

The Splitting with Silver Salts of the Cysteine-porphyrin Bonds in Cytochrome c

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When acid acetone is added to hemoglobin, myoglobin, catalases, horse radish peroxidase, cytochrome a and b, the prosthetic group is released from the protein part, which precipitates, while the prosthetic group remains in the dissolved state. In the cases of hemoglobin¹ and horse radish peroxidase¹ this fact has been used for studies of the linkages between protein and prosthetic group. If the same treatment be applied to cytochrome c the whole molecule is precipitated without fission, and thus its porphyrin-protein linkages are in this respect more stable. The thio-ether bridges from the side chains 2 and 4 of the porphyrin skeleton to the protein² are very likely not affected by acid acetone. Starting with the assumption that, if these linkages could be broken, it should be possible to split cytochrome c into free protein and prosthetic group and to open possibilities to carry out further studies on the heme-linked groups, we have been searching for a tool for that splitting.

Holmberg³ used mercuric chloride to release thioglycolic acid from S-methylthioglycolic acid. Because of the oxidizing power of the mercuric ions, the acid deteriorated. Peters and Wakelin⁴ reported the splitting of thioethers with silver salts under mild conditions.

In preliminary experiments, we found the ability to break the cysteine-porphyrin bonds in cytochrome c to be common to several metals. We tried silver, mercurous, mercuric, lead, cupric, cadmium, ferric, calcium, and magnesium salts, all of which except the three last mentioned liberated the prosthetic group so that it was extractable with ether in the presence of sufficient quantities of acetic acid or remained in the liquid phase upon the addition of an excess of acid acetone. We have used silver salts to avoid undesirable oxidation and double salt formation.

After splitting of cytochrome *c* at faintly acid reaction the acetone precipitated protein was soluble in water to give a slightly straw-coloured, clear solution of pH 4—5. An increase in pH to about 7 gave a slight opalescence, which grew to a thick precipitate at pH 9, the precipitation being reversed by acidification. If the excess of silver had been previously removed by dialysis, the precipitate formed at pH 9 redissolved at higher pH. The nitroprusside reaction was negative at room temperature and alkaline reaction, but turned to be positive immediately upon the addition of cyanide.

Since the reaction is carried out in an acid medium, the cytochrome *c*, completely oxidized, shows the absorption band at 610—640 $m\mu$. This band does not alter during the reaction. After the addition of acid acetone and centrifugation, the acetone solution of the iron-porphyrin shows a band of the »hemin» type with a maximum at 625 $m\mu$. The iron-porphyrin from cytochrome *c* shows the same solubility properties as most other hemins. Of a special interest is, however, its high solubility in alcohols, decreasing with increasing number of carbon atoms in the alcohols up to *n*-octanol (no higher alcohols tried). The iron-porphyrin could be crystallized from aqueous *n*-butanol and glacial acetic acid. During the isotope investigations at this institute to be reported elsewhere, use was made of this high solubility, which permits a solution of the hemin from cytochrome *c* in *n*-butanol to be washed with 5 % hydrochloric acid or water for the removal of inorganic and ferritin iron.

The velocity of the splitting has been studied under some different conditions. In the first experiments we used silver nitrate, but to avoid oxidation due to the nitric acid the sulfate was preferred for the final modification. In all cases the progress of the splitting, measured in terms of ether-acetic acid extractable porphyrin bound iron or light absorption of the acetone solution, was found to follow closely curves for first — order reactions. The velocity constants were obtained by plotting $\log a/(a-x)$ versus time and the slopes of the straight lines determined with the method of least squares, the velocity constants then being equal to $2.303 \times \frac{d \log a/(a-x)}{dt}$, where a is the total reacting amount and x the amount reacting in the time t .

The results are shown in Figs. 1—3, and details are given in the experimental part. The indices of k refer to the number of the experiment as given in the experimental part.

It is outside the scope of this paper to interpret kinetically these results and elucidate the reaction mechanism. The binding of silver ions by carboxyl groups and by enolized peptide bonds may be negligible at these pH values⁵,

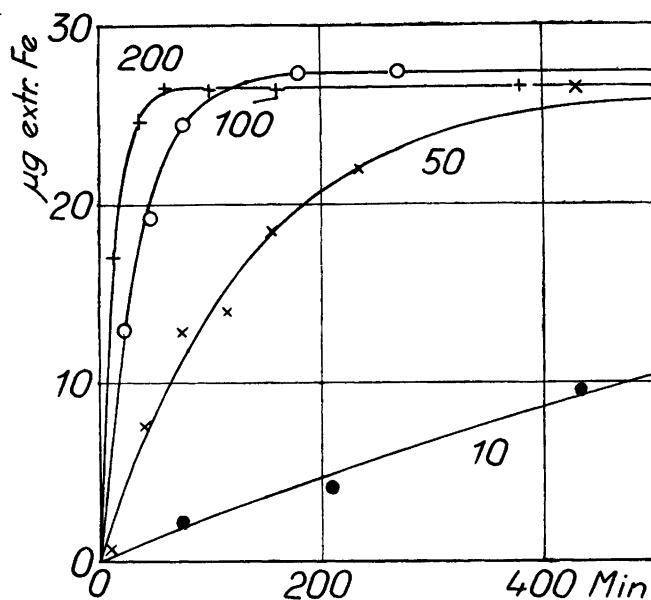


Fig. 1. Influence of ratio silver salt/cytochrome on the reaction velocity.
 $k_1 = 7.68 \times 10^{-2} \text{ min}^{-1}$. $k_2 = 3.08 \times 10^{-2} \text{ min}^{-1}$. $k_3 = 7.53 \times 10^{-3} \text{ min}^{-1}$.
 $k_4 = 9.6 \times 10^{-4} \text{ min}^{-1}$.

but the behaviour of the four residual sulphur atoms in cytochrome c, not linked to the porphyrin, is still unknown. In experiments 2, 5, and 6 the progress was followed simultaneously to the iron determinations by diluting samples with the buffers used in the experiments and determining the positions and optical densities of the Soret bands. The velocity constants obtained in this way were about 50 % higher than those from the iron determinations. In experiments 5 and 6 the top of the band migrated from 408 to 391—92 μ , whereas it in experiment 2 was consistently found at 391 μ .

We have not determined the rate of disappearance of silver ions from the solutions.

From experiments 2 and 7 the heat of activation was calculated according to the Arrhenius formula as 19 700 cal./mole, from experiments 8 and 9 as 19 700 cal./mole, and from experiments 9 and 10 as 16 400 cal./mole.

EXPERIMENTAL PART

Two different preparations of cow heart cytochrome c were used for the experiments reported in this paper, both of which had been obtained according to Keilin and Hartree⁶, and both of purity 0.95 (0.41 % iron).

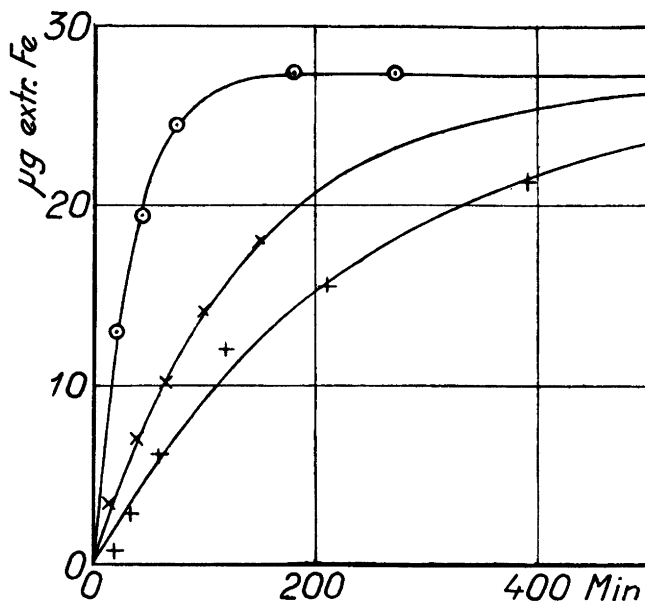


Fig. 2. Influence of acidity on the reaction velocity.

+ × ○ refer to expts. 5, 6, and 2 respectively.

$k_5 = 4.08 \times 10^{-3} \text{ min}^{-1}$. $k_6 = 7.35 \times 10^{-3} \text{ min}^{-1}$.

$k_2 = 3.08 \times 10^{-2} \text{ min}^{-1}$.

Influence of the ratio silver salt/cytochrome on the reaction velocity (Fig. 1.)

Expt. 1. 3.92 ml $1.37 \times 10^{-3} M$ cytochrome c + 1.074 ml 1.00 M silver nitrate + 5.00 ml glacial acetic acid (200 moles of silver nitrate/mole cytochrome). Temp. + 40° C. After certain times samples of 1.00 ml were withdrawn and extracted with 3×3 ml ether-glacial acetic acid 3 : 1, the combined extracts were then analyzed for iron. The splitting was carried out in a 40 ml closed vessel, protected against light.

Expt. 2. Cytochrome and glacial acetic acid as above, but with 0.536 ml silver nitrate + 0.54 ml distilled water. Extraction was carried out as above. Silver nitrate/cytochrome = 100.

Expt. 3. As above, but 0.268 ml silver nitrate + 0.81 ml distilled water. Silver nitrate / cytochrome = 50.

Expt. 4. As above, but 0.054 ml silver nitrate + 1.03 ml distilled water. Silver nitrate / cytochrome = 10.

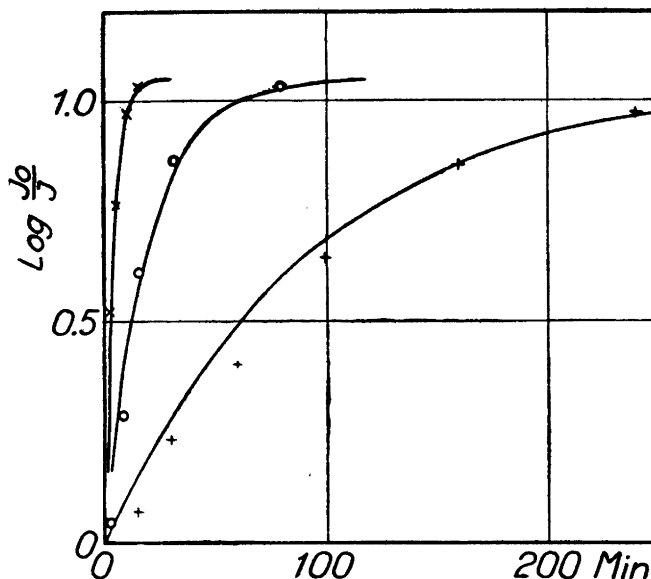


Fig. 3. Influence of temperature on the reaction velocity.

$k_8 = 2.79 \times 10^{-1} \text{ min}^{-1}$ (30°C , \times). $k_9 = 5.16 \times 10^{-2} \text{ min}^{-1}$ (60°C , \circ).
 $k_{10} = 1.06 \times 10^{-2} \text{ min}^{-1}$ (40°C , $+$).

Influence of acidity on the reaction velocity (Fig. 2)

Expt. 5. 3.92 ml $1.37 \times 10^{-3} M$ cytochrome + 0.536 ml 1.00 M silver nitrate + 0.54 ml distilled water + 5.00 ml acetate buffer of pH 4.6, ionic strength 0.1.

Expt. 6. As above, but with acetate buffer of pH 4.0, ionic strength 0.1. Compare experiment 2.

Influence of temperature on the reaction velocity

Expt. 7. As expt. 2, but temperature $+20^\circ \text{C}$. $k_7 = 3.52 \times 10^{-3} \text{ min}^{-1}$
 Compare experiment 2.

From these experiments we decided to use the following mixture for the standardised procedure. To 1 ml of the cytochrome solution should be added 0.2 ml glacial acetic acid and 1 ml of the silver salt solution. As mentioned above, the sulfate was substituted for the nitrate. Our silver sulfate solution contained 800 mg/100 ml ($M = 2.57 \times 10^{-2}$).

Some experiments on the influence of heat with the standardised reaction mixture and another preparation of cytochrome were then made. In 10 ml centrifuge tubes were taken 0.50 ml 9.09×10^{-4} M cytochrome solution, 0.10 ml glacial acetic acid, and 0.50 ml silver sulfate solution. The tubes were closed, immersed in a thermostat, removed after different times, cooled in tap water, and the contents precipitated with 10 ml acid acetone (10 ml 5 N sulfuric acid to 500 ml acetone). The protein residues were washed with 2 ml acid acetone. The light absorption at the wavelength for maximal absorption in the Soret band region (determined in each case and consistently found to be 392 m μ) of the combined extracts was measured after their dilution to 75 ml with acid acetone (Fig. 3).

In the following papers we will describe the analyses of the split products and a micromethod for the determination of cytochrome in tissues.

SUMMARY

In confirmation of the thio-ether configuration of cytochrome c we have found, that salts of some heavy metals release the prosthetic group from the protein part, so that the former can be extracted with ether-acetic acid or remains in the dissolved state upon the addition of an excess of acid acetone. Velocity constants for some given reaction conditions have been determined. This splitting seems to be useful in experiments, where the iron-porphyrin of cytochrome c has to be isolated, *e. g.* for isotope work.

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