on whether globin is dissociated from the haem at the pH of the original extraction. Thus in the usual Keilin & Hartree preparation of cytochrome c (pH of extraction 4·5) there are no more than traces of myoglobin, whereas if the extraction of the heart mince is carried out at neutral pH, as with 0·5 M ammonium acetate, most of the non-cytochrome fraction consists of myoglobin.

SUMMARY

1. A simple and reliable procedure using cation exchangers to prepare 0·43 % iron cytochrome c from the 0·34 % iron preparation from horse heart is described. The product is as active enzymically (per μmole of cytochrome c iron) as the preparation containing 0·34 % iron.

2. An application of this technique is made to the preparation of cytochrome c in either the reduced or oxidized state, in a stable form. This enables the preparation of solutions of reduced or oxidized cytochrome c in any desired concentration, whenever required and entirely free from reducing or oxidizing agents and their reaction products. After storage the dried products are enzymically fully active.

3. The non-cytochrome fraction of the 0·34 % iron cytochrome c preparations is identified as globin from myoglobin. Under suitable conditions cytochrome c can react with myoglobin or the globin prepared from it.

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The Chromatographic Behaviour of Cytochrome c on Cation Exchangers

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Paléus & Neilands (1950) first chromatographed an ox-heart cytochrome c preparation containing 0·34 % of iron on Amberlite IRC-50 (a synthetic, polycarboxylic cation exchanger). They obtained three fractions: the first was reduced cytochrome c, containing 0·401 % iron, the second oxidized cytochrome c, containing 0·466 % iron, while the third contained 0·353 % iron.

Boardman & Partridge (1953) studied quantitatively the effect of the cation concentration and pH on the chromatography of cytochrome c on Amberlite IRC-50. The cytochrome c could not be eluted below pH 6, the elution volume decreased rapidly from pH 6 to 7, remained stationary from pH 7 to 8, then decreased again to pH 10. This behaviour was explained by the summation of two forces affecting the protein—resin interaction, short-range forces which bind the cytochrome c irreversibly below pH 6, and the normal, electrostatic attraction depending both on the ionization of the resin, which is depressed as the pH decreases, and the ionization of the protein, which is depressed as the pH increases. At pH 7 cytochrome c could be eluted using univalent cations at concentrations above 0·22 g. ions/l.

In the course of work on the use of cation exchangers for the concentration and further purification of cytochrome c (Margoliash, 1952, 1954), it

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was found necessary to study the chromatographic behaviour of the protein, as well as the catalytic properties of the various fractions separated. This paper presents the results of this work.

MATERIALS AND METHODS

Amberlite IRC-50 (Rohm and Haas Co., Philadelphia, U.S.A.) as the ammonium salt, the heart-muscle preparation, the kidney preparation and the 0·34% iron cytochrome c were prepared as described in the previous paper (Margoliash, 1954).

The activity of cytochrome c fractions in the succinic-oxidase and cytochrome-oxidase systems, the total cytochrome c concentration and the percentage of reduced cytochrome c as well as the dry weight and the iron content of the various fractions were determined as given in the previous paper (Margoliash, 1954).

Ascorbic acid oxidation by cytochrome c preparations was measured in the test mixture used in the determination of the cytochrome c activity in the cytochrome-oxidase system, with ascorbic acid, but in the absence of the cytochrome-oxidase preparation. The pH was 7·3, and the final concentration of phosphate buffer was 0·066 M. All flasks contained 1×10^{-3} M ethylenediaminetetraacetic acid. A simultaneous blank determination was always carried out with ascorbic acid in the absence of cytochrome c, and the resulting measure of the rate of auto-oxidation of the ascorbic acid was subtracted from the values obtained with cytochrome c, to give the rate of oxidation of ascorbic acid by the cytochrome c preparation.

It should be emphasized that this is not equivalent to a measure of the rate of auto-oxidation of the cytochrome c but represents an oxidation of ascorbic acid catalysed by a small amount of an impurity in the preparation (see below, also Tsou, 1951).

RESULTS

Chromatography at pH 7

The effect of the cation concentration and the pH of the eluting solution on the movement of horse-heart cytochrome c adsorbed on a column of Amberlite IRC-50 was found to be the same as that described by Boardman & Partridge (1953). The elution fluids usually used in these experiments were: ammonium acetate + acetic acid buffers below pH 7, ammonium acetate at neutrality and ammonium acetate + ammonia buffers, as used by Paléus & Neilands (1950) above pH 7. Similar results were obtained using solutions of Na^+ or K^+ salts, with the appropriate salt of the resin (Boardman & Partridge, 1953).

Using 0·25 M ammonium acetate as elution fluid, chromatography of a 0·34% iron cytochrome c preparation, at pH 7, gave three fractions. First, a forerunning, non-cytochrome fraction which came off very soon after the beginning of elution, probably at the fluid front. This fraction corresponded to the non-adsorbed fraction when a 0·34% iron preparation is adsorbed on the resin (see previous paper, Margoliash, 1954). Secondly, *Fraction I* pH 7, a cytochrome fraction which contained from 75 to 90% of the total amount of cytochrome c put on the column, and came off in a fairly wide band. Thirdly, *Fraction II* pH 7, which was the remaining 10–25% of the cytochrome c which did not appear to move at all with the elution fluid containing 0·25 g. ions/l., and which was eluted as a single band with 0·5 N ammonium hydroxide.

Fig. 1 records a typical chromatogram at pH 7.

Chromatography at pH 9·6

When fraction I pH 7 was dialysed against distilled water until salt free and was rechromatographed at pH 9·6 (0·25 M buffer) it separated into three fractions. First, a very small, forerunning, non-cytochrome fraction which showed under the spectroscope the same compounds as the previous non-cytochrome fraction, except that there was no modified cytochrome c. It came off very soon after the beginning of elution like the non-cytochrome fraction obtained at pH 7. Secondly, *Fraction I* pH 9·6, which was a very small, distinct, fast-running cytochrome c band which constituted about 1–3% of the total cytochrome c. Thirdly, *Fraction II* pH 9·6, which contained the rest of the cytochrome put on the column. When this fraction was made to move slowly down the column it separated into a forerunning reduced fraction followed by the rest of the fraction in the oxidized form, as described by Paléus & Neilands (1950).

Fig. 2 records a typical chromatogram at pH 9·6, in which fraction II was eluted rapidly, so that there was no distinct separation of the reduced and oxidized parts.

Elution of reduced and oxidized cytochrome c

The separation of reduced and oxidized cytochrome c on columns of Amberlite IRC-50 was first achieved by Paléus & Neilands (1950). In all our experiments reduced cytochrome c separated as a distinct chromatographic fraction if the rate of elution were slow enough. Comparison of the elution curves for reduced and oxidized cytochrome c (Figs. 1, 2) shows that with every fraction eluted below the pH at which cytochrome c becomes readily auto-oxidizable (about pH 11) there was a peak of reduced cytochrome c slightly preceding the peak of the oxidized form. Fig. 3 shows the elution diagram of the total fraction II pH 9·6, containing both ferro- and ferri-cytochrome c, when rechromatographed at pH 7 at about one-third the rate used in the two previous runs. The reduced and oxidized forms have nearly separated, producing distinct peaks.

This separation is not a property peculiar to cation-exchange columns, as similar effects were noted on columns of calcium phosphate gel; indeed,

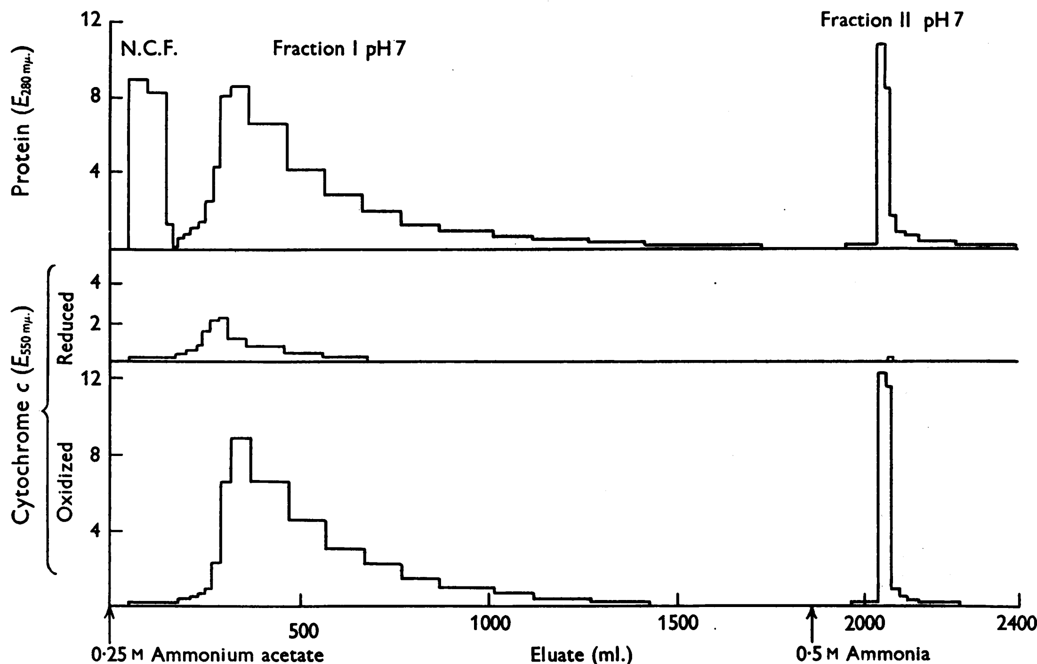

Fig. 1. Elution chromatogram of a 0·34% iron cytochrome *c* preparation at pH 7. N.C.F.: non-cytochrome fraction. Column of Amberlite IRC-50; ammonium salt, 35 × 3 cm. Rate of flow: 1·3–1·5 ml./min. Total of 142 μmoles of cytochrome *c* adsorbed on the column. Elution with 0·25 M ammonium acetate, then 0·5 N-NH_3. Ordinates: optical densities of eluate at 280 mμ. and at 550 mμ. for ferrocytochrome *c*, and for ferricytochrome *c* after reduction.

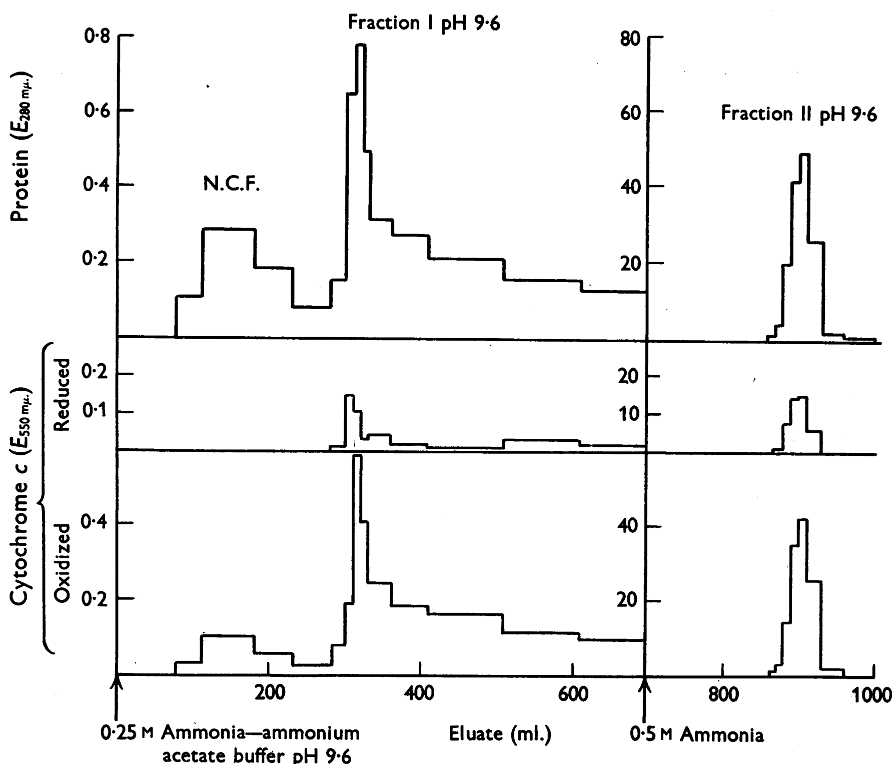

Fig. 2. Elution chromatogram of fraction I pH 7 at pH 9·6. Total of 75 μmoles of cytochrome *c* adsorbed on the column. Elution with 0·25 M ammonium acetate + ammonium hydroxide buffer pH 9·6, then with 0·5 N-NH_3. Column, rate of flow, ordinates and abbreviations as in Fig. 1.

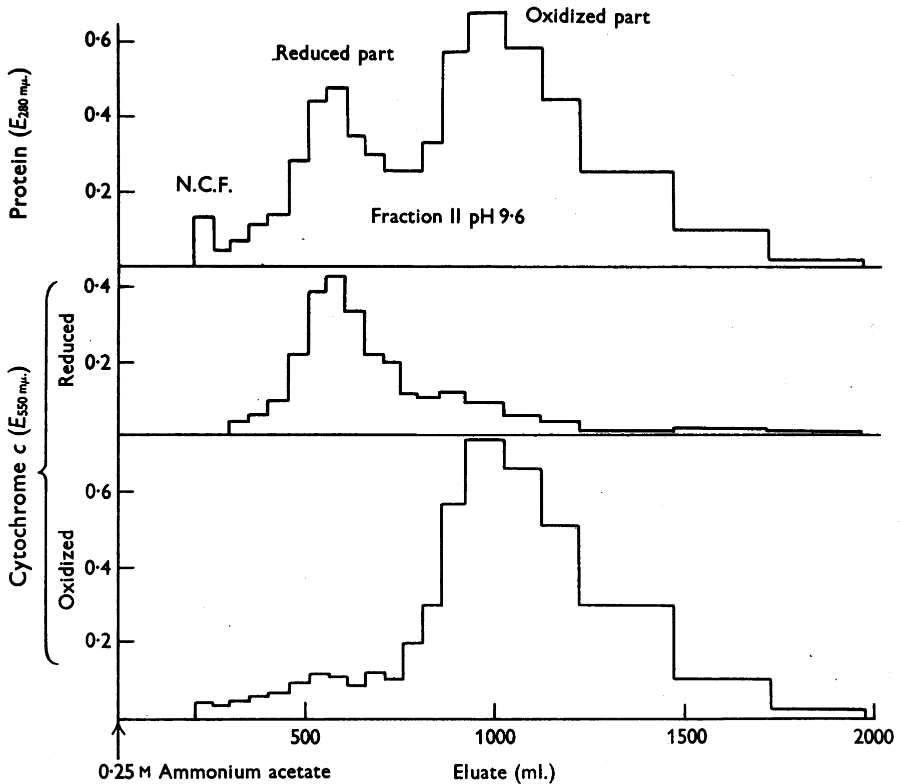

Fig. 3. Elution chromatogram of fraction II pH 9·6, at pH 7. Total of 20 μmoles of cytochrome c adsorbed on the column. Elution with 0·25 M ammonium acetate. Rate of flow 0·2–0·3 ml./min. Column, ordinates and abbreviations as in Fig. 1.

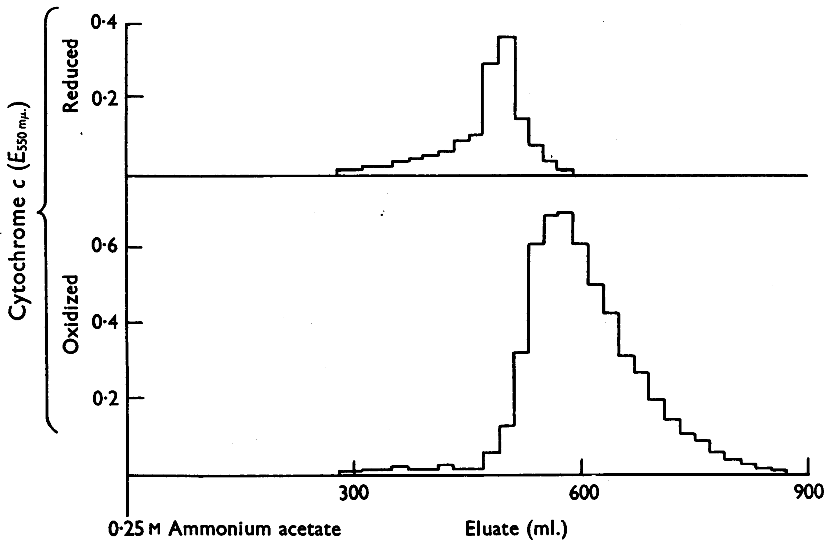

Fig. 4. Elution chromatogram at pH 7 of a fraction II pH 9·6 (oxidized), which had been artificially reduced to the extent of 21·5%. Column of Amberlite IRC-50, ammonium salt, 25 × 1 cm. Rate of flow 0·5 ml./min. Total of 4·9 μmoles of cytochrome c adsorbed on column. Elution with 0·25 M ammonium acetate. Ordinates: optical densities at 550 mμ. for ferrocytochrome c, and at 550 mμ. for ferricytochrome c after reduction.

the tendency of cytochrome *c* to precipitate when oxidized, and redissolve when reduced was used by Keilin (1930) in the preparation of yeast cytochrome *c*, while Zeile & Reuter (1933) showed that oxidized cytochrome *c* is adsorbed on kaolin, while under the same conditions the reduced form is not.

In order to test whether the reduced and oxidized parts of a fraction separated on the chromatogram entirely by virtue of their state of oxidation, the following experiment was performed. The starting material was the oxidized part of fraction II pH 9·6, which had been separated from the reduced part at pH 7 in a run similar to that recorded in Fig. 3, and chromatographed a second time at pH 7 to remove nearly all traces of the reduced material. It was then 99·6 % oxidized. A portion of this material was prepared in the reduced form by the cation-exchange procedure using sodium dithionite (Margoliash, 1954) and mixed with the original material to give a 21·5 % reduced fraction II pH 9·6. This was chromatographed at pH 7, and Fig. 4 records the elution diagram. It shows that although the reduced and oxidized cytochromes were originally the same chromatographic species, they separated when eluted slowly, the reduced fraction in the eluate being 19·1 % of the total.

Reduction of cytochrome c *during chromatography*

The original preparation used in the experiments reported in Figs. 1–3 was 3 % reduced; it is possible to calculate from the elution diagrams that fraction I pH 7 represented 89 % of the total cytochrome *c* put on the column at pH 7 and was 11·7 % reduced, while fraction II pH 9·6 represented 97 % of the total cytochrome *c* put on the column at pH 9·6 and was 24·9 % reduced. When the total fraction II pH 9·6 was rechromatographed at pH 7, so as to separate ferro- and ferri-cytochrome *c*, all the cytochrome *c* put on the column was recovered, 25·9 % in the reduced fraction, and 74·1 % in the oxidized fraction.

Thus, cytochrome *c* is partly reduced on a resin column, the degree of reduction increasing with the pH up to pH 11, where the auto-oxidation of cytochrome *c* becomes very rapid. When partial reduction had been caused by chromatography at a high pH, a second chromatography at a lower pH did not result in reoxidation of any of the reduced cytochrome *c* although the proportion of the latter was grossly above that which was normally reduced at the lower pH. In solution, however, reduction of cytochrome *c* did not take place even at pH 10. Reduction only occurred when cytochrome *c* was adsorbed on to some solid. This was not a property unique to the cation exchanger used, as the same phenomenon was readily observed on other cation exchangers such as Decalso F (Permutit Co. Ltd., London), and also on other adsorbents such as a calcium phosphate gel column.

Rate of ascorbic acid oxidation by the cytochrome c *fractions*

Table 1 records the rates of ascorbic acid oxidation by the original 0·34 % iron cytochrome *c* preparation used, and by the various fractions separated from it by chromatography at pH 7 and pH 9·6 on Amberlite IRC-50. There was a decrease of the rate of ascorbic acid oxidation as the cytochrome fractions were further chromatographed. This is consonant with the hypothesis that the oxidation of ascorbic acid by cytochrome *c* preparations is due to an impurity or to a modified form of cytochrome *c*, which is gradually removed by chromatography.

Table 1. *Activity in the cytochrome-oxidase system, rate of ascorbic acid oxidation and analysis of various chromatographic fractions of cytochrome* c

Cytochrome *c* fraction	Activity in cytochrome-oxidase system* (µl. O_2/hr./flask)	Rate of ascorbic acid oxidation* (µl. O_2/hr./flask)	Percentage of total preparation	Iron (%)	Reduced (%)	$\frac{E_{550\,m\mu.\,(reduced)}}{E_{280\,m\mu.\,(oxidized)}}$
Keilin & Hartree preparation	376	68	100	0·334	3	1·007
Fraction I pH 7	390	40	89	0·418	11·7	1·115
Fraction II pH 7	142	123	11	0·377	0·5	1·085
Fraction I pH 9·6	354	112	3	0·344	9·5	1·000
Fraction II pH 9·6 (total)	357	31	86	0·432	24·9	1·152
Fraction II pH 9·6 (reduced part)	351	42	22	0·413	65·8	1·120
Fraction II pH 9·6 (oxidized part)	367	19	64	0·452	5·3	1·185

* Both rates of O_2 uptake were measured in 0·066 M phosphate buffer pH 7·3, 1×10^{-3} M ethylenediaminetetraacetic acid, $2·72 \times 10^{-5}$ M added cytochrome *c* at 37°. 0·372 mg. Heart-muscle preparation (fat-free dry weight) was added to the flasks measuring cytochrome-oxidase activity. The O_2 uptakes of the flasks measuring ascorbic acid oxidation, containing all the ingredients except the oxidase, were used as the corresponding blank values.

Table 2. *Percentage of total cytochrome* c *separated as fraction II* pH 7 *from various preparations*

TCA represents trichloroacetic acid; NH_4Ac represents ammonium acetate.

Starting material	Extraction conditions: Solution used	pH	Time (hr.)	Amount of fraction II pH 7 as % of total cytochrome *c*
Horse-heart mince	2·5% TCA	4·5	3	11
Horse-heart mince	2·5% TCA	4·5	15	19
Washed horse-heart mince	1·5% TCA	4·5	3	0·7
Washed horse-heart mince	0·5M-NH_4Ac	7·0	3	0
Fraction I pH 7	2·5% TCA	4·5	3	56

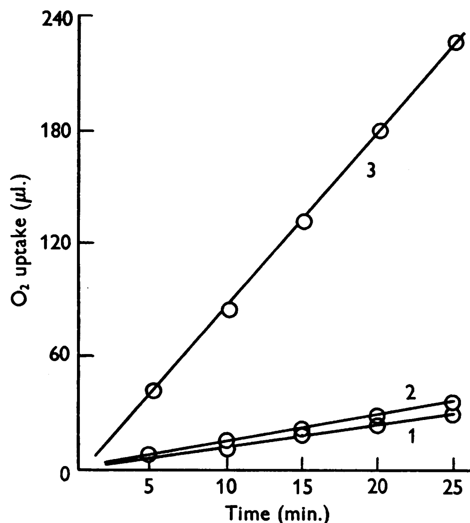

Fig. 5. Catalytic effects in the cytochrome-oxidase system of a normal 0·34% iron cytochrome *c* preparation (3), a fraction II pH 7 artificially prepared from a fraction I pH 7 by incubation with trichloroacetic acid (2), and a fraction II pH 7 separated from the original 0·34% iron preparation (1). Each flask contained: 0·066M phosphate buffer pH 7·3, 10^{-3}M ethylenediaminetetraacetic acid, 10 mg. ascorbic acid, 0·252 mg. heart-muscle preparation (fat-free dry wt.), 10 µg. of cytochrome *c* iron. Total volume: 3·3 ml. Temperature 37°. The values plotted were obtained by deduction of the O_2 uptake of the controls containing all the ingredients except the cytochrome oxidase, from that of the complete system.

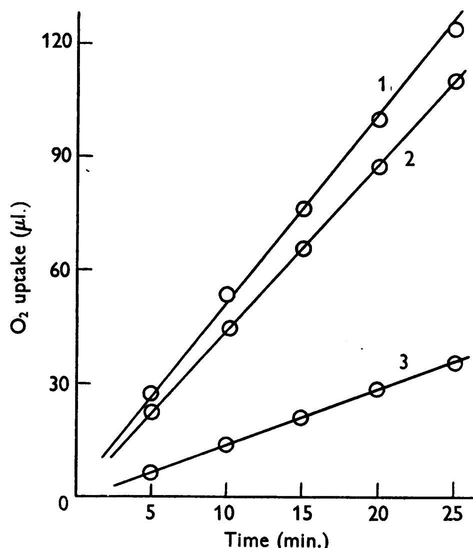

Fig. 6. Rates of ascorbic acid oxidation by a normal 0·34% iron cytochrome *c* preparation (3), a fraction II pH 7 artificially prepared from a fraction I pH 7 by incubation with trichloroacetic acid (2), and a fraction II pH 7 separated from the original 0·34% iron preparation (1). Each flask contained: 0·066M phosphate buffer pH 7·3, 10^{-3}M ethylenediaminetetraacetic acid, 10 mg. ascorbic acid, 10 µg. of cytochrome *c* iron. Total volume of fluid, 3·3 ml. Temperature 37°. The values plotted were obtained by deduction of the O_2 uptake of a control containing all the ingredients except cytochrome *c*, from that of the complete system.

Fraction II pH 7 showed an unusually high rate of ascorbic acid oxidation and low activity in the cytochrome-oxidase system. The properties of this fraction placed it in a class of its own, and it is discussed separately.

Enzymic activity of the various cytochrome c *fractions*

Table 1 records the activity of some of the various cytochrome *c* fractions in the cytochrome-oxidase system, using heart-muscle preparation, at a concentration of 5 µg. of added cytochrome *c* iron per flask. These fractions had similar activities and calculated on the basis of cytochrome *c* iron but not on total protein, these activities were the same as that of the original 0·34% iron preparation, which contained about 27% non-cytochrome protein. The only exception to this generalization is fraction II pH 7. A similar study of the activity of the various fractions in the succinic-oxidase system of kidney preparation gave identical results.

The nature of fraction II pH 7

Fraction II pH 7 differed considerably from all the other fractions and from the original cytochrome *c* preparation in having a high rate of ascorbic acid oxidation and low enzymic activity. Visual spectroscopy, however, did not show any difference between this fraction and the others. It did not appear to react appreciably with carbon monoxide and there was no intensification of the spectrum after treating with pyridine and dithionite. The question arose as to whether this unusual cytochrome *c* fraction was an artifact of the preparation, or was preformed in the tissue. In order to decide between these alternatives the following experiments were performed.

Table 3. *Data on the purest cytochrome* c *fraction obtained by chromatography on Amberlite IRC*-50

Iron (%)	0·465
$\epsilon_{550\,m\mu} \times 10^{-4}$ Reduced	2·77
$\epsilon_{550\,m\mu} \times 10^{-4}$ Oxidized	0·92
$\dfrac{E_{550\,m\mu\,(\text{reduced})}}{E_{280\,m\mu\,(\text{oxidized})}}$	1·192
Rate of ascorbic acid oxidation* (μl. O_2/hr./flask)	12
Equivalent weight†	12 000

* Determined as in Table 1.
† Calculated from the iron determinations.

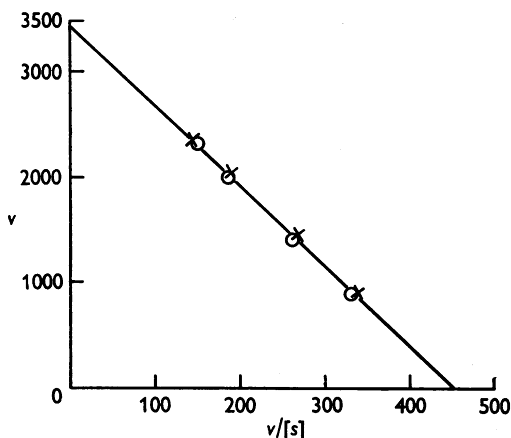

Fig. 7. Comparison of the activity of the cytochrome *c* in two preparations using the cytochrome-oxidase system of heart muscle: (1) the usual Keilin & Hartree (1945) preparation containing 0·34% iron (×), (2) the purest fraction obtained containing 0·465% iron (○). Modified Lineweaver & Burk (1934) type of plot, according to Hofstree (1952); *v* at each concentration of added cytochrome *c* was calculated as μl. O_2/hr./mg. fat-free dry weight of the heart-muscle preparation. [*s*] was taken as the estimated molar concentration of added cytochrome *c* and determined spectrophotometrically. Each flask contained: 0·066M phosphate buffer pH 7·3, 10^{-3}M ethylenediaminetetraacetic acid, 10 mg. ascorbic acid, 0·376 mg. heart-muscle preparation (fat-free dry weight) and from $2{\cdot}72 \times 10^{-5}$M–$16{\cdot}3 \times 10^{-5}$M added cytochrome *c*. Total volume of fluid 3·3 ml./flask. Temperature 37°. The O_2 uptake values were obtained by deduction of the O_2 uptake of controls containing, at each concentration of added cytochrome *c*, all the ingredients except the cytochrome oxidase, from that of the complete system.

Fresh horse-heart mince was divided into 1 kg. lots and extracted by different methods. The fluid was pressed out in a hydraulic press. The extracts were neutralized to pH 7·3, dialysed overnight against running tap water, filtered and adsorbed directly on to a column of Amberlite IRC-50. After being washed, the adsorbed cytochrome *c* was eluted with 0·25M ammonium acetate at pH 7 (which removed only fraction I pH 7) and then with 0·5M-NH_3 (which eluted fraction II pH 7). The percentage of the total present as fraction II pH 7 was calculated from spectrophotometric determinations. Simultaneously, 30 μmoles of a pure fraction I pH 7 which had been freed completely from fraction II pH 7, by chromatographing twice at pH 7, was kept at room temperature for 3 hr. in 2·5% trichloroacetic acid brought to pH 4·5 with 2N-NH_3. This caused the cytochrome *c* to be precipitated. It was then redissolved by neutralization and treated as the extracts above.

The results presented in Table 2 show that fraction II pH 7 appeared in extracts containing cytochrome *c* only when acid was used for extraction. The more concentrated the acid and the longer the time of extraction, the more of fraction II pH 7 appeared. Moreover, trichloroacetic acid at the

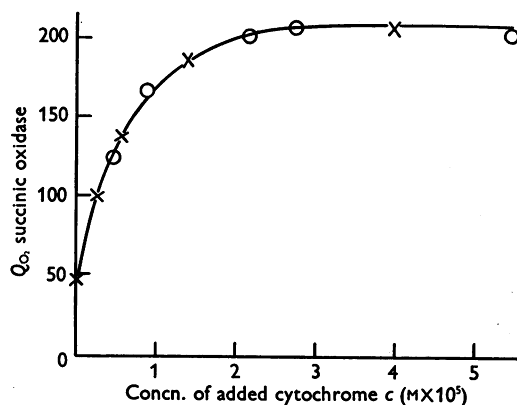

Fig. 8. Comparison of the activity of the cytochrome *c* in two preparations using the succinic-oxidase system of kidney: (1) the usual Keilin & Hartree (1945) preparation containing 0·34% iron (×), (2) the purest fraction obtained containing 0·465% iron (○). Final concentrations in the reaction mixture were: phosphate buffer pH 7·3, 0·10M; succinate 0·024M; added cytochrome *c* from 0 to $5{\cdot}5 \times 10^{-5}$M (estimated spectrophotometrically). Kidney preparation (fat-free dry weight) 0·865 mg./flask. Total volume 3·3 ml./flask. Temperature 37°. From the O_2 uptake between 5 and 25 min. after adding succinate, Q_{O_2} values were calculated as μl. O_2/hr./mg. fat-free dry weight of the kidney preparation.

same concentration and pH as used for the extraction of the heart-muscle mince transformed part of another chromatographic fraction into fraction II pH 7. This artificially prepared fraction II pH 7 was compared both as regards rate of ascorbic acid oxidation and enzymic activity to the fraction II pH 7 prepared from the 0·34 % iron cytochrome c preparation. The results presented in Figs. 5 and 6 show they had very similar properties.

Fraction II pH 7 was therefore an artifact produced by the trichloroacetic acid treatment at the time of extraction or possibly during the final precipitation (see procedure for the preparation of horse-heart cytochrome c, Keilin & Hartree, 1945). The fraction I pH 7 isolated in the above experiment, in which an artificial fraction II pH 7 was prepared by treatment of fraction I pH 7 with trichloroacetic acid, was found to have activities equal to those of the starting material in both the cytochrome-oxidase and succinic-oxidase systems, and the same rate of ascorbic acid oxidation. Thus, treatment with trichloroacetic acid modified only a proportion of the cytochrome c, depending on the concentration of the acid and the time of incubation in its presence. This occurred even under the relatively mild conditions used in the extraction of cytochrome c from heart mince. The modified form was easily separable from the native cytochrome c as fraction II pH 7.

Maximal purity obtainable by cation-exchange chromatography

A preparation with 0·450 % iron, and containing all the chromatographic fractions except the non-cytochrome fraction, was made by washing cytochrome c adsorbed on a column of Amberlite IRC-50, with 0·15 M ammonium acetate till the cytochrome front was three-quarters of the distance down the column. It was then eluted off with 0·5 M ammonia.

This already partially purified sample was subjected to chromatography at pH 7, then fraction I pH 7 was chromatographed at pH 9·6, and finally again at pH 7, as in the experiments recorded in Figs. 1–3.

Analyses showed that, as in experiments using the 0·34 % iron preparation, fraction II pH 9·6 (oxidized part) had the highest percentage of iron (0·465 %). Table 3 records some of the analytical and spectrophotometric data on this preparation, and Figs. 7 and 8 compare its activity in the cytochrome-oxidase and succinic-oxidase systems with that of the original 0·34 % iron preparation from which it was made, and which contained about 27 % of non-cytochrome protein. The figures show that there was no significant difference in the enzymic activities of the two preparations per μmole of cytochrome c iron.

It should be noted that the preparation containing 0·465 % iron does not necessarily represent the purest material obtainable by this method, since the ratio of the optical density at 550 mμ. (reduced) to 280 mμ. (oxidized) was 1·192 (see Table 3), whereas fractions having a ratio of 1·210 were obtained by taking small samples at the peak of the appropriate band during chromatography.

DISCUSSION

The results presented in this paper are in accord with those of Paléus & Neilands (1950), as regards the maximal purity obtainable by chromatography on cation exchangers, and with those of Boardman & Partridge (1953) on the effect of pH and cation concentration on the movement of the bulk of cytochrome c preparations on columns of Amberlite IRC-50. However, a study of the enzymic properties of the fractions isolated by chromatography shows that with the exception of fraction II pH 7 all the other fractions have the same activity in both the cytochrome-oxidase and succinic-oxidase systems. Calculated per μmole of cytochrome c iron, this activity is the same as that of the usual 0·34 % iron preparation which contains about 27 % non-cytochrome protein. Calculated per total protein present in the preparation, the activity of the pure cytochrome c is 27 % greater than that of the 0·34 % iron preparation. Thus, the protein impurity present in the 0·34 % iron preparation has neither catalytic nor inhibitory effects in the systems studied under the conditions used. Moreover, chromatography on cation exchangers under the conditions employed does not cause any change in the enzymic activity of cytochrome c per μmole of cytochrome c iron.

Fraction II pH 7 is unique among the chromatographic fractions of cytochrome c in showing a high rate of ascorbic acid oxidation and a low enzymic activity in the cytochrome-oxidase system. It causes most of the ascorbic acid oxidation of the 0·34 % iron cytochrome c preparations and was found to be an artifact of the acid extraction used in the ordinary procedure. The effect of trichloroacetic acid is to change a part of the extracted cytochrome c into a modified form, which is entirely indistinguishable spectroscopically from the normal cytochrome c, but which can easily be separated from it on cation exchangers, as fraction II pH 7, and has a low enzymic activity. The normal cytochrome c (fraction I pH 7) separated from a preparation which has been largely modified by a drastic treatment with trichloroacetic acid is, nevertheless, entirely normal with respect to its enzymic activity in the cytochrome-oxidase and succinic-oxidase systems and its oxidation of ascorbic acid.

Chromatography on cation exchangers causes a gradual decrease of the rate of ascorbic acid oxidation by the successive fractions eluted; this probably represents the separation from the bulk of the cytochrome *c* of a small amount of a modified cytochrome *c* which can act as a very effective catalyst in the oxidation of ascorbic acid. The purer cytochrome *c* fractions have a rate of ascorbic acid oxidation decreased to about 33–20 % of that of the usual 0·34 % iron preparation.

Apart from fraction II pH 7 and the very small fraction I pH 9·6, cytochrome *c* falls entirely into two chromatographic species, reduced and oxidized cytochrome *c*. These two species are reversibly transformable one into the other by reduction and oxidation, their relatively small difference in chromatographic behaviour being entirely due to their state of oxidation. This separation of reduced and oxidized cytochrome *c* on a cation-exchange column, the reduced form acting as the stronger acid, agrees well with the difference of one equivalent in the titration curves of both forms of the protein, observed in the physiological range of pH's, by Theorell & Åkeson (1941). However, a forerunning, reduced fraction may be less pure (lower $E_{550\,m\mu}/E_{280\,m\mu}$ and higher rate of ascorbic acid oxidation) than the following, oxidized fraction (see Table 1).

Reduction of cytochrome *c* was observed during chromatography. It occurs only when the protein is adsorbed on and eluted off solid particles and not in solution. This is, however, not a unique property of the cation-exchange resin used, but occurs also on other adsorbents, such as an aluminium silicate cation exchanger and calcium phosphate gel. The higher the pH at which the adsorption and elution takes place, the higher the percentage of the cytochrome *c* reduced, up to pH 11, where cytochrome *c* becomes rapidly auto-oxidizable. A similar phenomenon has been observed by Paléus (1952) during the electrophoresis of cytochrome *c* preparations on paper.

SUMMARY

1. Native horse-heart cytochrome *c* shows only two chromatographic species on cation exchangers, reduced and oxidized cytochrome *c*. These two chromatographic species are reversibly transformable one into the other. Other fractions are artifacts of the preparation.

2. Trichloroacetic acid modifies a variable proportion of any sample of cytochrome *c* into a form indistinguishable spectroscopically from the normal cytochrome *c*, but easily separated from it on cation-exchange columns, and having a low enzymic activity, and a high rate of ascorbic acid oxidation.

3. The degree of reduction of ferricytochrome *c* on adsorption and elution from a number of adsorbents increases with the pH up to 11, where cytochrome *c* becomes rapidly auto-oxidizable.

4. The rate of ascorbic acid oxidation of various cytochrome *c* preparations varies inversely with their degree of purity.

5. The enzymic activity per μmole of cytochrome *c* iron of the purest preparation obtained (0·465 % iron), both in the succinic-oxidase and the cytochrome-oxidase systems, is the same as that of the 0·34 % iron type of preparation containing about 27 % non-cytochrome protein, the non-cytochrome protein impurity having neither catalytic nor inhibitory effects in the systems and under the conditions used.

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