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The Effect of Disulphides on Mitochondrial Oxidations

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1. Nicotinamide nucleotide-linked mitochondrial oxidations were inhibited by the disulphides *NNN'N'*-tetraethylcystamine, cystamine and cystine diethyl ester, whereas L-homocystine, oxidized mercaptoethanol, oxidized glutathione, *NN'*-diacetylcystamine and tetrathionate were only slightly inhibitory. Mitochondrial oxidations were not blocked by the thiol cysteamine. 2. NAD-independent oxidations were not inhibited by cystamine. The oxidation of choline was initially stimulated. 3. The inactivation of isocitrate, malate and β-hydroxybutyrate oxidation of intact mitochondria could be partially reversed by external NAD. For the reactivation of α-oxoglutarate oxidation a thiol was also required. 4. A leakage of nicotinamide nucleotides from the mitochondria is suggested as the main cause of the inhibition. In addition, a strong inhibition of α-oxoglutarate dehydrogenase by cystamine was observed. A mixed disulphide formation with CoA and possibly also lipoic acid and lipoyl dehydrogenase is suggested to explain this inhibition.

Many thiols and disulphides of low molecular weight are known to be toxic to animals. Most disulphides are reduced by glutathione reductase (Pihl, Eldjarn & Bremer, 1957; Eldjarn, Bremer & Börresen, 1962), as well as by mitochondria (Eldjarn & Bremer, 1963). Therefore it has been difficult to establish whether the toxicity *in vivo* should be ascribed to the disulphide itself or to the thiol form of the compound.

Under certain conditions, a particular toxicity of a disulphide compound, distinctly different from that caused by the corresponding thiol, can be demonstrated. This is the case, e.g., for tetrathionate, a disulphide that is poorly reduced by the above systems. When administered to animals, this disulphide causes severe lesions of the proximal renal tubules (Gilman, Philips, Koelle, Allen & St John, 1946). Systems *in vitro* offer several possibilities for the demonstration of a specific disulphide toxicity. In studies on isolated cells disulphides have been shown to inhibit oxygen uptake (Kieler, 1962; Ciccarone & Milani, 1964) and cell division (Chevremont & Chevremont, 1953). In experiments with isolated mitochondria, disulphides are more potent than thiols in causing swelling (Neubert & Lehninger, 1962) and uncoupling of oxidative phosphorylation (Park, Meriwether, Park, Mudd & Lipman, 1956). Indications also exist that disulphides inhibit mitochondrial oxygen consumption (Lelievre, 1963; Eldjarn & Bremer, 1963), but no explanation of the mechanism underlying this inhibition has been given. Several mitochondrial functions thus appear to be particularly sensitive towards disulphides.

The detailed mechanism of the disulphide toxicity is unknown. Some of the effects may be due to inhibition of thiol enzymes by mixed disulphide formation (Eldjarn & Pihl, 1957). Such a mechanism has been proposed for the inhibition of hexokinase (Nesbakken & Eldjarn, 1963) and papain (Sanner & Pihl, 1963) by cystamine, as well as for the inhibition of several other enzymes by different disulphides (Graham, 1951; Nygaard & Sumner, 1952; Henderson & Eakin, 1960; Walker & Walker, 1960; Pihl & Lange, 1962). It should be stressed, however, that, in most of the cases of inhibition of enzymes by disulphide described, a disulphide with an oxidation potential far above that of cystamine has been used.

The present paper describes the toxic effects of some disulphides on mitochondrial oxidations. All nicotinamide nucleotide-dependent oxidative steps are inhibited through a cofactor loss, whereas NAD-independent oxidations are not or are only slightly affected. In addition, a specific inhibition of α-oxoglutarate dehydrogenase is shown.

MATERIALS AND METHODS

AMP, NAD, $NADH_2$, NADP and CoA were products of C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany. Cysteamine (2-mercaptoethylamine) and cysteamine derivatives were obtained from Fluka A.G. Chemische Fabrik, Buchs S.G., Switzerland. The corresponding disulphides were prepared by oxidation with iodine and

purified by recrystallization from ethanol–HCl–ether. The diacetylcystamine was prepared as described by Eldjarn, Pihl & Sverdrup (1956). All other chemical products used were commercial products of high purity.

Cysteamine is rapidly oxidized by air at pH 7·4. This autoxidation is initially slow, but is catalysed by the disulphide formed (Jellum, 1964). In experiments of short duration (less than 10 min.), the effect of the thiol may be observed, since the amounts of disulphide formed are small. In experiments of longer duration with actively metabolizing mitochondria (cf. Table 1), the autoxidation is counteracted by the ability of the mitochondria to reduce the cystamine thus formed (Eldjarn & Bremer, 1963). If the mitochondrial metabolism has been inhibited, however, their reduction capacity is lost and the tendency of added thiols to autoxidize must be counteracted by other means. Thiolated Sephadex has recently been introduced for this purpose (Jellum, 1964). When thiolated Sephadex and cysteamine (5 mM) were incubated with inhibited mitochondria at pH 7·4 a constant thiol concentration could be maintained for several hours. Thiolated Sephadex was prepared according to the method developed by Eldjarn & Jellum (1963). Before each experiment, the material was treated with cysteine (10 mM) to remove trace metals, washed repeatedly with distilled water and resuspended in KCl (0·15 M). Thiolated Sephadex, which is an insoluble particulate material, was found not to interfere with mitochondrial oxidations.

Rat-liver mitochondria were prepared as described by Eldjarn & Bremer (1963). Disintegration of mitochondria, when required, was accomplished by ultrasonic vibrations (20 kcyc./sec.) in the cold with a Branson Sonifier (model S75) for 15 or 30 sec. at 6 A.

Incubations in Warburg flasks were performed at 30° with air as the gas phase. Unless stated otherwise, the following additions were made (expressed as μmoles/3 ml.): KH_2PO_4 adjusted to pH 7·4 with 1 N-KOH, 45; AMP (potassium salt), 10; $MgCl_2$, 15. Mitochondria were added in amounts corresponding to approx. 0·5–1·0 g. of fresh liver tissue (0·5–1·0 ml. of mitochondrial suspension in 10% sucrose). The pH of all stock solutions was adjusted to 7·4 before the preparation of the incubation mixture. All reagents were added as 0·1 M solutions. The volume of the incubation mixture was adjusted to 3 ml. with 0·15 M-KCl. The centre well of each flask contained 0·2 ml. of 15% (w/v) KOH. Substrates and the appropriate thiol or disulphide were added in final concentrations as stated under the individual experiments. In some of the incubations a simultaneous addition of disulphide and substrate from the side vessel to the rest of the reaction mixture was done after a period of temperature equilibration. In other experiments mitochondria were preincubated with the thiol or disulphide to be tested for 5–7 min. at 30° before the addition of substrate from the side arm. In all experiments the control flasks were preincubated for the same time as those flasks to which thiols or disulphides were added.

Mitochondrial swelling was measured by following the decrease in extinction at 520 mμ in a Zeiss RPQ 20A Recording Spectrophotometer at 20°. Mitochondria were added in amounts giving an initial extinction value of 0·500–0·600 in a medium consisting of KCl (0·15 M) and $MgCl_2$ (5 mM). No swelling occurred in this medium, whereas a slight swelling (ΔE_{520} less than $-0{\cdot}100$ in 1 hr.) was observed in 0·15 M-KCl alone. After a control period of 10 min., the various agents were added alone or in a mixture as stated in the legend to Table 4. With all additions present this medium was identical with the preincubation medium of the Warburg experiments.

Spectrophotometric enzyme assays were performed with mitochondrial subfractions by following the rate of reduction or oxidation of added nicotinamide nucleotides at 340 mμ in a Zeiss RPQ 20A Recording Spectrophotometer. Mitochondria, disrupted by ultrasonic vibrations, were centrifuged at 120000 g for 120 min. at 0°. The pellet was resuspended in sucrose and used for the assay of β-hydroxybutyrate dehydrogenase. The supernatant was used for the assay of malate dehydrogenase and isocitrate dehydrogenase. Enzyme used for the assay of α-oxoglutarate dehydrogenase was partially purified from pig heart muscle according to the procedure of Sanadi, Littlefield & Bock (1952).

To obtain results comparable with the Warburg experiments, preincubations of enzyme (0·05–0·2 mg. of protein/ml.) in the presence or absence of cystamine or cysteamine at 30° for 7 min. were performed without substrate and cofactors. The medium was: potassium phosphate buffer, pH 7·4, 15 mM; $MgCl_2$, 5 mM; NaCN, 1 mM (except for the α-oxoglutarate dehydrogenase assay); KCl 0·11–0·13 M,. The final volume was 1·5 ml. AMP was omitted, as no interference with the disulphide inhibition could be demonstrated. After this preincubation the reaction mixture was rapidly cooled and the enzymes were assayed at 20° after the following additions: β-hydroxybutyrate dehydrogenase assay: NAD (1 μmole) and DL-β-hydroxybutyric acid (10 μmoles); isocitrate dehydrogenase assay: NADP (0·25 μmole) and DL-isocitric acid (5 μmoles); malate dehydrogenase assay: $NADH_2$ (0·25 μmole) and oxaloacetic acid (5 μmoles); α-oxoglutarate dehydrogenase assay: NAD (1 μmole), CoA (0·25 μmole) and α-oxoglutaric acid (5 μmoles). For the activity of the α-oxoglutarate dehydrogenase preparation the addition of CoA was essential. The reactions were started by the addition of substrate. The contents of the blank cuvette were identical with the reaction cuvette, except for substrate.

The specific activities of isocitrate dehydrogenase, malate dehydrogenase and β-hydroxybutyrate dehydrogenase were calculated from the readings obtained during the first 2 min. of the reaction. The activity of α-oxoglutarate dehydrogenase was calculated according to the method of Searls & Sanadi (1960) from the rate of reduction of NAD during the first 10 sec.

Protein was determined by the biuret method. The thiol content of the reaction mixture was estimated by amperometric silver titration at the rotating platinum electrode (Benesch, Lardy & Benesch, 1955). The modification of the method introduced by Börresen (1963) was used.

RESULTS

Table 1 shows that *NNN'N'*-tetraethylcystamine, cystamine and cystine diethyl ester cause a pronounced inhibition of the mitochondrial citrate oxidation. Sodium tetrathionate, oxidized mercaptoethanol, L-homocystine, oxidized glutathione and *NN'*-diacetylcystamine, on the other hand, are only slightly or not at all inhibitory. These disulphides, in contrast with cystamine, are poorly

Table 1. *Effect of thiols and disulphides on mitochondrial respiration*

Mitochondria were preincubated for 5min. at 30° with the test compound in a final concentration of 2mM in the absence of substrate. Then 20μmoles of citric acid were added from the side arm. The oxygen uptake of a control preincubated without cystamine and substrate was 33 μ-moles during 120min.

	O_2 uptake (% of control)	
	0–60 min.	60–120 min.
Cysteamine	103	135
NNN'N'-Tetraethylcystamine	18	0
Cystamine	30	0
Cystine diethyl ester	40	3
Sodium tetrathionate	92	58
Mercaptoethanol (oxidized)	90	91
L-Homocystine	98	90
Glutathione (oxidized)	97	99
NN'-Diacetylcystamine	101	99

reduced by erythrocytes as well as by mitochondria (Eldjarn *et al.* 1962; Eldjarn & Bremer, 1963). The lack of inhibition by these disulphides as well as their slow reduction may be explained by a low rate of penetration through biological membranes.

When cysteamine was added to mitochondria, a stimulation of the oxygen uptake was found (Table 1). As this increase was greater than that required for quantitative oxidation of the added cysteamine, an increased oxidation of substrate must have taken place. It is likely that this increase is caused by a continuous re-reduction by the mitochondria of cystamine formed during the experiment by autoxidation of cysteamine (Eldjarn & Bremer, 1963). Cystamine will under these conditions function as an artificial electron acceptor.

Of the compounds tested, cystamine was chosen for a closer study of the inhibitory mechanism. Fig. 1 shows the effect on the oxidation of citrate induced by preincubating mitochondria with different concentrations of cystamine without substrate for 5min. at 30°. Almost complete inhibition of the oxygen uptake from the beginning of the incubation period is obtained with 5mM-cystamine. At a final concentration of 0·1mM no initial inhibition is observed, but after 30min. the oxygen uptake also in this case is almost completely inhibited. This delay in the onset probably depends on the disulphide-reducing capacity of the mitochondria (Eldjarn & Bremer, 1963). It is likely that the respiration continues as long as the reducing capacity of the mitochondria is greater than the rate of diffusion of cystamine through the mitochondrial membrane. However, when this capacity is exceeded, a rapid disulphide poisoning occurs.

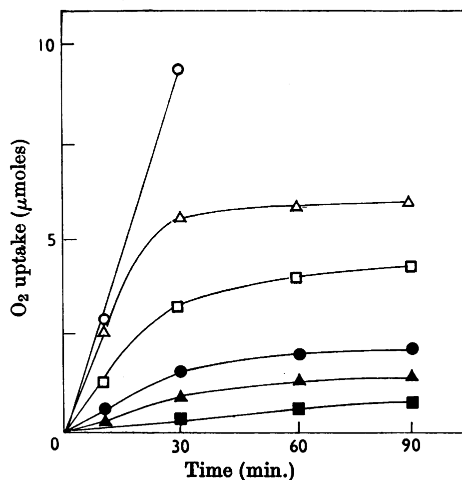

Fig. 1. Inhibition of citrate oxidation after preincubation with cystamine. Mitochondria corresponding to 0·5g. of fresh tissue were preincubated with cystamine for 5min. at 30°. Then 20μmoles of citric acid were added from the side arm. ○, Control preincubated without cystamine or substrate. In the other experiments the mitochondria were preincubated with the following final concentrations of cystamine: △, 0·1mM; □, 0·5mM; ●, 1mM; ▲, 2·5mM; ■, 5mM.

When citric acid-cycle intermediates and other mitochondrial substrates were added to mitochondria preincubated with cystamine (Figs. 2 and 3), a total inhibition of the oxygen uptake was revealed with all substrates except those oxidized directly by a flavoprotein (succinate, choline, sarcosine and α-glycerophosphate).

When cystamine and substrate were added simultaneously (Figs. 2 and 3) the mitochondria were much more resistant towards the disulphide. In such experiments the oxygen uptake during the first 30min. of incubation with some substrates was more rapid than in the controls. With further incubation, however, the oxygen consumption ceased completely.

When succinate is added to mitochondria inhibited by cystamine, the oxygen consumption stoicheiometrically corresponds to the first, flavoprotein-dependent, oxidative step (Fig. 4). This is in accordance with the observation that the oxidation of malate is blocked by cystamine (Fig. 2). Thus no cystamine inhibition of succinate dehydrogenase can be demonstrated, despite the fact that this enzyme contains essential thiol groups that are readily blocked by different thiol inhibitors (Singer & Kearney, 1963). However, only a few thiol enzymes have their thiol groups blocked by cystamine and other disulphides of low oxidation potential (Pihl & Eldjarn, 1958).

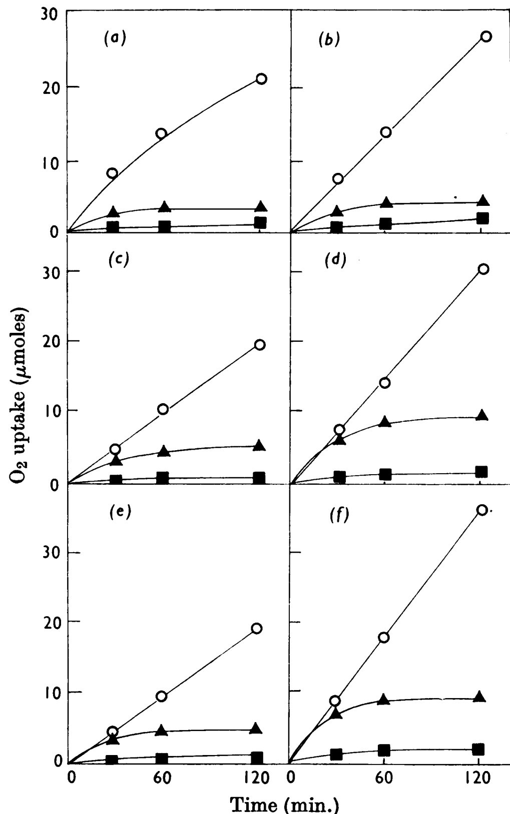

Fig. 2. Cystamine inhibition of nicotinamide nucleotide-dependent mitochondrial oxidations. Mitochondria corresponding to 0·5g. of fresh tissue were used. ○, Control preincubated for 5min. at 30° without cystamine or substrate; ■, mitochondria preincubated with cystamine (final concn. 5mM) before the addition of substrate; ▲, substrate and cystamine (final concn. 5mM) added simultaneously after the preincubation. The substrates (20μmoles of each) used were: (*a*) oxaloacetic acid; (*b*) pyruvic acid; (*c*) fumaric acid; (*d*) citric acid; (*e*) malic acid; (*f*) α-oxoglutaric acid.

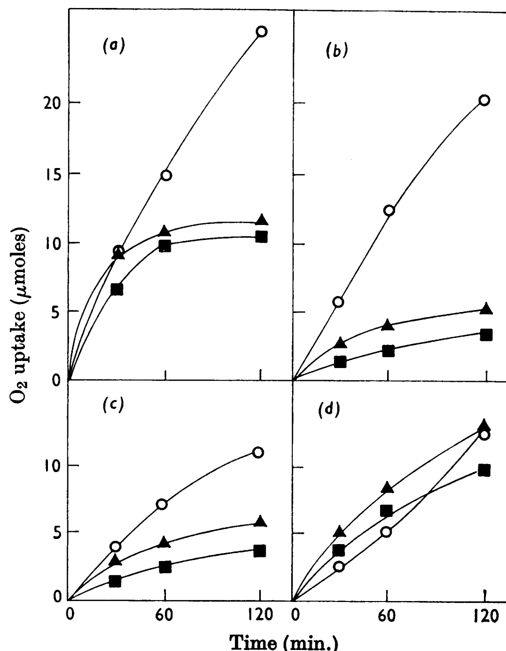

Fig. 3. Effect of cystamine on mitochondrial oxidations independent of nicotinamide nucleotides. Mitochondria corresponding to 0·5g. of fresh tissue were used. ○, Control preincubated for 5min. at 30° without cystamine or substrate; ■, mitochondria preincubated with cystamine (final concn. 5mM); ▲, substrate and cystamine (5mM) added simultaneously after the preincubation. The substrates (20μmoles of each) used were: (*a*) succinic acid; (*b*) sarcosine; (*c*) α-glycerophosphate; (*d*) choline.

The components of the electron-transport chain involved in the oxidation of succinate and $NADH_2$ by molecular oxygen appear to resist the disulphide poisoning (Fig. 4 and Table 2). This is evident from the finding that disrupted mitochondria preincubated with cystamine (Table 2) oxidize $NADH_2$ with an oxygen uptake stoicheiometrically corresponding to the amount of substrate. That cystamine does not function as an artificial electron acceptor in the oxidation of these substrates is indicated by the fact that no significant reduction to cysteamine takes place (Table 2). Thus, despite an apparently normal electron flow along the electron-transport chain, no cystamine is shown to be reduced. Our observations are therefore in agreement with the assumption, put forward by Eldjarn & Bremer (1963), that the mitochondrial disulphide reduction takes place before the generation of $NADH_2$.

When choline is the substrate the oxygen uptake is initially stimulated by cystamine (Fig. 3), suggesting that permeability factors may be rate-limiting in the utilization of this substrate by intact mitochondria. This observation is in agreement with the finding of Williams (1960) that choline is well utilized by mitochondria suspended in a sucrose medium, known to cause swelling, but not when suspended in an iso-osmotic potassium chloride medium.

To investigate the mechanism of the inhibition of mitochondrialnicotinamidenucleotide-linkedoxidations by disulphides, reactivation experiments were performed. Table 3 shows that cystamine caused a complete inhibition of the mitochondrial oxidation of endogenous substrate, with practically no reactivation by NAD alone. Therefore the oxygen uptake after the addition of substrate and NAD to mitochondria inhibited by cystamine needs only a

minor correction for the oxidation of endogenous substrate. Table 3 further shows that the oxidation of pyruvate and α-oxoglutarate was not effectively reactivated by external NAD alone. The first step in the oxidation of isocitrate or malate, however, was to a large extent reactivated by NAD. Only a minor part of the oxygen uptake of inhibited mitochondria can be accounted for by the second step in the oxidation of these substrates, since this is an α-oxo acid oxidation. When the oxidation of isocitrate and malate was reactivated by NAD, the oxygen uptake levelled off after approx. 1hr. at a value corresponding to 80% of a complete one-step oxidation of these substrates.

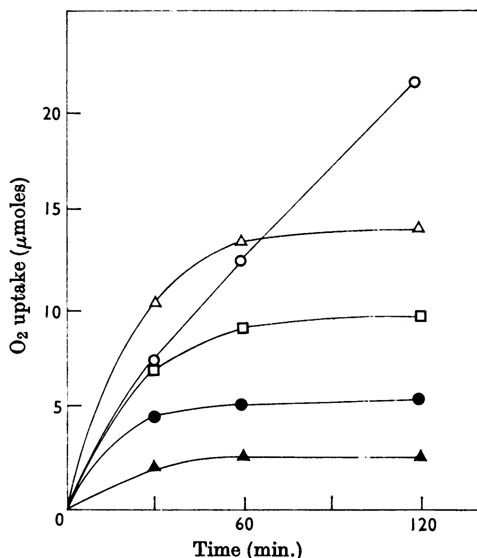

Fig. 4. Effect of cystamine on succinate oxidation. Mitochondria corresponding to 0·5g. of fresh tissue were preincubated with cystamine (final concn. 5mM) at 30° for 5min. before the addition of substrate. ○, Control preincubated without cystamine or substrate. The following amounts of succinic acid were added in the other experiments: △, 30μmoles; ○ and □, 20μmoles; ●, 10μmoles; ▲, 5μmoles.

In the oxidation of DL-β-hydroxybutyrate, an oxygen consumption approaching that corresponding to the oxidation of one isomer is obtained when cystamine-inhibited mitochondria are reactivated with NAD; only the D(−)-isomer is oxidized as such by a mitochondrial NAD-linked dehydrogenase (Lehninger, Sudduth & Wise, 1960). The L(+)-isomer is also oxidized by a mitochondrial NAD-linked dehydrogenase, but only after activation by ATP to β-hydroxybutyryl-CoA (Lehninger & Greville, 1953). As observed by Park *et al.* (1956), and also found by us, the oxidative phosphorylation of the mitochondria is completely uncoupled by cystamine. Therefore a reactivation of the oxidation of the L(+)-isomer after cystamine inhibition could not be expected.

The reactivation experiments thus indicated that the cystamine inhibition of the mitochondrial oxidations of isocitrate, malate and β-hydroxybutyrate could be accounted for mainly by a loss of nicotinamide nucleotides from the mitochondria. To elucidate whether this loss could be related to a simultaneously occurring mitochondrial swelling, the effects on swelling by the components of our preincubation medium were studied. Table 4 shows that cystamine, AMP and phosphate in the concen-

Table 2. *Inhibitory effect of preincubation with cystamine on the mitochondrial disulphide reduction*

During the reaction period, mitochondria corresponding to 1g. of fresh tissue were incubated for 45min. at 30° and the reaction was stopped by acidification to pH5 with 30% (w/v) trichloroacetic acid. During the preincubation period (5min.), cystamine (3·3mM) and substrate were present as shown.

Substrate (μmoles)	Preincubation period (5min.)		Reaction period (45min.)		O_2 uptake (μmoles)	Thiol at end of experiment (μmoles)
	Substrate	Cystamine	Substrate	Cystamine		
None	−	+	−	+	0·5	1·8
Succinic acid (20)	+	−	+	−	19·2	0
Succinic acid (20)	−	+	+	+	9·8	1·5
Succinic acid (20)	+	+	+	+	20·2	16·8
$NADH_2$* (20)	−	+	+	+	9·5	2·7
$NADH_2$* (10)	+	+	+	+	4·9	1·5

* Mitochondria disrupted by ultrasonic vibrations.

Table 3. *Reactivation of NAD-dependent mitochondrial oxidations after inhibition by cystamine*

Mitochondria (corresponding to 0·5g. of fresh tissue) were preincubated with cystamine (2mM) for 7min. at 30°. Controls were preincubated for 7min. without cystamine. Substrate and reactivators were then added from the side arm (final concns.: cysteamine, 5mM; NAD, 2mM; thiolated Sephadex, 40mg. dry wt./3ml.; substrate, 6·7mM, except for DL-β-hydroxybutyric acid, 13·3mM). The oxygen uptake was measured for 120min. after the addition of substrate.

	O_2 uptake (μmoles)					
Substrate (μmoles)	Uninhibited control	Reactivators added to inhibited mitochondria ... None	NAD	Cysteamine + thiolated Sephadex*	Cysteamine + NAD	NAD + cysteamine + thiolated Sephadex*
Endogenous	5·8	0	0·6	0·9	0·6	1·6
α-Oxoglutaric acid (20)	23·7	1	2·6	3·1	4·7	24·8
Pyruvic acid (20)	23·2	1	2·6	1·8	5	9·9
Isocitric acid (20)	21·5	4	8·4	3·9	16	21·3
Malic acid (20)	33	2·6	7·9	4	22	38
DL-β-Hydroxybutyric acid (40)	19	1·4	7·9	3	10·5	10·5

* A material counteracting the autoxidation of thiols (for details, see the text).

Table 4. *Mitochondrial swelling caused by cystamine alone and in combination with AMP and phosphate*

Experimental conditions are given in the Materials and Methods section. Initially the medium contained KCl (0·15M) and $MgCl_2$ (5mM). The swelling agents were added to this medium in the following concentrations: cystamine, 2mM; AMP, 3·3mM; phosphate buffer, pH 7·4, 15mM. The final volume was 1·5ml. The values give the decline in extinction at 520mμ.

	$-10^3 \times E_{520}$	
Additions	5min.	10min.
None	0	0
Cystamine	80	140
AMP	110	150
Cystamine + AMP	150	220
Phosphate	210	290
Cystamine + phosphate	330	380
AMP + phosphate	310	360
Cystamine + AMP + phosphate	330	360

trations used are potent swelling agents. Cystamine gives a swelling additional to that caused by AMP or phosphate when added together with either of these compounds. AMP and phosphate together cause a rapid swelling that is not further stimulated by cystamine. However, the release of nicotinamide nucleotides from the mitochondria when preincubated with phosphate and AMP without cystamine is not great enough to lower the oxygen uptake after the addition of substrate (cf. controls of Table 3 and Fig. 2). A further indication that cystamine acts by a mechanism additional to swelling lies in the observation (Hunter & Ford, 1955) that Mg^{2+} or AMP counteract the NAD release caused by phosphate, whereas in our experiments they are insufficient to prevent the inhibitory action of cystamine. Possibly cystamine, in addition to bringing about swelling, also interferes with the binding of NAD in the mitochondria.

With pyruvate or α-oxoglutarate as substrate, externally added NAD increased the oxygen uptake to only a minor degree (Table 3). Attempts to reactivate the mitochondrial oxidations of α-oxoglutarate with a combination of NAD and cysteamine were also unsuccessful. However, a complete spontaneous oxidation of cysteamine to the inhibitor cystamine was demonstrated to take place during the experiments. When the autoxidation of cysteamine was counteracted by the addition of thiolated Sephadex, a complete restoration of the mitochondrial oxygen uptake lasting for more than 2hr. was obtained, indicating a reactivation of all citric acid-cycle oxidations (Table 3). With pyruvate the reactivation is incomplete, possibly because of a depletion during the preincubation period of the endogenous oxaloacetate needed for the condensing-enzyme reaction.

A closer study of the effect of cystamine on some of the NAD-linked dehydrogenases was performed with partially isolated enzymes (Table 5). Isocitrate dehydrogenase, which is inhibited by a number of thiol-binding agents (Lotspeich & Peters, 1951),

Table 5. *Effect of cystamine and cysteamine on partially isolated mitochondrial nicotinamide nucleotide-linked dehydrogenases*

Experimental details are given in the Materials and Methods section. The enzymes were assayed spectrophotometrically and the values are given as mμmoles of nicotinamide nucleotide reduced or oxidized/min./mg. of protein.

Enzyme	Control without thiol or disulphide	Cystamine (2mM)	Cystamine (4mM)	Cysteamine (10mM)	Cystamine (2 mM) reactivated with cysteamine (10mM)
Isocitrate dehydrogenase	63	56	54	87	78
Malate dehydrogenase	93	91	90	96	—
α-Oxoglutarate dehydrogenase	1450	290	0	1600	1230
β-Hydroxybutyrate dehydrogenase*	33	14	14	46	43

* DL-β-Hydroxybutyric acid was used as substrate.

was only slightly inhibited by cystamine (2–4mM) after 7min. of preincubation at 30°. The enzyme was stimulated by cysteamine, and the inhibition by cystamine could be completely reversed by cysteamine. Malate dehydrogenase is not inhibited by cystamine under our experimental conditions. However, inhibition of this enzyme by lipoic acid and related disulphides (Henderson & Eakin, 1960), as well as by stronger thiol inhibitors such as *p*-chloromercuribenzoate (Barron & Singer, 1945), has been demonstrated. D(–)-β-Hydroxybutyrate dehydrogenase has been shown to have very labile thiol groups (Green, Dewan & Leloir, 1937; Singer & Barron, 1945; Lehninger *et al.* 1960; Sekuzu, Jurtshuk & Green, 1961). Under the conditions of the experiment shown in Fig. 5 probably no oxidation of the L(+)-isomer takes place even in the absence of cystamine, as this reaction needs the presence of CoA and an intact phosphorylation system. The inhibition by cystamine is therefore interpreted as an inhibition of D(–)-β-hydroxybutyrate dehydrogenase. A stimulation of D(–)-β-hydroxybutyrate dehydrogenase by thiols has been noted previously (Singer & Barron, 1945; Lehninger *et al.* 1960; Sekuzu *et al.* 1961). In accordance with this, a stimulation by cysteamine is shown. The disulphide inhibition is readily reversed by cysteamine (Table 5).

The incomplete reactivation of pyruvate and α-oxoglutarate oxidation with NAD alone in the Warburg experiments indicated an inhibition of the dehydrogenase complexes also. With the partially purified α-oxoglutarate dehydrogenase from pig heart a complete inactivation occurred after a short preincubation (7min.) at 30° with cystamine (4mM) in the absence of substrate and cofactors. A total inhibition was also found when CoA was preincubated under the same conditions and uninhibited enzyme was then added immediately before assay. The inhibition of the α-oxoglutarate dehydrogenase could be almost completely reversed with cysteamine (Fig. 5). Cysteamine alone stimulated the enzyme reaction slightly.

DISCUSSION

Effect on the electron-transport chain. The lack of inhibition by cystamine of $NADH_2$ and succinate oxidation indicates that this disulphide does not interfere with the electron-transport chain. The cystamine inhibition of nicotinamide nucleotide-linked oxidations must therefore be brought about by a blockage of the electron flow before the formation of $NADH_2$.

Effect on nicotinamide nucleotide-linked oxidations. It is well known that a total inhibition of nicotinamide nucleotide-linked oxidations occurs when mitochondria are depleted of their NAD by agents such as arsenate or phosphate (Hunter & Ford, 1955). Kaufman & Kaplan (1960) have shown that phosphate-swollen mitochondria lose their nicotinamide nucleotides only in the oxidized form. Reduced nucleotides, on the other hand, normally appear to be bound to intramitochondrial protein (Chance & Baltscheffski, 1958). Fresh mitochondria are only slowly penetrated by external NAD (Purvis & Loewenstein, 1961), in contrast with phosphate-swollen mitochondria, which can incorporate external NAD (Hunter, Malison, Bridgers, Schutz & Atchison, 1959). Nicotinamide nucleotide-linked oxidations can therefore be reactivated in swollen cofactor-depleted mitochondria by the addition of NAD to the medium (Hunter & Ford, 1955).

Whether the nicotinamide nucleotides are normally bound to citric acid-cycle dehydrogenases or to other mitochondrial components has not been settled (Shifrin & Kaplan, 1960). A number of dehydrogenases, however, require thiol groups for the binding of $NADH_2$ (Theorell & Bonnichsen, 1951; Shifrin & Kaplan, 1960). Hunter, Davis & Carlat (1956) have also postulated that thiol groups are essential for the binding of nicotinamide nucleo-

tides in the mitochondria, but the nature of this binding mechanism has not been definitely established. In our study cystamine did not give any swelling in addition to that obtained with magnesium chloride and AMP in combination, whereas only cystamine caused an inhibition of NAD-linked oxidations. Our results therefore suggest that cystamine, in addition to causing swelling, also interferes with the binding of the nicotinamide nucleotides in the mitochondria, possibly through an interaction with thiol groups essential for the binding.

Effect on partially isolated isocitrate dehydrogenase, malate dehydrogenase and β-hydroxybutyrate dehydrogenase. From the Warburg experiments with intact mitochondria it was concluded that, when isocitrate, malate or β-hydroxybutyrate was the substrate, NAD loss was the main cause of the disulphide inhibition of the oxygen uptake. The enzyme studies with mitochondrial subfractions (Table 5) in addition revealed a slight inhibition of isocitrate dehydrogenase by cystamine, whereas malate dehydrogenase was not inhibited under the conditions used. Only with β-hydroxybutyrate dehydrogenase was a substantial inhibition found. The experiments with whole mitochondria (Table 3), however, did not reveal this inhibition of β-hydroxybutyrate dehydrogenase, possibly because the electron-transport chain may be the rate-limiting factor in the mitochondrial oxidation of this substrate.

Effect on α-oxoglutarate oxidation. The requirement for a thiol in addition to NAD for the reactivation of the α-oxoglutarate oxidation in the experiments with whole mitochondria (Table 3) indicated an inhibition of the α-oxoglutarate dehydrogenase complex by disulphide in addition to the cofactor loss. The α-oxoglutarate dehydrogenase complex is likely to have highly reactive thiol groups, involved in the transfer of electrons to NAD, since cystamine is known to form mixed disulphides only with highly reactive thiol groups (Nesbakken & Eldjarn, 1963). In the α-oxoglutarate dehydrogenase complex lipoic acid, lipoyl dehydrogenase and CoA all contain essential thiol groups.

In the spectrophotometric assays the partially purified α-oxoglutarate dehydrogenase was completely inhibited when either the enzyme or CoA was preincubated for a short time with cystamine (4mM). CoA is known to form mixed disulphides (Jaenicke & Lynen, 1960). An inactivation of CoA by cystamine has been shown by Norum (1965) in the CoA-dependent exchange reaction between carnitine and acylcarnitine.

Lipoyl dehydrogenase has a disulphide–dithiol group essential to the transfer of electrons from the reduced flavine to NAD (Massey, 1963). After reduction of the disulphide group by $NADH_2$ the enzyme is sensitive to inhibition by arsenite, which can be reversed by excess of the dithiol BAL (2,3-dimercaptopropanol) (Searls, Peters & Sanadi, 1961). An inactivation of the lipoyl dehydrogenase by cystamine might therefore occur. Reed, De Busk, Hornberger & Gunsalus (1953) have shown the formation of a mixed disulphide from oxidized α-lipoic acid and cysteamine. In unpublished work L. Eldjarn & A. Pihl have shown that the addition of cystamine causes polymerization of free reduced lipoic acid by the formation of intermolecular disulphide bridges. A reaction of cystamine with the probably lysine-bound lipoic acid of the mitochondrial α-oxoglutarate dehydrogenase complex is also likely to occur.

On this background it is considered likely that the inhibition of α-oxoglutarate dehydrogenase by cystamine is caused by a mixed disulphide formation with CoA and probably also with essential thiol groups of the lipoyl dehydrogenase and the lipoic acid of the dehydrogenase complex. The inhibition of pyruvate oxidation by cystamine (Table 3) is thought to be analogous to the inhibition of α-oxoglutarate oxidation, because of the closely related composition of these two dehydrogenase complexes.

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