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Effects of Cystamine and Cysteamine on the Peroxidation of Lipids and the Release of Proteins from Mitochondria

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1. Cystamine slightly stimulated the peroxidation of lipids in mitochondria. Maximal effects were obtained at low concentrations (0·5mM). 2. Cysteamine, when allowed to autoxidize, had much stronger effects than cystamine. 3. Cysteamine and GSH did not induce peroxidation when their autoxidation was counteracted. 4. When kept reduced, cysteamine prevented the ascorbate-induced peroxidation of lipids. GSH was less efficient. 5. Cystamine as well as cysteamine prevented the loss of proteins from mitochondria induced by ascorbate, whereas cadaverine, GSSG and GSH were inefficient.

Cystamine, in contrast with cysteamine, induces a type of mitochondrial swelling that is not reversed by ATP (Neubert & Lehninger, 1962), inactivates mitochondrial NAD-linked reactions (Skrede, Bremer & Eldjarn, 1965) and causes a loss of bivalent cations from the mitochondria (Skrede, 1966). These effects may be attributed to mitochondrial permeability changes induced by the disulphide. It was proposed that this permeability increase might be due to the reaction of cystamine with thiol groups of the mitochondrial membrane. Similar thiol–disulphide interchange reactions in membrane structures probably are of considerable physiological importance (Lehninger & Neubert, 1961; Schwartz, Rasmussen, Schoessler, Silver & Fong, 1960).

Another possible mechanism for the permeability increase might be that cystamine induces the peroxidation of mitochondrial lipids, by analogy with the observation that such changes may precede the extensive swelling caused by a mixture of GSH and GSSG (Hunter *et al.* 1964*b*).

In the present study the effects of cystamine and cysteamine as inducers of lipid peroxidation in mitochondria were compared with the corresponding effects of GSSG and GSH. The release of proteins from the mitochondria was also studied and was used as an index of structural disintegration. In contrast with GSH, cysteamine strongly stimulated lipid peroxidation, probably via a more rapid formation of autoxidation intermediates. When autoxidation of the thiols was counteracted, neither of them induced lipid peroxidation. Cystamine and GSSG stimulated the peroxidation of mitochondrial lipids only slightly, and it is concluded that cystamine probably does not increase mitochondrial permeability by this reaction.

EXPERIMENTAL

Materials. Cysteamine and cadaverine were products of Fluka A.-G., Buchs SG, Switzerland. Cystamine was obtained from Dr Theodore Schuchardt, München, Germany, GSSG was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and GSH and L(+)-ascorbic acid were from E. Merck A.-G., Darmstadt, Germany. Thiolated Sephadex was prepared according to the method of Eldjarn & Jellum (1963) as described by Skrede (1966). All other reagents were commercial products of high purity.

Methods. Rat-liver mitochondria were prepared in 0·25M-sucrose by the method of Myers & Slater (1957). For the lipid peroxidation experiments, mitochondria were washed one additional time with 0·15M-KCl and resuspended in KCl, since sucrose interferes with the thiobarbituric acid method.

Incubations were performed as stated in the legend to Table 1. In the experiments on protein release, the reaction was slowed by the addition of 5ml. of ice-cold 0·15M-KCl solution and cooling in an ice bath. The mitochondria were then sedimented at 8000*g* for 10min. and the proteins in the supernatant precipitated with 0·5ml. of 35% (v/v) $HClO_4$. The protein content of the pellets and supernatants was determined by a micro-Kjeldahl method. In the lipid peroxidation experiments the reaction was stopped with 1·5ml. of 20% (w/v) trichloroacetic acid, and malonaldehyde immediately estimated by the thiobarbituric acid method as described by Ottolenghi (1959).

RESULTS

Table 1 shows that when freshly prepared rat-liver mitochondria were incubated in a tris–potassium chloride medium at 37° for 1hr. only small amounts of lipid peroxides were formed. Concomitantly there was some loss of mitochondrial protein into the incubation medium.

The disulphides cystamine and GSSG at 10mM

Table 1. *Effects of cystamine, cysteamine, GSSG and GSH on lipid peroxidation and protein release from rat-liver mitochondria*

Mitochondria (10mg. of protein in Expt. 1; 6·8mg. in Expt. 2) were incubated at 37° for 1hr. in tris–HCl buffer, pH7·5 (0·05M), containing KCl (0·06–0·08M) with additions as stated.

Additions	Malonaldehyde formed (mμmoles) Expt. 1	Mitochondrial protein released (%) Expt. 2
None	1·9	6
Cystamine (10mM)	9·2	0
Cysteamine (5mM)	39·1	1
Cysteamine (5mM) + thiolated Sephadex (40mg.)	4·3	1
Cysteamine (5mM) + cystamine (10mM)	17·8	2
GSSG (10mM)	10·5	17
GSH (5mM)	6·0	8
GSH (5mM) + thiolated Sephadex (40mg.)	1·3	9
GSH (5mM) + GSSG (10mM)	80·8	55
Ascorbate (0·2mM)	62·1	51
EDTA (5mM)	0	0
Cystamine (10mM) + EDTA (5mM)	0	—

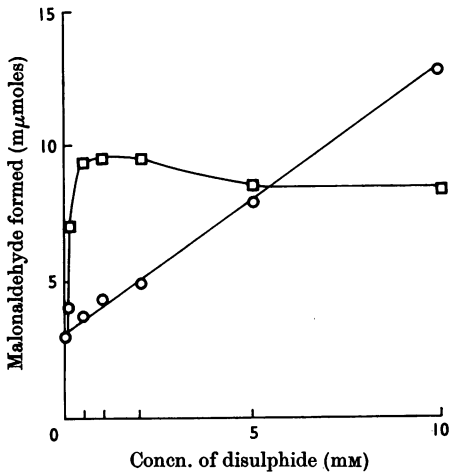

Fig. 1. Effects of cystamine and GSSG on lipid peroxidation in rat-liver mitochondria. Mitochondria corresponding to 9·2mg. of protein were incubated as indicated in Table 1. □, Cystamine; ○, GSSG.

each stimulated lipid peroxidation only slightly. Their effects on the loss of proteins from the mitochondria, however, were oppositely directed.

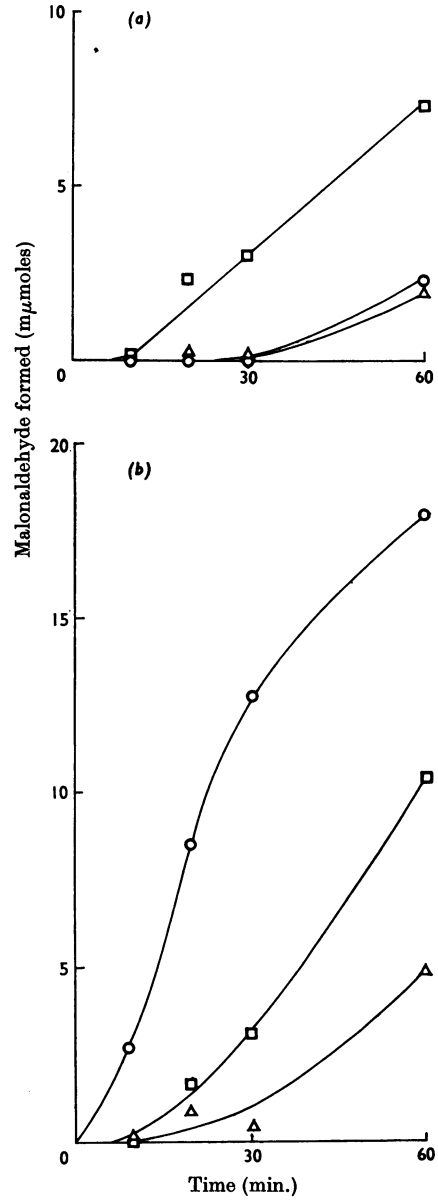

Fig. 2. Time-course of mitochondrial lipid peroxidation induced by cystamine or GSSG. Rat-liver mitochondria (10·5mg. of protein in *a*; 8·9mg. in *b*) were incubated as indicated in Table 1. The concentration of the disulphides was 1mM in (*a*) and 10mM in (*b*). □, Cystamine; ○, GSSG; △, control.

GSSG increased this loss, whereas cystamine counteracted the protein leakage as effectively as EDTA did. In contrast with EDTA and GSSG, cystamine caused a visible sedimentation of mitochondrial aggregates in a few minutes.

Fig. 1 shows that when the disulphide concentration was varied a clear difference between cystamine and GSSG in the tendency to stimulate lipid peroxidation was also revealed. With cystamine the maximal effect was obtained at 0·5mM and a significant stimulation at concentrations as low as 0·05mM. With GSSG, on the other hand, lipid peroxidation increased almost linearly with rising concentrations.

An additional difference between the effects of cystamine and GSSG was shown by the dependence on time for the induction of lipid peroxidation. At 10mM (Fig. 2*b*) a lag period was evident for the effect of cystamine, whereas with GSSG the curve was nearly linear from the start of the experiment. Also, at 1mM (Fig. 2*a*) there was a small lag period for the stimulatory effect of cystamine, whereas GSSG did not increase peroxidation significantly.

The thiols cysteamine and GSH, when kept reduced in the presence of thiolated Sephadex (Jellum, 1964), each caused only insignificant lipid peroxidation (Table 1). The protein loss could be counteracted by cysteamine but not by GSH.

In the absence of a disulphide-reducing system, cysteamine autoxidized completely during the experimental period. Cysteamine (5mM) under these conditions stimulated lipid peroxidation strongly (Table 1). GSH (5mM) caused only a slightly increased lipid peroxidation and protein loss even in the absence of an electron reservoir. It should be recalled that GSH autoxidizes more slowly than does cysteamine.

In accordance with previous results (Hoffsten, Hunter, Gebicki & Weinstein, 1962) the combination of GSH and GSSG caused extensive lipid peroxidation and protein loss. Much less peroxidation and no protein loss was caused by cysteamine in the presence of cystamine (Table 1). Altogether, Table 1 shows that in the presence of GSH, GSSG or ascorbate there was a correlation between lipid peroxidation and protein loss, whereas there was no such correlation with cysteamine and cystamine, which decreased the protein loss as compared with the controls irrespective of the peroxidation induced.

As observed previously (Ottolenghi, 1959; Hunter *et al.* 1964*a*), ascorbate caused extensive lipid peroxidation and protein loss. Both effects could be counteracted by EDTA (Table 2). The present study shows that cystamine also counteracted the protein loss induced by ascorbate. This protective effect was considerable even at a cystamine concentration of 0·5mM (Fig. 3) and was complete at 10mM. It should be stressed that the peroxidation of lipids by ascorbate was counteracted only to a minor degree by cystamine. GSSG, in contrast with cystamine, offered no protection against protein loss, and sometimes slightly stimulated the peroxidation induced by ascorbate, in accordance with previous observations (Hunter *et al.* 1964*a*).

Cysteamine, when combined with thiolated Sephadex, protected completely against both the above effects of ascorbate (Table 2). Also, GSH counteracted peroxidation to some extent when kept completely reduced, but still stimulated the protein loss.

The protection by cysteamine, without a thiol-regenerating system, against the peroxidation of lipids induced by ascorbate was incomplete in

Table 2. *Counteraction of mitochondrial lipid peroxidation and protein release induced by ascorbate*

Rat-liver mitochondria (6·5mg. of protein in Expt. 1; 8·5mg. in Expt. 2) were incubated as stated in Table 1. Ascorbate (0·2mM) was present in all tubes except the control.

Additions	Malonaldehyde formed (mμmoles)		Mitochondrial protein released (%)
	Expt. 1	Expt. 2	Expt. 1
Control without ascorbate	4·4	5·3	7
None (ascorbate alone)	169	94	53
Cystamine (10mM)	107	—	0
Cysteamine (10mM)	3·4	34	1
Cysteamine (10mM)+thiolated Sephadex (40mg.)	2·2	1·9	1
GSSG (10mM)	168	—	53
GSH (10mM)	189	—	65
GSH (10mM)+thiolated Sephadex (40mg.)	36	—	65
Thiolated Sephadex (40mg.)	136	—	55
Cadaverine (10mM)	159	—	41
EDTA (5mM)	0	—	0

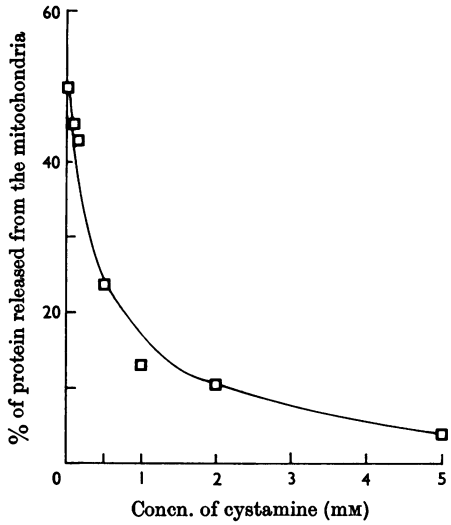

Fig. 3. Counteraction by cystamine of ascorbate-induced protein loss from mitochondria. Mitochondria corresponding to 6·2mg. of protein were incubated with ascorbate (0·2mM). Otherwise the conditions were as indicated in Table 1.

some experiments (Table 2, Expt. 2). The variability of the protection by this thiol was probably related to its tendency to autoxidize at the pH (7·5) of the experiments in the present study.

The diamine cadaverine, which structurally is closely related to cystamine, offered only a slight protection against the effects of ascorbate. This observation suggests that the effects of cystamine on the protein loss were due to its disulphide group rather than to its amino groups.

DISCUSSION

Previous studies have suggested that cystamine, but not cysteamine, increases mitochondrial permeability to nucleotides and bivalent cations (Skrede *et al.* 1965; Skrede, 1966). This effect was evident even during short incubations at 30°. The aim of the present study was to elucidate the possible role of peroxidation of mitochondrial lipids for the permeability increase caused by cystamine. From the present results, however, it appears unlikely that cystamine increases membrane permeability in this way, since this disulphide stimulates lipid peroxidation only slightly even at 37°. A lag period in the appearance of peroxidation products was also evident at different cystamine concentrations that rapidly induced the above permeability changes. The markedly stimulating effect on lipid peroxidation obtained with cysteamine also supports the conclusion that this process is not involved in the permeability increase, since this thiol did not inactivate NAD-linked oxidations and did not induce the loss of bivalent cations (Skrede *et al.* 1965; Skrede, 1966).

One of the most striking findings of the present investigation was the protective effect of cysteamine, and to a smaller extent of GSH, against lipid peroxidation induced by ascorbate, when the thiols were kept completely reduced by means of thiolated Sephadex. Previous studies have shown that GSH protects against lipid peroxidation of mitochondria and microsomes induced by compounds in particle-free supernatant (Christophersen, 1966). Together these findings suggest that GSH and cysteamine can prevent the formation of or destroy intermediary products in the peroxidation process.

Thiolated Sephadex alone also protected slightly against lipid peroxidation, probably because of its thiol content or because of its ability to bind certain heavy-metal ions (Jellum, 1964). Since, also, cysteamine forms complexes with certain heavy-metal ions (Knoblock & Purdy, 1961) this property may contribute to the protective effect of the latter thiol. With EDTA, the counteraction of peroxidation has been explained solely by its chelating properties (Ottolenghi, 1959; Fortney & Lynn, 1964).

The present paper as well as previous studies (Hopkins, 1925; Fortney & Lynn, 1964; Ottolenghi, 1959) shows that lipid peroxidation in mitochondria is strongly stimulated during the autoxidation of reducing compounds such as thiols and ascorbate. For the peroxidation of mitochondrial lipids by ascorbate, the leakage of endogenous iron has been thought to be essential (Ottolenghi, 1959; Fortney & Lynn, 1964). Ascorbate, as well as thiols, might act by offering reducing equivalents for a recycling of Fe^{3+} to Fe^{2+}. However, our results make it unlikely that thiols only react by reduction of Fe^{3+}, since a completely reduced thiol system did not maintain peroxidation, but on the contrary protected against this reaction. Another explanation, which appears more likely to us, is that intermediates in thiol autoxidation are directly involved. This concept explains why cysteamine caused more peroxidation than GSH, since the former thiol oxidizes more rapidly. It also explains the fact that GSSG potentiates the ability of GSH to induce lipid peroxidation in mitochondria (Hoffsten *et al.* 1962), since the autoxidation of a thiol is accelerated by disulphides (Dixon & Tunnicliffe, 1923; Jellum, 1964; Schneider, Smith & Hunter, 1964). The reason why the combination of cysteamine and cystamine is not more efficient in inducing peroxidation than is cysteamine alone (Table 1) may be because a catalytic concentration of disulphide will

spontaneously be reached rapidly in a solution containing cysteamine. In accord with these concepts, the extent of lipid peroxidation caused by a thiol compound will depend both on its tendency to oxidize in air and on the lability of its autoxidation intermediates.

The observation that cysteamine, when kept reduced, offers a complete protection against lipid peroxidation may have bearings on the protection of sulphur components *in vivo* against ionizing radiation, since the peroxidation of lipids in cellular membranes may be involved in radiation damage (Horgan, Philpot, Porter & Roodyn, 1957; Tappel, 1965). In the living organism, radioprotective sulphur compounds are likely to be mainly in the thiol form (Sørbo, 1962; Eldjarn, 1965), in accord with the occurrence of several disulphide-reducing systems (Pihl, Eldjarn & Bremer, 1957; Eldjarn, Bremer & Børresen, 1962; Eldjarn & Bremer, 1963). When kept reduced by biological mechanisms, cysteamine thus could counteract peroxidation damage in the cell in analogy with the present experiments *in vitro*.

Experiments with mitochondria *in vitro* have shown that extensive lipid peroxidation induced by different agents will coincide with lysis leading to massive protein loss (Hunter *et al.* 1964*a*; McKnight, Hunter & Oehlert, 1965). In the present study cystamine strikingly prevented the protein loss from the mitochondria. The fact that EDTA and cystamine, which had completely different actions on lipid peroxidation when ascorbate was the inducing agent, both prevented the protein loss suggests that they protect against protein release by different mechanisms. EDTA may maintain mitochondrial integrity primarily by preventing peroxidation. Cystamine probably acts by its disulphide group (since cadaverine is inefficient) and mitochondrial thiol groups are the most likely point of attack. GSSG did not protect against the protein loss even though most mitochondrial thiol groups are reactive towards GSSG (Riley & Lehninger, 1964). However, the mitochondrial membrane appears to be less permeable to GSSG than to cystamine (Eldjarn & Bremer, 1963) and further the disulphide group of GSSG is more slowly reacting than that of cystamine (Pihl & Eldjarn, 1958). The completely different effects of GSSG and cystamine in the present study suggest that cystamine penetrates the mitochondrial membrane and reacts with other thiol groups than does GSSG, and that this reaction in some way prevents lysis. One possibility is that cystamine may induce protein polymerization via the formation of disulphide bridges. This might prevent the protein loss, but still might cause an increased permeability to compounds of low molecular weight.

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