

## The Reversal of Phenylarsenoxide Inhibition of Keto Acid Oxidation in Mitochondrial and Bacterial Suspensions by Lipoic Acid and other Disulphides

BY MARGARET ARMSTRONG AND M. WEBB  
*Strangeways Research Laboratory, Cambridge*

(Received 2 September 1966)

1. Inhibition of pyruvate oxidation in suspensions of *Aerobacter aerogenes* cells and of isolated mitochondria from rat heart and liver by phenylarsenoxide is prevented by an excess of lipoic acid, whereas inhibition due to certain bivalent cations is not. 2. In both systems inhibition persists when the bacteria and mitochondria are recovered and resuspended in fresh media in the absence of the inhibitor. Persistent inhibition due to preincubation with phenylarsenoxide, but not with the metal ions, is reversed by lipoic acid and by certain other disulphides. 3. 2,3-Dimercaptopropan-1-ol prevents the inhibition of pyruvate oxidation by phenylarsenoxide and by bivalent cations in both mitochondria and bacterial cells. 4. In aerobic suspensions of mitochondria and bacteria disulphides such as lipoic acid are reduced rapidly to dithiols. Reduction is inhibited by  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$ , but not by phenylarsenoxide. 5. It is concluded that the inability of lipoic acid to prevent the action of the metal ions on pyruvate oxidation is due to the inhibition of its reduction to the effective dithiol.

The inhibitory action of organic derivatives of trivalent arsenic and of the arsenite ion on keto acid oxidation is attributed to the common ability of these compounds to interact with the dithiol form of enzymically bound lipoic acid. Sanadi, Langley & White (1959b) have summarized the relevant earlier literature on this subject and, in studies with oxoglutarate dehydrogenase, have established that turnover of the enzyme, with the generation of the reactive dithiol, is essential for effective inhibition by arsenite. The direct interaction of an organic arsenical, 4-(*p*-arsenosophenyl)-*n*-butyrate, and dihydrolipoic acid has been demonstrated spectrophotometrically by Reiss (1958).

We have found that the effects of organic arsenicals, unlike those of a number of bivalent cations, are both prevented and reversed by lipoic acid. Reiss & Hellerman (1958) have described partial reversal by lipoic acid of the inhibitory action of 4-(*p*-arsenosophenyl)-*n*-butyrate on pyruvate oxidation in suspensions of rat-heart mitochondria. The mechanism of this reversal has not been established, although Reiss & Hellerman (1958) and Deitrich & Hellerman (1964) consider that reduction of the exogenous lipoic acid to the dithiol, which then acts as a competitor for the arsenoso compound, is more likely than replacement of the inactivated covalently bound coenzyme. Dihydrolipoic acid and 2,3-dimercaptopropan-1-ol (BAL) are known to reverse inhibition by 4-(*p*-

arsenosophenyl)-*n*-butyrate (Reiss & Hellerman, 1958; Deitrich & Hellerman, 1964). The production of dihydrolipoic acid by reduction of lipoic acid, however, would be expected also to prevent the inhibition of keto acid oxidation in biological systems by some heavy-metal cations. The inhibition of the succinate-oxidase system (Baron & Kalnitsky, 1947) and of oxoglutarate dehydrogenase (Sanadi *et al.* 1959b) by  $\text{Cd}^{2+}$ , and of pyruvate oxidation in *Streptococcus faecalis* 10Cl by  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Pb}^{2+}$  (Grunert & Rohdenburg, 1960), is relieved by 2,3-dimercaptopropan-1-ol. This compound, as well as 1,3-dimercaptopropane, also reverses the uncoupling of oxidative phosphorylation by low concentrations of  $\text{Cd}^{2+}$  in isolated mitochondria (Jacobs, Jacob, Sanadi & Bradley, 1956).

Although DL- $\alpha$ -lipoic acid prevents the inhibition by arsenite of pyruvate oxidation by *S. faecalis* 10Cl, this protective activity is not shared by the higher homologue 3-(6-carboxyhexyl)-1,2-dithiolane, the corresponding dithiol (8,10-dimercaptodecanoic acid), or the intermediate dithiol homologue 7,9-dimercaptononanoic acid (Grunert & Rohdenburg, 1960). In contrast, 3-(6-carboxyhexyl)-1,2-dithiolane is as effective as DL- $\alpha$ -lipoic acid in the prevention of arsenic toxicity in mice (Grunert, 1960). Also, the partial reversal of 4-(*p*-arsenosophenyl)-*n*-butyrate inhibition of pyruvate utilization in the mitochondria of rat heart and brain

by lipoic acid is restricted to the dextro-rotatory form (Reiss, 1958; Deitrich & Hellerman, 1964). Undoubtedly, if the reversal of inhibition is due to reduction of the disulphide to the dithiol, the observed specificity must lie in the reductive step, since (+)- and (-)-dihydrolipoic acid are equally effective (Reiss, 1958). Such specificity in the reduction of lipoic acid isomers is apparent in the observation (Sanadi, Langley & Searls, 1959a) that, in the reversible oxidation of dihydrolipoic acid by NAD, catalysed by  $\alpha$ -oxoglutarate dehydrogenase from pig heart, (+)-lipoic acid is far more active than the (-)-isomer.

The present paper is concerned with the difference in response of metal ion- and phenylarsenoxide-inhibited mammalian mitochondria and bacterial cells to lipoic acid, and the mechanism whereby the disulphide reverses the action of the organic arsenical.

## MATERIALS AND METHODS

**Bacterial cultures.** Most experiments were done with the laboratory strain of *Aerobacter aerogenes*, the source and conditions of maintenance of which have been given by Webb (1958). Cultures were grown, with shaking, either in 2.0% (w/v) peptone (British Drug Houses Ltd., Poole, Dorset)-0.5% NaCl-0.2% glucose in distilled water, or in an inorganic salts-glucose medium (Monod, 1942). Some measurements were made also with a cobalt-resistant strain that was derived from the parent *A. aerogenes* by serial subculture in the chemically defined medium supplemented with increasing concentrations of  $\text{Co}^{2+}$ . Resistance was acquired rapidly and from the initial cultures, the growth of which was inhibited completely by  $80 \mu\text{M-Co}^{2+}$ , strains resistant to  $1 \text{ mM-Co}^{2+}$  (the limiting concentration at which the cation remained soluble) were obtained after five or six subcultures. All cultures were harvested after 15 hr. at  $37^\circ$ , and the cells were washed three times with  $0.15 \text{ M-NaCl}$  at  $4^\circ$  and resuspended in  $0.15 \text{ M-NaCl}$  at a density of 10 mg. dry wt. of organisms/ml.

**Isolation of mitochondria.** All preparations were made in a cold room at  $4^\circ$ . Mitochondria were isolated from rat liver by Schneider's (1948) method, as described by Dingle, Heath, Webb & Daniel (1962), and from rat heart by the following procedure. The animals were killed in groups of three by cervical dislocation, and the hearts were excised immediately and placed in ice-cold  $0.15 \text{ M-NaCl}$ . After the removal of unwanted tissue each heart was cut into four pieces. These were washed in fresh  $0.15 \text{ M-NaCl}$  and then in medium A, which is the homogenization medium described by Reiss & Hellerman (1958). The pooled tissue was blotted, weighed and cut into small fragments. These were washed twice with small volumes (less than 10 ml.) of medium A and ground with sand (acid-washed, fine grade; British Drug Houses Ltd.) in a precooled mortar as described by Azzone & Carafoli (1960). The slurry was stirred with medium A (9 ml./g. of original tissue), and the supernatant fraction was decanted from the sand and centrifuged at 600g (MSE refrigerated centrifuge, rotor no. 17) at  $0^\circ$  for 10 min. The sedimented material was dispersed in the same volume of medium A and recentrifuged as before. The two

supernatant fractions were combined and centrifuged at 8500g at  $1^\circ$  for 8 min. The deposit was dispersed in  $1 \text{ mM-EDTA-4 mM-tris-0.25 M-sucrose}$ , pH 7.2, and the suspension was diluted with the same solution to a volume equal to three times the weight of the original tissue. This suspension was centrifuged at 8500g and  $1^\circ$  for 8 min. and the mitochondrial pellet was resuspended in either  $\text{EDTA-tris-sucrose}$  medium or  $0.25 \text{ M-sucrose}$ . A small volume of this suspension was diluted appropriately with  $0.25 \text{ M-sucrose}$  for the turbidimetric determination of protein nitrogen. This was done in a Hilger Spekker Absorptiometer, previously calibrated with suspensions of heart mitochondria of known nitrogen content. The main fraction of the mitochondrial suspension was then diluted to contain 1 mg. of N/ml.

For the preparation of 'water-treated' mitochondria of increased permeability (Reiss & Hellerman, 1958), a portion of the suspension in  $\text{EDTA-tris-sucrose}$  medium was centrifuged (at 8500g at  $1^\circ$  for 8 min.) and the pellet was dispersed in one-fifth of the initial volume of glass-distilled water. After 10 min. at  $4^\circ$  an equal volume of  $0.5 \text{ M-sucrose}$  was added and the suspension was diluted to the original volume with  $0.25 \text{ M-sucrose}$ .

**Manometric procedure.** Oxygen consumption was measured in the conventional Warburg apparatus at  $37^\circ$  with air as the gas phase and 0.2 ml. of  $5 \text{ N-NaOH}$  in the centre well. Bacterial cells (5 mg. dry wt. of organisms) were incubated in a fluid volume of 2 ml. with phosphate buffer, pH 6.0 (75  $\mu\text{moles}$ ), and sodium pyruvate (10  $\mu\text{moles}$ ), the substrate being added from the side arm after temperature equilibration. The basal medium for mitochondria contained KCl (80  $\mu\text{moles}$ ), potassium phosphate buffer, pH 7.4 (4  $\mu\text{moles}$ ), tris buffer, pH 7.4 (5  $\mu\text{moles}$ ),  $\text{MgCl}_2$  (10  $\mu\text{moles}$ ), ATP (4  $\mu\text{moles}$ ), cytochrome *c* (3  $\mu\text{moles}$ ), sodium pyruvate (30  $\mu\text{moles}$ ), sodium fumarate (3  $\mu\text{moles}$ ) and NAD (see the text) in a volume of 1.5 ml. Unless stated otherwise, 0.5 ml. of mitochondrial suspension (containing 0.5 mg. of protein N) was added to each flask. When smaller amounts of mitochondria were used, the fluid volume was adjusted to 2.0 ml. with  $0.25 \text{ M-sucrose}$ . Other additions were made as indicated in the Results section.

In the preincubation experiments, which required larger amounts of bacteria and mitochondria, the molar concentrations of the components of the media were maintained when the volumes were increased.

**Disulphide reduction.** (a) By *A. aerogenes*. Duplicate flasks, each containing sodium pyruvate (120  $\mu\text{moles}$ ), potassium phosphate buffer, pH 8.0 (900  $\mu\text{moles}$ ), and *A. aerogenes* cells (8 mg. dry wt. of organisms) in a total volume of 24 ml. were incubated with shaking in a water bath at  $37^\circ$ . Lipoic acid (24  $\mu\text{moles}$ ) or other disulphide was added to the experimental system at zero time. At intervals, 2.8 ml. portions of both suspensions were treated with 0.2 ml. of 10 mM-bis-(3-carboxy-4-nitrophenyl) disulphide in 0.1 M-phosphate buffer, pH 8.0 (Ellman, 1959). After 5 min. at room temperature the  $E_{412}$  value of the experimental sample was determined with the control as blank. Disulphide reduction was expressed as  $\mu\text{moles}$  of SH formed/min./mg. dry wt. of organisms,  $E_{412}$  1.0 being equivalent to an SH concentration of 73.5  $\mu\text{moles/ml}$ .

(b) By mitochondria. The incubation medium contained, in a volume of 5.2 ml., potassium phosphate buffer, pH 7.5 (30  $\mu\text{moles}$ ), KCl (500  $\mu\text{moles}$ ),  $\text{MgCl}_2$  (60  $\mu\text{moles}$ ), sodium fumarate (3  $\mu\text{moles}$ ), sodium pyruvate (30  $\mu\text{moles}$ ), ATP

(6  $\mu$ moles) and NAD (6  $\mu$ moles). After the addition of 4 ml. of the mitochondrial suspension (containing 0.25 mg. of protein N/ml. in 0.25 M-sucrose), the system was brought to 37° in a metabolic water bath before the addition of 0.8 ml. of a solution of lipoic acid (8  $\mu$ moles) in 0.1 M-tris buffer, pH 8.0. Immediately after preparation, and at intervals thereafter, 0.5 ml. samples were transferred to 3.5 ml. of 0.5 mM-bis-(3-carboxy-4-nitrophenyl) disulphide in 0.1 M-phosphate buffer, pH 8.0. After 5 min. at room temperature the  $E_{412}$  value was measured. In each series of measurements a blank, from which the disulphide substrate was omitted, was incubated in parallel with the experimental series. Duplicate portions (0.5 ml.) of this suspension were removed at the appropriate times and added to (a) 3.5 ml. of Ellman's reagent, and (b) 3.5 ml. of 0.1 M-phosphate buffer, pH 8.0. The latter was used in the reference cell of the spectrophotometer to correct for changes in turbidity of the mitochondrial suspension with time.

**Chemicals.** Phenylarsenoxide was kindly provided by Sir Rudolph Peters, F.R.S. A weighed amount of the compound was dissolved in the minimum volume of N-NaOH, the solution was diluted with 0.1 M-tris buffer, pH 7.4, and the pH was readjusted to this value with 0.1 N-HCl. This solution was diluted with water and tris buffer to give 0.1 M-phenylarsenoxide in 10 mM-tris buffer, pH 7.4. Fresh solutions were prepared for each experiment.

Commercial preparations of lipoic acid [3-(4-carboxybutyl)-1,2-dithiolane] (Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.; L. Light and Co. Ltd., Colnbrook, Bucks.) and of lipoamide (Aldrich Chemical Co. Inc.) were used without further purification. Dithiodiglycollic acid was obtained from L. Light and Co. Ltd. and was recrystallized from benzene-light petroleum. 3-(3-Carboxypropyl)-1,2-dithiolane and 3-(5-carboxypentyl)-1,2-dithiolane were gifts from Dr Lester J. Reed (Clayton Foundation Biochemical Institute, University of Texas, Austin, Texas, U.S.A.).

In preliminary experiments, lipoic acid was dissolved in a small volume of N-NaOH, and the solution was immediately neutralized with 0.1 N-HCl (with phenol red as internal indicator) and diluted to volume. Frequently, however, solutions prepared in this way inhibited oxygen consumption when added to mitochondrial and bacterial suspensions to give lipoate concentrations 50  $\mu$ M and above. Such inhibition was not observed at concentrations less than 1 mM when the lipoic acid was dissolved in the minimum volume of ethanol and the solution diluted to volume with 10 mM-tris buffer, pH 7.4. Unless stated otherwise the latter procedure was used in all experiments with lipoic acid and other disulphides.

## RESULTS

**Properties of water-treated mitochondria.** In general, the rate of pyruvate oxidation by rat-heart mitochondria (250–420  $\mu$ l. of oxygen/hr./mg. of N) was increased by 5–30% when they were treated with water. These water-treated preparations had remarkable stability and showed only a slow loss of oxidative activity on storage in 0.25 M-sucrose at 4°. As is well known, however, treatment with water increases the rate of efflux of diffusible cofactors from the mitochondria, and in the ionic medium at

37° the initial high rate of oxygen consumption was not maintained. Oxygen uptake was increased by 40–60% by NAD which, in concentrations greater than 2 mM, also prevented the progressive decrease in oxidative activity in suspensions of freshly isolated mitochondria. With aged (20 hr.) preparations, NAD produced less stimulation (26%) and did not prevent the fall-off in activity.

The omission from the incubation medium of cytochrome c, which is known to diffuse from mitochondria in certain ionic solutions (Chance & Williams, 1955; Estabrook, 1958; Jacobs & Sanadi, 1960), had little effect on the oxidative activities of the water-treated preparations.

**Inhibition of mitochondrial oxidation of pyruvate by bivalent cations and phenylarsenoxide.** The patterns of heavy-metal inhibition of pyruvate oxidation by sucrose suspensions of rat-heart mitochondria resembled those described for mitochondria from rat liver and skeletal muscle (Dingle *et al.* 1962). With  $\text{Co}^{2+}$ , significant inhibition of oxygen uptake (10–20%, and occasionally 40%) was apparent at low concentrations (50  $\mu$ M) of the cation, but sensitivity to inhibition was slightly greater in water-treated preparations. With both water-treated and untreated mitochondria inhibition was maximal (75–85%) with 0.25–0.5 mM- $\text{Co}^{2+}$ , and even 5 mM- $\text{Co}^{2+}$  failed to inhibit oxygen consumption completely. Lipoic acid (50–500  $\mu$ M) had no effect on the inhibition by 50  $\mu$ M- $\text{Co}^{2+}$ .

Inhibition of pyruvate oxidation by phenylarsenoxide was observed at concentrations as low as 0.1  $\mu$ M and was maximal (70–90%) at 5  $\mu$ M. As with  $\text{Co}^{2+}$ , complete inhibition was not obtained. These inhibitory concentrations of phenylarsenoxide are similar to those reported for the inhibition of pyruvate oxidation in suspensions of rat-heart and rat-brain mitochondria by 4-(*p*-arsenosopentyl)-*n*-butyrate (Reiss & Hellerman, 1958; Deitrich & Hellerman, 1964). According to the latter authors failure of the arsenicals to cause complete inhibition of pyruvate utilization, even at considerably higher concentrations, indicates an alternative pathway for metabolism of the keto acid.

The inhibitory action of 5  $\mu$ M-phenylarsenoxide on pyruvate oxidation by rat-heart mitochondria was decreased by over 80% by 0.5 mM-2,3-dimercaptopropan-1-ol (Table 1). This concentration of the dithiol, in the absence of the arsenical, caused a significant inhibition (44%) in the rate of pyruvate oxidation; in Table 1 this decreased rate has been used as the control value in calculations of percentage inhibition. Inhibition was also prevented by lipoic acid (Table 1) provided that the latter was present in a five- to ten-fold excess over phenylarsenoxide. 3-(5-Carboxypentyl)-1,2-dithiolane was as active as lipoate (Table 1), although in its

Table 1. *Effects of 2,3-dimercaptopropan-1-ol, lipoic acid, 3-(3-carboxypropyl)-1,2-dithiolane and 3-(5-carboxypentyl)-1,2-dithiolane on pyruvate oxidation, and its inhibition by phenylarsenoxide, in suspensions of rat-heart mitochondria*

Additions were made to the experimental system as defined in the Materials and Methods section. Each flask contained 0.5 ml. of a suspension of water-treated rat-heart mitochondria (1.0 mg. of N/ml.). Rates of oxygen consumption were calculated from measurements made over the first 20 min. of incubation. The experiments were done with separate preparations of mitochondria and the results recorded are the means of at least two determinations. In the first series, the lipoic acid was dissolved in *n*-NaOH and then neutralized with 0.1 *N*-HCl. This solution slightly inhibited oxidation of pyruvate (see the text).

Mitochondrial preparation	Additions	Oxygen consumption ( $\mu$ l. of O <sub>2</sub> /hr./mg. of N)	Inhibition (% of appropriate control)
I	None	392	
	2,3-Dimercaptopropan-1-ol (0.5 mM)	221	44
	Phenylarsenoxide (5 $\mu$ M)	31	92
	Phenylarsenoxide (5 $\mu$ M), 2,3-dimercaptopropan-1-ol (0.5 mM)	178	20
	Lipoic acid (50 $\mu$ M)	313	20
	Phenylarsenoxide (5 $\mu$ M), lipoic acid (50 $\mu$ M)	275	12
II	None	326	
	Lipoic acid (50 $\mu$ M)	390	
	3-(3-Carboxypropyl)-1,2-dithiolane (50 $\mu$ M)	146	61
	3-(5-Carboxypentyl)-1,2-dithiolane (50 $\mu$ M)	344	
	Phenylarsenoxide (1 $\mu$ M)	166	49
	Phenylarsenoxide (1 $\mu$ M), lipoic acid (50 $\mu$ M)	298	24
Phenylarsenoxide (1 $\mu$ M), 3-(5-carboxypentyl)-1,2-dithiolane (50 $\mu$ M)	294	15	

presence the rate of oxygen consumption decreased more rapidly during incubation than in either the control or the lipoate system; in some cases the rate fell by as much as 50% in 50 min. In all experiments with mitochondria 3-(3-carboxypropyl)-1,2-dithiolane inhibited oxygen consumption.

*Effect of preincubation on the oxidative activities of rat-liver and rat-heart mitochondria.* Mitochondria that were recovered after incubation at 37° for short periods (10–20 min.) under the standard conditions, and that at the time of centrifugation were actively metabolizing pyruvate, were unable to oxidize either this substrate or  $\alpha$ -oxoglutarate when resuspended in fresh sucrose and added to the ionic medium. Although succinate was oxidized by these preincubated preparations the rate of oxygen consumption decreased rapidly with time. When Co<sup>2+</sup> (0.83 mM) was included in the preincubation system, the activities of the recovered mitochondria with succinate were 12–40% greater than those of the controls, whereas the decline in oxidation rate was much diminished. It therefore appears that Co<sup>2+</sup>, which does not inhibit succinate oxidation by rat-liver mitochondria (Dingle *et al.* 1962), decreases the efflux of diffusible cofactors that must occur in the presence of the ionic medium.

Increased amounts of NAD (5  $\mu$ moles) partially restored the ability of preincubated mitochondria to oxidize pyruvate and  $\alpha$ -oxoglutarate; cytochrome *c* had no effect. Even in the presence of

supplementary NAD, however, the initial rate of keto acid oxidation decreased by about 50% within 50 min. Activity of the preincubated suspensions was restored more completely (50–75%) by addition of 0.8 ml. of an extract of water-treated mitochondria. The extract was obtained by centrifuging a mitochondrial preparation that had been incubated in 3 vol. of the ionic medium for 30 min. at 37°. Under these conditions the rate of pyruvate oxidation by preincubated control suspensions of mitochondria remained essentially linear for at least 60 min.

*Persistence of inhibition of pyruvate oxidation in preincubated mitochondria by Co<sup>2+</sup> and phenylarsenoxide.* It was concluded above that preincubation of mitochondria with 0.83 mM-Co<sup>2+</sup> decreased the rate of loss of cofactors when the mitochondria were resuspended in fresh incubation medium supplemented with additional NAD. Thus, in contrast with the controls, the rates of pyruvate oxidation by Co<sup>2+</sup>-treated preparations remained constant for at least 60 min. Comparison of the initial rates of oxygen consumption of the control and treated preparations, however, indicates that the inhibition of pyruvate oxidation that occurred on preincubation with Co<sup>2+</sup> remained essentially unchanged when the particles were recovered and resuspended in fresh medium without further addition of the cation. This was confirmed by the use of the above-mentioned mitochondrial extract

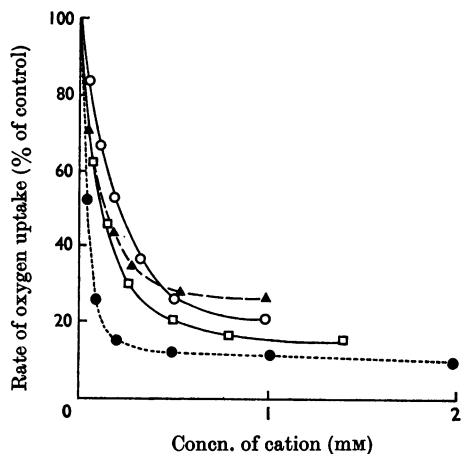


Fig. 1. Inhibition of pyruvate oxidation in suspensions of *A. aerogenes* cells (5 mg. dry wt. of organisms) by Zn<sup>2+</sup> (▲), Cd<sup>2+</sup> (○), Co<sup>2+</sup> (□) and Ni<sup>2+</sup> (●) at 37°. The experimental conditions were as described in the Materials and Methods section.

in place of extra NAD. Under these conditions the inhibition (80%) of pyruvate oxidation that was produced during preincubation of the mitochondria with 0.25 mM-Co<sup>2+</sup> persisted in the recovered mitochondria and was unaltered by 1 mM-lipoamide, which increased the rate of oxygen uptake in the preincubated control preparation (112  $\mu$ l. of oxygen/hr./mg. of N) by 36%. In contrast, the persistent inhibition (40%) produced by preincubation of the mitochondria with 1  $\mu$ M-phenylarsenoxide (inhibition during preincubation being 46%) was decreased to 5% by 50  $\mu$ M-lipoic acid. Both inhibitions were reversed by 0.1 mM-2,3-dimercaptopropan-1-ol.

*Inhibition of pyruvate oxidation in suspensions of A. aerogenes cells by bivalent cations and phenylarsenoxide.* Oxidation of pyruvate by *A. aerogenes* cells was inhibited by Zn<sup>2+</sup>, Cd<sup>2+</sup>, Co<sup>2+</sup> and Ni<sup>2+</sup> (Fig. 1). With increasing cation concentration, inhibition increased rapidly and then became constant. The level of residual respiration was dependent on the inhibitory cation; for example, oxidation non-susceptible to Zn<sup>2+</sup> was inhibited further by Ni<sup>2+</sup>. Inhibition by 0.1 mM concentrations of any one of the above cations was prevented by 0.5 mM-2,3-dimercaptopropan-1-ol, but was unaffected by either 0.5 mM-lipoic acid or 1 mM-lipoamide.

Oxidation of pyruvate by *A. aerogenes* cells was also sensitive to inhibition by low concentrations of phenylarsenoxide (Table 2). Inhibition increased rapidly with concentration up to 5  $\mu$ M and then remained essentially constant. The effects of this concentration of phenylarsenoxide were prevented

Table 2. *Inhibition by phenylarsenoxide of pyruvate oxidation by A. aerogenes cells*

Measurements were made at 37° as described in the Materials and Methods section.

Concn. of phenylarsenoxide ( $\mu$ M)	Oxygen uptake ( $\mu$ l. of O <sub>2</sub> /hr./mg. dry wt. of organisms)	
	Without lipoic acid	With 20 $\mu$ M-lipoic acid
0	58.8	57.8
2	33.4	56.0
5	14.0 (76%†)	55.0 (4.8%†)
8	11.0	17.6*

\* Calculated from measurements made over the first 10 min. of incubation; with this combination of phenylarsenoxide and lipoic acid, inhibition decreased with the period of incubation and after 20 min. the rate of oxygen uptake was 29.8  $\mu$ l. of O<sub>2</sub>/hr./mg. dry wt. of organisms.

† Percentage inhibition of control rate.

almost completely by 20  $\mu$ M-lipoic acid. Both 3-(5-carboxypentyl)-1,2-dithiolane acid and 3-(3-carboxypropyl)-1,2-dithiolane were as effective as lipoic acid.

The inhibition of pyruvate oxidation produced in suspensions of *A. aerogenes* cells after incubation with phenylarsenoxide or Co<sup>2+</sup> persisted when the cells, after being harvested and thoroughly washed in 0.15 M-sodium chloride, were resuspended at pH 6.0 in fresh substrate (Table 3). Some decrease in oxidative rate (from 58.8 to 46.0  $\mu$ l. of oxygen/hr./mg. dry wt. of organisms) occurred on preincubation of control suspensions, but, as shown in Tables 2 and 3, the inhibition due to phenylarsenoxide was essentially unchanged (76 and 72% before and after preincubation respectively.) This was found also for cells that were preincubated with Co<sup>2+</sup>, and in which the persistent inhibition was unaffected by lipoic acid (Table 3). In contrast, inhibition of cells that were preincubated with 5  $\mu$ M-phenylarsenoxide was partially reversed by 20  $\mu$ M-lipoic acid (Table 3). The fact that the latter compound was less effective under these conditions than under those of the experiments of Table 2 may be due to loss of cofactors, essential for the reduction of the disulphide, during preincubation (see above).

*Disulphide reduction by mitochondria.* The measurement of disulphide reduction by mitochondria was complicated by an increase in the SH content of the system in the absence of exogenous substrate. This was due to liberation of SH-containing material, part of which at least was heat-coagulable protein. Even with low concentrations of mitochondria in the absence of the disulphide substrate, the initially high concentration of free SH increased during incubation to reach a

Table 3. Persistence of the inhibition of pyruvate oxidation in *A. aerogenes* cells after preincubation with  $\text{Co}^{2+}$  and phenylarsenoxide

Suspensions of *A. aerogenes* cells (10mg. dry wt. of organisms/ml.) in 0.15 M-NaCl (5.0 ml.) were preincubated at 37° with sodium pyruvate (100  $\mu\text{moles}$ ) and potassium phosphate buffer, pH 6.0 (750  $\mu\text{moles}$ ), in a total volume of 20 ml. Additions of  $\text{Co}^{2+}$  (5  $\mu\text{moles}$ ) and phenylarsenoxide (0.1  $\mu\text{mole}$ ) were made to separate suspensions. After 20 min. the cells were harvested from a portion (16 ml.) of each incubation mixture, washed three times in 0.15 M-NaCl (10 ml.) and resuspended in 0.15 M-NaCl at a density of 10mg. dry wt. of organisms/ml. Pyruvate oxidation by these cells, with and without the addition of lipoic acid (20  $\mu\text{M}$ ), was determined as described in the Materials and Methods section. The values in parentheses give the inhibitions expressed as percentages of the corresponding control.

Addition	Rate of pyruvate oxidation ( $\mu\text{l. of O}_2/\text{hr./mg. dry wt. of organisms}$ )		
	Preincubated control cells	Cells preincubated with $\text{Co}^{2+}$	Cells preincubated with phenylarsenoxide
None	46.0	13.8 (70%)	12.8 (72%)
Lipoic acid (20 $\mu\text{M}$ )	50.2	15.0 (70%)	30.0 (40%)

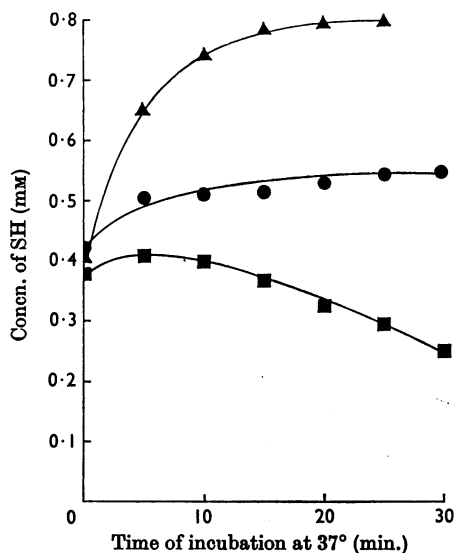


Fig. 2. Reduction of lipoic acid by isolated rat-liver mitochondria. The Figure shows the change in SH content on incubation of dilute suspensions of mitochondria (0.1 mg. of N/ml.) at pH 7.5 and 37° as described in the text (a) alone (●), (b) in the presence of 0.8 mM-lipoic acid (▲), and (c) after the addition of  $\text{Co}^{2+}$  (0.4 mM) (■). The results obtained when lipoic acid and  $\text{Co}^{2+}$  were present together at the above concentrations were identical with those shown for  $\text{Co}^{2+}$  alone.

1  $\mu\text{M}$ -phenylarsenoxide, but was inhibited completely by 0.4 mM- $\text{Co}^{2+}$ . The changes in the SH content of the system were identical with those observed when the cation was added to control suspensions of either liver or heart mitochondria in the absence of the disulphide substrate (Fig. 2). Under the latter conditions there was a significant decrease in the free SH content during 30 min., possibly through catalysis by  $\text{Co}^{2+}$  of the oxidation of the SH compounds. It is unlikely, however, that any reduction of lipoic acid occurred in the presence of  $\text{Co}^{2+}$ , since there was no increase in  $E_{510}$ . Dihydrolipoic acid reacts with  $\text{Co}^{2+}$  to give an intensely coloured chelate with an absorption maximum at this wavelength, and micro quantities of the dithiol can be detected in this way (Webb, 1962). It was not possible, however, to obtain satisfactory proof of inhibition by  $\text{Co}^{2+}$  of lipoic acid reduction by its persistence in mitochondria after preincubation with the cation. Thus reductive activity was lost completely from control preparations when these were recovered after incubation for 10–15 min. in the ionic medium. Reactivation of preincubated suspensions with an extract from other mitochondria, a procedure at least partially effective in the restoration of respiratory activity, was not practicable in these experiments, since such preparations had excessively high SH contents.

**Disulphide reduction by *A. aerogenes* cells.** Reduction of lipoic acid occurred extremely rapidly in the presence of *A. aerogenes* cells (Fig. 3). The rate of reduction of substrate by the cobalt-resistant strain (107 m $\mu\text{moles}$  of SH/min./mg. dry wt. of organisms) was almost double that of the parent, and was decreased by only 20% when cultures were grown in the presence of 1 mM- $\text{Co}^{2+}$ . In contrast with mitochondrial suspensions, there was no significant increase in free SH content when the

maximum in about 30 min. Frequently the SH content then decreased, presumably through oxidation to disulphides under the aerobic conditions of the experiments.

Reduction of lipoic acid with the production of free SH groups was not affected measurably by

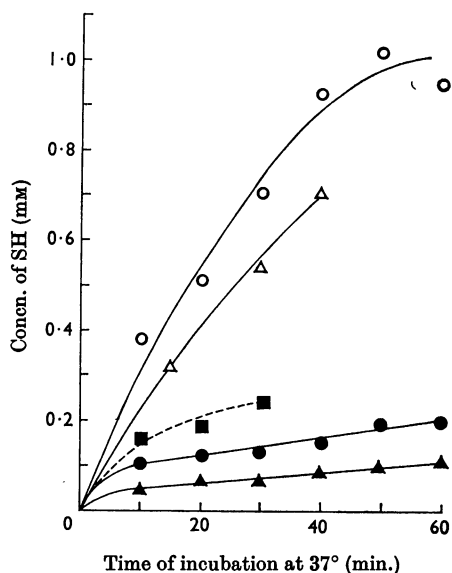


Fig. 3. Reduction of lipoic acid (1.01 mM) (○), 3-(3-carboxypropyl)-1,2-dithiolane (0.8 mM) (Δ), cystine (0.94 mM) (■), bis-(3-carboxy-4-nitrophenyl) disulphide (0.107 mM) (●) and dithiodiglycolic acid (0.94 mM) (▲) by suspensions of *A. aerogenes* cells (0.33 mg. dry wt. of organisms/ml.) at 37° in the presence of 5 mM-pyruvate and 37.5 mM-phosphate buffer, pH 8.0.

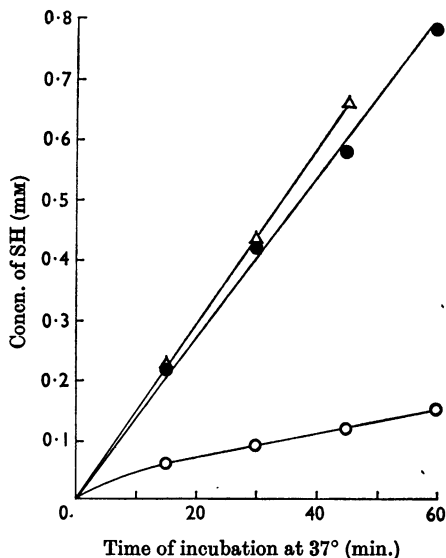


Fig. 4. Reduction of lipoic acid (1.0 mM) by *A. aerogenes* cells (0.33 mg. dry wt. of organisms/ml.) after preincubation alone (●), with phenylarsenoxide (4  $\mu$ M) (Δ) and with  $\text{Co}^{2+}$  (0.5 mM) (○). The preincubated preparations were obtained as described in the text. Each was resuspended in 0.15 M-NaCl at a density of 10 mg. dry wt. of organisms/ml., and a portion (0.8 ml.) then incubated at 37° with lipoic acid under the conditions given in the Materials and Methods section.

bacterial cells were incubated in the absence of lipoic acid or other disulphide. However, activity was dependent on the presence of a metabolizable substrate. 3-(3-Carboxypropyl)-1,2-dithiolane and cystine, but not dithiodiglycolic acid, were also reduced readily by *A. aerogenes* cells, and in the absence of other disulphides some activity was observed with bis-(3-carboxy-4-nitrophenyl) disulphide (Fig. 3).

Some loss (about 20%) of disulphide-reducing activity occurred when control cell suspensions were preincubated for 2 hr. at 37°. After preincubation with 0.5 mM- $\text{Co}^{2+}$  their activity was considerably diminished (Fig. 4), whereas after preincubation with 4  $\mu$ M-phenylarsenoxide it was usually somewhat greater and occasionally more than 70% higher than in the controls. Activity was restored to the initial level (i.e. before preincubation) in the control, but not in the  $\text{Co}^{2+}$ -treated cells, by the addition of the supernatant fraction from the preincubated control suspension. This latter fraction was free from 'disulphide reductase'. Neither NAD nor NADH<sub>2</sub> (0.1 mM) affected the rate of lipoic acid reduction by either the controls or the  $\text{Co}^{2+}$ -preincubated cells.

Reduction of lipoic acid was inhibited when *A.*

*aerogenes* cells were preincubated with  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  (Table 4). Of these cations,  $\text{Ni}^{2+}$  was the most effective. Cations taken up by the cells during preincubation were firmly bound since the inhibition of lipoic acid reduction was not relieved when the cells were washed with EDTA (Table 4). This treatment with EDTA lowered the rate of lipoic acid reduction in the control preparations (Table 4). The rate of reduction by the EDTA-washed cells was greatly stimulated by 10 mM- $\text{Fe}^{2+}$  (Table 5), and the removal of this cation appears to be the most likely cause of the loss in activity, although some activation of both 0.15 M-sodium chloride-washed and EDTA-washed control cells was produced also by  $\text{Mg}^{2+}$ . High concentrations of  $\text{Mg}^{2+}$  decreased or overcame the persistent inhibition due to preincubation with  $\text{Co}^{2+}$  (Table 5), whereas in the absence of additional  $\text{Mg}^{2+}$  inhibition (as a percentage of the corresponding control) was not decreased significantly by  $\text{Fe}^{2+}$  (Table 5).  $\text{Mn}^{2+}$  was consistently inhibitory to disulphide reduction by both 0.15 M-sodium chloride-washed and EDTA-washed control cells and also depressed further the residual activities of cells that were preincubated with  $\text{Co}^{2+}$  (Table 5).

Table 4. *Inhibition of pyruvate oxidation and of lipoic acid reduction in A. aerogenes cells by preincubation with bivalent cations*

*A. aerogenes* cells (2.5 mg. dry wt. of organisms/ml.) were preincubated for 2 hr. at 37° in 37.5 mm-phosphate buffer, pH 6.0, with 2.5 mm-pyruvate and the additions shown below. Each suspension was then divided into two fractions; these were centrifuged and the cell pellets washed by resuspension to half the original volume (a) with 0.15 M-NaCl (three times), and (b) with 20 mm-EDTA in 0.1 M-tris buffer, pH 8.0 (twice), and in 0.15 M-NaCl (once). The washed cells were resuspended in 0.15 M-NaCl at 10 mg. dry wt. of organisms/ml. The rates of pyruvate oxidation and of lipoic acid reduction by these cell suspensions were determined as described in the Materials and Methods section.

Addition to preincubation system	Preincubated <i>A. aerogenes</i> cells washed with 0.15 M-NaCl		Preincubated <i>A. aerogenes</i> cells washed with 20 mm-EDTA, pH 8	
	Lipoic acid reduction (mμmoles of SH/min./mg. dry wt. of organisms)	Q <sub>O<sub>2</sub></sub> (pyruvate) (μl. of O <sub>2</sub> /hr./mg. dry wt. of organisms)	Lipoic acid reduction (mμmoles of SH/min./mg. dry wt. of organisms)	Q <sub>O<sub>2</sub></sub> (pyruvate) (μl. of O <sub>2</sub> /hr./mg. dry wt. of organisms)
None	36.0	45.8	24.3	44.6
Co <sup>2+</sup> (0.5 mm)	3.3	16.6	1.8	17.6
Co <sup>2+</sup> (50 μM)	6.0	22.0	7.2	25.2
None	38.9	37.6	23.0	40.4
Zn <sup>2+</sup> (0.5 mm)	24.6	17.6	27.5	25.4
Zn <sup>2+</sup> (50 μM)	30.9	31.8	27.8	43.8
None	45.5	38.0	24.0	40.4
Ni <sup>2+</sup> (0.5 mm)	0.0	5.6	0.0	5.6
Ni <sup>2+</sup> (50 μM)	0.0	5.6	0.0	5.6
None	35.1	36.8	23.4	31.2
Cu <sup>2+</sup> (0.5 mm)	*	2.6	*	4.8
Cu <sup>2+</sup> (50 μM)	18.6	31.6	15.4†	31.0
None	31.0	42.8	26.1	37.2
Cd <sup>2+</sup> (0.5 mm)	13.5	17.4	8.8	15.4
Cd <sup>2+</sup> (50 μM)	28.4	21.2	33.8	21.6

\* At this concentration of Cu<sup>2+</sup> the cells were precipitated during the preincubation.

† This value was increased to 22.2 after incubation for 45 min.

Table 5. *Effects of Fe<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> on the reduction of lipoic acid by preincubated A. aerogenes cells*

The experiments were done as described in the legend for Table 4.

Addition to assay system	Lipoic acid reduction (mμmoles of SH/min./mg. dry wt. of organisms)			
	Control cells		Cells preincubated with 0.5 mm-Co <sup>2+</sup>	
	Washed with 0.15 M-NaCl	Washed with EDTA	Washed with 0.15 M-NaCl	Washed with EDTA
None	53.5	38.9	18.5	13.0
Mg <sup>2+</sup> (1 mm)	62.5	44.0	51.0	31.3
Mg <sup>2+</sup> (10 mm)	53.5	37.3	54.9	*35.0
Mn <sup>2+</sup> (0.1 mm)		26.0		10.0
Mn <sup>2+</sup> (0.5 mm)	16.7			
Fe <sup>2+</sup> (0.1 mm)		61.0		30.7

## DISCUSSION

In suspensions of mammalian mitochondria and of *A. aerogenes* cells, pyruvate oxidation is inhibited by phenylarsenoxide and by Co<sup>2+</sup> in addition to

certain other bivalent cations. Although some similarities are apparent in the response to these inhibitors, inhibition by phenylarsenoxide is prevented by a five- to ten-fold excess of lipoic acid whereas that due to Co<sup>2+</sup> is not. It is probable that



in both systems phenylarsenoxide interacts with reduced enzymically bound lipoic acid to give a stable ring structure. Inhibition of pyruvate oxidation by 4-(*p*-arsenosphenyl)-*n*-butyrate, for example, is unaffected by the presence of an excess of monothiols (Reiss & Hellerman, 1958). In contrast, for the same degree of inhibition, the bivalent cations are required in concentrations much higher than those of phenylarsenoxide. Although one would expect these ions to be bound at multiple sites, their uptake by both mitochondria and bacteria, at concentrations that inhibit keto acid oxidation, is small (M. Webb, unpublished work.) Both the phenylarsenoxide and the bivalent cations that are bound by mitochondria and bacteria during a preliminary incubation are retained, since the inhibition of pyruvate oxidation persists when the particles and cells are recovered and resuspended in fresh medium in the absence of the inhibitor. With both bacteria and mitochondria lipoic acid relieves the persistent inhibition due to phenylarsenoxide, but is ineffective against that produced by  $\text{Co}^{2+}$ .

To demonstrate this persistent inhibition in rat-liver and rat-heart mitochondria additional supplements are required since, after preincubation in an ionic environment, these particles are unable to oxidize the keto acid when resuspended in the normal medium. Activity is restored by an extract of water-treated mitochondria and, less effectively, by increased amounts of NAD. This loss of co-factors is diminished when  $\text{Co}^{2+}$  is included in the preincubation system, since in such preparations the rate of oxidation of succinate, which is not inhibited by the cation (Dingle *et al.* 1962), is greater than in the corresponding controls. It therefore seems possible that part of the  $\text{Co}^{2+}$  that is taken up by the mitochondria during preincubation may be bound at the surface membrane and alter the permeability.

In aerobic suspensions of both mitochondria and *A. aerogenes* cells certain disulphides, including lipoic acid, are reduced rapidly to dithiols. The colorimetric measurement of this reduction by mitochondria is complicated by the liberation from the particles of material rich in SH groups. This liberation is not apparent when measurements of disulphide reduction are confined to trichloroacetic acid-soluble fractions (Eldjarn & Bremer, 1963).

Reduction of lipoic acid by both mitochondrial and bacterial suspensions is inhibited by  $\text{Co}^{2+}$ , but not by phenylarsenoxide. These observations, coupled with the fact that dithiols such as 2,3-dimercaptopropan-1-ol prevent the inhibition of keto acid oxidation by both phenylarsenoxide and  $\text{Co}^{2+}$  in these systems, provide good evidence that the reversal of the action of the former, but not of the latter, inhibitor by lipoic acid is correlated with the

reduction of the disulphide to a competitive dithiol. Our results therefore provide some confirmation of the suggestion by Reiss & Hellerman (1958) and Deitrich & Hellerman (1964) that lipoic acid antagonizes the inhibitory action of 4-(*p*-arsenosphenyl)-*n*-butyrate on the utilization of pyruvate in suspensions of rat-heart mitochondria.

It is probable that the reduction of lipoic acid by mitochondria is caused by the 'disulphide-reducing system' that is present in rat-liver mitochondria (Eldjarn & Bremer, 1963). This system, which is independent of glutathione reductase, catalyses the reduction of a number of low-molecular-weight disulphides, the reaction being dependent on the presence of substrates of the citric acid cycle, stimulated by  $\text{Mg}^{2+}$ , oxygen, AMP and ADP, but unaffected by NAD, NADP or nicotinamide. Eldjarn & Bremer (1963) conclude that  $\text{NADH}_2$  probably does not participate in the reduction, but that the disulphides are reduced through exchange reactions with the dithiol form of enzymically bound lipoic acid. This now appears less likely, since we have shown that disulphide reduction occurs in mitochondria in which keto acid oxidation is inhibited, at least initially, by phenylarsenoxide, an effect attributed to the interaction of the arsenical with the lipoic acid coenzyme. Even if some turnover of the latter were possible through incomplete combination with the inhibitor, reduction of exogenous substrate would be expected to be autocatalytic, whereas in the presence, as in the absence, of phenylarsenoxide the reaction has its maximum velocity during the initial stages of incubation. Therefore, if it is accepted that lipoic acid and other effective disulphides can reverse phenylarsenoxide inhibition only after reduction, it seems that the hydrogen necessary for the generation of the dithiols must be derived from a pathway of oxidative metabolism that is insensitive to the arsenical.

The above considerations apply also to disulphide reduction by *A. aerogenes* cells, which is stimulated by  $\text{Mg}^{2+}$  and  $\text{Fe}^{2+}$ , inhibited by  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$ , unaffected by phenylarsenoxide and requires a metabolizable substrate. In this organism there is a parallel between the activities of the bivalent cations in the inhibition of disulphide reduction and of pyruvate oxidation,  $\text{Ni}^{2+}$  being the most active inhibitor of the two processes, and  $\text{Zn}^{2+}$  the least. Further, under the appropriate conditions  $\text{Mg}^{2+}$  reverses  $\text{Co}^{2+}$  inhibition of both disulphide reduction and of pyruvate oxidation. It is still possible, however, that despite this indirect evidence these processes are not interdependent but are inhibited separately by the metal ions.

Reduction of disulphides by *A. aerogenes* cells is similar to that by yeast (Nickerson & Falcone, 1956; Black, Harte, Hudson & Wartofsky, 1960). The

latter authors separated the enzyme system that catalyses a specific reduction of L-(–)-methionine sulphoxide to methionine into three components, and found that two of these proteins, when present together, also catalyse a non-specific reduction of disulphides to thiols by NADPH<sub>2</sub>. Substrates for this combination of arsenite- and iodoacetate-sensitive enzymes included DL-lipoic acid, D- and L-cystine and bis-(3-carboxy-4-nitrophenyl) disulphide, which are reduced by *A. aerogenes* cells (Fig. 3), in addition to bis-(2-hydroxyethyl) disulphide, homocystine, GSSG, oxytocin and the three disulphide bonds of insulin.

Although Black *et al.* (1960) regard the disulphide-reducing system of yeast as non-specific, the present results provide examples of disulphides that are not reduced by either the bacterial or the mitochondrial system. Dithiodiglycollic acid, for example, is not a suitable substrate for *A. aerogenes* cells (Fig. 3). Moreover, the failure of certain disulphides to reverse the action of organic arsenicals in suspensions of bacteria and mitochondria, mentioned in the introduction, implies that these compounds are not reduced to dithiols. Eldjarn & Bremer (1963) found that GSSG, homocystine, tetrathionate and cystamine-*NNN'*-tetra-acetic acid, in contrast with cystine diethyl ester, cystamine and its *N*-alkyl derivatives, are not reduced by rat-liver mitochondria. These authors point out that such differences cannot be explained by differences in oxidation-reduction potential, and they suggest that the mitochondrial membrane must be impermeable to certain disulphides. This, however, is unlikely to apply to water-treated mitochondria. No satisfactory explanation can be given, therefore, for the finding that 3-(3-carboxypropyl)-1,2-dithiolane, which reverses phenylarsenoxide inhibition and is reduced to the dithiol in suspensions of *A. aerogenes* cells, inhibits oxygen consumption by rat-heart mitochondria. Although this compound is a lower homologue of lipoic acid it is unlikely to act as an antagonist of the covalently bound coenzyme. One possibility, for which there is yet no

experimental evidence, is mixed-disulphide formation, as suggested by Skrede, Bremer & Eldjarn (1965) to explain the inhibition of mitochondrial  $\alpha$ -oxoglutarate dehydrogenase by cystamine. In this connexion, it may be significant that 3-(5-carboxypentyl)-1,2-dithiolane, which initially reverses the effects of phenylarsenoxide, strongly inhibits pyruvate oxidation by mitochondria after a relatively short period of incubation.

## REFERENCES

- Azzone, G. F. & Carafoli, E. (1960). *Exp. Cell Res.* **21**, 447.  
 Baron, E. S. G. & Kalnitsky, D. R. (1947). *Biochem. J.* **41**, 346.  
 Black, S., Harte, E. M., Hudson, B. & Wartofsky, L. (1960). *J. biol. Chem.* **235**, 2910.  
 Chance, B. & Williams, G. R. (1955). *J. biol. Chem.* **217**, 395.  
 Deitrich, R. A. & Hellerman, L. (1964). *J. biol. Chem.* **239**, 2735.  
 Dingle, J. T., Heath, J. C., Webb, M. & Daniel, M. (1962). *Biochim. biophys. Acta*, **65**, 34.  
 Eldjarn, L. & Bremer, J. (1963). *Acta chem. scand.* **17** (Suppl. 1), 59.  
 Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.  
 Estabrook, R. W. (1958). *J. biol. Chem.* **230**, 735.  
 Grunert, R. R. (1960). *Arch. Biochem. Biophys.* **86**, 190.  
 Grunert, R. R. & Rohdenburg, E. L. (1960). *Arch. Biochem. Biophys.* **86**, 185.  
 Jacobs, E. E., Jacob, M., Sanadi, D. R. & Bradley, L. B. (1956). *J. biol. Chem.* **223**, 147.  
 Jacobs, E. E. & Sanadi, D. R. (1960). *J. biol. Chem.* **235**, 531.  
 Monod, J. (1942). *Recherches sur la Croissance des Cultures Bacteriennes*, p. 32. Paris: Hermann et Cie.  
 Nickerson, W. J. & Falcone, G. (1956). *Science*, **124**, 318.  
 Reiss, O. K. (1958). *J. biol. Chem.* **233**, 789.  
 Reiss, O. K. & Hellerman, L. (1958). *J. biol. Chem.* **231**, 557.  
 Sanadi, D. R., Langley, M. & Searls, R. L. (1959a). *J. biol. Chem.* **234**, 178.  
 Sanadi, D. R., Langley, M. & White, F. (1959b). *J. biol. Chem.* **234**, 183.  
 Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.  
 Skrede, S., Bremer, J. & Eldjarn, L. (1965). *Biochem. J.* **95**, 838.  
 Webb, M. (1958). *Biochem. J.* **70**, 472.  
 Webb, M. (1962). *Biochim. biophys. Acta*, **65**, 47.