蛋白质与酶学研究

PROTEIN AND ENZYME RESEARCH

Selected Publications by

Chen-Lu Tsou

1949-1992

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序　言

这本选集选录了从 1949 年我在英国 Nature 杂志上发表第一篇短文开始，到 1992 年为止，四十多年来在国内外发表百余篇论文中的 30 篇。 其中多数是研究论文，也包括少数综合介绍我自己工作的论文。按照内容和我自己从事这一领域工作的先后，分为 1. 呼吸链，2. 胰岛素，3. 蛋白质化学修饰和分子酶学，4. 酶活性不可逆修饰动力学，以及 5. 蛋白质的折叠与去折叠等五个部分，是我四十多年来从事研究工作曾涉及的主要方面。

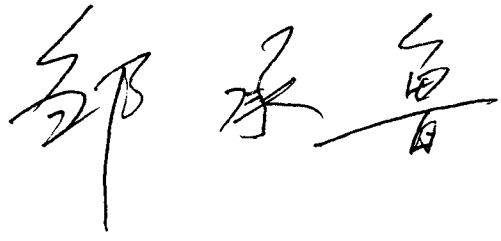
在这三十篇论文中，除了早期的 1. 1 和 1. 2 两篇论文的实验部分完全是我自己完成的，3. 1，4. 1，4. 2，5. 4 等文完全不包括实验部分之外，其他各篇论文，即使有些是我单独署名的综述性论文，全都包含了四十多年来先后和我共同工作过的同事们的大量实验结果。 虽然有些同事们的工作没有包括在这三十篇论文之中，但是这五个部分的工作本身都是连续性的，前后密切相关的，即使是在这本集子里未收录的论文所提供的实验结果，也同样影响了我的思索，同样是所得出的一些科学结论的基础。 所有先后和我共同工作过的同事，都对这些科学结论做出了贡献。 能够和他们一起工作，看他们逐渐成长为成熟的科学家，不少在自己的学术领域中做出了重要的贡献，在国际科学界建立了自己的声誉，有的现在已经是中国科学院学部委员，是我一生从事科学研究工作中最值得欣慰的。 上述五个领域的工作，曾先后获得陈嘉庚生命科学奖，国家和中国科学院的自然科学一、二、三等奖多次。这些都包含着他们的辛勤劳动，我谨在此向他们表示诚挚的谢意。

自然科学基础研究是世界性的。 读者也许会注意到这里收集的论文，特别是近年来的论文，绝大部分是在国外重要刊物上发表的。 只有不断在国外重要刊物上发表论文，才能逐渐在自然科学基础研究的世界舞台上占领一席之地。 由于国外同行较多，在国外重要刊物上发表论文要经过本领域中同行的审查，所提意见常常是中肯的，关键的，有时更是很严格的。 根据这些意见对论文进行必要的补充和修改，对提高论文水平很有好处。 即使是审稿人的意见不正确，或不完全正确，真理总是越辩越明的，在和审稿人反复讨论的过程中，我也感到获益良多。 我恳切希望，今后我国有更多科学家在国外重要刊物上发表论文，使我国生物化学与生物物理学研究成果能更为广泛地被国际同行了解，总体水平更快地进入世界先进行列。

C. L. Tsou

Preface

In this collection, 30 papers published between 1949 and 1992 were selected and grouped into 5 fields namely respiratory chain, insulin, chemical modification of proteins and molecular enzymology, kinetics of irreversible modification of enzyme activity, and folding and unfolding of proteins, roughly in the order of the time of my beginning to work in these fields. Many of the papers here collected appeared in home journals as it has been possible only since 1979 to publish abroad. I believe that some of the papers published in Scientia Sinica(now Science in China) may not be easily available and hence familiar to my colleagues in the west. In most of the experimental studies, it has been my good fortune to be assisted by my colleagues. As the papers here collected represent a random selection from a series of interconnected papers, many colleagues whose names did not appear on the papers here selected, nevertheless made significant contributions in our studies of the above fields. It is one of my greatest pleasure in my 45 years of scientific life, to be able to associate with such talented people and to see many of them now established scientists with international reputations and in responsible positions. I wish to express my sincere thanks to them all.



Contents

3. Chemical Modification of Proteins and Molecular Enzymology

4. Kinetics of Irreversible Modification of Enzyme Activity

5. Folding and Unfolding of Proteins

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Cytochrome c Modified by Digestion with Pepsin

ALTHOUGH much attention has been given to the importance of prosthetic groups of certain enzymes for their activity, the part played by the protein portion of the molecule is still little understood. For example, how far a modification in the protein portion of an enzyme molecule without affecting its prosthetic group would be reflected in its enzymic and other properties is scarcely known. For the study of this problem the extremely stable protein cytochrome *c* seems most suitable. In the following experiments, cytochrome *c* was modified by splitting off a great part of the protein portion of the molecule with pepsin. The resulting substance was then isolated and studied.

Cytochrome *c*, containing 0·34 per cent iron, was prepared by the method of Keilin and Hartree[1]. Pepsin, crystallized from Hopkin and Williams's pepsin

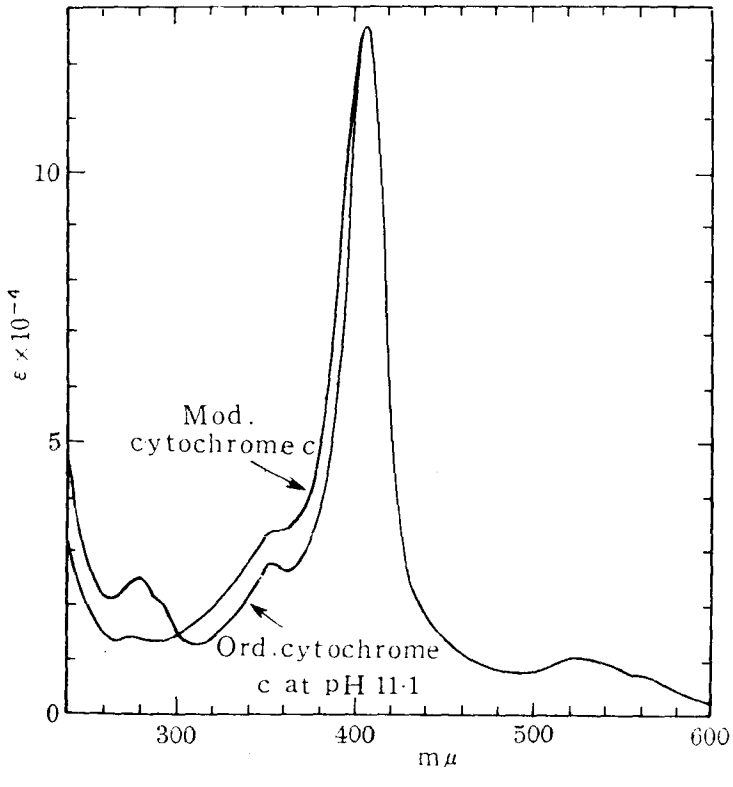

Fig. 1. Absorption spectra of ordinary ferricytochrome *c* at pH 11·1 and modified ferricytochrome *c*. The two spectra coincide between 400 and 600 mμ. Ordinate: molecular extinction coefficient; abscissa: wave-length in mμ

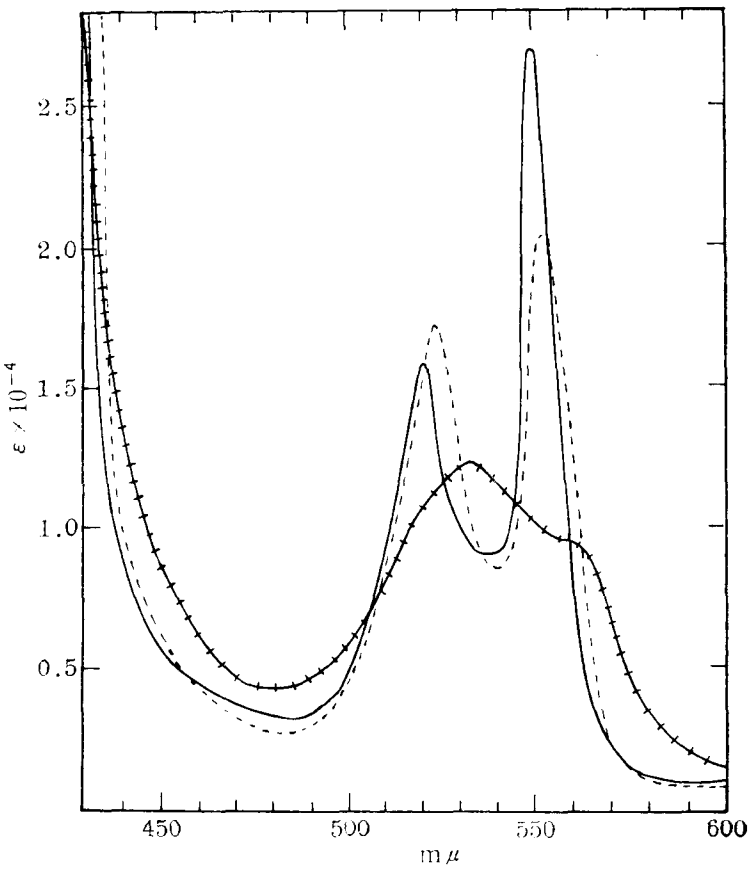

Fig. 2. Absorption spectra of modified ferrocytochrome *c* ———; cyanide compound of modified ferrocytochrome *c* – – – – –; and carbon monoxide compound of modified ferrocytochrome *c* + + +. Ordinate and abscissa as in Fig. 1

by the method of Northrop[2], had an activity[3] of 0·29 hæmoglobin unit per mgm. nitrogen.

By digestion of 1 per cent cytochrome *c* solution with pepsin (0·15 unit per gm. of cytochrome *c*), at *p*H 1·5 and 24° C. for 20 hr., a modified cytochrome *c* was obtained which was purified by fractionation with ammonium sulphate, and repeated precipitation in a dialysing sac against 0·01 *M* phthalate buffer *p*H 5, at which *p*H the solubility of modified cytochrome *c* is at a minimum. The final preparation with an iron content of 2·21 per cent resisted further attempts at fractionation. Assuming one iron atom per molecule, the molecular weight would be about 2,500.

The absorption spectrum of modified ferrocytochrome *c* was identical with that of ordinary ferrocytochrome *c* in the visible region, including the γ-band at 417 mμ, whereas the absorption spectrum of modified ferricytochrome *c* resembled most closely that of type IV of ordinary ferricytochrome *c* described by Theorell and Akesson[4], being almost identical

between 400 and 600 mμ, but considerably different in the ultra-violet region (Fig. 1). It contained no grouping or groupings that would combine with hæmatin in *N* sodium hydroxide to give a hæmochromogen even after being heated for 5 min. at 95° C. It was autoxidizable, combined with hydrogen cyanide and nitric oxide in both the ferric and ferrous state and with carbon monoxide, methyl *iso*cyanide and nitrosobenzene in the ferrous state. The absorption spectra of the carbon monoxide and hydrogen cyanide compounds with modified ferrocytochrome *c* are shown in Fig. 2. It was inactive in both the cytochrome oxidase system with *p*-phenylene diamine as substrate and in the succinic oxidase system. However, it strongly catalysed the oxidation of ascorbic acid, with the reduction of oxygen to hydrogen peroxide and evolution of carbon dioxide. Hydrogen peroxide thus produced could be demonstrated by a coupled oxidation of alcohol in the presence of catalase[5]. As an artificial ascorbic oxidase it had a Q_{O_2} (μl. oxygen per mgm. dry weight per hr.) at *p*H 7·2 and 39° C. of about 10,000. Hydrogen cyanide inhibited this catalysed oxidation of ascorbic acid by combining with modified ferricytochrome *c*, thus preventing its reduction, while carbon monoxide, by combining with modified ferrocytochrome *c*, prevented its oxidation. The carbon monoxide inhibition was somewhat light-sensitive. This reaction was also inhibited by methyl *iso*cyanide and nitrosobenzene. Modified cytochrome *c* also catalysed the decomposition of hydrogen peroxide, being itself destroyed in this reaction. Cyanide inhibited this catalysed decomposition of hydrogen peroxide.

The result of this experiment agrees with an observation of Keilin and Hartree[6] that when cytochrome *c* is made autoxidizable it loses its catalytical activity in biological systems.

I wish to thank Prof. D. Keilin for his interest in this work and advice, and Dr. E. C. Slater for reading the manuscript.

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July 29.

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[2] Northrop, J. H., *J. Gen. Physiol.*, **30**, 177 (1946).
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Exogenous and Endogenous Cytochrome *c*

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Keilin (1930) first suggested that extracted cytochrome *c* might be different from the cytochrome *c* existing in the living cell. Later, Keilin & Hartree (1940) showed that exogenous cytochrome *c* is much less active catalytically than the endogenous form. This has been re-emphasized by Keilin & Hartree (1945, 1949) and by Slater (1949*b*, 1950*b*). Keilin & Hartree (1940) also pointed out that their heart-muscle preparation contained a cytochrome system in a very high degree of organization comparable to that existing in the living cell, and this was later demonstrated quantitatively by Slater (1949*b*). In the present investigation, the quantitative difference in catalytic activities between exogenous and endogenous cytochrome *c* is further examined, a qualitative difference between the two forms is demonstrated and it is also shown that by suitable means the soluble exogenous form of cytochrome *c* can be transformed into a bound form which is identical with the endogenous form in every respect. Some of the findings have been briefly reported elsewhere (Tsou, 1951*c*).

MATERIALS AND METHODS

Cytochrome c of iron contents of 0·34 and 0·43% was prepared by the methods of Keilin & Hartree (1945). Unless otherwise specified, the preparation with 0·34% iron was used.

Dihydrocozymase. Cozymase was obtained by an unpublished method of Ochoa and reduced enzymically as described by Slater (1950*a*).

Light absorption in the visible and ultraviolet regions was measured with a Beckman photoelectric spectrophotometer.

Heart-muscle preparation containing the complete cytochrome system and the enzymes responsible for the reduction of cytochrome *c* by succinate and dihydrocozymase was prepared according to the method of Keilin & Hartree (1947) as previously described (Tsou, 1951*a*).

Extraction of cytochrome c *from washed heart-muscle mince*. Samples (6 g.) of heart-muscle mince, well washed with water until haemoglobin-free, were weighed into a series of test tubes to which were added 6 ml. portions of the extracting solutions of known concentrations. The water content of the same washed muscle mince was determined by drying a separate 6 g. sample to constant weight at 100°. The mixtures were allowed to stand at room temperature overnight, filtered, and the cytochrome *c* content of the filtrates was determined as follows: to 3 ml. of the filtrate was added 0·1 ml. of diluted (1 : 10) heart-muscle preparation in 0·25 M-phosphate buffer, pH 7·3, thoroughly mixed, and the optical density (D_o) at 550 mμ. read in a 1 cm. cell against a blank containing 3 ml. of 0·1 M-phosphate buffer, pH 7·3, and 0·1 ml. of the same diluted heart-muscle preparation; 0·1 ml. of a solution containing 0·4 M-succinate and 0·05 M-cyanide was then added to both the cytochrome *c*-containing solution and the blank. After mixing, the optical density was again read until a steady value (D_r) was reached. The concentration of cytochrome *c* in the extract was given by the formula

$$\frac{D_r(3{\cdot}2/3{\cdot}0) - D_o(3{\cdot}1/3{\cdot}0)}{1{\cdot}92} \times 10^{-4}\,\text{M},$$

in which the difference ($D_r - D_o$) is corrected for dilution by the added reagents and divided by the difference between the

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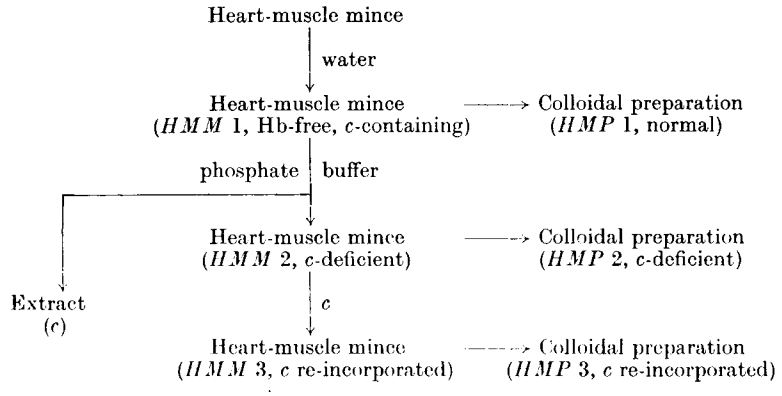
Table 1. *Relation between various colloidal heart-muscle preparations*



Hb = haemoglobin, *c* = cytochrome *c*.

molecular extinction coefficients of reduced and oxidized cytochrome *c* ($1{\cdot}92 \times 10^4$).

Cytochrome c*-deficient heart-muscle preparation* was obtained as follows: heart-muscle mince, 150 g., well washed with water, was stirred with 2 l. 0·15 M-phosphate buffer, pH 7·3, for 2–3 hr. The mince was collected on muslin, squeezed hard to remove the phosphate buffer which contained extracted cytochrome *c*, and the process repeated three times. During the last extraction, the stirring was carried out at 0° overnight. Next morning the mince was collected and squeezed as before, and washed once with water, and the cytochrome *c*-deficient muscle mince was then treated in the same way as for a normal heart-muscle preparation.

Incorporation of exogenous cytochrome c *into the cytochrome* c*-deficient muscle mince.* Heart-muscle mince (50 g.) was thoroughly washed with water and made deficient in cytochrome *c* by repeated extraction with phosphate buffer as described above. After the last extraction, the muscle mince, washed once again with water, was incubated 3 hr. at room temperature with 100 ml. of a solution containing 0·01 M-phosphate buffer, pH 7·3 and 3×10^{-5} M-cytochrome *c*. The mixture was then ground with sand and treated as for a normal heart-muscle preparation except that the precipitate obtained at pH 5·5 was washed once with water before the final suspension in phosphate buffer. The relation between various colloidal heart-muscle preparations is summarized in Table 1.

RESULTS

The cytochrome c*-cyanide complex.* The light absorption of the ferricytochrome *c*-cyanide complex in the visible region of the spectrum was determined by Potter (1941) and by Horecker & Kornberg (1946). The results obtained by these workers have been confirmed in the present study, and measurements have extended to the ultraviolet region. Incubation with 0·01 M-cyanide for 20 min. at room temperature shifts the Soret band of ferricytochrome *c* at 408 mμ. by more than 5 mμ. towards the red (Fig. 1). Cytochrome *c* preparations of both 0·34 and 0·43 % iron were used and identical results obtained.

Potter (1941) found that unlike cytochrome *c* itself, the cyanide complex is not reduced either by succinate or by dihydrocozymase in presence of the appropriate enzyme systems. It has now been found that the complex is not perceptibly reduced by *p*-phenylenediamine, ascorbic acid, cysteine, quinol or adrenaline, all of which readily reduce the untreated pigment.

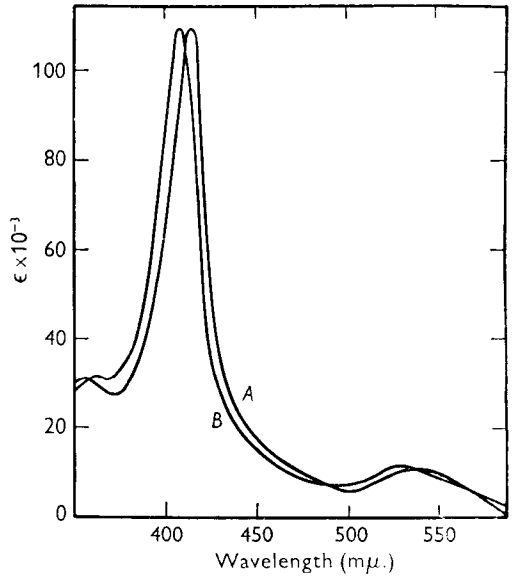


Fig. 1. The absorption spectrum of the ferricytochrome *c*-cyanide complex (*A*) compared with that of ferricytochrome *c* (*B*). Cyanide concentration, 0·05 M; readings taken after 20 min. incubation at room temperature.

The ferricytochrome *c*-cyanide complex is stable throughout the pH range 2·5–14 with the same absorption spectrum persisting. At very low pH, no change in the absorption spectrum of ferricytochrome *c* can be observed despite prolonged incubation with strong cyanide.

Behaviour of endogenous cytochrome c towards cyanide. Although the catalytic activities of endogenous and exogenous cytochrome *c* are of very different order, no qualitative difference between them has been reported. It has now been shown that whereas exogenous cytochrome *c* combines with cyanide to form a stable complex which is not reduced by the succinic dehydrogenase system, by the dihydrocozymase-diaphorase system, or by simple organic reducing agents such as *p*-phenylenediamine or ascorbic acid, endogenous cytochrome *c* is not affected by prolonged incubation with cyanide. The absorption spectrum of endogenous ferricytochrome *c* as present in the heart-muscle preparation cannot easily be seen. Consequently, any small changes in its absorption spectrum on incubation with cyanide cannot be detected spectroscopically. The lack of effect of prolonged incubation with cyanide on endogenous cytochrome *c* was demonstrated by the experiments set out in Table 2. It must be pointed out that: (1) In all cases when cytochrome *c* was in a partly oxidized and partly reduced state (tube no. 5) both exogenous and endogenous cytochrome *c* were present. Moreover, the intensity of the α-band of reduced cytochrome *c* corresponded exactly with the intensity of that of the endogenous cytochrome *c* had there been no exogenous cytochrome present, whereas the intensity of the band at 537 mμ. corresponded to that of the cytochrome-cyanide complex had there been no endogenous cytochrome present. Therefore, it was concluded that cyanide had combined exclusively with the exogenous form. (2) The addition of three drops of heart-muscle preparation to a total volume of 2 ml. makes no difference to the absorption spectrum as viewed in the microspectroscope. A mixture of buffer (2 ml.) with three drops of heart-muscle preparation plus a little solid sodium dithionite showed no absorption bands whatsoever when examined in the microspectroscope. (3) The absence of reduction of endogenous cytochrome *c* by succinate in tubes 5 and 7 was not due to the combination of cyanide with endogenous cytochrome *c*, but to the inactivation of succinic dehydrogenase by cyanide (Tsou, 1951*b*). (4) The reduction of endogenous cytochrome *c* by succinate plus three drops of untreated heart-muscle preparation was rather slow. This was expected because succinic dehydrogenase is destroyed on long incubation with cyanide (Tsou, 1951*b*), and the amount of active succinic dehydrogenase in three drops of heart-muscle preparation was much less than was originally present. In any case, the succinic dehydrogenase added was situated on one set of particles which, naturally, would react with the endogenous cytochrome *c* situated on another set of particles with some difficulty (see Keilin & Harpley, 1941).

From these results, it is clear that incubation with cyanide does not interfere with the reduction of endogenous cytochrome *c* by chemical or enzymic reactions. Under the same conditions, as shown above, the reduction of exogenous cytochrome *c* would be blocked completely within 20 min. It will also be noticed that the presence of denatured protein does not protect exogenous cytochrome *c* from the action of cyanide. Moreover, when both

Table 2. *Effect of incubation with cyanide on exogenous and endogenous cytochrome c*

(The filling of each tube is listed in the first part of the table. The final concentration of cyanide in all cases was 0·02 M which would suffice to convert practically all the exogenous cytochrome *c* into the complex within 20 min. Immediately before the experiments the cytochrome *c* used was acidified to pH 3, aerated to ensure complete oxidation and reneutralized. The tubes were allowed to stand at room temperature for 24 hr. Portions (2 ml.) were then removed from each tube, treated as described in the second part of the table and examined in the microspectroscope. The results are denoted by +, − and ± signs showing that the cytochrome *c* was in a reduced state, oxidized state and a partly oxidized and partly reduced state respectively.)

Filling of the tubes (ml.)	Tube no. 1	2	3	4	5	6	7
HMP 1 in 0·1 M-phosphate buffer, pH 7·4	–	–	–	8	8	8	8
Cyt. *c*, 0·5 mM, in 0·5% NaCl	2	2	2	2	2	–	–
KCN, 0·12 M	–	2	2	–	2	–	2
NaCl, 0·5%	–	–	–	–	–	2	2
KCl, 0·12 M	2	–	–	2	–	2	–
0·1 M-Phosphate buffer, pH 7·4	8	8	8	–	–	–	–
Denatured globin, mg.	–	–	50	–	–	–	–
Treated with	Observed reduction						
Three drops Co I H_2	–	–	–	+	±	+	+
Three drops Co I H_2 and 3 drops of *HMP* 1	+	–	–	+	±	+	+
Three drops 0·4 M-succinate	–	–	–	+	–	+	–
Three drops 0·4 M-succinate and 3 drops *HMP* 1	+	–	–	+	±	+	+
Small crystal *p*-phenylenediamine	+	–	–	+	±	+	+
Three drops 0·5 M-sodium ascorbate	+	–	–	+	±	+	+

HMP 1 = heart-muscle preparation, 1 (Table 1) undiluted. Cyt. *c* = cytochrome *c*. Co I H_2 = dihydrocozymase.

exogenous and endogenous cytochrome *c* are present, cyanide acts exclusively on the exogenous form.

As the activity of the heart-muscle preparation is rapidly destroyed at low and high pH (Keilin & Hartree, 1940), it has not been possible to determine within what pH range endogenous cytochrome *c* is resistant to the attack of cyanide. However, experiments carried out as above at pH 6·2 and 8·4 yielded identical results, hence it is concluded that endogenous cytochrome *c* does not combine with cyanide within the pH range where the colloidal succinic system is active.

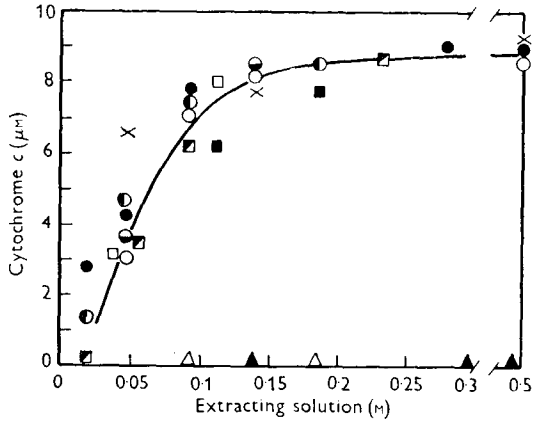


Fig. 2. Extraction of cytochrome *c* from heart-muscle mince (for method see text). ●, KCl; ○, NaCl; ◐, LiCl; ◒, KNO_3; phosphate buffer: ■, pH 6·2; ◪, 7·3; □, 8; ×, Na_2SO_4; △, boric acid titrated to pH 7·3; ▲, sucrose.

Extraction of cytochrome c *from water-washed heart-muscle mince.* In spite of very thorough washing of the heart-muscle mince with water, very little, if any, cytochrome *c* is removed, although it is well known that exogenous cytochrome *c* is extremely soluble in water. However, the bound cytochrome *c* which is present in the heart-muscle mince could be extracted by salt solutions as is shown in Fig. 2. (The bound cytochrome *c* in the final colloidal preparation was, however, not extracted by similar treatment, nor was it extracted by trichloroacetic acid in the pH range 1·5–5.) All the salt solutions tested had the same order of effectiveness except borate buffer which was completely non-effective. Non-electrolytes, such as sucrose, were without effect. It appears that the extent of extraction is mainly dependent on the anion concentration of the extracting solutions. Cytochrome *c* thus extracted has the same activity in biological systems as the pigment prepared in the usual way and, like the latter, it is also susceptible to the attack of cyanide.

Cytochrome c*-deficient heart-muscle preparation and the difference in activity between endogenous and exogenous cytochrome* c

The study of the effect of added cytochrome *c* on respiration has been somewhat handicapped by the fact that the most active cell-free preparation

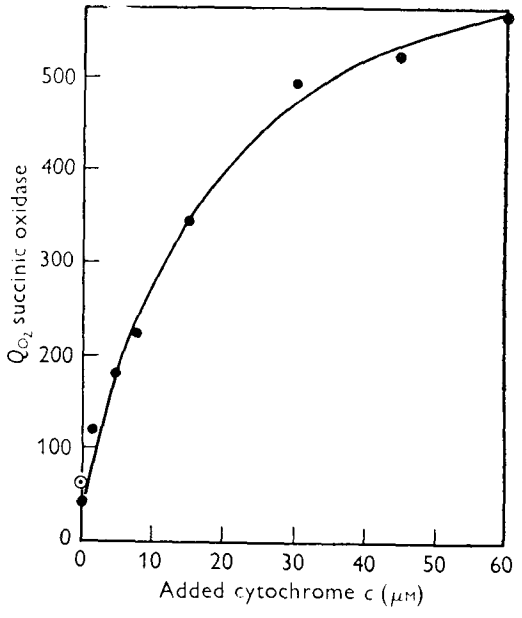


Fig. 3. Effect of added cytochrome *c* on the succinic oxidase activity of a cytochrome *c*-deficient heart-muscle preparation. Succinic oxidase activity measured at 38° as described by Slater (1949*b*). The point (⊙) shows the effect of added denatured globin. (Q_{O_2} = μl. O_2 uptake/mg. fat-free dry wt. of heart-muscle preparation/hr.)

(Keilin & Hartree, 1940), which behaves almost exactly like the natural complex oxidation system of the cell, already contains enough cytochrome *c* for its normal function. For instance, under optimal conditions, the addition to such preparations of an excess of cytochrome *c* only raises the rate of oxidation of succinate by about 40% (Slater, 1949*b*). Although exogenous cytochrome *c* is freely soluble in water, the particle-bound endogenous pigment of the heart-muscle preparation survived thorough washing with water and remained firmly attached to the particles. So far it has not been possible to remove the bound cytochrome *c* from the heart-muscle preparation without harming the latter.

By extraction with phosphate buffer, it has now been found possible to remove the greater part of the bound cytochrome *c* from heart-muscle mince (*HMM* 1, Table 1) without affecting the ability of the final preparation (*HMP* 2, Table 1) to oxidize succinate when cytochrome *c*, extracted and purified in the usual way, is added. On reduction with

succinate, such a preparation (*HMP* 2) shows strong *a* and *b* bands but a very weak *c* band. The cytochrome *c* content, estimated as described by Slater (1949*b*), is about 0·1 μmol./g. fat-free dry weight of heart-muscle preparation as compared with 0·76 of a normal preparation. The Q_{O_2} (μl. O_2 uptake/mg. fat-free dry wt./hr.) of this preparation (*HMP* 2) in the oxidation of succinate with different concentrations of added cytochrome *c* is given in Fig. 3.

It will be seen that the cytochrome *c*-deficient preparation, in presence of excess added cytochrome *c*, has a $Q_{O_2} = 565$, which is of the same order as that of a normal preparation. Although the addition of denatured globin has little effect on the activity of the cytochrome *c*-deficient preparation, the addition of 6×10^{-5}M-cytochrome *c* to this preparation caused a more than 10-fold increase as compared with 1·3- to 1·4-fold in a normal preparation which in absence of any added cytochrome *c*, already has a Q_{O_2} about 450. The addition of about $2{\cdot}7 \times 10^{-5}$M-cytochrome *c* is necessary in order to raise the Q_{O_2} of this preparation (*HMP* 2, Table 1) to about 450 which is the activity under the same experimental conditions of a normal preparation containing about $2{\cdot}3 \times 10^{-7}$M-endogenous cytochrome *c*. These results are similar to those obtained by Keilin & Hartree (1949) while using certain modified types of preparation and support their conclusion that cytochrome *c* acts as a specific oxido-reduction catalyst and not as an indifferent protein in the succinic oxidase system.

The cytochrome oxidase activity of the cytochrome *c*-deficient preparation was about double that of a normal heart-muscle preparation (Table 3). This is probably due to the removal of a considerable amount of indifferent protein by phosphate buffer extraction. However, the succinic dehydrogenase activities of these two preparations were about the same (Table 3), which indicates that besides indifferent protein, part of the dehydrogenase had also been removed or destroyed.

Table 3. *Comparison of a normal and a cytochrome c-deficient heart-muscle preparation*

(The succinic oxidase and succinic dehydrogenase activities were measured as described by Slater (1949*b*). The cytochrome oxidase activities were measured by both the Keilin & Hartree method (1940) and the Slater method (1949*a*). Q_{O_2} = μl. O_2 uptake/mg. dry wt. of enzyme preparation.)

	Heart-muscle preparations	
	Normal	Cytochrome *c*-deficient
Succinic dehydrogenase (Q_{O_2})	250	290
Cytochrome oxidase (Q_{O_2}):		
Keilin & Hartree	1470*	2800*
Slater	3300	6550
Succinic oxidase (Q_{O_2})	590	565

* These values are based on total dry weight, whereas the others are based on fat-free dry weight of the enzyme preparations.

Transformation of exogenous cytochrome c into the bound form

By incubation of the cytochrome *c*-deficient heart-muscle mince (*HMM* 2, Table 1) with cytochrome *c* as described above, a final colloidal preparation (*HMP* 3, Table 1) was obtained into which cytochrome *c* was re-incorporated (Table 4). This cytochrome *c* was firmly bound to the colloidal particles and could not be removed by thorough washing with water, it therefore behaved like endo-

Table 4. *Relation of cytochrome c content and succinic oxidase activity of different heart-muscle preparations*

(Three different preparations* were used: a normal preparation (normal), a cytochrome *c*-deficient preparation (*c*-def.) and a preparation with re-incorporated cytochrome *c* (*c*-re-incorp.). The determination of cytochrome *c* content and the measurement of succinic oxidase activity were carried out as described by Slater (1949*b*). The amount of enzyme preparation used per flask and the concentration of added cytochrome *c* in each case are listed in the table.)

		Heart-muscle preparations		
		Normal	*c*-def.	*c*-re-incorp.
Fat-free dry wt./ml. (mg.)		19·2	22·6	17·4
Cytochrome *c* content (μmol./g. fat-free dry wt. of preparation)		0·76	0·092	0·62
Amount of preparation/flask (mg.)		0·96	1·13	0·87
Concentration of endogenous cytochrome *c* under experimental conditions (μM)		0·22	0·031	0·164
	Added cytochrome *c* (μM)			
Succinic oxidase (Q_{O_2})	0	455	62	355
	5	520	200	470
	15	—	370	—
	60	620	555	605

* All these three preparations were made from the same batch of heart-muscle mince (see Table 1).

genous cytochrome *c* in a normal heart-muscle preparation. Moreover, like the normal, but unlike the cytochrome *c*-deficient preparation, this preparation with re-incorporated cytochrome *c* (*HMP* 3, Table 1) already contained enough cytochrome *c* for the near optimal functioning of the whole succinic oxidase system. Thus the addition of excess cytochrome *c* raised the succinic oxidase activity from a Q_{O_2} of 355 to 605, as is shown in Table 4.

The turnover number, i.e. the oxygen uptake/min./oxygen equivalent of cytochrome *c* (Keilin & Hartree, 1940), of bound cytochrome *c* can be calculated from the data given in Table 4. It was obtained by dividing the succinic oxidase activity without added cytochrome *c* (in μl. O_2 uptake/min./mg. fat free dry weight) of the preparation by its bound cytochrome *c* content (in oxygen equivalent/mg. fat-free dry weight of the total preparation). The values were 1780 and 1710 for the bound cytochrome *c* present in the normal preparation (*HMP* 1) and in the preparation with re-incorporated cytochrome *c* (*HMP* 3, Table 1) respectively. The turnover number of exogenous cytochrome *c*, on the other hand, was 24 (obtained from Table 4 by dividing the increase in oxygen uptake of the cytochrome *c*-deficient preparation by the oxygen equivalent of cytochrome *c* added). Similar results were obtained with dihydrocozymase as substrate. The activity of the re-incorporated cytochrome *c* is thus of the same order as that of endogenous cytochrome *c* but far greater than that of exogenous cytochrome *c*. Experiments similar to those of Table 2 also showed that the re-incorporated cytochrome *c*, like endogenous cytochrome *c*, does not combine with cyanide.

DISCUSSION

In the preparation with re-incorporated cytochrome *c* (*HMP* 3, Table 1), and in the normal preparation (*HMP* 1, Table 1) the activity of bound cytochrome *c* is lower than the figure given by Keilin & Hartree (1940) for the cytochrome *c* of yeast cells, i.e. turnover number, 3850. This is understandable, since it can hardly be expected that the heart-muscle preparation has retained completely the high state of organization and efficiency of the living cell (Slater, 1949*b*), or that cytochrome *c* present in the heart-muscle preparation is entirely in the endogenous form. In support of this view, the reducible fraction of cytochrome *c* in the heart-muscle preparations, after prolonged incubation with cyanide, is always somewhat less than the total cytochrome *c* present.

Quantitative differences between exogenous and endogenous cytochrome *c* have been previously pointed out. Keilin & Hartree (1940) showed that even under optimal conditions, the exogenous pigment is only about one-third as active as the endogenous form. Keilin & Hartree's results on the exogenous pigment were obtained by adjusting the concentrations of heart-muscle preparation and of added cytochrome *c* so as to make the latter the limiting factor. On the other hand, the activity of the endogenous pigment was measured under such conditions that there was no reason to suppose that cytochrome *c* was the limiting factor. In the present work, it is demonstrated that using approximately the same amount of heart-muscle preparations (Table 4) the difference in activities of the two forms of cytochrome *c* is far greater. Slater (1950*b*) has shown that exogenous cytochrome *c* is reduced by succinate or dihydrocozymase in presence of the appropriate enzyme systems at least 1200 times more slowly than the endogenous pigment. In addition, exogenous cytochrome *c* is reduced by cysteine (Keilin, 1930) or ascorbic acid (Slater, 1949*a*) more readily than the endogenous enzyme. However, their different behaviour towards incubation with cyanide seems to be the first record of any qualitative difference. In view of the fact that cytochrome *c* occupies an important position in the electron transferring chain of biological oxidation, and since it is the only component of the cytochrome system so far prepared in a soluble and pure form, a considerable amount of work has been done to link various oxidative enzymes to the cytochrome system via exogenous cytochrome *c*. It would appear, therefore, that the demonstration of any qualitative difference between the two forms of the pigment might assist towards a clearer understanding of the part played by cytochrome *c* in biological oxidation. The present work emphasizes the necessity for caution in interpreting results obtained with exogenous cytochrome *c*, and for confirmation of the results, whenever possible, with an enzyme material, such as the heart-muscle preparation of Keilin & Hartree, where the cytochromes are in a more organized state. Experiments with a cytochrome *c*-deficient heart-muscle preparation indicate that it is much more difficult to 'saturate' the oxidase with exogenous cytochrome *c* than with the endogenous form (Fig. 3). In other words, the presence of 0·22 μM-endogenous cytochrome *c* in the manometer flask is already enough for the near optimal function of the succinic oxidase system of a normal preparation, whereas a far greater concentration of exogenous cytochrome *c* was necessary before the curve in Fig. 3 (where a cytochrome *c*-deficient preparation was employed) approached an asymptotic value.

There are two possible explanations for the differences between exogenous and endogenous cytochrome *c*. (1) During extraction, cytochrome *c* was slightly modified so that the soluble 'pure' cytochrome *c* obtained was chemically different from the endogenous pigment present in the living