberated when photolysis was carried out at higher ambient temperatures. Cole (1963) has also observed a combined light and temperature response in the dissociation and interchange of some disulphides.

The effects of light-treated lipoic acid on mitochondrial enzyme systems are not secondary to some general effect on mitochondrial structure since intact mitochondria (Tables 2 and 3), swollen mitochondria, mitochondrial fragments (Table 4), and contracted mitochondrial systems (Table 5) all respond similarly. These effects may be due to a disulphide–sulphydryl interchange between the photolytic product of lipoic acid and sulphydryl or disulphide groups associated with the active sites of the enzymes as was previously suggested by Wiskich and Morton (1960), since washing of the mitochondrial system after a pretreatment with light-treated lipoic acid failed to remove the response (Table 6). There is ample evidence to indicate functional sulphydryl or disulphide groups in electron-transport oxidation of NADH (Minakami, Schindler, and Estabrook 1964; Tyler *et al.* 1965) and high-energy phosphate fixation (Fluharty and Sanadi 1960; Newton 1962). However, detailed mechanisms cannot be formulated until the reactive components of light-treated lipoic acid are identified.

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