would occur between the two marked carbon atoms.

If this is in fact the case, the first aromatic compound formed would be a half-anhydride of a completely acetylated phloroglucinol carboxylic acid and acetic acid, the reaction being analogous to that whereby phenols are obtained from hexadiene-carboxylic acids by reaction in acetic anhydride.[5] The different colour components then have to be formed through further condensations with the phloroglucinol derivative, together with deacetylation. This could explain why the amines are bonded to the coloured reaction products.

1. Ohkuma, S. *J. Pharm. Soc. Japan* **75** (1955) 1124.
2. Feigl, F. *Spot tests in organic analysis*, 6th Ed., Elsevier, Amsterdam-London-New York 1960, p. 281.
3. Groth, A. B. and Dahlén, M. E. *Acta Chem. Scand.* **21** (1967). *In print.*
4. Roeder, G. *J. Am. Pharm. Assoc.* **30** (1941) 74.
5. Chiusoli, G. P. and Agnès, G. *Proc. Chem. Soc. (London)* **1963** 310.

Received November 2, 1966.

^{35}S-Cystamine as a Thiol Reagent for the Study of Oxidative Phosphorylation

SVERRE SKREDE

Institute of Clinical Biochemistry, Rikshospitalet, University of Oslo, Oslo, Norway

Rat liver mitochondria contain approximately 0.1 μmole of thiol groups per mg of protein.[1] A number of investigators have felt that thiol groups may be involved in oxidative phosphorylation.[1-3] This conclusion has mainly been reached through the study of the uncoupling effect of different thiol reagents, whereas variations in mitochondrial thiol levels in different metabolic states have not been demonstrated. A possible explanation for this may be that silver ions and other reagents used for the estimation of thiol groups are so reactive that they cause a rapid lysis of mitochondria even at concentrations amounting to only 10 % of mitochondrial thiol groups.

^{35}S-Cystamine by its ability to form mixed disulphides can be regarded as a specific and mild thiol-blocking agent.[4] Preliminary experiments showed that cystamine was bound to mitochondrial proteins much more slowly than were silver ions. Since cystamine, even at high concentrations (5 mM), does not inhibit the respiratory chain,[5] nor uncouples oxidative phosphorylation provided substrate is present,[6] this disulphide might prove to be a useful reagent in the study of mitochondrial thiol groups. It might be possible to maintain different states of electron transport and oxidative phosphorylation even during the reaction period which is required with cystamine (5−15 min at pH 7.0−7.5). Since Eldjarn and Bremer [7] showed that disulphides (*e.g.* cystamine) which penetrate into mitochondria slowly will become reduced to thiols, it was regarded essential to secure by thiol estimation that the disulphide concentration is not too much altered during experiments on the binding of cystamine to mitochondria.

In the present work it is shown that ^{35}S-cystamine is suitable as a reagent for the study of mitochondrial thiol groups, and that a relation seems to exist between the levels of reactive thiol groups and different states of oxidative phosphorylation.

Methods. ^{35}S-Cystamine was obtained from the Radiochemical Centre, Amersham, England. 0.1 M stock solutions of cystamine (specific activity approximately 50 000 cpm/μmole) were prepared by the addition of carrier cystamine purchased from Calbiochem, Los Angeles, California, USA (lot No. 30 177). Oligomycin and Antimycin A were products of Sigma Chemical Co., St. Louis, Mo., USA. All other reagents were commercial products of high purity. Rat liver mitochondria were prepared according to Myers and Slater.[8] A Zeiss RPQ 20 A Recording Spectrophotometer was used in the swelling experiments. The oxygen uptake was measured by Warburg techniques, and the reaction was stopped with 0.25 ml of 6 M $HClO_4$. Disappearance of P_i was determined by the method of Martin and

Table 1. Rat liver mitochondria (6.6 mg protein) were preincubated for 7 min at 30° with Tris/HCl (10 mM, pH 7.15); $MgCl_2$ (5 mM); ADP (0.8 mM) and KCl (0.07–0.10 M) and other additions as stated. The experimental period (10 min at 30°) was started by adding agents as indicated. Swelling experiments were performed immediately on the same mitochondrial batch. In the latter experiments, mitochondria were added in amounts giving an initial extinction value of 0.5–0.6 (approximately 0.14 mg protein/1.5 ml) and the decrease in optical density was followed for 5 min at 20°. The additions were as those during the experimental period of the Warburg experiments.

Agents (μmoles) present during the preincubation	Added at the start of the experimental period (μmoles)	O_2 uptake (μatoms)	P_i esterified (μmoles)	P/O ratio	Cystamine reduced to thiol (%)	^{35}S bound		Swelling
						mμmoles per mg protein	increase (%)	($10^3 \times E_{520}$)
None	Cystamine (6)	1.3	—	—	2.5	9.2	—	65
Succinate (20)	Cystamine (6)	5.0	—	—	5	9.4	2	80
None	Cystamine (6) phosphate (25) hexokinase/glucose*	1.3	3.2	2.5	5	16.8	83	105
Succinate (20)	Cystamine (6) phosphate (25) hexokinase/glucose	11.4	21.0	1.8	11	11.4	24	150
Succinate (20) + oligomycin (2 μg)	Cystamine (6) phosphate (25) hexokinase/glucose	6.8	1.2	0.2	12	11.6	26	25
Succinate (20) + antimycin A (4 μg)	Cystamine (6) phosphate (25) hexokinase/glucose	1.8	0	0	7.5	16.6	80	0
Succinate (20) + 2,4-DNP (1)	Cystamine (6) phosphate (25) hexokinase/glucose	6.5	0.3	0.1	7	13.8	50	0

* When added, the amounts were: Hexokinase (1.5 mg); glucose (50 μmoles).

Doty.[9] The thiol content at the end of the incubations was estimated by amperometric silver titration at the rotating platinum electrode.[10] The precipitated proteins were washed repeatedly with 0.3 M $HClO_4$ and finally resuspended in 1 ml of water. Protein-bound radioactivity was estimated in a TRI-CARB Liquid Scintillation Counter. By internal standard technique the small degree of quenching was corrected for. By a second incubation of the mitochondria with 10 mM thiol (cysteamine) 90–95 % of the protein-bound ^{35}S-cystamine could be removed, indicating that the binding was of the mixed disulphide type. Proteins were determined by a micro-Kjeldahl method.

The conditions of Table 1 were chosen to give different levels of "non-phosphorylated high energy intermediates" in the mitochondria. The amount of ^{35}S-cystamine bound corresponded to 9.2 mμmoles of thiol groups/mg of protein with endogenous substrate, but without P_i and acceptor system. No difference in binding was observed when succinate was also added. With endogenous substrate, the addition of P_i and acceptor system nearly doubled the binding of ^{35}S-cystamine (16.8 mμmoles/mg of protein). This effect of P_i was to a considerable degree counteracted by substrate, probably because oxidative phosphorylation then became the rate limiting reaction. The latter interpretation was supported by the observation that oligomycin, which inhibited phosphorylation nearly completely, did not reduce the binding of cystamine more than did substrate alone. The binding of ^{35}S was stimulated by 2,4-DNP, which inhibited the oxygen consumption, probably by causing an accumulation of oxaloacetate. With Antimycin A in addition to substrate and P_i, the binding was as high as with endogenous substrate and P_i.

Thus, when the conditions were chosen to remove nonphosphorylated intermediates of oxidative phosphorylation (excess P_i, uncoupler or respiratory inhibitor), more thiol groups were made accessible to mixed disulphide formation with ^{35}S-cystamine.

Before interpreting the results, it should be considered whether different states of swelling might occur and influence the exposure of thiol groups. However, Table 1 shows that swelling was maximal with substrate, P_i, acceptor and cystamine, whereas the binding of ^{35}S-cystamine in this case was low. With Antimycin A or DNP, the swelling was completely counteracted, but the binding of ^{35}S was increased. It thus appears unlikely that the stimulatory effect of P_i on the binding of ^{35}S-cystamine is related to its swelling effect.

The metabolic reduction of cystamine to the corresponding thiol, which also should be considered as a possible source of error, is shown in Table 1. It is not probable that this reduction is of any significance, since in all experimental conditions the degree of reduction was small (less than 12 %). Further, no correlation was found between the extent of reduction and the amounts of ^{35}S-cystamine bound. It thus appears that the levels of cysteamine reached did not represent any serious source of error in these experiments.

In conclusion, the experiments with ^{35}S-cystamine have shown that this disulphide may be used as a thiol-blocking agent even in studies with actively metabolizing mitochondria. It is shown that when mitochondria are uncoupled or otherwise depleted of non-phosphorylated intermediates of oxidative phosphorylation, thiol groups become unmasked and are made accessible for reaction with ^{35}S-cystamine, whereas these thiol groups are covered up or masked when the levels of such intermediates are expected to be high. It appears to be unlikely that the variations in binding of ^{35}S-cystamine are caused by mitochondrial swelling or by metabolic reduction of the disulphide.

As an explanation of the present findings, it might be suggested that those thiol groups which vary with different metabolic states of the mitochondria, are located on a protein which undergoes conformative changes, thus preventing the access of cystamine to the reactive thiol group(s) when high energy intermediates are formed. Alternatively, an SH/SS-shift might occur in such a protein simultaneously with its participation in the formation of a high energy intermediate.

The most probable explanation, however, appears to be that protein thiol groups participate directly in the binding of nonphosphorylated intermediates of oxidative phosphorylation, *e.g.* in thioester bonds, and that the levels of free thiol groups in this way are inversely related to the accumulation of such intermediates.

1. Riley, M. V. and Lehninger, A. L. *J. Biol. Chem.* **239** (1964) 2083.
2. Kielley, W. W. *Proc. 5th Intern. Congr. Biochem.*, Moscow, Vol. 5 (1961) 378.
3. Fluharty, A. L. and Sanadi, D. R. *Biochemistry* **2** (1963) 519.
4. Pihl, A. and Eldjarn, L. *Proc. 4th Intern. Congr. Biochem., Vienna*, Vol. **13** (1958) 43.
5. Skrede, S., Bremer, J. and Eldjarn, L. *Biochem. J.* **95** (1965) 838.
6. Skrede, S. *Biochem. J.* **98** (1966) 702.
7. Eldjarn, L. and Bremer, J. *Acta Chem. Scand.* **17** (1963) 59.
8. Myers, D. K. and Slater, E. C. *Biochem. J.* **67** (1957) 558.
9. Martin, J. B. and Doty, D. M. *Anal. Chem.* **21** (1949) 965.
10. Børresen, H. C. *Anal. Chem.* **35** (1963) 1096.

Received October 24, 1966.

Preliminary IR and NMR Investigations on the Alkali Soap-Carboxylic Acid-Water Systems

STIG FRIBERG

Research Institute of National Defence, Department 1, Sundbyberg, and Institute of Physical Chemistry, University of Stockholm, Stockholm,

LEO MANDELL and PER EKWALL

Laboratory for Surface Chemistry, The Royal Swedish Academy of Engineering Sciences, Stockholm, Sweden

The system sodium caprylate-caprylic acid-water was investigated by Ekwall and co-workers,[1-6] who gave evidence for the presence of a crystalline acid soap $2NaC_8{\cdot}HC_8$, of several mesomorphous phases and of two homogeneous isotropic solution phases (L_1 and L_2) (Fig. 1).

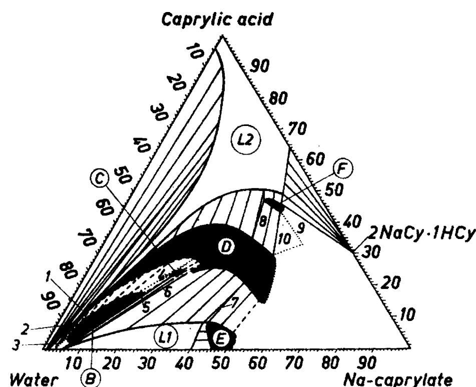

Fig. 1. The three component system water-sodium caprylate-caprylic acid.

Since molecular spectroscopy has not previously been used on systems like this with the exception of the IR investigation on solid acid soaps by Goddard [7] and Dunken [8] an investigation of the chemical bonds and molecular arrangements in the sodium caprylate-caprylic acid-water system by means of IR and NMR spectroscopy was thought to be of value. The present investigation has been limited to the region L_2 with a homogeneous isotropic solution. Considering the various acid salts and acid soaps which have been reported by Ekwall,[9,10] McBain,[11] and Dunken [8,12] the detection of complexes between acid and soap in the L_2-solution would be of highest interest.

The investigations were carried out by means of an infrared spectrophotometer Perkin Elmer 221 and a high resolution nuclear magnetic spectrometer Varian A 60A.

Infrared spectra of caprylic acid solutions of sodium caprylate are given in Fig. 2. Addition of the soap to the acid decreases the frequency of the absorption of the OH stretching vibration in the region 2500−3300 cm^{-1} and at the same time a new absorption at 1900 cm^{-1} grows.

The absorption of the carbonyl group at 1710 cm^{-1} does not change appreciably while the frequency of the absorption of the antisymmetric vibration of the ionized carboxyl group at 1540 cm^{-1} is increased somewhat when soap is added. The absorption of the out-of-plane bending vibration of the OH groups of the carboxylic acid at 930 cm^{-1} decreases linearly with the soap content and reaches zero when the ratio acid to soap is 2:1. NMR measurements of the hydroxylic proton of the acid showed a down-field shift of the signal frequency of about 100 cps which varied linearly with added soap.