Cytochrome *c* Modified by Digestion with Proteolytic Enzymes

1. DIGESTION

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Although the importance of prosthetic groups to the activity of certain enzymes has been much studied, the part played by the protein portion of the molecule has received little attention. Haemoglobin, catalase and peroxidase all have the same prosthetic group, namely, protohaematin, yet the attachment of different proteins gives them distinct and characteristic properties. Thus haemoglobin is a respiratory carrier, capable of combining reversibly with oxygen without undergoing oxidation; catalase, an enzyme catalysing the decomposition of hydrogen peroxide and peroxidase, an enzyme utilizing hydrogen peroxide for the oxidation of a number of substances. Similarly, some flavoprotein enzymes, having the same prosthetic group, catalyse different reactions. In other words, the difference in their enzymic properties is a reflexion of the difference in the protein portion of the molecule.

It would seem interesting, therefore, to find out to what extent a modification in the protein portion of an enzyme which has a well defined prosthetic group would be reflected in the catalytic and other properties of the enzyme. For this type of experimental approach cytochrome *c* has the following advantages. It is stable within a wide range of pH and temperature, it is easily isolated and purified on a comparatively large scale and its biological activity can be estimated by simple methods.

MATERIALS

Cytochrome c

(1) *Cytochrome c with iron content of 0.34%.* This was prepared by the method of Keilin & Hartree (1945) from horse hearts. Fe content was usually 0.34%. In cases where a lower figure was found, it was always brought up to this value by diluting the solution to 1 l. and repeating the $(\text{NH}_4)_2\text{SO}_4$ -trichloroacetic acid precipitation.

(2) *Cytochrome c with an iron content of 0.43%.* This was prepared from cytochrome *c* with Fe content of 0.34% according to the method of Keilin & Hartree up to the point of the filtration of the mixture of saturated $(\text{NH}_4)_2\text{SO}_4$ and cytochrome at pH 10. Instead of being dialysed, the filtrate was diluted with 80% saturated $(\text{NH}_4)_2\text{SO}_4$ to 1 l. and precipitated with 25 ml. 20% trichloroacetic acid. The precipitate was suspended in as little 0.05 N-NH₃ as possible and

dialysed first against 0.005 N-NH₃ followed by 0.5% NaCl solution.

Provided that the starting material had Fe content of not less than 0.34% and a concentration of not less than 1% protein/ml., cytochrome *c* of Fe content of 0.43% was invariably obtained.

(3) *A simple method of preparation of cytochrome c of iron content 0.43% from cytochrome of iron content 0.34%.* Cytochrome *c* solution (12 ml.) (1%, Fe content, 0.34%) was placed in a 50 ml. centrifuge tube containing a few drops of CHCl_3 . The solution was heated in a water bath so that the CHCl_3 boiled vigorously. The heating was continued until the precipitation of cytochrome *c* was virtually complete. The precipitate was centrifuged down, washed once with distilled water, and dissolved in 5 ml. of 0.1 N-NaOH. Exactly 5 ml. 0.1 N-HCl were then added and the solution kept in the refrigerator overnight. Next morning the light-brown precipitate was filtered off to give a clear filtrate containing cytochrome *c* (Fe content 0.43%) in 0.6% NaCl. The yield, measured spectrophotometrically, was usually 50–60%.

The cytochrome *c* thus obtained was found to have the same activity in the succinic oxidase and cytochrome oxidase systems as cytochrome *c* prepared by other methods. The Fe content was usually 0.43%, although occasionally preparations with slightly lower content (0.40–0.42%) were obtained.

Pepsin. This was crystallized from Hopkin & Williams pepsin by the method of Northrop (1946). The crystals obtained were collected on a filter, redissolved in water, dialysed until salt-free and freeze-dried to a colourless light powder. This material was kept *in vacuo* over CaCl_2 and retained its activity for over a year.

Trypsin and chymotrypsin. These were crystallized from fresh ox pancreas by the method of Kunitz & Northrop (1936) except that the conversion of trypsinogen to trypsin was carried out according to McDonald & Kunitz (1946). The enzymes were stored as described by these workers.

Papain. A commercial product of British Drug Houses Ltd. was used.

Heart-muscle preparation. This was prepared from horse heart according to Keilin & Hartree (1947) except that the following modification was introduced in the washing procedure. Minced heart muscle (350 g.) was placed in a muslin sac of about 4 l. capacity supported on an iron ring. A jet of tap water was then run into the sac at such a rate that the minced muscle was kept well stirred and the water level inside the sac was maintained about three-fifths full. Occasionally the running tap water was stopped, the water inside the sac was allowed to drain completely and the sac was squeezed hard to get rid of as much water as possible. After about 4 hr. washing and eight squeezings, the muscle pulp was usually free from myoglobin. It was then treated as described by Keilin & Hartree (1947). This preparation was always used within 5 days.

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Kidney preparation. This was prepared from horse kidney using essentially the same method as described by Slater (1949). This preparation was also used within 5 days.

METHODS

Cytochrome c. The Fe content of cytochrome *c* and of the autoxidizable cytochrome fractions was estimated according to the method of Keilin & Hartree (1945). The activity in the cytochrome and succinic oxidase systems was estimated as described by Slater (1949) except that in the cytochrome oxidase system 0.05 M-*p*-phenylenediamine was used as the substrate.

Proteolytic enzymes. The activity of the proteolytic enzymes was estimated by the method of Anson (1938) and expressed in haemoglobin units.

Spectrophotometric control of the course of digestion. The digestion of cytochrome *c* with the formation of an autoxidizable derivative was studied in the following way: portions of the digestion mixture were withdrawn at suitable intervals and mixed with a measured volume of 0.25 M-phosphate, pH 7.3. In the case of pepsin digestion, where the mixture contained 0.06 N-HCl, an equivalent amount of NaOH was also added to the portions withdrawn. The total amount of cytochrome *c*, autoxidizable and non-autoxidizable, was estimated by measuring the intensity of the absorption at 550 m μ . after reduction by Na₂S₂O₄. A current of pure CO was then passed through the solution and the absorption at 550 m μ . again measured. The molar concentration of autoxidizable cytochrome *c* is given by $(D_r - D_{co})/(\epsilon_r - \epsilon_{co})$, where D_r = density after reduction at 550 m μ ., D_{co} = density after reduction and passing CO at 550 m μ ., ϵ_r = molecular extinction coefficient of ferrocytochrome *c* at 550 m μ ., 2.8×10^4 , and ϵ_{co} = molecular extinction coefficient of the CO compound of autoxidizable cytochrome *c* at 550 m μ ., 1.07×10^4 (Tsou, 1949).

The concentration of autoxidizable cytochrome *c* present can also be determined from the absorption at 550 m μ . of the reduced pigment before and after a current of air has been passed through the solution. Such results are less certain than those obtained by the CO method described above owing to the fact that aeration in presence of the reducing agent invariably leads to the formation of H₂O₂, which slowly oxidizes non-autoxidizable cytochrome *c*. Nevertheless, results were obtained which confirmed the general picture given by the CO method.

Isolation of a pepsin-modified cytochrome *c*

Digestion was carried out in 1% cytochrome *c* solution to which was added a quarter of its volume of 0.3 N-hydrochloric acid and a measured amount

(about 0.3 ml./100 ml. of cytochrome *c* solution) of 1% pepsin solution, so that the amount of pepsin used was 0.15 haemoglobin unit per g. of cytochrome. The final pH of the solution was usually about pH 1.5–1.7 as measured with a glass electrode. The digestion was allowed to proceed for 16–20 hr. at 24° after which the reaction was stopped by the addition of an amount of N-sodium hydroxide equivalent to that of the acid initially added.

It was found that the component of the digest which showed the characteristic cytochrome *c* spectrum could be precipitated by either of the following methods: (1) by making the solution 85% saturated with (NH₄)₂SO₄; (2) by freeing the solution almost completely of salt at pH 5. The procedure finally adopted for the isolation of this component was as follows. The neutralized solution was made 85% saturated with (NH₄)₂SO₄, the mixture was cooled to 0° and filtered cold. The precipitate was dissolved from the filter using 1 l. of water per 2 mg. cytochrome *c*-Fe. The solution was then made 70% saturated with (NH₄)₂SO₄ and 25 ml. of 20% trichloroacetic acid were added per l. of solution. The precipitate was centrifuged down, suspended in as little 0.05 N-NH₃ as possible, transferred to a small cellophan tube and dialysed against 0.01 M-phthalate, pH 5. After three or four changes of the outer solution, the precipitation of modified cytochrome was almost complete. The contents of the cellophan tube were then rinsed with the same phthalate buffer into a centrifuge tube and the precipitate collected by centrifugation. The supernatant, still showing the cytochrome *c* type of spectrum, probably contained various breakdown products of cytochrome *c*. The precipitate, after being washed once with phthalate buffer, was dissolved in 0.02 N-NH₃ and the precipitations by (NH₄)₂SO₄, trichloroacetic acid and phthalate were repeated. After the second precipitation in the cellophan tube, the supernatant was almost colourless. The precipitate, washed once with phthalate buffer and once with a little distilled water, was dissolved in 2% Na₂SO₄ to give a dark-red solution containing 3–4 mg. protein per ml. This solution was kept saturated with CHCl₃ at 0°. It contained a pepsin-modified cytochrome *c*, hereafter referred to as PMc.

Digestion with papain, trypsin and chymotrypsin

Conditions used in the digestion of cytochrome *c* with papain, trypsin and chymotrypsin are summarized in Table 1.

Separation of autoxidizable from non-autoxidizable cytochrome c. The mixture, containing about 50 μ g. cytochrome-Fe per ml., was made 80% saturated with (NH₄)₂SO₄, and left at 0° overnight. Next morning it was filtered cold (filtrate A).

Table 1. Conditions used in the digestion of cytochrome *c* (cyt. *c*) by proteolytic enzymes

(Concentration of cytochrome *c* in all cases, 1% protein/ml. Fe content, 0.34% except where specified in text.)

Proteolytic enzymes	Activator	pH	Buffer	Temp. (°)	Time of digestion (hr.)
Pepsin	—	1.5	—	24	20
Papain (50 mg./g. cyt. <i>c</i>)	—	4.9	0.1 M-Phthalate	4	72
Papain (50 mg./g. cyt. <i>c</i>)	Cyanide or cysteine	4.9	As above	24	20
Trypsin (0.05 Hb unit/g. cyt. <i>c</i>)	—	8.5	0.1 M-Borate	24	20
Chymotrypsin (0.05 Hb unit/g. cyt. <i>c</i>)	—	8.5	As above	24	20

The precipitate was then dissolved in water, reprecipitated by $(\text{NH}_4)_2\text{SO}_4$ and trichloroacetic acid and dialysed as usual to give a solution of autoxidizable cytochrome. Since the precipitation of autoxidizable cytochrome c in 80% saturated $(\text{NH}_4)_2\text{SO}_4$ is not always complete, the method can only be applied when a considerable proportion of the pigment is in the autoxidizable form.

Separation of non-autoxidizable from autoxidizable cytochrome c. Filtrate A was dialysed against 0.5% NaCl until free from $(\text{NH}_4)_2\text{SO}_4$ and then placed upon a chromatographic column prepared by mixing kieselguhr into a calcium phosphate gel until a suitable consistency was obtained. The intense red band was developed by alternate washings with 0.25 M Na_2HPO_4 and water. A brownish-red substance was found to move down more rapidly than the main band which was red in colour. The brownish-red material on the column was rather diffuse, whereas the main red band was quite well defined. As soon as the two pigments were clearly separated, the column was cut through while still in the glass tube, the portion containing the main red band was eluted with sodium oxalate and precipitated from the eluate by means of $(\text{NH}_4)_2\text{SO}_4$ and trichloroacetic acid. The precipitate was finally resuspended in dilute NH_3 and dialysed as usual.

RESULTS

Digestion by pepsin

The change in the total cytochrome *c* concentration and the formation of an autoxidizable cytochrome is shown in Fig. 1. A curious result of this experiment was the fact that in presence of pepsin the total amount of cytochrome *c*, autoxidizable and non-autoxidizable, dropped to 60% in about 5 min. and then slowly returned to about 90% of the original amount in 1 hr. On the other hand, the amount of non-autoxidizable cytochrome decreased very rapidly so that in about 20–30 min. the cytochrome became completely autoxidizable. Similar results were obtained with cytochrome *c* preparations of both 0.34 and 0.43% iron.

The effects of temperature and of concentration of pepsin on the course of digestion were also examined. The results obtained made it clear that by varying temperature and pepsin concentration only the rate of the initial drop and the subsequent rise in the total concentration of cytochrome *c* were affected: the general shape of the curves remained unchanged. In all cases there was a quick initial decrease of the total cytochrome concentration to 60–65% of the original and a slow return to about 90%. The conversion of non-autoxidizable to autoxidizable cytochrome *c* was invariably complete as soon as the total cytochrome had passed the minimal point. The results obtained at 0, 20 and 38° are presented in Fig. 2.

With increasing pepsin concentration the time for reaching the minimal point was shortened, whereas the general shape of the curves remained unchanged.

Pepsin is very rapidly inactivated in neutral solutions; therefore, the digestion of cytochrome *c*

Fig. 1. The digestion of cytochrome *c* by pepsin showing the change in total cytochrome (autoxidizable + non-autoxidizable) concentration in terms of percentage of original concentration (curve B), and in autoxidizable cytochrome (curve C) against time (in min.) as compared with a control without pepsin (curve A). Temp., 38°, pH = 1.6, amount of pepsin = 0.15 Hb unit/g. of cytochrome *c*, original cytochrome *c* concentration = 0.4 mm.

Fig. 2. The effect of temperature on the digestion of cytochrome *c* by pepsin showing the change in total cytochrome concentration (% of original concentration against time). Curve A, temp., 38°. Curve B, temp., 20° and curve C, temp., 0°. Amount of pepsin, pH, and original cytochrome concentration as in Fig. 1.

by pepsin can be stopped at any desired stage by the addition of alkali to bring the pH to neutrality. Making use of this property it was found that pepsin was responsible for the whole course of digestion. In other words, the presence of pepsin was necessary for both the initial drop and subsequent rise in the total amount of cytochrome *c*. A typical example to illustrate this point is shown in Fig. 3 in which the

Fig. 3. Digestion of cytochrome *c* by pepsin interrupted by neutralization. Temperature, pH, amount of pepsin and original cytochrome concentration as in Fig. 1. The reaction was interrupted at the point indicated by the arrow. Curve *A* shows the change in total cytochrome concentration by re-acidification and addition of the same amount of pepsin; curve *B* shows a slow rise of total cytochrome concentration by re-acidification immediately after neutralization due probably to a partial re-activation of pepsin; curve *C* shows that the total cytochrome concentration remained unchanged if re-acidification was carried out 24 hr. after neutralization or if the reaction mixture was allowed to remain neutral.

digestion was stopped when the total amount of cytochrome *c* was very nearly at the minimum. If the reaction was interrupted at points other than the minimum, on re-acidification and addition of more pepsin the digestion always followed the same course it would have taken had the reaction not been interrupted by neutralization.

Digestion with papain

Digestion with papain in absence of activator. A pigment with the characteristic cytochrome *c* spectrum was isolated from the reaction mixture (Table 1, cytochrome *c* iron, 0.34%) after the digestion and found to have an iron content of 0.48%

as measured by both the absorption at 550 m μ . of the reduced pigment or by the 2:2'-dipyridyl method. This pigment was non-autoxidizable and did not combine with carbon monoxide. It had approximately the same activity per iron atom as ordinary cytochrome *c* in the cytochrome oxidase system with either ascorbic acid or *p*-phenylenediamine as substrate, although if anything it slightly inhibited the succinic oxidase system of heart-muscle preparations.

Using the carbon monoxide method it was found that this pigment contained about 10% autoxidizable cytochrome *c*. The non-autoxidizable fraction, when separated from the autoxidizable fraction, gave an iron content of 0.427% and was indistinguishable from ordinary cytochrome *c* (iron, 0.43%) in every respect. Thus the high iron content and the slight inhibitory effect of this pigment were due to the autoxidizable cytochrome present.

Digestion with papain in presence of an activator (Table 1). The pigment isolated in the usual way, by ammonium sulphate-trichloroacetic acid precipitation, at the end of the digestion contained 1.05% iron. It was autoxidizable, combined with carbon monoxide in the reduced state and was biologically inactive.

Digestion with trypsin (Table 1)

At the end of digestion the reaction mixture was rapidly heated in a boiling-water bath to 95° and held at this temperature for 3 min. to destroy the trypsin activity. The solution was then filtered, diluted tenfold and the pigment isolated in the usual way by precipitation with ammonium sulphate-trichloroacetic acid. Cytochrome *c* thus modified, with an iron content of 0.87%, was autoxidizable and combined with carbon monoxide.

Digestion with chymotrypsin (Table 1)

At the end of the digestion an estimation by the carbon monoxide method showed that the product was a mixture of autoxidizable and non-autoxidizable pigments. The solution was then heat-treated as above, filtered and the autoxidizable and non-autoxidizable fractions separated.

The non-autoxidizable fraction was found to have an iron content of 0.345% and was indistinguishable from ordinary cytochrome *c* in every respect. If the starting material used was a cytochrome *c* preparation of iron content of 0.43%, the non-autoxidizable fraction obtained also had the same iron content, 0.43%. The autoxidizable fraction obtained had an iron content of 0.71%. Like the other autoxidizable pigments obtained by pepsin, papain and trypsin, it also combined with carbon monoxide.

The course of digestions by papain, trypsin and chymotrypsin were also followed in respect of the total cytochrome concentration and the formation of

the autoxidizable fraction. The initial drop and the subsequent rise in total cytochrome *c* concentration, observed in the case of pepsin digestion has not been encountered in digestions by papain, trypsin or chymotrypsin. Moreover, after the initial drop in total cytochrome *c* concentration produced by pepsin digestion none of the proteolytic enzymes examined except pepsin could bring about the subsequent rise in the total cytochrome *c* concentration.

*Activity of autoxidizable cytochrome c
in biological systems*

The autoxidizable fractions of cytochrome *c* modified by digestion with proteolytic enzymes were not reduced either by the succinic dehydrogenase system or by the dihydrocozymase-diaphorase system. They were not active in the succinic oxidase system nor in the cytochrome oxidase system with *p*-phenylenediamine as substrate. Details of the results obtained with PMc are shown in Table 2.

Table 2. *Activity of pepsin-modified cytochrome c (PMc) in biological systems*

(O₂ uptake was measured at 38° in Barcroft differential manometers and expressed as Q_{O₂} (μl. oxygen uptake/mg. fat-free dry wt./hr.). Details of measurement were essentially the same as described by Slater, 1949.)

System	Components	Activity with	
		Heart-muscle preparation (Q _{O₂})	Kidney preparation (Q _{O₂})
Cytochrome oxidase	Enzyme + <i>p</i> -phenylenediamine	590	250
	Enzyme + <i>p</i> -phenylenediamine + PMc, 0.055 mM	575	265
	Enzyme + <i>p</i> -phenylenediamine + cytochrome <i>c</i> , 0.055 mM	1540	635
Succinic oxidase	Enzyme + succinate	515	75
	Enzyme + succinate + PMc, 0.055 mM	460	60
	Enzyme + succinate + cytochrome <i>c</i> , 0.055 mM	665	215

DISCUSSION

By treating ordinary cytochrome *c* with *n*-potassium hydroxide for 18 hr. at 20°, Keilin & Hartree (1940) obtained an autoxidizable cytochrome *c* which was catalytically inactive in biological systems. They concluded that when cytochrome *c* becomes autoxidizable, it loses its catalytic activity. The findings of the present investigation amply confirm the conclusion reached by Keilin & Hartree.

All the autoxidizable cytochrome *c* fractions obtained in the present investigation have many properties in common. They are all biologically inactive, they combine with carbon monoxide in the reduced state and, moreover, they all have the same type of absorption spectrum which is very similar to that of ordinary cytochrome *c* in both the oxidized and reduced states (see Tsou, 1951). The results of these experiments seem to suggest that the protein portion of a cytochrome *c* molecule containing 0.43 % iron cannot be further reduced without affecting its catalytic activity. This does not necessarily mean,

however, that cytochrome with 0.43 % iron is any 'purer' than the pigment with an iron content of 0.34 % as is believed by Theorell & Åkeson (1941). In any case, the question whether one of them is any purer than the other does not seem to be of much importance in view of the fact that neither of them is as active as the endogenous form (Keilin & Hartree, 1940, 1945).

In the light of the present investigation it is not difficult to understand that Altschul & Hogness (1938) observed only a very slight effect of carbon monoxide on the absorption spectrum of ferrocytochrome *c*. Keilin & Hartree (1939), using the manometric method, showed that although a concentrated solution of ferrocytochrome *c* took up some carbon monoxide, the amount was far less than could have been expected from a chemical combination between carbon monoxide and ferrocytochrome *c*. They attributed the slight effect obtained by Altschul & Hogness either to a small fraction of

denatured cytochrome *c* or to some other haematin impurity. All the present autoxidizable cytochrome *c* fractions have been found to combine reversibly with carbon monoxide at all pH values and, moreover, the absorption spectrum of reduced autoxidizable cytochrome *c* has been found to be indistinguishable from that of the unmodified pigment. Consequently its presence in an ordinary cytochrome preparation could not be detected by the purity test devised by Altschul & Hogness. It is therefore likely that the cytochrome *c* preparation of these workers contained a fraction of autoxidizable cytochrome *c* whose carbon monoxide compound was responsible for the observed change in the absorption spectrum.

The findings of Paul (1948) that the loss of activity of cytochrome *c* in strong acid solution is not reflected in a corresponding decrease in the absorption at 550 mμ. of ferrocytochrome *c* can also be explained. The first stage of destruction of cytochrome *c* by acid is probably the slow transformation of cytochrome *c* into an autoxidizable derivative

which, although not biologically active, still has the same absorption spectrum as that of non-autoxidizable cytochrome *c*. By extraction with hot 0.1N-acetic acid, Goddard (1944) obtained cytochrome *c* from wheat germ and found that it was less active than the pigment isolated from animal tissues. It is reasonable to assume that as a result of the drastic treatment his preparation contained a considerable amount of autoxidizable cytochrome *c* which would account for the low activity. It is also possible that the apparently high iron content of cytochrome *c* preparations obtained by Tint & Reiss (1950) was not due, as they believed, to any species differences but rather to the presence of an autoxidizable fraction.

The intensity of absorption at 550 m μ . of ferro-cytochrome *c* has commonly been used as a measure of its concentration. However, the presence of an inactive autoxidizable fraction cannot be distinguished by this method. Although the amount of this autoxidizable material is usually negligible it can sometimes lead to a high iron content and thus create a false impression of the purity of the preparation. As a precaution it is therefore recommended, for estimation of cytochrome *c* concentrations by the spectrophotometric method, that a further set of readings be taken after a current of carbon monoxide has been bubbled through the

solution. In this way not only the presence but also a rough estimation of the amount of autoxidizable cytochrome *c* present can be obtained.

SUMMARY

1. Digestion of cytochrome *c* by pepsin produced an autoxidizable pigment. During the digestion the total amount of cytochrome present, autoxidizable and non-autoxidizable, showed a rapid initial drop and a slow subsequent rise.

2. Digestion with papain in absence of an activator raised the iron content of cytochrome *c* from 0.34 to 0.43 % without affecting its catalytic activity. In presence of an activator papain produced an autoxidizable pigment.

3. Digestion with trypsin produced an autoxidizable pigment while chymotrypsin produced a mixture of autoxidizable and non-autoxidizable pigments. The non-autoxidizable part of this mixture, when isolated, contained 0.34 % iron.

4. None of the autoxidizable pigments was active in the succinic oxidase system or in the cytochrome oxidase system with *p*-phenylenediamine as substrate.

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Cytochrome *c* Modified by Digestion with Proteolytic Enzymes

2. PROPERTIES OF PEPSIN-MODIFIED CYTOCHROME *c*

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In the preceding communication (Tsou, 1951) the digestion of cytochrome *c* by proteolytic enzymes and the isolation of autoxidizable fractions of cytochrome *c* thus modified have been described. These autoxidizable cytochrome *c* fractions have been shown to possess several properties in common, namely they are all autoxi-

dizable, they combine with carbon monoxide in the ferrous state and they are not active in biological systems. The properties of one of them, that obtained by digestion with pepsin, will now be examined in greater detail. Some of the findings have already been reported briefly elsewhere (Tsou, 1949).