Effects of Cystamine and Cysteamine on the Adenosine-Triphosphatase Activity and Oxidative Phosphorylation of Rat-Liver Mitochondria

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(Received 15 July 1965)

1. Cystamine (2,2'-diaminodiethyl disulphide) caused an unmasking of mitochondrial adenosine triphosphatase and a leakage of Mg^{2+} from the mitochondria, and decreased the stimulation of adenosine triphosphatase by 2,4-dinitrophenol. When Mg^{2+} was added, cystamine potentiated the activation of adenosine triphosphatase by 2,4-dinitrophenol. 2. Cystamine was without effect on the adenosine triphosphatase of disrupted mitochondria. 3. Cystamine was moderately potent as an uncoupling agent and as an inhibitor of the $[^{32}P]P_i$ -ATP exchange reaction. 4. Cysteamine (2-aminoethanethiol) was without the above effects, when special precautions were taken to counteract its autooxidation. 5. The effects of cystamine should probably be ascribed to its disulphide group, since the diamine cadaverine protected slightly against the loss of Mg^{2+} and the decrease of 2,4-dinitrophenol-stimulated adenosine-triphosphatase activity caused by aging of the mitochondria. It is suggested that cystamine acts by a breakdown of mitochondrial permeability barriers.

Disulphides and thiols of the cysteine-cystamine group are well-known protectors against the effects of ionizing radiation. Further information on the biochemical effects of these compounds has recently been requested (Eldjarn, 1962; Bacq & Alexander, 1964), as such knowledge might contribute to a better understanding of their mode of action.

Some observations on their effects on cellular and subcellular functions have already been made. Thus disulphides of low molecular weight inhibit cellular respiration (Ciccarone & Milani, 1964) and mitochondrial oxygen consumption (Eldjarn, 1962; Lelievre, 1963; Eldjarn & Bremer, 1963). Cystamine and related disulphides apparently interfere with the binding of nicotinamide nucleotides in the mitochondria (Skrede, Bremer & Eldjarn, 1965), and block all NAD-dependent oxidations by isolated mitochondria through a loss of cofactors. Cystamine also uncouples oxidative phosphorylation (van Bekkum & de Groot, 1956; Park, Meriwether, Park, Mudd & Lipmann, 1956) and induces mitochondrial swelling (Neubert & Lehninger, 1962).

Some of the effects of cystamine are probably due to a formation of mixed disulphides between cystamine and the thiol groups of mitochondrial proteins. By this type of reaction cystamine, which is of a low oxidation potential, acts as a specific and mild thiol-blocking agent, combining

with highly reactive thiol groups only (Pihl & Eldjarn, 1958; Pihl & Lange, 1962). Further studies on the interaction of cystamine with mitochondria might therefore contribute to the elucidation of the reactivity of mitochondrial thiol groups and their role in mitochondrial functions. Mitochondrial membrane proteins contain substantial amounts of thiol groups (Riley & Lehninger, 1964); the blockage of such groups causes a rapid swelling of the mitochondria (Tapley, 1956; Riley & Lehninger, 1964). It has been suggested that thiol groups have intimate association with oxidative phosphorylation (Kielley, 1961; Fluharty & Sanadi, 1963; Riley & Lehninger, 1964), and that mitochondrial ATPase* has 'inhibitory' thiol groups as well as thiol groups essential for the function of the enzyme (Cooper, 1960).

The present paper deals with the effects of the disulphide cystamine (2,2'-diaminodiethyl disulphide) on oxidative phosphorylation, mitochondrial ATPase, $[^{32}P]P_i$ -ATP exchange reaction and the retention of Mg^{2+} by the mitochondria. The effects of the chemically related diamine cadaverine (1,5-diaminopentane) and the corresponding thiol cysteamine (2-aminoethanethiol) were also investigated. The main effect of cystamine appears to be to cause an increase of the permeability of the mitochondrial membrane(s), whereas cadaverine

* Abbreviation: ATPase, adenosine triphosphatase.

and cysteamine are without this effect. The results support the concept that the effects of cystamine are due to its disulphide group.

EXPERIMENTAL

Materials. ATP (disodium salt) was a product of Sigma Chemical Co., St Louis, Mo., U.S.A. Hexokinase was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Cystamine and cysteamine were obtained from Fluka A.-G., Chemische Fabrik, Buchs SG, Switzerland. Cadaverine was a product of British Drug Houses Ltd., Poole, Dorset. [32 P]P_i was obtained from the Institute of Atomic Energy, Kjeller, Norway. Naphthalene and dioxan were white-label reagents from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. 2,5-Diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were obtained from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. Sephadex was supplied by AB Pharmacia, Uppsala, Sweden. 2-Aminoethyl hydrogen sulphate was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and was recrystallized from aqueous ethanol. *N*-Acetyl-DL-homocysteine thiolactone was a product of Schwarz Bioresearch Inc., Mount Vernon, N.Y., U.S.A. All other commercial reagents were products of high purity.

Thiolated Sephadex was prepared by the method of Eldjarn & Jellum (1963). Slight modifications of the method were worked out in co-operation with E. Jellum. The starting material was Sephadex G-25 of medium porosity (65g.) that was rapidly stirred into a chilled solution of NaOH (60g.) and 2-aminoethyl hydrogen sulphate (20g.) in water (156ml.). After a few minutes the swollen material was spread out on a glass plate and dried at 120° overnight. The aminated Sephadex thus formed was repeatedly washed with water until free from alkali. To the total amount of swollen slurry was then added *N*-acetyl-homocysteine thiolactone (12g.) in water (800ml.). Under vigorous stirring successive small volumes of *m*-AgNO₃ were added followed by *n*-NaOH to maintain the pH at 7.5–8.0, until a total amount of 75.5 ml. of AgNO₃ had been added. It is essential that the pH be checked frequently at this stage of the procedure, since the Ag⁺ ions may be reduced by the end groups of the cross-linked dextran if the pH exceeds 9. The suspension was allowed to stand with frequent stirring for 2 hr. at room temperature, while the pH was maintained at 8.0 by the frequent addition of small amounts of *n*-NaOH. The Ag⁺ ions were removed with thiourea (140g. in the minimum amount of water) added together with *n*-HNO₃ (250ml.). The thiolated Sephadex was washed a further 3 times with thiourea (45g.) in 0.01 *n*-HNO₃ and finally several times with water. The swollen suspension was stored at 0–4°, preferably in water, as homocysteine is slowly split off in neutral or alkaline buffers. If dried with ethanol followed by ether and kept in the cold, the material may be stored for months. The number of thiol groups was determined by adding an excess of a neutral solution of tetrathionate to a sample of the thiolated Sephadex and titrating the thiosulphate formed with iodine. The thiolated Sephadex usually contained approx. 0.55–0.45% of thiol groups. Before use the material was treated with cysteine (10mm) to remove trace metals, washed repeatedly with water and resuspended in 0.15 *M*-KCl.

Methods. Rat-liver mitochondria were prepared by the method of Myers & Slater (1957). The mitochondria were washed once and resuspended in 0.25 *M*-sucrose. Disintegration of the mitochondria, when required, was accomplished by treating ice-cold suspensions with ultrasonic vibrations (20000 cyc./sec.) with a Branson Sonifier (model S75) for 6 × 10 sec. at 6A.

ATPase assay. The method used for determining ATPase activity was based on that given by Myers & Slater (1957). All experiments were carried out at pH 7.4 in a medium containing tris-HCl buffer (0.05 *M*), EDTA (1mm) and sucrose (0.05 *M*), with or without MgCl₂ (5mm) or 2,4-dinitrophenol (0.25 or 0.13mm). The thiol or the disulphide was added as stated in the individual experiments. The incubation volume was made up to 2 ml. with KCl (0.07–0.105 *M*). The reaction was started with ATP (2.5 or 5mm) except for the preincubation experiments, where the addition of mitochondria initiated the experimental period. All incubations were performed at 30° and the assay period was always 10 min. The reaction was stopped by the addition of 1 ml. of 10% (w/v) trichloroacetic acid. The proteins were removed by centrifugation, and P_i was determined in the supernatant fluid by the method of Berenblum & Chain (1938) as modified by Martin & Doty (1949).

Cysteamine autoxidizes rapidly in air at pH 7.4. During incubations exceeding 10–15 min., a significant fall in the thiol titre was observed. In experiments with cysteamine where the preincubation period and incubation period together exceeded 15 min., the autoxidation of the thiol was counteracted by thiolated Sephadex, which is a particulate material functioning as an electron reservoir for the continuous reduction of cystamine formed by the autoxidation of the thiol (Jellum, 1964). The material itself was shown not to affect the ATPase activity, but maintained the thiol titre at the initial level for more than 2 hr. The thiol content of the reaction mixture at the end of the incubations was estimated by amperometric silver titration at the rotating platinum electrode (Benesch, Lardy & Benesch, 1955). The modification of the method introduced by Børresen (1963) was used.

Oxidative phosphorylation. The oxygen uptake was measured in a Warburg apparatus. The esterification of phosphate was determined by the disappearance of P_i in the presence of a phosphate-trapping system. The assay medium contained the following. The main chamber contained: potassium phosphate buffer, pH 7.4, 30 μ moles; MgCl₂, 15 μ moles; ATP, 2.5 μ moles; mitochondria, approx. 9 mg. of protein (7 mg. in Expt. 3 in Table 5) suspended in 0.15 *M*-sucrose; and (in Expts. 1 and 3 in Table 5) cystamine or cysteamine in the concentrations stated. The volume was made up to a total of 3 ml. with 0.15 *M*-KCl. The side arm contained: hexokinase, 1.5 mg.; glucose, 100 μ moles; succinate, 20 μ moles; and (in Expts. 2 and 4 in Table 5) cystamine or dinitrophenol in the concentrations stated. When cysteamine was present, thiolated Sephadex (30 mg. dry wt., suspended in 0.15 *M*-KCl) was also added. The contents of the side arm were added after temperature equilibration for 7 min. The reaction was run for 10 or 15 min. at 30°, and was stopped by tipping in 0.2 ml. of 6 *N*-HClO₄ from the second side arm. The proteins were spun down, and P_i was determined in the supernatant by the method of Martin & Doty (1949).

[32 P]P_i-ATP exchange reaction. A slight modification of the method given by Wadkins & Lehninger (1963) was

used. The reaction system contained: ATP, 5 μ moles; tris-HCl buffer, pH 7.4, 50 μ moles; phosphate buffer, 10 μ moles containing approx. 600000 counts/min. of 32 P; and mitochondria, 1.2–1.5 mg. of protein. The total volume was 1 ml. Incubations were performed at 30° for 10 min. The reaction was started by the addition of mitochondria and stopped with 1 ml. of 10% trichloroacetic acid. The proteins were removed by centrifugation, and 0.5 ml. of the supernatant fluid was used for the analysis of 32 P-labelled ATP. The extraction and washing procedures were undertaken in accordance with the method of Rose & Ochoa (1956). The radioactivity of the sample was determined by counting 1 ml. of the aqueous phase in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co Inc.), with 15 ml. of scintillator consisting of 12.5% naphthalene, 0.75% 2,5-diphenyloxazole and 0.0375% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in dioxan. The extent of incorporation of [32 P]P_i into ATP was calculated as described by Wadkins & Lehninger (1963).

Ca²⁺ and Mg²⁺ determinations. In these experiments (indicated in Fig. 3) mitochondria containing approx. 50 mg. of protein were used/incubation tube. The mitochondria were incubated at 30° for 5, 10 or 15 min. in a medium containing: tris-HCl buffer, pH 7.4, 0.10 M; sucrose, 0.01–0.04 M; and the following where indicated: cystamine, 5 mM; cysteamine, 10 mM; cadaverine, 5 mM; dinitrophenol, 0.25 mM. The reaction was stopped by rapid cooling in ice to 0°, and the mitochondria were sedimented by centrifugation at 22000 g for 10 min. After decantation the tubes were blotted dry, and the pellet was treated with 1 ml. of 10% HClO₄. The proteins were spun down, and the Ca²⁺ and Mg²⁺ content of the neutralized HClO₄ extract was determined by photoelectric titration with EDTA (Gjessing, 1959). Mg²⁺ was also determined in the ashes of the HClO₄-insoluble material but only insignificant amounts were usually found.

Protein was determined by a biuret method (Goa, 1955).

RESULTS

In the experiments given in Table 1, the ATPase activity of intact mitochondria was low, in accordance with previous observations (Kielley & Kielley, 1951; Myers & Slater, 1957). Only a slight stimulation by Mg²⁺ could be observed. 2,4-Dinitrophenol stimulated the ATPase 10–20-fold when freshly prepared mitochondria were used. Mg²⁺ sometimes caused an additional stimulation of dinitrophenol-stimulated ATPase (Expts. 2 and 3 in Table 1), but in most experiments no such effect was present.

Cystamine, cysteamine or cadaverine at concentrations below 5 mM caused no significant stimulation of ATPase activity in the presence or absence of Mg²⁺ when the preincubation period was less than 5 min. (Table 1). When cystamine was added together with dinitrophenol, the ATPase activity was strongly inhibited as compared with the values with dinitrophenol alone (Table 1). On the other hand, cysteamine or cadaverine did not have an inhibitory action on dinitrophenol-stimulated ATPase.

The inhibition of dinitrophenol-stimulated ATPase induced by cystamine could be completely reversed when Mg²⁺ was also added (Table 1). In fact, the ATPase activity of intact mitochondria in the presence of cystamine, dinitrophenol and Mg²⁺ was higher than the values obtained with dinitrophenol alone. Cysteamine or cadaverine, on the other hand, did not cause any increase of the activity obtained in the presence of dinitrophenol and Mg²⁺ (Table 1).

Table 1. *Effects of cystamine, cysteamine and cadaverine on the ATPase activity of fresh mitochondria*

Freshly prepared mitochondria (approx. 0.5 mg. of protein/tube) were subjected to temperature equilibration for 5 min. in the presence of all additions except ATP. ATP was added at zero time. The following concentrations were used: cystamine, 5 mM; cysteamine, 10 mM; cadaverine, 5 mM; dinitrophenol, 0.25 mM; MgCl₂, 5 mM; ATP, 2.5 mM (Expts. 1 and 2) or 5 mM (Expt. 3).

Expt. 1 (with cystamine)		Expt. 2 (with cysteamine)		Expt. 3 (with cadaverine)	
Addition	ATPase activity (μ moles/hr./ mg. of protein)	Addition	ATPase activity (μ moles/hr./ mg. of protein)	Addition	ATPase activity (μ moles/hr./ mg. of protein)
None	0.5	None	1.0	None	3.8
Mg ²⁺	0.8	Mg ²⁺	2.7	Mg ²⁺	6.1
Cystamine	1.5	Cysteamine	0.9	Cadaverine	4.2
Mg ²⁺ + cystamine	1.2	Mg ²⁺ + cysteamine	1.7	Mg ²⁺ + cadaverine	6.2
Dinitrophenol	11.4	Dinitrophenol	9.2	Dinitrophenol	12.8
Dinitrophenol + cystamine	2.2	Dinitrophenol + cysteamine	9.8	Dinitrophenol + cadaverine	12.2
Dinitrophenol + Mg ²⁺	11.6	Dinitrophenol + Mg ²⁺	12.2	Dinitrophenol + Mg ²⁺	17.6
Dinitrophenol + cystamine + Mg ²⁺	15.9	Dinitrophenol + cysteamine + Mg ²⁺	10.5	Dinitrophenol + cadaverine + Mg ²⁺	17.3

The effect of different concentrations of cystamine on the ATPase activity of fresh mitochondria pre-incubated in the presence of the disulphide for 5min. is shown in Fig. 1. Cystamine alone caused no significant stimulation. When Mg^{2+} was also added, the ATPase activity was moderately increased at a cystamine concentration of 10mm (Fig. 1). Fig. 1 also shows that the inhibition of dinitrophenol-stimulated ATPase by cystamine

was enhanced with increasing concentrations of the disulphide. The activation of dinitrophenol-stimulated ATPase by cystamine in the presence of Mg^{2+} was accentuated at higher concentrations. When the preincubation period was increased to 10min. at 30°, cystamine caused a complete unmasking of Mg^{2+} -activated ATPase. Cadaverine did not accelerate the unmasking, nor did cysteamine, when its autoxidation was counteracted. My results thus did not confirm the brief report by Åkerfeldt (1963) that cysteamine causes a moderate stimulation of mitochondrial ATPase. In the present study, a moderate inhibition by all three compounds was observed when the Mg^{2+} -activated ATPase of the controls was completely unmasked after 2hr. of aging (Table 2). It is well known that the ATPase-stimulating effect of dinitrophenol is diminished and finally lost by aging. It has also been shown that mitochondria lose Mg^{2+} during aging in the presence of deoxycholate (Siekevitz, Löw, Ernster & Lindberg, 1958) or P_i (Siekevitz & Potter, 1955a). One of the requirements for dinitrophenol-induced stimulation of the ATPase activity of fresh mitochondria is obviously the presence of bound Mg^{2+} in the mitochondria (Cooper, 1958). It has therefore been suggested that the loss of activation by dinitrophenol on aging is due to a release of bound Mg^{2+} from the mitochondria. Fig. 2(a) shows that mitochondria preincubated ('pre-aged') in the presence of dinitrophenol had lost 70% of their dinitrophenol-stimulated ATPase activity after 20min. Cystamine (5mm) increased the loss of dinitrophenol-stimulated ATPase activity significantly, so that the residual activity after 20min. of aging was only 10%. The inactivation of dinitrophenol-stimulated ATPase was not accelerated by cystamine, and was even delayed by

Fig. 1. Effect of cystamine on the ATPase activity of fresh mitochondria. The experimental conditions were the same as those given in Table 1; 10 μ moles of ATP were used. Additions: Δ , none; $\square$, Mg^{2+} ; $\circ$, dinitrophenol; $\blacksquare$, Mg^{2+} + dinitrophenol.

Table 2. ATPase activity of mitochondria aged in the presence of cystamine, cysteamine or cadaverine

Intact mitochondria were incubated at 30° for 10 or 120 min. in an iso-osmotic tris-sucrose medium, with or without the addition of cystamine, cysteamine or cadaverine. Then 10ml. of ice-cold 0.25 M-sucrose was added, and the mitochondria were spun down at 22000g for 10 min. and resuspended in 0.25 M-sucrose. The assay was then immediately performed as described in the Methods section, with 10 μmoles of ATP/tube. The addition of mitochondria (0.4–0.5 mg. of protein) started the experiments.

		ATPase activity (μmoles of P _i /mg. of protein/hr.)				
		Unaged control	Agents present during aging			
			None	Cystamine (5 mm)	Cysteamine (10 mm)	Cadaverine (5 mm)
10 min.	Without Mg ²⁺	3.6	3.0	2.6	3.6	3.1
(Expt. 1)	With Mg ²⁺ (5 mm)	6.1	12.3	29.3	13.4	13.0
120 min.	Without Mg ²⁺	—0.8	0	0	0	0
(Expt. 2)	With Mg ²⁺ (5 mm)	—1.5	30.1	26.6	24.0	23.3

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Fig. 2. (a) Significance of the preincubation time for the inhibitory effect of cystamine on dinitrophenol-stimulated ATPase. Intact mitochondria were preincubated in the reaction tubes at 30° for various times. ATP (5 μ moles) was then added, and the incubation continued for 10 min. Additions during the preincubation and assay period: ○, dinitrophenol (0.25 mM); ■, dinitrophenol (0.25 mM) + cystamine (5 mM); △, dinitrophenol (0.25 mM) + cysteamine (10 mM); □, dinitrophenol (0.25 mM) + cadaverine (5 mM). The values are given as percentages of those of controls with mitochondria added at the start of the assay period. The activity of the controls with dinitrophenol was 15 μ moles of P_i liberated/mg. of protein/hr. (b) Significance of the preincubation time for the stimulation by cystamine of the ATPase assayed in the presence of dinitrophenol and Mg^{2+} . The experimental conditions were the same as those described in the legend to Fig. 2(a), except that Mg^{2+} (5 mM) was present in all tubes during preincubation and assay period. The symbols are those used in Fig. 2(a).

cadaverine. The inhibitory effect of cystamine on dinitrophenol-stimulated ATPase could be completely reversed by the addition of Mg^{2+} (Table 1 and Fig. 2b). These observations suggested that the disulphide caused an accelerated loss of Mg^{2+} from the mitochondria.

In a series of experiments where the duration of the preincubation period was varied, the stimulation of ATPase by cystamine in the presence of dinitrophenol and Mg^{2+} was found to be increased when the preincubation period was prolonged to 20 min. (Fig. 2b). Cysteamine or cadaverine did not cause any significant stimulation in the presence of dinitrophenol and Mg^{2+} , but tended to counteract the decrease of enzyme activity occurring when only the latter agents were present. This decrease is in accordance with observations by Potter, Siekevitz & Simonson (1953).

Fig. 3 shows that freshly prepared rat-liver mitochondria, when incubated at 30° in a tris-sucrose medium, lost Mg^{2+} . The loss was usually less than 30% after 15 min., however. Cystamine caused a pronounced increase of the Mg^{2+} loss, leaving no detectable Mg^{2+} in the mitochondria after 15–20 min. (Fig. 3). A similar, but less pronounced,

Fig. 3. Release of Mg^{2+} from mitochondria incubated in the presence of cystamine, cysteamine, cadaverine or dinitrophenol. Experimental details are given in the Methods section. The values are given as percentages of the amount of Mg^{2+} initially present (33–40 μ moles of Mg^{2+} /mg. of mitochondrial protein). The additions were: ●, none; □, dinitrophenol (0.25 mM); ■, cystamine (5 mM); △, cysteamine (10 mM); ○, cadaverine (5 mM).

effect was elicited by dinitrophenol. Cysteamine did not influence the release of Mg^{2+} . Cadaverine retarded the loss slightly. In uncited experiments it was shown that the combination of cystamine and dinitrophenol did not cause a loss of Mg^{2+} more rapid than that obtained with cystamine alone.

Studies on disrupted mitochondria were undertaken to elucidate whether cystamine has any direct stimulating effect on the ATPase activity. Mitochondria disrupted by a short exposure to ultrasonic vibrations evidently had lost their Mg^{2+} , as they did not exhibit any significant ATPase activity without the addition of Mg^{2+} (Table 3). The addition of dinitrophenol, cystamine or cysteamine to such preparations did not elicit any significant ATPase activity. However, the activating effect of dinitrophenol was in some experiments still observable in ultrasonically treated mitochondria in the presence of Mg^{2+} . These results are comparable with those obtained by Lardy & Well-

man (1953) with fresh mitochondria and acetone-dried powder extracts, or with those obtained by Cooper (1958) with 'digitonin particles', and are in accordance with the concept that dinitrophenol acts by stimulating Mg^{2+} -activated ATPase. No further stimulation by cystamine or cysteamine in addition to that obtained with dinitrophenol and Mg^{2+} was observed in disrupted mitochondria.

Table 4 shows that cystamine was a moderate inhibitor of the $[^{32}P]P_i$ -ATP exchange reaction, giving a 40% inhibition at 5mm. Cysteamine, on the other hand, did not give any significant inhibition of the exchange reaction. The inhibitory effect of cystamine was very moderate, in contrast with the effect of dinitrophenol, which is indicated for comparison. Dinitrophenol gave a nearly complete inhibition at concentrations as low as 0.05mm.

It has been reported that cysteamine causes uncoupling of oxidative phosphorylation (van Bekkum & de Groot, 1956; Park *et al.* 1956). Park

Table 3. *Effects of cystamine, cysteamine, Mg^{2+} and dinitrophenol on the ATPase activity of disrupted mitochondria*

The mitochondria were disrupted by ultrasonic vibrations. The protein content of each tube was approx. 0.5mg./2ml. The incubations were performed at 30° for 10min. after temperature equilibration for 5min. The concentrations of the additions were: cystamine, 5mm (Expt. 1); cysteamine, 10mm (Expt. 2); $MgCl_2$, 5mm; dinitrophenol, 0.13mm; ATP, 5mm.

Expt. 1 (with cystamine)		Expt. 2 (with cysteamine)	
Addition	ATPase activity (μ moles/hr./mg. of protein)	Addition	ATPase activity (μ moles/hr./mg. of protein)
None	0.2	None	0.0
Cystamine	0.6	Cysteamine	1.4
Dinitrophenol	0.7	Dinitrophenol	0.9
Dinitrophenol + cystamine	1.4	Dinitrophenol + cysteamine	1.0
Mg^{2+}	15.8	Mg^{2+}	20.3
Mg^{2+} + cystamine	15.6	Mg^{2+} + cysteamine	16.2
Mg^{2+} + dinitrophenol	18.5	Mg^{2+} + dinitrophenol	19.5
Mg^{2+} + dinitrophenol + cystamine	18.6	Mg^{2+} + dinitrophenol + cysteamine	21.0

Table 4. *Effects of cystamine and cysteamine on mitochondrial $[^{32}P]P_i$ -ATP exchange activity*

Experimental details are given in the Methods section. The experiment was started with the addition of mitochondria and was run for 10min. at 30°. The results are given as loss in activity as a percentage of that in uninhibited controls. The exchange activity of the controls was in the range 0.05–0.1 μ mole of P_i incorporated into ATP/min./mg. of protein.

Addition	Concn. of addition (mm).....	Inhibition (%)					
		0.05	0.5	1.0	2.5	5.0	10.0
Cystamine	—	12	8	24	41	66	—
Cysteamine	—	0	0	0	0	5	5
Dinitrophenol	93	98	99	—	—	—	—

Table 5. *Effects of cystamine and cysteamine on oxidative phosphorylation*

Rat-liver mitochondria (approx. 9mg. of protein; 7mg. in Expt. 3) were used with succinate (20 μ moles) as substrate in Warburg experiments. In Expts. 2 and 4, mitochondria were preincubated in the main chamber for 7 min. Cystamine or dinitrophenol was then added from the side arm together with substrate, hexokinase and glucose. In Expts. 1 and 3, the mitochondria were preincubated for 7 min. with cystamine or cysteamine as indicated. The reaction was then run for 10 min. (Expts. 1, 2 and 4) or 15 min. (Expt. 3).

Addition	Concn. of addition (mm).....	0	0.05	0.5	1.0	2.5	5.0	10.0
Cystamine (Expt. 1)	P _i esterified (μ moles)	21.7	—	18.0	12.4	2.7	0	0
	O ₂ uptake (μ g.atoms)	10.3	—	9.9	7.6	7.7	7.4	7.1
	P/O ratio	2.1	—	1.8	1.6	0.3	0	0
Cystamine (Expt. 2)	P _i esterified (μ moles)	18.1	—	17.0	14.0	16.7	16.5	14.5
	O ₂ uptake (μ g.atoms)	9.7	—	9.6	10.0	9.0	10.3	9.6
	P/O ratio	1.9	—	1.8	1.4	1.8	1.6	1.5
Cysteamine (Expt. 3)	P _i esterified (μ moles)	21.4	—	—	—	—	20.9	17.5
	O ₂ uptake (μ g.atoms)	12.0	—	—	—	—	11.6	9.8
	P/O ratio	1.8	—	—	—	—	1.8	1.8
Dinitrophenol (Expt. 4)	P _i esterified (μ moles)	14.9	2.5	—	—	—	—	—
	O ₂ uptake (μ g.atoms)	8.4	4.3	—	—	—	—	—
	P/O ratio	1.8	0.6	—	—	—	—	—

et al. (1956) report that cysteamine produces a detectable uncoupling at a concentration of 1mM, and that cystamine uncouples at concentrations 50-fold lower. They suggest that the uncoupling observed with cysteamine might be due to cystamine formed by the autooxidation of the thiol. Oxidation of cysteamine occurs easily at pH 7.4 and 30°. In my experiments, after 15 min. of incubation of a freshly prepared cysteamine solution (5 or 10mM) 5–10% of the thiol was already oxidized. In the experiments given in Table 5, the tendency of cysteamine to autoxidize was counteracted by the addition of thiolated Sephadex (Jellum, 1964). No uncoupling by cysteamine could then be observed, in accordance with the observation that the concentration of thiol was unchanged at the end of the experimental period.

Mitochondria reduce several small-molecular disulphides in the presence of added citric acid-cycle intermediates (Eldjarn & Bremer, 1963). If the true effect of cystamine on oxidative phosphorylation is to appear, conditions must therefore be chosen to ensure that the disulphide is not reduced by the mitochondria. This is the case when the mitochondria are preincubated with cystamine for 5–10 min. Their disulphide-reducing capacity is then lost (Skrede *et al.* 1965). In Expt. 1 in Table 5, the mitochondria were preincubated with cystamine for 7 min. in the absence of substrate, and it is shown that this disulphide caused a complete uncoupling at 5mM. Under the conditions of Expt. 1 in Table 5, cystamine also caused a moderate inhibition of the oxygen uptake. Possibly, cystamine, by analogy with dinitrophenol (see also

Expt. 4 in Table 5), because of its uncoupling effects, caused an accumulation of oxaloacetate (Slater & Hülsmann, 1961), which is a powerful inhibitor of succinate oxidation.

Under conditions where the mitochondria were able to reduce a significant part of the disulphide to cysteamine, which does not cause uncoupling, only a slight uncoupling effect could be seen (Expt. 2 in Table 5). In the latter experiment, cystamine and succinate were added to the mitochondria simultaneously at the start of the experimental period after the usual temperature equilibration for 7 min.

DISCUSSION

It has been discussed whether the ATPase unmasked by 2,4-dinitrophenol (Lardy & Wellman, 1953) and the ATPase revealed by Mg²⁺ in aged mitochondria (Kielley & Kielley, 1951) are different entities (Racker, 1961; Lehninger & Wadkins, 1962). Mg²⁺ seem to be necessary also for the stimulation of ATPase by dinitrophenol (Lehninger & Wadkins, 1962). Therefore dinitrophenol-stimulated ATPase and Mg²⁺-activated ATPase seem to have at least some step in common (Siekevitz *et al.* 1958), or may represent the same enzyme system activated in different ways (Cooper, 1958; Racker, 1961). The role of Mg²⁺ in the activation process is not clear, but probably a chelate between Mg²⁺ and ATP is the 'active substrate' for the enzyme (Kielley, 1951; Cooper & Lehninger, 1957; Ulrich, 1964).

In the present study, the following effects of

cystamine were shown: (1) an unmasking of the ATPase activity of fresh mitochondria; (2) a release of Mg^{2+} from the mitochondria; (3) a decrease in the activation of the ATPase activity of fresh mitochondria by dinitrophenol in the absence of Mg^{2+} ; (4) a stimulation of the activation of the ATPase of fresh mitochondria by dinitrophenol in the presence of Mg^{2+} ; (5) uncoupling of oxidative phosphorylation; (6) an inhibition of the $[^{32}P]P_i$ -ATP exchange reaction.

In these investigations, the effects of cystamine were compared with those of the chemically closely related diamine cadaverine and the corresponding thiol cysteamine. Since the latter two compounds did not show the listed effects, cystamine probably acts by its disulphide group.

The above effects of cystamine may appear to be rather complex. However, it might prove profitable to discuss all observations from the angle that the effects may be due to primary structural changes in the mitochondria caused by the disulphide. This explanation gains some support from a previous observation that cystamine stimulates choline oxidation by fresh mitochondria, and inhibits nicotinamide nucleotide-linked oxidations, probably by causing a loss of nicotinamide nucleotides from the mitochondria (Skrede *et al.* 1965). Other authors have shown that a number of surface-active agents activate the ATPase of fresh mitochondria by causing damage to the mitochondrial structure; such compounds are digitonin (Cooper & Lehninger, 1956), bilirubin and deoxycholate (Pressman & Lardy, 1956; Siekevitz *et al.* 1958). The unmasking of the ATPase of fresh mitochondria by cystamine may also well be explained by such a mechanism. One of the most striking observations in the present study is the accelerated Mg^{2+} loss from the mitochondria during incubation with cystamine. Under normal conditions, about half of the Mg^{2+} of mitochondria is tightly bound to the mitochondrial structure (Lehninger, 1964), but the nature of the binding mechanism is not known. That cystamine causes a leakage of Mg^{2+} from the mitochondria suggests that this disulphide reacts with thiol groups involved in the maintenance of mitochondrial morphology, first studied by Tapley (1956). Such thiol groups are apparently required for the retention of Mg^{2+} , even that part which is tightly bound. In the present study, cadaverine was shown to retard the loss of Mg^{2+} slightly, and accordingly to delay the inactivation of dinitrophenol-stimulated ATPase which appears on prolonged preincubation with dinitrophenol. These effects of cadaverine are probably analogous to the effects of spermine on mitochondria. The latter polyamine, by an unknown mechanism, offers protection against swelling and loss of material absorbing at $260m\mu$

from the mitochondria (Tabor, Tabor & Rosenthal, 1961).

The importance of permeability effects in the study of mitochondrial ATPase has been pointed out (Perry & Chappell, 1957), as fresh mitochondria may have a limited permeability to ATP (Siekevitz & Potter, 1955b). This view is supported by observations suggesting that, in fresh mitochondria, the combined effect of Mg^{2+} and an agent which causes an increased permeability of the membrane(s) seems to be necessary to give maximal ATPase activity. Thus Potter *et al.* (1953) observed maximal stimulation of the ATPase of fresh mitochondria when Ca^{2+} was added together with Mg^{2+} . Ca^{2+} is (like thyroxine) believed to give primary structural changes of the mitochondria (Tapley & Cooper, 1956). In the present experiments, maximal stimulation of the ATPase activity of fresh mitochondria was obtained with cystamine and dinitrophenol in the presence of Mg^{2+} , whereas dinitrophenol and Mg^{2+} , in accordance with the observations by Potter *et al.* (1953), caused a submaximal stimulation (Expt. 1 in Table 1 and Fig. 2b). Also, these findings may well be explained by membrane effects of cystamine, allowing a facilitated diffusion of ATP and Mg^{2+} to the ATP-hydrolysing sites in the mitochondria.

An attempt was made to eliminate some of the effects of cystamine on mitochondrial permeability by studying the interference of this disulphide with mitochondrial ATPase in ultrasonically disrupted mitochondria. Such particles had apparently lost their Mg^{2+} . The results also indicated that cystamine is not a direct activator of mitochondrial ATPase, and that the disulphide does not interfere with the activation of the ATPase of such mitochondrial particles by Mg^{2+} . From these results, some conclusions on the effects of cystamine apart from the effects on mitochondrial permeability may be suggested. In a study with thiol-binding agents, Cooper (1960) showed that ATPase obtained from digitonin extracts of mitochondria has one 'inhibitory' set of thiol groups, as well as another set essential for the activity of the enzyme. The present observation that the ATPase activity of ultrasonically disrupted mitochondria is not changed by cystamine also suggests that neither of these two sets of thiol groups of mitochondrial ATPase possesses significant reactivity towards cystamine. This finding was not very surprising, however, since this disulphide is of a low oxidation potential, whereas the inhibitory set of thiol groups of the ATPase of mitochondrial particles obtained by ultrasonic oscillations are of a relatively low reactivity as judged from experiments with *p*-chloromercuribenzoate (Kielley, 1963).

When the above effects of cystamine are compared with those of dinitrophenol, it seems likely

that these agents act by distinctly different mechanisms. Cystamine has only a moderate effect on oxidative phosphorylation, ATPase activity of fresh mitochondria and [^{32}P]P_i-ATP exchange, when compared with dinitrophenol. On the other hand, the release of Mg^{2+} from the mitochondria is accelerated more by cystamine than by dinitrophenol, and cystamine is also required for the maximal stimulation of the ATPase of fresh mitochondria.

As a conclusion, it appears likely that, whereas dinitrophenol acts on the chain of oxidative phosphorylation by reacting with a 'high-energy' intermediate (Hemker, 1964), cystamine diminishes mitochondrial permeability barriers. The uncoupling effect of cystamine may be more 'indirect' and mediated by an effect on the mitochondrial structure, resembling the effects of thyroxine or Ca^{2+} (Tapley & Cooper, 1956). However, the effects of cystamine on mitochondrial permeability obviously cannot be directly related to its swelling effects. Thus the thiol cysteamine is nearly as effective as cystamine as a swelling agent (Neubert & Lehninger, 1962). This observation has been confirmed in this Laboratory (unpublished results), with special precautions taken to prevent the autoxidation of cysteamine to cystamine.

The author is indebted to Professor L. Eldjarn and Dr J. Bremer for helpful discussions and suggestions. The technical assistance of Mrs Kari Havnen is acknowledged. The author is a Research Fellow of the Norwegian Council for Science and the Humanities.

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