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Short Communications

The Distribution of Sulphur in Cytochrome c

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In connection with other investigations [1] we have found it necessary to reinvestigate the distribution of sulphur in cytochrome c. In a recent review [2] it was concluded that the cytochrome c molecule contains six sulphur atoms, two of which belong to methionine [3], two to the cysteine residues in the thioether bridges [4,5], and two to cystine. These conclusions were based on data obtained on cytochrome c preparations purified according to Theorell and Åkeson [6] with the use of electrophoresis at different pH levels. We have now redetermined the content of sulphur and methionine in cytochrome c prepared on ion exchangers [7,8] and tested the cytochrome c for the presence of sulfhydryl and disulphide groups.

Experimental and results. Cytochrome c preparation. Cytochrome c was prepared from beef hearts on ion exchangers according to the principles given by Paléus and Neilands [7] and Margoliash [8]. The preparation contained 0.429 % iron.

Sulphur analysis. Three different methods were used, namely those of Josephson [9], Kirsten [10] and Zimmermann [11]. The figures show that cytochrome c contains 4 atoms of sulphur per molecule. The values obtained with the methods of Kirsten and Zimmermann agree very well, while the method of Josephson seems to give too low values (Table 1).

Determination of methionine. Methionine was determined by a modification of the Baernstein method [12]. We have only determined the methyl iodide formed and not the homocysteine thiolactone, which is the other product of the digestion. The determination was carried out in a Pregl micromethoxyl apparatus according to Vieböck and Brecher [13]. The yield of methyl iodide for a pure sample of methionine was found to be 96 % and the corresponding factor was thus 1.04. When 40.6 mg of cytochrome c with the iron content of 0.429 % was digested with hydriodic acid for 12 hours 0.922 mg CH_3I was obtained, corresponding to 2.16 methionine residues per molecule cytochrome c. Cytochrome c thus contains two methionine residues in the molecule.

Sulfhydryl- and disulphide group determination. The sulfhydryl group determination in the intact cytochrome was carried out with the mercurimetric amperometric titration method as published by Kolthoff *et al.*[14]. No mercury consumption could be detected in up to 28.5 mg of preparation used (2.4×10^{-6} moles cytochrome c). As this method determines as little as 5×10^{-8} moles of cysteine, cysteine must be absent from cytochrome c. The native cytochrome and preparations hydrolyzed in 20 % HCl for up to 20 hrs were titrated in the presence of sulphite with mercury ions according to Stricks *et al.*[15]. Up to 35 mg hydrolyzed cytochrome (2.9×10^{-6} moles) was titrated but in none of the samples could mercury consumption be demonstrated. As the sensitivity in this case is about 2×10^{-7} moles cystine, less than 1/10 mole of cystine is present in 1 mole of cytochrome c.

In addition, the nitroprusside test for sulfhydryl groups was negative on the preparation used in this work. This confirms the results obtained by Theorell and Åkeson [16]. The nitroprusside test for disulphide groups [17] was also negative.

Åkeson [3] found two methionine residues and two cysteines involved in the thioether linkages to the heme prosthetic group and could thus account for the presence of four of the six S atoms/molecule. The methionine analysis in the present work only verifies the data of Åkeson. The sulphur analyses, however, have revealed only four

Table 1. Results of the sulphur analyses.

Preparation	% Sulphur	Methods	Atoms sulphur per mole cyt. c (calculated from the iron content)
0.429 % iron	0.87, 0.87	Josephson	3.52
0.429 » »	1.00, 0.95	Kirsten	3.97
0.429 » »	0.94	Zimmermann	3.81
0.409 » » *	0.98	»	4.17

* This preparation was kindly provided by Drs H. Tuppy and G. Bodo, II. chem. Laboratorium der Universität, Wien.

sulphur atoms/molecule, and we have therefore been able to account for all the sulphur occurring in this preparation of cytochrome c.

The discrepancy of the present work with the results of Carruthers [18] is difficult to explain. Using electrophoretically purified cytochrome c with an iron content of 0.411 % he found a catalytic double wave in an ammoniacal solution of hexamine cobalt ions, which he attributed to cysteine or cystine in the cytochrome. There is a possibility that the cobalt ions present in the experiments of Carruthers might split the thioether bond of cytochrome c in the same way as silver ions [19], the double catalytic wave thus being caused by the two heme-linked cysteine residues.

Paul [20] dissociated a specially purified preparation of cytochrome c (iron content = 0.424 %) with silver and found that the protein moiety combined with 3.5—3.6 atoms of silver, probably due to two thiol groups (derived from the thioether bridges) and one disulphide group.

There is still the difference of two sulphur atoms between the preparation of Theorell and Åkeson [16] and the preparation of the present work to be explained. Perhaps that difference might be due to the preparation method used in each case. Further investigations of this difference would be of great interest.

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