with the infusion of the reactive fraction appeared to be that of hemoglobinemia due to hemolysis *in vivo*. This can be accounted for on the basis of intolerance of the organism for low molecular weight fatty acids and their esters such as were contained in fraction A. The relative solubility of the latter and their ease of dissociation hastened the process of saponification in the blood and the surface action effect on the red blood cells causing hemolysis and the resultant hemoglobinemia. Better toleration obtained with the injections of the B and C fractions can be accounted for on the relative insolubility of the long chain fatty acid esters retarding in the blood any chemical interaction or hydrolysis.

Summary. 1. Emulsions of the glycerides of the short chain fatty acids were toxic when injected intravenously into human subjects. 2. The glycerides of the high molecular weight fatty acids, lauric, myristic, palmitic and stearic acids were non-toxic when injected in emulsion form. 3. Inclusion with the latter of small concentrations of the fatty acid esters of the type of oleic and linoleic acids did not affect the toxicity of the emulsions.

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α-Lipoic Acid in Oxidative Reactions of a *Corynebacterium*. (19499)

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α-Lipoic acid is a highly active growth factor for several microorganisms(1,2) and functions in the oxidative conversion of pyruvate to acetate by *Streptococcus faecalis*(1). Acetate will replace the factor for the growth of several lactic acid bacteria. On the other hand, acetate will not substitute for a lipoic acid-like material in the growth of a *Corynebacterium*(2,3). This difference prompted the present study of the *Corynebacterium*, since it suggested that α-lipoic acid possesses a function or functions in addition to its role in the formation of acetate from pyruvate.

Methods. The organism,* maintained on yeast extract-glucose-agar slants, was grown in a medium similar to that described by Stokstad, *et al.*(3). Instead of "protogen", a concentrate of lipoic acid was used; this material, potency 50,000 acetate equivalents/mg(4), gave suitably deficient cells at a level of 50 μg/liter or less. To permit aeration of the medium, 1.5 mg/liter of Dow-Corning Antifoam A were added. A 10% inoculum, 24 hours old, was used for 14 liter batches. After 24 hours at 25°C, the cells were harvested with a Sharples Super-Centrifuge, washed with 0.9% saline, suspended in water, lyophilized, and stored at −18°C. The experiments described below were performed with these dried, deficient cells, utilizing the usual manometric methods. The cells and crystalline α-lipoic acid† were mixed just before equilibrating the Warburg flasks.

Lipoic acid effects. A preliminary survey revealed that a variety of substances was oxidized by the dried cells and that the rate of oxygen uptake for many of these was stimulated by α-lipoic acid (Table I, Columns

* Our thanks are due to Dr. E. L. R. Stokstad for a culture of this organism.

† We are indebted to Dr. L. J. Reed for samples of α-lipoic acid.

TABLE I. Effect of Crystalline α-Lipoic Acid on Oxidation of Various Substrates.

	Oxygen uptake (μatoms)			
	Deficient cells		"Aged" cells	
	Without lipoic acid (1)	With lipoic acid (2)	Without lipoic acid (3)	With lipoic acid (4)
Endogenous	14.8	20.9	18.1	27.7
			9.7*	5.8*
Substrates:†				
Pyruvate	15.5	21.6	14.6	14
Lactate	18.8	25.9		
l-Malate	6.6	20.8	1.6	18.5
Fumarate	7.4	13.8	6.1	23.6
Acetate	9.2	30	2	7.3
α-Ketoglutarate	.8	3.4		
Glutamate	2.2	20.7	.0	11.1
Ornithine	7.5	18.2		
Arginine	45.8	52.6		
Proline	27.7	62.3	8.2	29.2
			8*	22.8*
γ-Aminobutyrate	71.1	69	62.4	67.3

* Substrate tipped in after 2 hr. † Oxygen uptakes corrected for endogenous respiration. Conditions: 7 mg cells and .07 μg α-lipoic acid in 1.8 ml .055 M phosphate pH 5.9; .2 ml .2 M substrate tipped in after 15-20'. Duration of exp.: 180'.

TABLE II. Response of "Aged" Cells to Varying Amounts of Crystalline α-Lipoic Acid.

Wt of α-lipoic acid (μg)	Oxygen uptake (μatoms)		
	Endogenous	Proline*	l-Malic acid*
.0	26.2	16.2	1.9
.002	27	22.3	5.9
.01	30.6	37.4	19
.05	33.4	45.5	35.1

* Substrate uptakes corrected for endogenous uptake.
Conditions as in Table I, but exp. was 300 min.

1 and 2). Distinct stimulations were observed with as little as 0.002 μg lipoic acid per Warburg flask (Table II). The endogenous activity is high and is stimulated by the growth factor under the conditions used in most experiments; however, it should be noted that low lipoic acid levels produce substrate stimulations without appreciable effect on the endogenous activity (Table II).

The finding that pyruvate is oxidized by this organism raises the possibility that pyruvate oxidation is the rate-limiting step in the oxidation of the other substrates. Since α-lipoic acid functions in pyruvate oxidation (1) and shows a stimulatory effect with pyruvate here, the possibility then exists that the stimulatory effects observed with the other substrates are the result of a single function. However, the results described below appear to rule out this explanation.

Deficient "aged" cells. Table I, Columns 3 and 4, presents results of experiments performed with a batch of cells which had been in use over a period of 3 months. These "aged" cells could still oxidize pyruvate, but the rate of oxygen uptake was no longer stimulated by α-lipoic acid. Nevertheless, the stimulatory effect on the other substrates is still in evidence. This finding suggests a role for lipoic acid in the oxidation of these substances which is distinct from its known role in pyruvate oxidation. Attention is drawn to the observation that preliminary aeration of the cell suspension reduces the blank activities considerably without greatly affecting the stimulatory effect of lipoic acid on proline oxidation (starred values, Table I). In fact, the stimulation was observed with cells in which the endogenous activity was now *lower* in the presence of lipoic acid (because of greater depletion of endogenous substrates during the aeration).

Effect of high pH. All experiments described above were performed at pH 5.9. At pH 9.2, with fresh cells, pyruvate oxidation was reduced to a very low level, yet proline oxidation was considerable and showed stimulation by lipoic acid (Table III, Columns 1 and 2). The activity of the blanks at this pH was of minor proportions. The pyruvate

TABLE III. Effect of α-Lipoic Acid on the Oxidation of Various Substrates at High pH.

Substrate	Oxygen uptake (μatoms) Deficient cells — Without lipoic acid (1)	Deficient cells — With lipoic acid (2)	"Aged" cells — Without lipoic acid (3)	"Aged" cells — With lipoic acid (4)
Pyruvate	.3	2	.0	.0
Proline	11.1	23.3	2.8	11.2
γ-Aminobutyrate			3.1	8.7
None	2.8	5.9	5.7	9.2

Conditions: 7 mg dry cells, .07 μg α-lipoic acid, and 50 μmoles K_2HPO_4 in 1.8 ml tris(hydroxymethyl)aminomethane buffer, .11 M. .2 ml .2 M substrate tipped in after equilibration. Columns 1, 2: pH 9.2; duration 120′. Columns 3, 4: pH 8.8; duration 150′. Oxygen uptake corrected for endogenous respiration.

activity appears to be much too small to account for the lipoic acid effect on proline. With "aged" cells at pH 8.8, there is no pyruvate oxidation at all, yet proline and γ-aminobutyric acid are oxidized and respond to the growth factor (Table III, Columns 3 and 4). At this pH, acetate, malate, and glutamate are also not attacked, and it may be concluded that lipoic acid operates in proline and aminobutyrate oxidation at a point distinct from its function in the oxidation at the lower pH of the other substrates mentioned here.

Non-accumulation of Pyruvate. If in this organism α-lipoic acid were involved only in pyruvic acid oxidation, one would expect pyruvate (under the conditions used) to *accumulate* during substrate oxidation in the absence of the factor. Analysis of the reaction mixtures(5) revealed no accumulation of pyruvate as a result of acetate, proline, or endogenous oxidation, with or without added lipoic acid. This supports the previous evidence and indicates a non-pyruvate function for lipoic acid.

Glutamate oxidation. A trapping agent, 2,4-dinitrophenylhydrazine, was used to seek carbonyl products of glutamate oxidation. The reagent was incubated with dried deficient cells at pH 5.9 buffer, with and without sodium glutamate, and with and without α-lipoic acid. The cells were centrifuged off and the supernatants acidified and extracted with ethyl acetate. The organic extracts were analyzed by paper chromatography, using two different solvents(6). Spots corresponding in position to pyruvate and α-ketoglutarate were found in all samples. The spots were all equally faint except the ketoglutarate spot obtained from the glutamate-lipoic acid combination; this was markedly more intense. This agrees with the manometric data and suggests that formation of ketoglutaric acid from glutamic acid is stimulated by lipoic acid.

γ-Aminobutyric acid oxidation. Since the metabolism of this compound has been studied so little, and because the *Corynebacterium* shows such a high rate of oxidation, further studies were made of the mode of oxidation. A trapping experiment like the one above resulted in pyruvate and ketoglutarate spots which were more intense than the controls, the pyruvate considerably so. The pyruvate spot was identified further by elution with pH 7.4 phosphate and examination with a spectrophotometer. In agreement with a sample of pyruvate similarly treated, an absorption peak was found at 370 mμ; addition of alkali moved this to 445 mμ. Succinaldehydic and formylacrylic acids, possible intermediates, were ruled out by comparison of R_F values and absorption spectra. The closely related compounds, γ-hydroxybutyric acid, δ-aminovaleric acid, and β-aminopropionic acid are not attacked.

Other metabolites. Glycolic, glyoxylic, citric, itaconic, and succinic acids were inert as substrates, or nearly so; similarly, with glycine, alanine, leucine, phenylalanine, lysine, and hydroxyproline. Formate was oxidized rapidly, but without influence by α-lipoic acid.

Putrescine was the most active substrate tested; oxygen uptake by deficient cells proceeded rapidly until 5 atoms/mole putrescine were taken up. The uptake then equaled that of the control. α-Lipoic acid did not speed

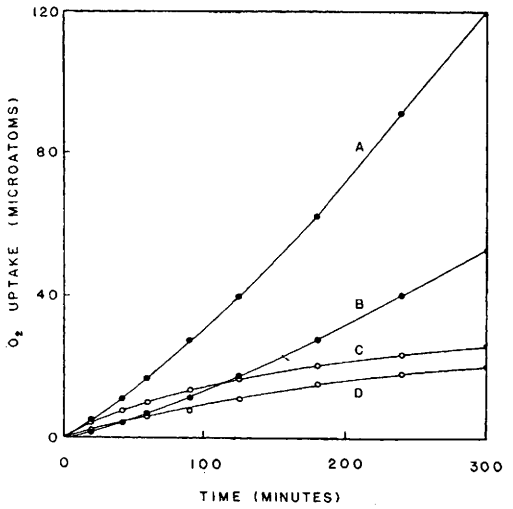

FIG. 1. Effect of α-lipoic acid on the oxidation of proline by deficient cells. Conditions as in Table I. Curves A and B represent response to proline as the substrate in the presence (A) and absence (B) of α-lipoic acid. Oxygen uptake values are corrected for endogenous respiration, represented by curves C (α-lipoic acid present) and D (α-lipoic acid absent).

the oxidation, but prolonged it so that ultimately the ratio was 6 instead of 5. The number of atoms required for complete oxidation is 11. As would be expected from these ratios, very little carbon dioxide was produced: about 1.5 moles/mole putrescine, with or without lipoic acid. Cadaverine was not attacked. *Anaerobically*, very little carbon dioxide is produced by the cells. Pyruvate yields a trifle, but glutamate, proline, and ornithine are inert.

Discussion. The high blanks found in most experiments and the complexity of the enzyme system used, of necessity, make tentative the conclusion that lipoic acid has multiple functions. However, it has been shown that the endogenous activity appears to be independent of the qualitative effects of lipoic acid on various substrates. Moreover, extension of the experiment duration produces a lower ratio of blank:substrate activity without affecting the qualitative results, as illustrated in Fig. 1. Furthermore, in practically all experiments α-lipoic acid revealed its stimulatory effect within the first 10 minutes after tipping in the substrate. The rapid effect appears also when there is no pre-incubation of the growth factor with the cells, as found in comparisons with acetate, malate, and proline. This, and the use of dried cells, would appear to rule out some indirect effect, such as adaptive formation of enzymes.

Summary. The metabolism of dried cells of a *Corynebacterium* has been studied and evidence presented that indicates α-lipoic acid is involved in the oxidation of a variety of substrates and that the locus of action is at several independent sites.

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