[31a] L. LEDOUX, *Arch. intern. physiol.*, 64 (1956) 134.
[32] L. LEDOUX, *Biochim. Biophys. Acta*, 23 (1957) 121.
[33] L. LEDOUX, *Biochim. Biophys. Acta*, 13 (1954) 121.
[34] G. A. LEPAGE, *Cancer Research*, 13 (1953) 178.
[35] F. GAVOSTO ET A. FICQ, *Nature*, 172 (1953) 406.
[36] R. M. S. SMELLIE, dans E. CHARGAFF ET J. N. DAVIDSON, *The Nucleic Acids*, Vol. II, Academic Press, Inc., New York, 1955, p. 392.
[37] G. B. BROWN ET P. M. ROLL, dans E. CHARGAFF ET J. N. DAVIDSON, *The Nucleic Acids*, Vol. II, Academic Press, Inc., New York, 1955, p. 000.
[38] R. V. POTTER, L. I. HECHT ET E. HERNERT, *Biochim. Biophys. Acta*, 20 (1956) 439.
[39] C. HEIDELBERGER, *Biochim. Biophys. Acta*, 20 (1956) 445.
[40] R. MARKHAM ET J. D. SMITH, *Biochem. J.*, 60 (1955) 8.
[41] L. A. HEPPEL ET P. R. WHITFELD, *Biochem. J.*, 60 (1955) 1.
[42] L. A. HEPPEL, P. R. WHITFELD ET R. MARKHAM, *Biochem. J.*, 60 (1955) 8.
[43] R. MARKHAM ET J. D. SMITH, *Biochem. J.*, 49 (1951) 401.
[44] G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
[45] D. D. VAN SLYKE, D. A. MACFADYEN ET P. HAMILTON, *J. Biol, Chem.*, 141 (1941) 671.
[46] O. K. LOWRY, N. J. ROSEBROUGH, L. FARR ET R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[47] S. E. KERR, K. SERAIDARIAN ET M. WARGON, *J. Biol. Chem.*, 181 (1941) 761.

Reçu le 6 décembre 1956

CYSTALDIMINE: THE PRODUCT OF OXIDATION OF CYSTAMINE BY DIAMINE-OXIDASE

D. CAVALLINI, C. DE MARCO AND B. MONDOVÌ

Institute of Biological Chemistry, University of Rome, and Centro di Enzimologia del C.N.R., Rome (Italy)

INTRODUCTION

It has been shown that cystamine is oxidized by diamine-oxidase extracted either from pig kidney cortex or from pea seedlings[1]. The rate of oxidation of this sulfur-containing diamine is in the range of that found, under the same conditions, using a typical substrate such as cadaverine. Although the analytical values are in agreement with a typical diamine-oxidase reaction, the total O_2 uptake, with cystamine as substrate, is higher than that obtained with cadaverine. This probably indicates a high reactivity of the reaction product of cystamine which is further metabolized.

Cadaverine and putrescine have been shown to be oxidized by diamine-oxidase to the cyclized internal Schiff bases instead of their free amino-aldehyde derivatives[2,3]. In this paper we present evidence on the analogous intermediate formation of a cyclized disulfide-containing azo-methine derivative, by the interaction of cystamine with diamine-oxidase.

METHODS AND MATERIALS

Diamine-oxidase extracted from pea seedlings, according to the method of KENTEN AND MANN[4], has been used as enzyme throughout this work. 20 mg of the final acetone powder, or higher proportional amounts, have been homogenized with 2 ml of 0.1 *M* phosphate buffer, at the ap-

References p. 358.

propriate pH, and filtered from the insoluble residue. Typical experiments have been performed with 2 ml buffered enzyme extract (*i.e.* 20 mg dry enzyme) plus 1 ml water containing 10 μmoles of neutralized cystamine dihydrochloride, and eventual additions. A few experiments have been performed in the absence of buffer, in which case the enzyme was homogenized in water.

The conventional Warburg apparatus was used for the estimation of the O_2 uptake. Working temp., 25°; air was used as gas. Substrates dissolved in 0.5 ml water were placed in the side arm; the center well contained 0.2 ml 10% NaOH.

Descending chromatography on Whatman No. 4 paper was employed for the detection of intermediate compounds. Solvent: butanol, acetic acid, water (4, 1, 5; v/v). Spray reagents are described in the text. Deproteinization was obtained by adding to the sample one tenth of its volume of 100% trichloroacetic acid. After filtration, the precipitant was partly removed by extracting the filtrate five times with equal amounts of ether. 0.15–0.2 ml of the final aqueous layer was dried on the top of the paper sheet.

Cystamine and cadaverine dihydrochlorides were commercial products; *o*-aminobenzaldehyde was prepared by the method of BAMBERGER *et al.*[5].

EXPERIMENTAL AND RESULTS

The effect of change of pH on the O_2 uptake

The amount of O_2 consumed in the oxidation of cadaverine by the pig-kidney diamine-oxidase is very close to the theoretical value of 0.5 moles O_2 per mole of substrate; cystamine under the same conditions takes up more than twice this amount[1]. With the plant enzyme (Fig. 1) at pH 7.4 cadaverine also shows a higher O_2 uptake when used in 10 μmoles amount. This is in agreement with the higher value found for cadaverine at this pH by HASSE AND MAISACK[3], even in the presence of catalase. This extra consumption of O_2 has been explained by the same authors as being caused

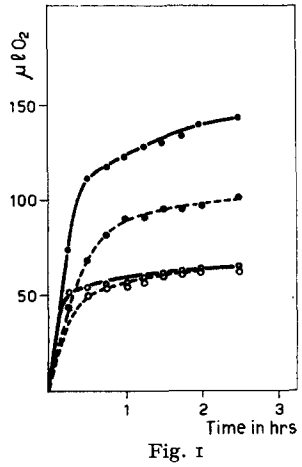


Fig. 1

Fig. 1. Oxidation of cadaverine by 20 mg diamine-oxidase. pH 7.4, ———; pH 5.6, – – – –; with 10 μmoles substrate, ●; with 5 μmoles substrate, ○. Buffer, 2 ml 0.1 *M* phosphate; temp., 25°; gas, air; final vol., 3 ml.

Fig. 2. Oxidation of cystamine by 20 mg diamine-oxidase. pH 7.4, ———; pH 5.6, – – – –; with 10 μmoles substrate, ●; with 5 μmoles substrate, ○. All other conditions as in Fig. 1.

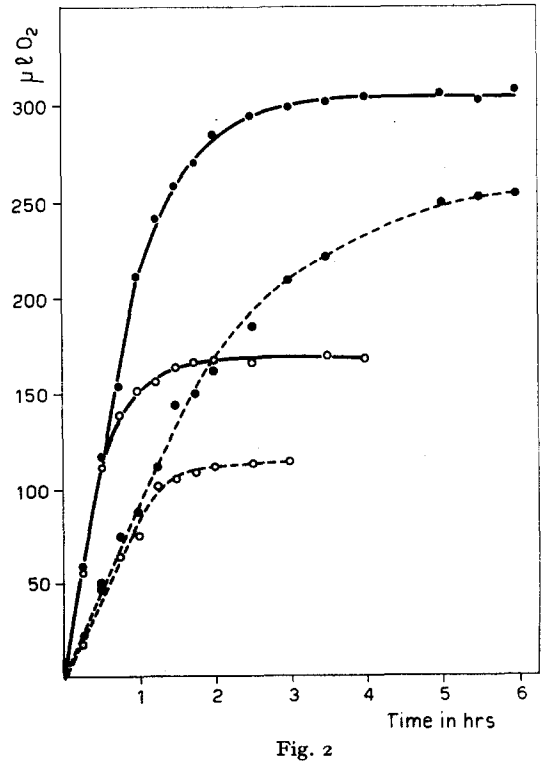


Fig. 2

References p. 358.

by a secondary oxidation of the reaction product; the secondary oxidation is eliminated by working at a low pH (pH 5.7).

As is shown in Fig. 2, the O_2 uptake of cystamine in the presence of diamine-oxidase at pH 7.4 is more than twice the theoretical value with both the concentrations of the substrate, in analogy with the higher values obtained with the animal oxidase[1]. Working at pH 5.6 the total O_2 uptake is notably depressed in the case of cystamine also, indicating an inhibition of the secondary oxidation of the reaction product. The inhibition of secondary reactions is also indicated by the lack of colour change at low pH. In contrast to cadaverine, the incubation mixtures with cystamine quickly assume a dark brown colour at pH 7.4, as was found also with animal oxidase[1]. No coloration or only a very faint colour appears when working at pH 5.6.

The effect of o-aminobenzaldehyde on the O_2 uptake

o-aminobenzaldehyde has the property of combining spontaneously and specifically with cyclized azo-methine compounds yielding a yellow or orange dihydroquinazolinium derivative[2,3,6-8]. The incubation of diamine-oxidase with cystamine in the presence of added *o*-aminobenzaldehyde resulted invariably in the production of a golden yellow colour in the place of the dark brown colour that is usually obtained at pH 7.4 without any addition. Since cystamine, alone or incubated with boiled enzyme extract, failed to give any coloration, this seems to indicate that the oxidation product of cystamine reacts with the added aminobenzaldehyde. Furthermore, the total O_2 uptake in the presence of the aminobenzaldehyde is notably reduced; it is depressed to the theoretical value of 0.5 moles O_2 per mole substrate. The theoretical value is obtained by extrapolating to zero time the second part of the oxidation curve of Fig. 3.

These results are highly significant for the production of an azomethine derivative in the case of cystamine also. Of particular interest is the depression of the total O_2 uptake induced by the aminobenzaldehyde. This indicates that the oxidation in excess of the theoretical value may be imputable to the further oxidation of the azomethine product, which in the presence of aminobenzaldehyde is trapped by the reagent and made unavailable for further oxidation. The slower oxidation which follows in the second part of the curve reported in Fig. 3, may possibly indicate a dissociation of the dihydroquinazolinium derivative. These compounds are in fact reported to be easily hydrolysable to the parent reactants[8].

Paper chromatography

Further evidence on the production of a cyclized azo-methine compound has been obtained by paper chromatography. To avoid desalting of the samples to be chromatographed, no buffer was used in these experiments: the dry enzyme preparation was dissolved in water and treated in the same way as the buffered enzyme. The pH of the reacting mixture was initially adjusted to 5.6 by careful introduction of diluted HCl. In the absence of buffer the pH did not change appreciably at the end of the experiment; the O_2 uptake was of the same order as that obtained with the buffered mixture. The low pH was preferred, with the aim of minimizing the higher oxidation and side reactions of the oxidation product.

Various samples taken at intervals from the incubation mixture of cystamine with the oxidase, were deproteinized and spotted on the top of the same paper sheet.

References p. 358.

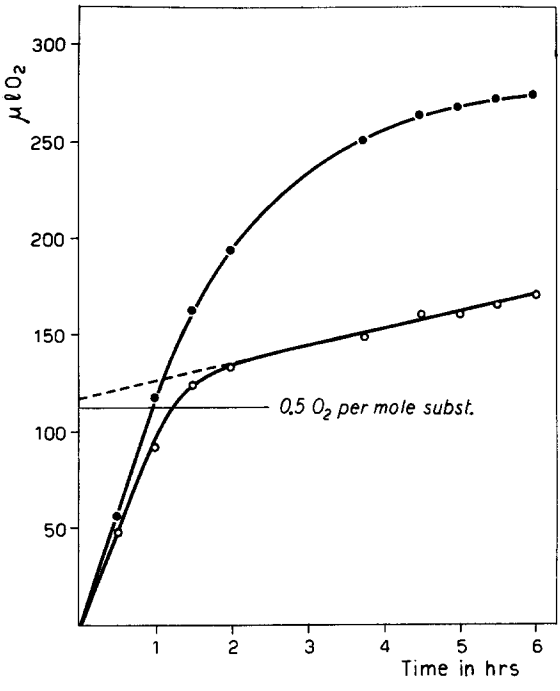


Fig. 3

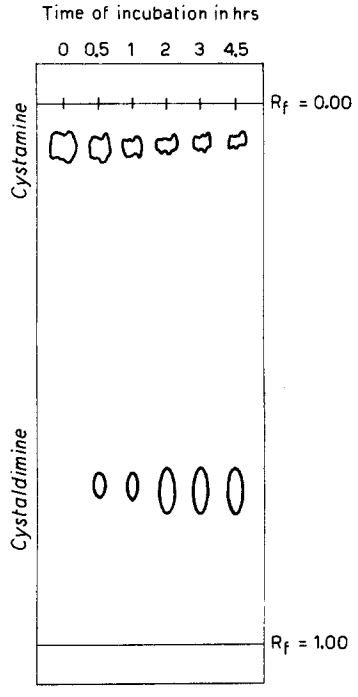


Fig. 4

Fig. 3. Oxidation of cystamine by 20 mg diamine-oxidase; in the presence (—○—○—), and inthe absence (—●—●—) of 12 μmoles of *o*-aminobenzaldehyde. Substrate, 10 μmoles; pH 7.4. All other conditions as in Fig. 1.

Fig. 4. Progressive chromatogram of the incubation mixture of 20 mg diamine-oxidase with 10 μmoles of cystamine. No buffer was used; pH initially adjusted at 5.6. Other conditions as in Fig. 1. At the indicated time, 0.15 ml of the deproteinizate was dried at R_F 0.00. Solvent, butanol-acetic acid; spray reagent, Folin-Marenzi with bisulfite.

The chromatograms were run in butanol-acetic solvent, dried, and sprayed with the Folin-Marenzi reagent containing bisulfite as used for the detection of disulfide compounds[9,10].

Fig. 4 reports the result of one of these experiments. A spot in the region of R_F 0.67 invariably appeared after 30 min incubation and increases in surface and intensity only in the two first hours of incubation. By the use of appropriate reagents this compound could be identified as the azo-methine derivative of cystamine. It gave the following reactions on the dried paper:

(a) Iodoplatinate	positive
(b) Folin-Marenzi + bisulfite	positive
(c) Folin-Marenzi without bisulfite	negative
(d) Ninhydrin	positive (weak yellow)
(e) *o*-aminobenzaldehyde (1 % in acetone)	positive (yellow)
(f) *p*-dimethylaminobenzaldehyde (2 % in pyridine)	positive (orange)

Reactions (a–c) are indicative of the presence of an —SS— group in the compound[9–11]; reactions (d–f) are indicative of the presence of a cyclized Schiff base[2,6–8,12]. Thus paper chromatography confirms the assumption that an internal Schiff base is the product of the primary oxidation of cystamine by diamine-oxidase.

References p. 358.

Isolation of the compound

Pyrroline and piperideine produced by the oxidation of putrescine and cadaverine by diamine-oxidase have been isolated by HASSE AND MAISACK[3] as the picrate of their respective dihydroquinazolinium derivative. A similar procedure has been applied to the isolation of the Schiff base produced with cystamine. In order to increase the yield we preferred the incubation of the substrate with the enzyme in the presence of a suitable amount of *o*-aminobenzaldehyde.

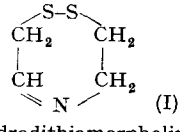
31 incubation mixtures each containing 20 mg enzyme and 10 μmoles cystamine were shaken in Warburg vessels at pH 7.4 in the presence of 12 μmoles *o*-aminobenzaldehyde until a little more than the theoretical amount of O_2 was taken up. At the end of the incubation all the liquids were pooled and the vessels carefully washed with a small amount of water. The pH was adjusted to 4.5 with acetic acid, the liquid filtered and 400 mg of picric acid were stirred in. After being left overnight at 0° the precipitate was centrifuged, washed with water, suspended in 30 ml ethanol and boiled on a water bath. The suspension was filtered, the solution concentrated in vacuum to 5–6 ml and left to crystallize at 0°. The supernatant was poured out and the residue was dissolved by boiling with 30 ml ethanol. After filtration the solution was again concentrated to 2–3 ml and left to crystallize at 0°. Crystallization from ethanol was repeated once, leaving a crystalline residue of 20 mg of red needles which melted at 155–156°. The microanalysis gave the following values: C, 44.69%; H, 3.61%; S, 13.48%. Calculated for the picrate of the dihydroquinazolinium derivative (mol. wt. = 237.35): C, 43.86; H, 3.24; S, 13.77.

DISCUSSION

The results of the present work indicate that one of the products of the oxidation of cystamine by diamine-oxidase is a typical cyclized azo-methine derivative. This finding extends to cystamine the properties found for other diamines that are oxidized by the same enzyme to the cyclized form. The compound produced with cystamine should have structure (I).

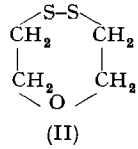
It is unknown in the chemical literature and would be correctly named 1,2-dehydro dithiamorpholine. However we propose for it the more euphonious name of *cystaldimine*.

Seven-membered disulfide-containing rings have been prepared and are therefore not merely hypothetical. DAVIS AND FETTES[13] have synthesized a cyclic disulfide of formula (II), FROMM AND JOERG[14] a disulfide of formula (III), and AFFLECK AND DOUGHERTY[15] the compound (IV).

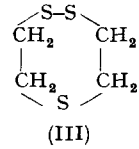


(I)

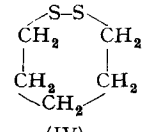
1,2-dehydrodithiamorpholine (cystaldimine)



(II)



(III)



(IV)

Moreover DAVIS AND FETTES indicated that these compounds may undergo reversible polymerization. Cystaldimine not only contains a disulfide bond, but also an azo-methine bond, which is well known to cause polymerization[6,7]. Its structure could

References p. 358.

explain the peculiar excess oxidation encountered with cystamine: indeed it is known that polymers of piperideine are easily oxidized by the enzyme, owing to the presence in the preparation of as yet unidentified cofactors[2,4].

Cystaldimine appears to be the first product of oxidation of cystamine by the oxidase. When it is not trapped by *o*-aminobenzaldehyde it undergoes further modifications as indicated by the final brown coloration of the liquid, by the excess oxidation, and by the increase of the sulfur content of the trichloroacetic acid precipitate of the enzymic preparation at the end of the oxidation of cystamine[16]. With regard to the last-mentioned effect, 64% of the sulfur initially present in the incubation mixture as cystamine is recovered bound to the proteins at the end of the incubation period. The increase in protein sulfur explains the disappearance of the —SS— groups from the trichloroacetic filtrate observed in a previous work[1]. We do not possess at present any reliable information on the kind of modifications to which cystaldimine is submitted once it is formed; neither do we understand the mechanism involved in the linkage of cystaldimine to the proteins. As a result of the present work we know that both a low pH and the presence of *o*-aminobenzaldehyde have an inhibitory effect on the secondary reactions, although they leave the production of cystaldimine unaffected.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Professor D. MAROTTA for permitting the microanalysis to be carried out at his Institute by Dr. MARZADRO, and to the Rockefeller Foundation and to the Consiglio Nazionale delle Ricerche for grants.

SUMMARY

The product of oxidation of cystamine by diamine-oxidase extracted from pea seedlings has been identified as the cyclized form of its aminoaldehyde. The name of *cystaldimine* has been proposed for this compound. Cystaldimine is the primary product of the oxidation, it undergoes further reactions as it is produced. Low pH and the presence in the reaction mixture of *o*-aminobenzaldehyde have an inhibitory effect on the secondary reactions of cystaldimine, although they leave its production unaffected.

REFERENCES

1 D. CAVALLINI, C. DE MARCO AND B. MONDOVÌ, *Experientia*, 12 (1956) 377.
2 P. J. G. MANN AND W. SMITHIES, *Biochem. J.*, 61 (1955) 89.
3 K. HASSE AND H. MAISACK, *Biochem. Z.*, 327 (1955) 296.
4 R. H. KENTEN AND P. J. G. MANN, *Biochem. J.*, 50 (1952) 360.
5 E. BAMBERGER AND E. DEMUTH, *Ber.*, 34 (1907) 1330; 60 (1927) 319.
6 C. SCHOEPF AND F. OECHLER, *Ann.*, 523 (1936) 1.
7 C. SCHOEPF, A. KOMZAK, F. BRAUN AND E. JACOBI, *Ann.*, 559 (1948) 1.
8 H. J. VOGEL AND B. D. DAVIS, *J. Am. Chem. Soc.*, 74 (1952) 109.
9 D. CAVALLINI, B. MONDOVI AND C. DE MARCO, *Biochim. Biophys. Acta*, 18 (1955) 122.
10 B. MONDOVI, G. MODIANO AND C. DE MARCO, *Giorn. biochim.*, 4 (1955) 324.
11 H. M. WINEGARD, G. TOENNIES AND R. J. BLOCK, *Science*, 108 (1948) 506.
12 J. R. S. FINCHAM, *Biochem. J.*, 53 (1953) 313.
13 F. O. DAVIS AND E. M. FETTES, *J. Am. Chem. Soc.*, 70 (1948) 2611.
14 E. FROMM AND H. JOERG, *Ber.*, 58 (1925) 304.
15 J. G. AFFLECK AND G. DOUGHERTY, *J. Org. Chem.*, 15 (1950) 865.
16 D. CAVALLINI, C. DE MARCO AND B. MONDOVÌ, (in the press).

Received December 12th, 1956