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The Stability of Cytochrome c at Extreme pH Values

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In an earlier publication[1] the author has reported an investigation on the oxidation-reduction potential of cytochrome c* in the region pH 4—9. In that paper the existency of two new dissociation constants was shown, one in oxidized c at pH 6.86, and one in reduced c at pH 9.28. From earlier investigations [2] on c is known, however, that some further dissociations occur outside this region, and it would be of interest to confirm these constants by recording the curve E_0' *versus* pH over the complete pH scale. For this reason it was necessary to know the stability of c at different pH values. Generally it takes six hours for a platinum electrode to reach a stable potential in a solution of a partly reduced hemin proteid if no accelerating mediator is present. The aim of this paper is therefore to determine the highest acid and lowest alkaline pH value where no inactivation has taken place after six hours.

c, which has been exposed to pH < 1 or > 12 can not with certainty be distinguished spectroscopically from non-exposed c. A slight shift to the red in the absorption spectrum of reduced, neutralized c is sometimes seen after treatment with strong alkali [3], but never after exposure to even relatively high concentrations of acids [2]. For that reason we considered it necessary to follow the destruction of the enzyme, if it does take place, by measuring its activity in an enzyme system. Not very much has been done in this field, as most investigators have followed the fate of c by the changes in its light absorption. Keilin and Hartree [3], however, using an enzymic method, state that c can stand treatment with 0.3 *M* hydrochloric acid for 18 hours at room temperature without loss of activity and 0.1 *M* potassium hydroxide for 18 hours at the same temperature with a loss of 50 % of the activity.

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* For the sake of brevity we will call cytochrome c only c in this paper.

METHODS AND PREPARATIONS

The stability experiments were arranged as follows. To a small sample of c in a test tube was added hydrochloric acid or potassium hydroxide to a desired concentration, the tube closed and placed in a waterbath at 20° C. Small samples were withdrawn at certain time intervals and neutralized with potassium hydroxide or hydrochloric acid. The activities of these samples were compared manometrically with the activities of corresponding amounts of untreated c.

We have chosen the succinic acid dehydrogenating system for the determinations of the catalytic activities of the c samples. If the other enzymes in this chain are present in excess of c, the oxygen consumption should vary only with the concentration of active c. We found kidney cortex to be the best source for these enzymes. There are two reasons which support this choice: 1:o Kidney cortex in itself has a low content of c [3], and 2:o. c seems to be easier to remove from this tissue than from for instance heart muscle. The kidney cortex preparations were obtained according to a slight modification of the method of Keilin and Hartree [3]. Slices of about three mm. thickness of pig kidney cortex were ground in a common meatmill and then washed several times with tap water. The residue was homogenized in a »Turmix» together with some crushed ice. The following part of the preparation was carried out at + 5° C. To follow the removal of c from the tissues we washed the homogenate with acetic acid — water mixtures at different pH values. After each acid addition pH was determined with a glass electrode, the suspension centrifuged down (10 min at 3500 r. p. m.) and supernatant and residue investigated spectroscopically, the residue then being used for the washing at the next pH. Parts of the residue were withdrawn immediately after each washing, suspended in *M*/15 phosphate buffer of pH 7.3, and subjected to activity measurements. If the washing with tap water had been very careful, *i. e.* for several hours with running water, nothing unusual was observed. The residues showed after reduction bands at 550—565 mμ and 602—605 mμ, and so did the turbid supernatants but weaker. In the supernatants the band at 550—565 mμ was slightly strengthened at 550 mμ, indicating that the supernatant was enrichened in c in relation to the residue. If, however, the washing with tap water not had been so intensive, some interesting observations could be made. These are collected in Table 1. From Table 1 some conclusions can be drawn.

1. No oxyhemoglobin bands were visible in the insoluble residue after washing at pH 5.2, but the red supernatant after washing the tissue at pH 5.05 obviously contained considerable quantities of what proved to be hemo-

Table 1. Spectroscopical investigation of homogenized kidney cortex (see text). »Dir.» means observation before and »Red.» after addition of dithionite.

pH after add. of acetic acid	Supernatant	Insoluble residue
6.0	Dir. HbO_2-bands Red. Faint band 550—560.	Dir. No bands Red. Faint band 550—560.
5.2	Faintly yellowish-brown. Dir. Faint HbO_2-bands. Red. Band 550—560, reinforcement at 550.	Dir. No bands. Red. Bands 550—565, no reinf. at 550. Faint band at 605.
5.05	Sharply red. clear. Dir. Strong HbO_2-bands. Red. Band 545—565, reinforcement at 550.	Dir. No bands. Red. Band at 550—565, no reinforcement at 550. Strong band 602—605.
4.8	Yellowish-brown like supernatant after pH 5.2. Dir. Faint HbO_2-bands. Red. Faint band at 550.	Dir. No bands. Red. Band at 550—565, no reinforcement at 550. Strong band at 602—605.

globin, since the bands of its CO-compound agreed with those of (human) carbon monoxide hemoglobin. The bands of this oxygenated hemoglobin disappeared upon reduction and were replaced by a broad band at 550—565 mμ.

2. Neither residue showed the c band at 550 mμ after reduction, but in all supernatants this band was found. The absorption spectrum of extracted c from kidney cortex could not be distinguished spectroscopically from that of heart muscle c.

3. In spite of the fact that we never obtained preparations which were free of a blank determination (succinate but not c added), we believe that this residual oxygen uptake in the blanks was due to the presence of traces of c in the homogenates, since the supernatants from pH 4.8 contained c. Obviously c can not be removed completely at a pH where the dehydrogenating system is not completely destroyed. One might call this c »occult», as it can not be seen spectroscopically in the tissue.

4. If residue from pH 5.2 were suspended in phosphate buffer as mentioned above to give a dry weight of about 25 mg/ml and 20 μg of heart muscle c were added to one ml of this suspension, the band at 550 mμ was easily visible after reduction.

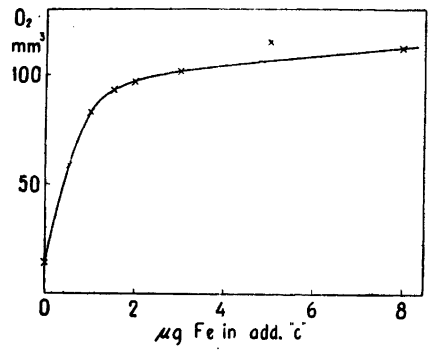

Fig. 1. Calibration curve for kidney cortex preparation number P 11/2. Dry weight 8.1 mg/ml.

With regard to these observations we consider it to be dangerous to decide from spectroscopical data on tissues about the absence of hemin proteids in them or about the spectra and natures of these proteids. Thus we doubt the correctness of stating *from spectroscopy* that kidney cortex and heart muscle c are non-identical. A comparison of washed cow heart muscle and washed kidney cortex confirmed the observation made by Keilin and Hartree [3] that the two distinct bands at 550 and 565 mμ in heart muscle are replaced by one broad band at 550—565 mμ in kidney cortex. If, however, c was extracted from these two organs, we could not spectroscopically find any difference between them. In any case further investigations of this question are required. Bonnichsen found catalases from different organs of one animal to be identical [4].

When the pH of the washings was decreased, the activities progressively declined. Preparations washed at pH 4.8 had almost no activity. Therefore we used washing at pH 5.2 as a standard procedure, since at that pH the majority of c was removed without complete inactivation of the other enzymes in the succinodehydrogenase system. The residue was washed once with distilled water and then suspended in phosphate buffer (*M*/15, pH 7.3). The preparations were stable for 2 days. Before they were used for activity measurements, they were tested with varied amounts of c in order to find the most linear part of the oxygen uptake *versus* added c. A typical curve is shown in Fig. 1.

Dry weights were determined after removal of the buffer salts by dialysis.

The preparations of c which were used for the stability experiments had been prepared according to Keilin and Hartree [5]. This method is a very convenient one even for large scale preparations, but we have never succeeded in reaching an iron content of more than 0.40—0.41 %. The impurities do not contain iron, since the total iron content always agrees with the light absorption values. One electrophoresis at pH 10.5 brings up the iron content to 0.43 %. Other authors had the same experience of the degree of purity

after the ammonia-ammonium sulphate treatment [6]. It may be mentioned here, that when a modification of the usual method of Keilin and Hartree was used (addition of 750 instead of 550 grams of ammonium sulphate per liter of the crude, neutralized trichloroacetic acid extract) an iron content of 0.36—0.37 % was obtained instead of the usual 0.34 %.

A Beckman spectrophotometer type DU, the wavelength accuracy of which is ± 1 mμ, was used for the light absorption measurements.

pH was determined with a hydrogen gas electrode (except in the preparation of homogenate).

The determinations of oxygen uptakes were made in a Warburg apparatus with constant volume manometers. The oxygen uptakes were read after shaking for 30 min at 37° C with a frequency of 78 oscillations per minute. The vessels were filled with: Well: 0.20 ml 2 *M* KOH. Main chamber: 2.00 ml *M*/15 phosphate buffer of pH 7.3 + 0.50 ml kidney preparation + c + water to 3.15 ml. Side bulb: 0.30 ml 10 % sodium succinate.

Iron was determined in the following way, the principle features of which derive from Lorber [8], except the combustion technique *. A sample, containing 5—70 μg iron was boiled in a Pyrex test tube over a free gas flame with 1.0 ml sulphuric acid (sp. g. 1.84) and three drops of 30 % hydrogen peroxide until the color disappeared and reappeared. This generally took 2—5 min. Then the boiling was continued with successive addition of hydrogen peroxide at a rate of one drop every minute until the solution remained colorless. The boiling must be continued ten minutes after the last addition of hydrogen peroxide. This combustion method has been in use in this institute for the last year [1,4], and we have found it to be by far the most reliable and convenient method for iron analyses in hemin proteids. The solution was then cooled in ice water, diluted with a few ml. of water and neutralized with 35 % ammonia. 0.30 ml of 20 % sulphosalicylic acid and more ammonia were added to a yellow color. The solution was then transferred to a 15 ml volumetric flask, diluted with water to the mark and measured in the spectrophotometer at 424 mμ. 1.00 μg iron per ml per 1 cm optical depth gives a density of $\log I_0/I = 0.103$.

RESULTS

It was found that c, treated with very dilute acid or base, accelerated the oxygen uptake more than a corresponding amount of non-treated c did. Even in the experiments with stronger solutions an increased activity could be traced (see discussion). For that reason numerous experiments were made on the influence of addition of potassium chloride to untreated c upon the oxygen uptake (Fig. 2), but in no case we could find that the salt influenced in those concentrations it actually had in the manometer vessels with treated c. At a concentration above 0.01 *M* the respiration was inhibited. The same inhibition was caused by sodium chloride and potassium sulphate at the same concentrations (the concentration of the latter calculated in terms of the potassium ion). Potter [8] observed inhibition by some neutral salts in 0.1 *M* solu-

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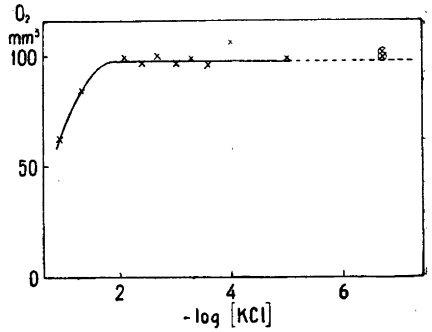

Fig. 2. Influence of KCl upon oxygen uptake. To the main chamber had been added c corresponding to 1.0 µg iron and KCl to give the abscissa concentrations. Three times more of c did not change the shape of the curve, nor that -log[KCl] where inhibition started. Crossed circles: No addition of KCl (place on abscissa quite arbitrarily chosen).

tions. The physiological concentration of total potassium in cells is of that order of magnitude where inhibition just began in our experiments. It must be kept in mind, however, that under the experimental conditions used by us, the contents of the vessels presumably were poorer in c than the intact tissue is, and that a part of the succinate dehydrogenating activity was destroyed during the preparation. In some stability experiments, where the concentration of potassium chloride would have exceeded 0.01 *M* in the manometer vessels, the neutralized sample was dialyzed against distilled water until the chloride reaction became negative.

In some experiments the light absorption was recorded parallel with the activity.

A typical experiment with alkali was carried out as follows. 50.0 ml of 3.32×10^{-4} *M* c and 0.676 ml 6.00 *M* KOH were mixed to give pH 12.77 (0.08 *M* KOH). Samples of 10.0 ml were withdrawn at the time intervals listed in Table 2 and neutralized with 0.133 ml 6.00 *M* HCl. 0.0387 ml of each neutralized sample were used for activity determinations, corresponding to 0.7 µg iron, and compared with 0.0377 ml untreated c (Table 2). Of the neutralized sample 0.30 ml were diluted with 3.50 ml *M*/15 phosphate buffer pH 6.8 for the determinations of the densities of some characteristic bands (Table 2). (Some experiments, not belonging to this investigation were made with the samples, which explains the large volumes used.)

The following is an example of acid treatment of c. 8.00 ml 1.09×10^{-4} *M* c were acidified with 1.14 ml 4.00 *M* HCl to give pH 0.66. After the hours listed in Table 3 samples of 2.00 ml were taken and neutralized with 0.25 ml 4.00 *M* KOH. When all samples had been collected, they were dialyzed against distilled water as was some untreated c. During this procedure, some c passed through the cellophane membranes and some was adsorbed to it, so the concentrations had to be measured by iron analyses. The testing of the kidney preparation was carried out in the same experiment. For the light absorption

Table 2. Changes of activity and light absorption upon treatment with 0.08 M KOH. In the last vessel KCl was added to bring the concentration of the salt to the same value as in the vessels with treated »c». Light absorption determined after reduction. Dry weight of kidney preparation 8.1 mg/ml.

Time for treatm. with 0.08 *M* KOH	Blank	Untreat.	50 min	8 h	20 h	28 h	Untreat. + KCl
Oxygen uptake	58	146	145	126	95.5	76.5	132.5
in μl	—	133.5	148	126.5	101.5	83.5	—
Average	58	140	146.5	126.5	98.5	80	132.5
% remaining activity	—	100	108	83.5	49.5	27	91
Wavelength in mμ for max. of light abs. and dens. at that wavelength		414 1.65 520 0.195 549 0.341	416 1.60 519 0.194 549 0.336	414 1.51 520 0.181 549 0.310	416 1.53 520 0.179 549 0.296	416 1.51 520 0.179 549 0.288	

measurements 0.50 ml of the samples were taken before the dialysis and diluted to 4.00 ml with buffer (Table 3). The results of alkali treatment are given in Fig. 3 and those of acid treatment in Fig. 4. For calculating »per cent remain-

Table 3. Influence of treatment with 0.50 M HCl upon activity and light absorption of cytochrome c. Dry weight of kidney preparation 17.1 mg/ml. The wavelength of the maximal absorption of α-band of the reduced cytochrome was consistently found to be 550 mμ.

Time for treatm. with 0.50 *M* HCl	Blank	Untreated			3 h	6 h	12 h	24 h
μg iron in »c» used to act.detn.	—	0.3	0.7	1.0	0.7	0.7	0.7	0.7
Oxygen uptake in μl	32.5	144.5	250	320	239	226	196.5	163
% activity	—		100		95	89	75.5	60
Density of α-band of red. cytochrome	—		0.325		0.325	0.322	0.323	0.324

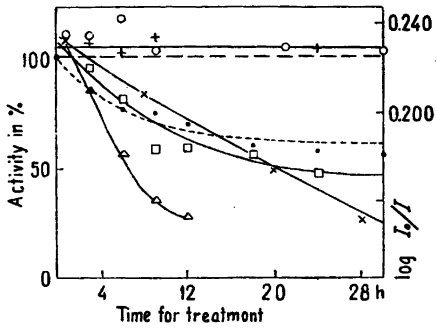

Fig. 3. Stability of »c» at high pH values. ○ 0.01 M KOH (pH 10.81). + 0.03 M KOH (pH 12. 30). □ 0.05 M KOH (pH 12.67). × 0.08 M KOH (pH 12.77). △ 0.10 M KOH (pH 13.04). ● values for log I_0/I at the top of the α-band of reduced »c» (treatment with 0.05 M KOH). ----- curve for a first order reaction with initial concentration corresponding to a density of log $I_0/I = 0.225$, and concentration at infinity corresponding to log $I_0/I = 0.185$, and with $\frac{1}{t} \ln \frac{a}{a-x} = 0.143$. — — »100 % activity». The upper curve is drawn from experiments at both pH 10.81 and pH 12.30.

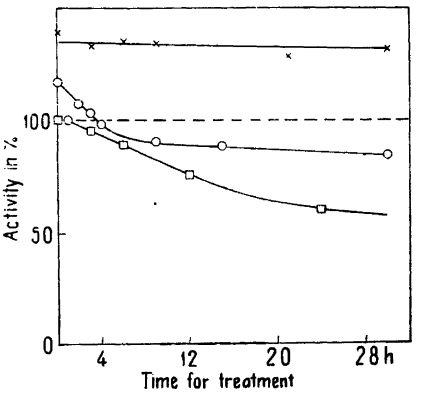

Fig. 4. Stability of »c» at low pH values. × 0.05 M HCl (pH 1.63). ○ 0.10 M HCl (pH 1.25). □ 0.50 M HCl (pH 0.66). — — — — »100 % activity».

ing activity», the oxygen uptake of the blank was subtracted from the values for treated and untreated c, and from the ratio between the two rests was obtained »per cent activity». This is of course not a strictly correct method, since there is no perfect linear relationship between oxygen consumption and amount of c in the vessels, but we believe the errors to be not very large.

DISCUSSION

Figs. 3 and 4 show, that if c is inactivated at a certain pH, this inactivation starts immediately. Therefore, we believe it to be correct to judge from the slopes of the curves »per cent activity» *versus* time, whether c has been stable or not at a given pH. Thus it is seen from Figs. 3 and 4 that c is destroyed at pH 13.04, 12.77, 12.67, 1.25 and 0.66. At pH 1.63 c is just at the lower limit of the stable region, but perfectly stable at pH 12.30.

It is difficult to say how the increase in activity, observed after mild treatment, should be explained. Barron and Kalnitsky[9] recently described the inhibition of succinate dehydrogenase by some heavy metals. However,

we do not believe that the increase in activity observed by us can be explained by the assumption that traces of some heavy metals present as an impurity in our c preparations could be removed by for instance a formation of some complex ion, since the activation occurred at both acidic and alkaline pH, was not reversible and was observed with several different c preparations. Keilin and Hartree [11] criticizing the work of Haas [12, 13] on cytochrome oxidase, have recently showed that proteins exert an unspecifically stimulating effect upon the enzyme system. In our experiments this can hardly be the explanation, since that would mean that destroyed c should be more active than native, and moreover comes that the c, destroyed or not, is quite vanishing regarded upon as a protein in comparison to the masses of inert protein in our kidney preparations (5—10 mg per vessel). The more or less pronounced sigmoid form of the inactivation curves in comparison to the exponential curves for light absorption decrease may be explained by the assumption that the former are composed of two different curves: One curve for inactivation, and one for increasing activity of the remaining, not destroyed c. We must admit, however, that we have not tried to penetrate this problem deeply.

SUMMARY

The stability of cytochrome c has been determined by measuring the activity of samples, which have been kept in hydrochloric acid or potassium hydroxide of various concentrations for various times. Cytochrome c has been found to be perfectly stable from pH 1.6 to pH 12.3.

The density of the absorption band mostly used for concentration determinations of cytochrome c, the α-band, has been followed parallel with the activity. Decrease in activity due to treatment with the acid was not accompanied by a decrease of the band, but during alkali treatment the changes in density and in activity run roughly parallel with each other. In neither case the shape of the spectrum was altered.

Observations made on a homogenate of kidney cortex prove that one can not with certainty judge about the presence or absence of hemin proteids in tissues only from spectroscopical data.

An appearance of a higher activity per μg cytochrome c after its treatment with acid or base has been noted and briefly discussed.

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