A Permeability Barrier in Mitochondria in 'High-Energy States' against Positively Charged Disulphides

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1. In rat liver mitochondria in state 1 or 4 there is a permeability barrier against cystamine, probably in the inner membrane. 2. The permeability barrier was broken (a) when oxidative phosphorylation was uncoupled, (b) when the respiratory chain was inhibited or in anaerobiosis, or (c) when phosphate was added in the absence of exogenous substrate. Under these conditions increased amounts of [^{35}S]cystamine residues were bound to matrix proteins. 3. It appears that the permeability barrier against cystamine in mitochondria reflects a 'high-energy state'. A gradual increase in the permeability for cystamine strikingly coincided with the loss of respiratory control induced by increasing concentrations of different uncoupling agents. 4. Cystamine caused uncoupling of oxidative phosphorylation in state 2 or 5, but not in state 1, 3 or 4. The uncoupling effect of cystamine was dependent on the phosphorylation potential. ATP counteracted, whereas ADP potentiated, the uncoupling by cystamine. 5. The variable penetration of cystamine appears to depend on its positive charge, since a dication derivative, $\text{NNN}'\text{N}'$ -tetramethylcystamine, has a similar pattern of penetration, whereas an uncharged derivative, NN' -diacetylcystamine, penetrates rapidly into mitochondria irrespective of their metabolic state. 6. It is suggested that a charge barrier is present in or across the inner mitochondrial membrane in 'high-energy states'.

The binding of cystamine to mitochondrial proteins was in a recent note shown to vary in different metabolic states (Skrede, 1966a).

The present study shows that the cause of these variations is that the penetration of cystamine into rat liver mitochondria is strikingly dependent on the metabolic state. The disulphide is able to penetrate the mitochondrial membrane(s) only at a low phosphorylation potential. The uncoupling effect of cystamine (Park, Meriwether, Park, Mudd & Lipmann, 1956) then becomes evident. The positive charge of cystamine appears to prevent its permeation into mitochondria in 'high-energy states'. The present results are thus not in accord with the statement by Blondin & Green (1967) that the rate of penetration of ions into mitochondria is not influenced by the metabolic state.

EXPERIMENTAL

Materials. [^{35}S]Cystamine was obtained from The Radiochemical Centre, Amersham, Bucks. Carrier cystamine was purchased from Calbiochem, Los Angeles, Calif., U.S.A. (lot no. 30177). To remove decomposition products, cystamine (3mg. of radioactive compound and 400mg. of carrier) was recrystallized two or three times from 10ml. of

water-conc. HCl-ethanol (1:1:8, by vol.) by the addition of diethyl ether. Stock solutions of 0.1M-cystamine with a specific activity of approx. 50000 counts/min./ μmole were used. NN' -Diacetylcystamine (m.p. 91°) was prepared by the method of Eldjarn & Pihl (1957). $\text{NNN}'\text{N}'$ -Tetramethylcystamine was a gift from Norsk Hydro's Institute for Cancer Research, Montebello, Norway. Oligomycin, antimycin A, ATP, ADP and AMP were products of Sigma Chemical Co., St Louis, Mo., U.S.A. Dicoumarol and 2,4-dinitrophenol were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. N -Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid was a product of Calbiochem, Lucerne, Switzerland. Carbonyl cyanide m -chlorophenylhydrazone was a generous gift from Dr P. G. Heytler, Central Research Department, du Pont de Nemours and Co., Wilmington, Del., U.S.A. All other reagents were commercial products of high purity.

Methods. Rat liver mitochondria were prepared by the method of Myers & Slater (1957). The mitochondria were washed one additional time in 0.15M-KCl and resuspended in 0.15M-KCl. Disintegration of the mitochondria, when required, was accomplished by treating ice-cold suspensions with ultrasonic vibrations (20kcyc./sec.) with a Branson Sonifier (model S75) for six 10sec. periods at 6A.

The oxygen uptake was measured by Warburg techniques as described by Skrede (1966b) or with a Clark electrode essentially as described by Chappell (1964). The esterification of P was determined by the disappearance of P_i in the

presence of hexokinase and glucose. P_1 was determined by the method of Martin & Doty (1949). Swelling was estimated by following the decrease in E_{520} in a Zeiss RPQ 20A recording spectrophotometer at 20°, or by measuring the wet weight of mitochondrial pellets that were spun down from the incubation medium at 25 000g for 5 min.

^{35}S radioactivity was measured in a Tri-Carb liquid-scintillation counter. The scintillant was: 2,5-diphenyl-oxazole (5g.), 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (0.05g.), naphthalene (80g.), dioxan (385ml.), xylene (385ml.) and ethanol (230ml.). When protein-bound ^{35}S radioactivity was measured, the addition of Hyamine was usually not necessary. The counting efficiency was approx. 40%. Without Hyamine, only a small degree of quenching occurred (2–4%), which was corrected for by internal-standard techniques.

The penetration of disulphides into mitochondria was studied by three different methods. (1) The distribution space of labelled disulphides was compared with that of $^3\text{H}_2\text{O}$ as described by Yates & Garland (1966). This method was suitable for the evaluation of the distribution of NN' -diacetylcystamine, but not for cystamine. The latter disulphide was loosely bound to the mitochondrial membranes, probably by its amino groups, thus giving a 'distribution space' much larger than that of $^3\text{H}_2\text{O}$. (2) The extent of binding of [^{35}S]cystamine residues to matrix proteins was used as an index of penetration. After incubation of whole mitochondria with labelled disulphides, free disulphide was removed by washing with ice-cold 0.15M-KCl. The mitochondria were then disrupted by ultrasonic vibrations, and separated into a membrane fraction (pellet) and a matrix fraction (supernatant) by centrifugation at 125 000g for 40 min. Protein-bound ^{35}S radioactivity was assayed in the washed HClO_4 precipitate of both fractions. (3) The decrease in thiol content of matrix proteins during incubation of whole mitochondria with unlabelled disulphides was determined. Incubation of mitochondria and isolation of matrix proteins was performed as in method (2). Thiols of low molecular weight were removed from the matrix proteins by gel filtration in the cold, and protein thiol groups were determined.

Thiol groups were determined by amperometric silver titrations at the rotating platinum electrode (Benesch, Lardy & Benesch, 1955) as modified by Børresen (1963). Freshly prepared solutions of GSH, cystamine and bovine serum albumin were used for standardization. Corrections for unspecific binding of Ag^+ ions were made (Jellum, 1966) by using a double titration with or without pretreatment with N -ethylmaleimide. Fresh rat liver mitochondria were then found to contain 65–85 μmoles of SH/mg. of protein. These values are slightly lower than those given by Riley & Lehninger (1964).

Cystamine reacts quite specifically with thiol groups, forming acid-stable mixed disulphides (Pihl & Eldjarn, 1958). However, the reaction of cystamine with the thiol groups of some proteins is incomplete, since this disulphide reacts only with highly reactive thiol groups (Eldjarn & Pihl, 1956; Pihl & Eldjarn, 1958). Approx. 40 μmoles of SH/mg. of protein, or 60–70% of the thiol groups of gel-filtered matrix or membrane proteins of rat liver mitochondria, could apparently be combined with cystamine residues on prolonged incubations. The experimental conditions of the present study (e.g. additions of uncoupling agents, phosphate or EDTA) did not interfere with

the reaction of cystamine with the thiol groups of microsomal proteins or serum albumin (results not shown).

When the binding of [^{35}S]cystamine to mitochondrial thiol groups was investigated, free disulphide was always present in great excess as compared with the amounts bound to protein thiol groups. Protein-bound [^{35}S]cystamine residues were determined in acid-precipitated proteins after repeated washings with 0.3M- HClO_4 and a final resuspension in water. A loose binding of cystamine to mitochondrial membranes was broken on precipitation of the proteins with HClO_4 (cf. Table 6), and was therefore not a source of error in the present results on acid-stable cystamine binding to proteins. When the acid-washed proteins were subjected to a second incubation with 10mM-cystamine at pH 7.5, approx. 90% of the [^{35}S]cystamine residues were removed, indicating that the binding to mitochondrial proteins was of the mixed-disulphide type. When the radioactivity was released from the precipitated proteins with a thiol, the sum of the radioactivity released and the residual protein-bound radioactivity was identical with the total protein-bound radioactivity before the release of ^{35}S , showing that no extra quenching of ^{35}S radioactivity occurred in the cystamine-protein mixed disulphide.

Fluorimetric determinations of reduced nicotinamide nucleotides were performed in dilute mitochondrial suspensions by the method of Estabrook (1962), with a Beckman Ratio Fluorimeter (excitation light 366 μ). Calibration was performed by the addition of a standardized solution of NADH to a mitochondrial suspension containing KCN (1mM).

Protein was determined by a micro-Kjeldahl method.

RESULTS

In the present study, the penetration of cystamine into mitochondria in different metabolic states was investigated. The metabolic states were established by using standard incubation conditions, and characterized by oxygen uptake or by fluorimetric assay of the extent of reduction of the nicotinamide nucleotides. The nomenclature of Chance and co-workers is used throughout (Chance & Williams, 1956; Chance & Hollunger, 1963).

Table 1 shows the permeability for cystamine as indicated by recovery of protein-bound [^{35}S]cystamine residues in mitochondrial subfractions after incubation of whole mitochondria. These values were compared with the binding of cystamine to mitochondrial subfractions isolated before incubation with the disulphide. From Expt. A it is evident that when mitochondria were incubated in state 1 comparatively small amounts of [^{35}S]cystamine were bound to matrix proteins. When 2,4-dinitrophenol was also added (state 2_u), however, matrix thiol groups appeared to be completely exposed for reaction with cystamine, since the binding of ^{35}S then was equal to the binding when the isolated subfraction was incubated. Also, the binding of [^{35}S]cystamine to membrane proteins of whole mitochondria was considerably increased in

Table 1. *Penetration of cystamine into mitochondria evaluated by its binding to matrix proteins*

In Expt. A, rat liver mitochondria (12.9 mg. of protein) were incubated at 30° for 10 min. with [³⁵S]cystamine (3.3 mM), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid-HCl buffer, pH 7.3 (10 mM), and KCl (0.12–0.14 M) making the volume up to 3 ml., and other additions as stated. Fluorimetric determinations of the apparent concentration of reduced nicotinamide nucleotides were performed in mitochondrial suspensions containing approx. 0.2 mg. of mitochondrial protein/ml. The reaction period was terminated by the addition of 10 ml. of ice-cold KCl (0.15 M), and the mitochondria were centrifuged at 25000g for 5 min. at 0°. ³⁵S radioactivity was measured in HClO₄-precipitated matrix or membrane proteins prepared by ultrasonic vibrations followed by centrifugation (for details see the Experimental section).

	Mitochondrial preparation incubated	Addition	Reduced nicotinamide nucleotides (μM)			³⁵ S bound to matrix proteins (mμmoles/mg. of protein)	³⁵ S bound to membrane proteins (mμmoles/mg. of protein)
			0 min.	½ min.	10 min.		
Expt. A	Whole mitochondria	None	5.2	5.0	4.2	7.8	2.5
		2,4-Dinitrophenol (0.3 mM)	5.4	3.0	1.7	35.4	14.9
Expt. B	Matrix fraction	None	—	—	—	36.0	—
		2,4-Dinitrophenol (0.3 mM)	—	—	—	33.5	—
Expt. C	Membrane fraction	None	—	—	—	—	27.3
		2,4-Dinitrophenol (0.3 mM)	—	—	—	—	24.7

Table 2. *Increased binding of [³⁵S]cystamine to mitochondria in nitrogen atmosphere (state 5)*

Mitochondria (10.3 mg. of protein) were preincubated for 5 min. in *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid-HCl buffer, pH 7.3 (10 mM), MgCl₂ (5 mM) and KCl (0.10–0.12 M) either in air or N₂ atmosphere. Cystamine (3.3 mM) was then added, and bubbling with air or N₂ was continued for 1 min. After the reaction vessels had been closed the reaction continued until 10 min. at 30°, and was then stopped with HClO₄ for determination of protein-bound ³⁵S. The P/O ratio was calculated by the 'stairway' technique of Chance & Williams (1956), by using ADP in a comparable experiment with a Clark electrode.

Atmosphere	Addition	³⁵ S bound (mμmoles/mg. of protein)	P/O ratio at end of incubation
Air	None	9.6	1.7
	ATP (1.8 mM)	7.4	—
Nitrogen	None	18.7	0.0
	ATP (1.8 mM)	14.5	—

the presence of 2,4-dinitrophenol. When isolated subfractions were incubated with cystamine, 2,4-dinitrophenol inhibited the binding of [³⁵S]-cystamine slightly. Table 1 indicates that there is a permeability barrier for cystamine in mitochondria in state 1, probably in the inner membrane, and that this barrier is broken in state 2_u (carbonyl cyanide *m*-chlorophenylhydrazine had the same effect as 2,4-dinitrophenol). In uncited experiments, the induction of state 2 (by addition of P_i

Table 3. *Effect of different permeant anions on the binding of [³⁵S]cystamine to mitochondrial proteins*

Mitochondria (8.6 mg. of protein) were incubated for 10 min. at pH 7.3 with [³⁵S]cystamine (3.3 mM) as described in Table 1.

Addition	[³⁵ S]Cystamine residues bound (mμmoles/mg. of protein)
None	9.8
Potassium phosphate (5 mM)	19.6
Potassium acetate (5 mM)	9.5
Potassium propionate (5 mM)	9.7

and acceptor system), as indicated by oxidation of the nicotinamide nucleotides, also caused an increase of the binding of cystamine residues to matrix proteins.

Table 1 thus indicates that the binding of cystamine to matrix proteins is an index of its penetration into mitochondria. Since most of the mitochondrial proteins (matrix and part of the inner membrane) are inaccessible for cystamine in state 1, an increased penetration of the disulphide will also be reflected by a significant increase of the binding of cystamine to the total proteins of mitochondria. This is evident from Table 2, in which the binding of cystamine to mitochondria in state 1 was compared with the binding in state 5 (nitrogen atmosphere). Increased amounts of [³⁵S]cystamine residues were bound in state 5, and the mitochondria then became uncoupled. The increased binding of

Fig. 1. Stimulation by P_1 of the binding of $[^{35}\text{S}]$ cystamine to mitochondrial proteins. Mitochondria (7.0 mg. of protein) were incubated at 30° with $[^{35}\text{S}]$ cystamine (2 mM), N -tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid-HCl buffer, pH 7.5 (10 mM), and KCl (0.12 M). Other additions: ○, none; Δ, EDTA (1 mM); ■, P_1 (10 mM, pH 7.5). —, Binding of $[^{35}\text{S}]$ cystamine residues; -----, fluorescence due to reduced nicotinamide nucleotides (measured as indicated in Table 1).

cystamine was to some extent counteracted by ATP.

The experiments of Tables 1 and 2 were performed in N -tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid-potassium chloride medium. Since Cl^- penetrates the mitochondrial membranes poorly (Chappell & Haarhoff, 1967), and since results similar to those of Table 2 were obtained even with mitochondria depleted of endogenous phosphate by a preincubation with succinate, ADP and hexokinase-glucose, the presence of a permeant anion seems to be of less importance for the penetration of cystamine than is the energy state of the mitochondria. This was shown to be the case (Table 3), since neither acetate nor propionate stimulated the binding of cystamine to the mitochondria, even though both anions are held to penetrate the mitochondrial membranes rapidly (Chappell & Haarhoff, 1967).

P_1 , on the other hand, strikingly increased the binding of cystamine (Table 3), probably by an effect on the metabolic state in addition to the ability to penetrate the mitochondrial membranes. Fig. 1 thus shows that P_1 induced an increased oxidation of the nicotinamide nucleotides, indicating a slow conversion into state 2 or 2_u , in agreement with the observation of Chance & Connelly (1957) on the effect of P_1 on the metabolic state. The binding of cystamine was concomitantly increased.

Table 4. *Uncoupling effect of cystamine in mitochondria in different metabolic states*

Rat liver mitochondria (5.2 mg. of protein) were preincubated at 30° in Warburg flasks for 7 min. in the presence of tris-HCl buffer, pH 7.15 (10 mM), MgCl_2 (5 mM) and KCl (0.10-0.12 M) making the volume up to 3 ml., and other additions as stated. Where indicated, 1.5 mg. of hexokinase and 50 μmoles of glucose were added. The experimental period (10 min. at 30°) was started by the addition of the contents of the side arm as stated, and was stopped by the addition of HClO_4 (0.5 M).

Preincubation period			Experimental period					
Agents present	Supposed state	Agents added	Supposed state	O ₂ uptake (μg.atoms)	P _i esterified (μmoles)	P/O ratio	³⁵ S bound (mμmoles/mg. of protein)	SH formed (μmoles)
Cystamine (3.3 mm)	1	HClO ₄	—	—	—	—	6.2	0.5
Cystamine (3.3 mm) + P _i (1.7 mm) + ADP (0.8 mm) + hexokinase-glucose	2	HClO ₄	—	—	—	—	10.7	0.5
None	1	Succinate (6.7 mm)	4	1.0	—	—	—	—
Cystamine (3.3 mm)	1	Succinate (6.7 mm)	4	1.2	—	—	6.7	0.6
Cystamine (3.3 mm)	1	Succinate (6.7 mm) + P _i (8.4 mm) + ADP (0.8 mm) + hexokinase-glucose	3	6.6	10.6	1.6	8.8	1.6
P _i (1.7 mm) + ADP (0.8 mm) + hexokinase-glucose	2	Succinate (6.7 mm) + P _i (6.7 mm)	3	7.6	12.2	1.6	—	—
Cystamine (3.3 mm) + P _i (1.7 mm) + ADP (0.8 mm) + hexokinase-glucose	2-2 _u *	Succinate (6.7 mm) + P _i (6.7 mm)	3 _u *	4.0	0.0	0.0	15.3	0.3

* States 2_u and 3_u are uncoupled states, without or with substrate, respectively (Chance & Hollunger, 1963).

Fig. 1 also shows that in state 1 (with or without EDTA) the reaction of cystamine with the thiol groups of mitochondrial proteins was restricted even during an incubation lasting for 30 min. The maintenance of a high concentration of reduced nicotinamide nucleotides indicated that no conversion into state 2_u occurred. This observation is inconsistent with the earlier view that cystamine is invariably an uncoupling agent (Park *et al.* 1956; Bacq, 1965).

Table 4 shows a reinvestigation of the uncoupling effect by cystamine. Preincubation with cystamine of mitochondria in a tris-potassium chloride medium without substrate (state 1) did not cause respiratory release, which was shown by the subsequent addition of succinate (state 4). Accordingly, when succinate and acceptor system was added to mitochondria preincubated with cystamine, the P/O ratio was equal to that of the controls. Therefore cystamine caused uncoupling neither in the preincubation period (state 1) nor in the experimental period (state 3). Mitochondria preincubated in state 2 with cystamine were completely uncoupled. The oxygen uptake was then inhibited, probably because of oxaloacetate accumulation, since rotenone counteracted this effect of cystamine (cf. Fig. 6).

The amounts of ³⁵S bound to mitochondria in state 1, 3 or 4 were small. When cystamine acted as an uncoupling agent (state 2), however, increased amounts of [³⁵S]cystamine residues were bound to mitochondrial proteins, indicating an increased penetration of the disulphide. Earlier, the penetration of cystamine into mitochondria was held to be free (Eldjarn & Bremer, 1963; Eldjarn, 1965). In accord with this assumption the mixed-disulphide formation with proteins could be kept low if the penetrating disulphide acted as an artificial electron acceptor by being reduced to the corresponding thiol (cf. Eldjarn & Bremer, 1963). However, Table 1 shows that this cannot be the explanation of the low binding of ³⁵S to mitochondria preincubated with cystamine (state 1) followed by succinate (state 4), since the sum of thiol formed was small. It appears that cystamine penetrates the mitochondrial membrane(s) poorly in these states. In the presence of succinate, P_i and acceptor system (state 3), however, greater amounts of disulphide were reduced. A certain extent of penetration of cystamine in this state therefore cannot be ruled out.

Experiments with the Clark electrode (not shown) confirmed that cystamine did not act as an uncoupling agent in state 1 or 4, but caused uncoupling in state 2. A longer preincubation with P_i was required to reveal the uncoupling effect of cystamine when ADP was omitted.

The above results suggest that the penetration of

Fig. 2. Binding of [³⁵S]cystamine to mitochondrial proteins in relation to respiratory release induced by 2,4-dinitrophenol. Mitochondria (11.8 mg. of protein) were preincubated for 7 min. at 30° with isocitrate (8.3 mM), tris-HCl buffer, pH 7.25 (10 mM), MgCl₂ (5 mM) and KCl (0.07–0.10 M). [³⁵S]Cystamine (3.3 mM) and 2,4-dinitrophenol were then added. The reaction period was 10 min. at 30°. In a control without 2,4-dinitrophenol, but with P_i (10 mM), ADP (0.8 mM) and hexokinase-glucose, the oxygen uptake was 5.4 μg.atoms; the P/O ratio was 2.7; 10.2 mμmoles of ³⁵S were bound/mg. of protein and 1.4 μmoles of SH were formed from the added disulphide. ○, Oxygen uptake; ■, ³⁵S bound; Δ, SH formed.

Fig. 3. Binding of [³⁵S]cystamine to mitochondrial proteins related to the respiratory release induced by dicoumarol. Mitochondria (12.8 mg. of protein) were preincubated for 7½ min. with dicoumarol, and otherwise as stated in the legend to Fig. 2. The reaction period (10 min.) was initiated by the addition of [³⁵S]cystamine (3.3 mM). In a control (cf. Fig. 2) the oxygen uptake was 5.4 μg.atoms, the P/O ratio was 2.2, 9.0 mμmoles of ³⁵S were bound/mg. of protein and 1.9 μmoles of SH were formed. ○, Oxygen uptake; ■, ³⁵S bound; Δ, SH formed.

cystamine into mitochondria is restricted in 'high-energy states' (state 1 or 4), but rapid in 'low-energy states' (e.g. state 2, 2_u or 5). Figs. 2-4 show the penetration of cystamine during gradual changes in mitochondrial energy states induced by uncoupling agents. Since isocitrate was used as substrate, and the mitochondria then might be able to reduce considerable amounts of the disulphide to the corresponding thiol (Eldjarn &

Bremer, 1963), the degree of disulphide reduction was also determined.

Figs. 2, 3 and 4 show that with 2,4-dinitrophenol, dicoumarol or carbonyl cyanide *m*-chlorophenylhydrazone an increasing respiratory release was accompanied by a gradual increase of binding of cystamine to mitochondrial proteins, indicating an increasing penetration of cystamine. Since the extent of disulphide reduction was only slight, and since only minor variations of this reaction occurred on changes in the concentration of uncoupling agents, this reaction seems to be excluded as a source of error in the interpretation. At higher concentrations of the uncoupling agents, the binding of cystamine again decreased somewhat. Since 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone can be bound to mitochondrial proteins (Weinbach & Garbus, 1965), and since 2,4-dinitrophenol also lowered the binding of cystamine to isolated mitochondrial subfractions (Table 1), a competition for the binding site or a steric hindrance by the uncoupling agents may be the explanation. With carbonyl cyanide *m*-chlorophenylhydrazone, only minor variations in the mitochondrial wet weight occurred. The observed stimulation of [³⁵S]cystamine binding by uncoupling agents could therefore not be due to a swelling-induced exposure of thiol groups.

Table 5 shows the binding of ³⁵S to mitochondrial proteins (reflecting the penetration of cystamine) during changes in mitochondrial energy states induced by the addition of adenine nucleotides. ATP counteracted the stimulation of cystamine penetration induced by P_i, though incompletely. ADP or AMP, on the other hand, markedly potentiated the effect of P_i. These effects of the nucleotides were even more pronounced at low (1.7 mM) concentrations of P_i (results not shown). Arsenate stimulated the binding of ³⁵S and to

Concn. of carbonyl cyanide *m*-chlorophenylhydrazone (μM)

Fig. 4. Binding of [³⁵S]cystamine to mitochondrial proteins in relation to respiratory release induced by carbonyl cyanide *m*-chlorophenylhydrazone. Mitochondria (10.3 mg. of protein) were preincubated for 7 min. as in the experiment shown in Fig. 2. [³⁵S]Cystamine and carbonyl cyanide *m*-chlorophenylhydrazone were then added. The reaction period was 10 min. In a control (cf. the legend to Fig. 2), the oxygen uptake was 5.9 $\mu\text{g. atoms}$, the P/O ratio 2.2, 9.0 μmoles of ³⁵S were bound/mg. of protein and 1.6 μmoles of SH were formed. O, Oxygen uptake; ■, ³⁵S bound; △, SH formed; □, wet wt. of pellet.

Table 5. Effect of adenine nucleotides on the binding of [³⁵S]cystamine to mitochondria

Mitochondria (11.3 mg. of protein in Expt. 1; 7.3 mg. in Expt. 2) were incubated at 30° for 8 min. (Expt. 1) or 10 min. (Expt. 2) as described in Table 1. The vessels were rapidly cooled on ice and centrifuged at 25 000g for 5 min. at 0°. The pellets were weighed, and 0.25 ml. of HClO₄ was added 30 min. after the start of the experiment.

	Additions	[³⁵ S]Cystamine bound (mμmoles/mg. of protein)	Wet wt. of pellet (mg.)
Expt. 1	None	6.7	55
	P _i (10 mM)	22.7	85
	P _i (10 mM) + ATP (3.3 mM)	18.3	102
	P _i (10 mM) + ADP (3.3 mM)	30.3	92
	P _i (10 mM) + AMP (3.3 mM)	32.1	78
Expt. 2	None	10.9	39
	Arsenate (1.7 mM)	18.1	55
	Arsenate (1.7 mM) + ATP (3.3 mM)	15.8	65
	Arsenate (1.7 mM) + ADP (3.3 mM)	20.1	—
	Arsenate (1.7 mM) + AMP (3.3 mM)	20.0	—

Fig. 5. Dependence on the phosphorylation potential of the uncoupling effect of cystamine. Mitochondria (10 mg. of protein) were preincubated for 7½ min. at 30° with [³⁵S]-cystamine (1 mM), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid-HCl buffer, pH 7.3 (10 mM), P_i pH 7.3 (1.7 mM), and KCl (0.12 M). The experimental period (10 min.) was initiated by the addition of P_i (6.7 mM), hexokinase (3 mg.), glucose (20 mg.), MgCl₂ (1.3 mM), isocitrate (13.3 mM) and NAD⁺ (2 mM). The adenine nucleotides (concentrations as shown) were always present in the preincubation period. At maximal concentration of ADP, hexokinase-glucose was also present in the preincubation period. In controls preincubated without adenine nucleotides, but otherwise as stated above, 1.9 μg.atoms of oxygen were consumed, the P/O ratio was 1.9 and 6.5 mμmoles of ³⁵S were bound/mg. of protein. In other controls without cystamine, but with adenine nucleotides, the oxygen consumption was 4.9 μg.atoms and the P/O ratio was 4.4. ○, Oxygen uptake; ▲, P/O ratio; ■, ³⁵S bound.

about the same extent as P_i. With arsenate, adenine nucleotides had the same effects on the binding of ³⁵S as with P_i, but less pronounced.

The experiments of Table 5 add evidence to the above conclusion that there is no constant relationship between the extent of swelling and the stimulation of binding of [³⁵S]cystamine. With P_i (10 mM) and cystamine, the mitochondria underwent large-amplitude swelling. ATP potentiated the swelling, probably by acting as an energy source, whereas ADP stimulated the swelling only slightly and AMP counteracted somewhat. The effects of adenine nucleotides in this system were similar to those observed by Azzi & Azzone (1965) on the swelling induced by P_i and *N*-ethylmaleimide.

Figs. 5 and 6 relate the variations of the 'phosphorylation potential' [ATP]/[P_i][ADP] (cf. Klingenberg, 1964) to the extent of uncoupling by cystamine and its binding to mitochondrial proteins.

Fig. 6. Dependence on the phosphorylation potential of the uncoupling effect of cystamine. Mitochondria (6.9 mg. of protein) were preincubated for 9 min. with [³⁵S]cystamine (3.3 mM) as in Fig. 5 except that rotenone (0.5 μM) was also present. During the experimental period (10 min.) succinate (6.7 mM) was present instead of isocitrate and NAD⁺. In controls preincubated without adenine nucleotides, but otherwise as stated in Fig. 5, the oxygen uptake was 7.8 μg.atoms, the P/O ratio 0.6 and 14.4 mμmoles of [³⁵S]cystamine were bound/mg. of protein. In controls without cystamine, the oxygen consumption was 8.8 μg.atoms and the P/O ratio was 2.1. ○, Oxygen uptake; ▲, P/O ratio; ■, ³⁵S bound.

In Warburg experiments, mitochondria were preincubated with cystamine, a low concentration of P_i (1.7 mM) and inversely varied concentrations of ATP and ADP. When compared with controls preincubated without adenine nucleotides (see legends to Figs. 5 and 6), increasing concentrations of ADP potentiated both the uncoupling effect and the stimulation of binding of ³⁵S-labelled residues. ATP, on the other hand, counteracted both effects. In the experiment shown in Fig. 5 (with isocitrate) the controls had an artificially high P/O ratio (4.4), probably because the oxidation of α-oxoglutarate contributed to the oxygen uptake, and because the oxygen consumption could be underestimated during the short incubation (cf. Haslam, 1965). In the experiment shown in Fig. 6 an increasing inhibition of the oxygen uptake occurred on changes of the phosphorylation potential towards high ADP concentration. Probably an inhibition of the α-oxoglutarate dehydrogenase complex occurred on increasing cystamine penetration (cf. Skrede, Bremer & Eldjarn, 1965). An additional cause might be that the capacity for oxidation of isocitrate decreases in mitochondria preincubated with ADP and P_i, probably because of depletion of endogenous substrate required for its oxidation (Chappell & Haerhoff, 1967). In conclusion, Figs. 5 and 6 show a dependency on the phosphorylation

Table 6. *Disappearance of thiol groups in matrix proteins as an index of the penetration of NNN'N'-tetramethylcystamine and cystamine*

Whole mitochondria (18.5 mg. of protein) were incubated for 10 min. and matrix proteins were isolated as indicated in Table 1. The thiol groups of matrix proteins were determined by silver titration.

Addition	SH in matrix proteins (mμmoles/mg. of protein)		
	Incubation without disulphide	Incubation with NNN'N'-tetramethyleystamine	Incubation with cystamine
None	74	73	71
Antimycin A (2.7 μg./ml.)	71	48	46
2,4-Dinitrophenol (0.17 mM)	74	53	54

Table 7. *NN'-Diacetyl[³⁵S]cystamine space in rat liver mitochondria*

Mitochondria (14.7 mg. of protein in Expt. 1, 14.3 mg. in Expt. 2) were incubated at 30° for 10 min. with *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid-HCl buffer, pH 7.3 (10 mM), MgCl₂ (5 mM) and KCl (0.10–0.14 M) making the volume up to 3 ml. (The μl. values indicate the volume of ³H₂O or disulphide present in the whole pellet.)

	Additions	Wet wt. of pellet (mg.)	³ H ₂ O space (μl.)	'Disulphide space'		³⁵ S bound (mμmoles/mg. of protein)
				(μl.)	(% of ³ H ₂ O space)	
Expt. 1	NN'-Diacetyl[³⁵ S]cystamine (3.3 mM)	53	27	30	111	2.0
	NN'-Diacetyl[³⁵ S]cystamine (3.3 mM) + 2,4-dinitrophenol (0.17 mM)	56	31	31	100	2.2
Expt. 2	[³⁵ S]Cystamine (3.3 mM)	54	33	50	151	6.8
	[³⁵ S]Cystamine (3.3 mM) + 2,4-dinitrophenol (0.17 mM)	62	39	74	190	21.7

potential for the uncoupling effect of cystamine as well as for the extent of binding of cystamine. Both parameters may reflect the penetration of cystamine through the inner mitochondrial membrane.

To shed light on the role of charge of cystamine for its restricted penetration into mitochondria, a comparison was made with a dication derivative, NNN'N'-tetramethylcystamine, and a neutral derivative, NN'-diacetylcystamine. Table 6 shows that there is a permeability barrier also against NNN'N'-tetramethylcystamine in *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid-potassium chloride medium (state 1), since practically no reaction with matrix-protein thiol groups then occurred. In analogy with cystamine, the penetration was facilitated by 2,4-dinitrophenol (state 2_u) or antimycin A (state 5), indicated by an increased disappearance of matrix-protein thiol groups.

Table 7 shows that the distribution of NN'-diacetyl[³⁵S]cystamine and ³H₂O is equal in rat liver mitochondria. 2,4-Dinitrophenol did not alter the space for this disulphide. The binding of ³⁵S to mitochondrial proteins was low with NN'-diacetylcystamine, which has a low reactivity (Eldjarn & Pihl, 1957). In uncited experiments with the

osmotic-swelling method of Chappell & Haarhoff (1967), NN'-diacetylcystamine induced an instantaneous swelling, indicating a free penetration through the mitochondrial membrane(s). Table 7 also shows that the 'distribution method' is unsuitable for evaluation of the penetration of cystamine, since 'cystamine space' was much greater than ³H₂O space. Probably cystamine is bound by its amino groups in a loose acid-labile binding to mitochondrial phospholipids. A similar interaction has been shown between the polyamine spermine and phospholipids (Razin & Rozansky, 1959).

DISCUSSION

It has been stated by Blondin & Green (1967) that the penetration of ions into mitochondria appears not to be influenced by the energy state of the mitochondria. The present results indicate that this is not always true, since the penetration of the dications cystamine and NNN'N'-tetramethylcystamine is rapid only in 'low-energy states'. The permeability barrier, which is evident in state 1 or 4, appears to reflect a 'high-energy state' of the membrane. The gradual induction of respiratory release by uncoupling agents coincided closely with

the gradually increased permeability for cystamine. Energy required for the maintenance of the barrier can be derived from coupled electron transport or, less effectively, from externally added ATP. The permeability barrier was broken by different uncoupling agents, by respiratory-chain inhibitors, by anaerobiosis or in the presence of P_i .

The permeability barrier is most probably located in the inner mitochondrial membrane, since the outer membrane in general appears readily permeable to various low-molecular-weight substances (Klingenberg & Pfaff, 1966). Moreover, as much as 15–20% of those mitochondrial thiol groups that are capable of reacting with cystamine are apparently located outside the barrier. When these thiol groups of the sucrose space were combined with cystamine residues (6–10 m μ moles/mg. of protein) by incubating mitochondria in state 1 with the disulphide, the capacity for oxidative phosphorylation was not impaired.

When cystamine is allowed to penetrate into the mitochondria (as in state 2), the disulphide reacts with thiol groups of the membrane. The permeability to endogenous nucleotides or ions then becomes increased (Skrede, Bremer & Eldjarn, 1965; Skrede, 1966b). The uncoupling effect of cystamine, which then becomes evident, can be explained by a modification of the membrane or by a block of the thiol groups of a specific protein carrier involved in the formation of hypothetical energy-rich intermediates. Because of the differing permeability for cystamine in different metabolic states, it is not possible to establish whether its uncoupling effect at low phosphorylation potentials merely is a reflection of its penetration into the mitochondria, or is due to the unmasking of thiol groups when a high-energy intermediate (e.g. a thiol ester) is removed.

The striking effect of P_i on the penetration of cystamine might be explained in several ways. It appears most likely that the increased penetration of cystamine is secondary to an effect of P_i on the metabolic state, since other permeant anions such as acetate or propionate did not stimulate the penetration of this disulphide. A shift from state 1 to state 2, indicated by the oxidation of intramitochondrial nicotinamide nucleotides, is instituted, not only by ADP, but also by P_i alone (Chance & Connelly, 1957). The conversion from state 1 into state 2 is far more rapid when ADP is also added, however (cf. Chance & Hollunger, 1963; Purvis, 1960), and accordingly the increase of permeability for cystamine then was found to have the shortest lag period. Since a certain degree of swelling always follows the oxidation of nicotinamide nucleotides induced by P_i (Azzi & Azzone, 1965; Kaufman & Kaplan, 1960), swelling might be a contributing cause in the stimulation by P_i of the penetration of

cystamine. However, the swelling caused by P_i in the presence of cystamine was potentiated by ATP or succinate (see also Skrede, 1966a), whereas the stimulation of cystamine penetration due to P_i in both instances was counteracted. Further, uncoupling agents or antimycin A (Skrede, 1966a) broke the permeability barrier for cystamine without inducing swelling, demonstrating that swelling is not the general mechanism for breaking the barrier. In contrast, the penetration of thio-sulphate into mitochondria is stimulated by swelling agents (included P_i), but not by 2,4-dinitrophenol or antimycin A (Greville & Chappell, 1959).

A linkage of the penetration of cystamine to a phosphate permease might also be suggested. Such a carrier has been postulated by Chappell & Crofts (1965). The following results argue against this interpretation. During succinate oxidation (state 3), P_i penetrated rapidly into the mitochondria without a concomitant increase of the binding of cystamine to mitochondrial proteins. If succinate was left out (state 2), cystamine penetrated and became bound to matrix proteins. On the other hand, cystamine penetrated the barrier in the absence of P_i if uncoupling agents or respiratory-chain inhibitors were present.

The use of the binding of disulphide residues to mitochondrial proteins as an index of the penetration of cystamine into mitochondria in state 3 is complicated by the increased reduction of the disulphide that then takes place. The explanation of the low binding might be that cystamine penetrates mitochondria more easily in state 3 than in state 1 or 4, but that its binding to matrix proteins is kept low by a reduction of the disulphide, which then may act as an artificial electron acceptor (Eldjarn & Bremer, 1963). Even this interpretation appears to be in accord with the concept that the penetration of cystamine is regulated by the energy state of the mitochondria, since the phosphorylation potential is held to be intermediate in state 3 (Chance & Hollunger, 1963).

The increase of binding of cystamine to mitochondrial proteins in the presence of P_i was less effectively counteracted by ATP than by succinate. The cause might be that mitochondria oxidizing endogenous substrate have a low capacity for disulphide reduction. This reaction is particularly evident when different citric acid-cycle intermediates are added (Eldjarn & Bremer, 1963). A second explanation is that the penetration of cystamine is most restricted when $X \sim I$ is generated by coupled electron transport, since the reactions:

may be the rate-limiting ones in respiratory-chain phosphorylation (Chappell, 1963; Høfer & Pressman,

1966). In uncited experiments, succinate counteracted the stimulation by P_1 of the permeation of cystamine into the mitochondria, even in the presence of oligomycin. Cystamine then did not relieve the inhibition of succinate oxidation by oligomycin. Also, the latter observation is in accordance with the view that the accumulation of energy is essential for the barrier against cystamine.

The restricted penetration of cystamine evident at high phosphorylation potentials might be due to a special conformation of the membrane. Conformational changes due to variations of the phosphorylation potential have been suggested by Lehninger (1964). However, a comparison with the dication $NNN'N'$ -tetramethylcystamine and the neutral derivative NN' -diacetylcystamine suggested that the permeability barrier against cystamine in 'high-energy states' of the mitochondria is dependent on the positive charge of the disulphide. The present study therefore leads to the concept that a charge barrier across or in the inner mitochondrial membrane is created in 'high-energy states'. This barrier appears to reject the positively charged disulphides, since in 'low-energy states' the mitochondrial membrane is permeable to all three disulphides studied.

The present results are not easily reconciled with the chemiosmotic hypothesis of oxidative phosphorylation (Mitchell, 1961, 1966a), according to which permeant cations are accumulated by mitochondria in a process of exchange diffusion with H^+ ions extruded by electron transport (Mitchell, 1966b). If such a mechanism were operating in ion transport, it appears that cystamine should be accumulated in 'high-energy states', and not be repelled by a charge barrier. If the charge barrier for cystamine is a transmembrane potential, its direction seems to be in reverse of that proposed by Mitchell (1966a) to operate together with a pH gradient. The present results give no indications, however, that the transition to a 'high-energy state' is accompanied by the creation of such a transmembrane potential, counteracting the influx of positively charged disulphides. The decreased permeability to these cations in 'high-energy states' might also depend on fixed charges within the membrane, unrelated to the equilibrium distribution of ions across the mitochondrial membrane.

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