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Cytochrome and cytochrome oxidase

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[Plate 1]

I. Introduction

It was previously shown that the addition of cytochrome *c* to a heart-muscle preparation greatly increases its power of catalysing the oxidation of such substances as *p*-phenylene diamine, hydroquinone, cysteine and ascorbic acid. It was demonstrated that the oxidations of these substances, which can be used for the detection and estimation of intracellular oxidase, are catalysed not directly by the oxidase but through the co-operation of cytochrome. The only direct function of the oxidase, so far ascertained, is the oxidation of reduced cytochrome, and the enzyme can therefore be considered as cytochrome oxidase (Keilin and Hartree 1938*a*). Several properties which have been previously ascribed to it do not belong, however, to this enzyme alone but are the properties of the complete cytochrome-oxidase system.

The object of this paper is the study of the mechanism of oxidation and reduction of cytochrome in order to determine the properties and the nature of cytochrome oxidase.

II. Heart-muscle preparation and some of its properties

The best material for this study is the heart-muscle preparation obtained by the method described in a previous paper (Keilin and Hartree 1938*a*). This preparation is free from haemoglobin but contains several enzymes

and carriers which are either insoluble or intimately bound to insoluble material.

On spectroscopic examination it shows five diffuse bands in the visible region: (1) a narrow band at about 605 mμ which belongs probably to a small fraction of cytochrome *a* in the reduced state; (2) a band at 567 mμ which corresponds to the fused α-bands of oxidized cytochrome components *b* and *c*; (3) a band at 529 mμ which represents the fused β-bands of the same two components; and finally (4) and (5) two bands at 495 and 455 mμ which belong to a flavoprotein compound (VII, fig. 2, Plate 1).*

The appearance of this absorption spectrum does not change even when the suspension is kept in pure nitrogen or when treated with cyanide which shows that it is completely devoid of reducing substances and metabolites. This is, moreover, corroborated by the facts that this preparation has no oxygen uptake and does not reduce methylene blue anerobically.

On addition of sodium succinate to this preparation cytochrome becomes reduced, and on shaking the mixture with air it becomes rapidly re-oxidized. Tested manometrically, the suspension now shows a rapid oxygen uptake, which is greatly accelerated by the addition of cytochrome *c* (10^{-3} to 10^{-4} M), and when tested anaerobically it rapidly reduces methylene blue.

Although this muscle preparation contains other enzymes, including a fairly strong catalase, the only reactions which take place on addition of succinic acid are: (1) its oxidation to fumaric acid, and (2) the formation of malic from fumaric acid catalysed by fumarase; both reactions being reversible.

Other enzymes are practically inactive owing to the absence of their specific substrates and of coenzymes essential for their activities.

III. Absorption spectrum of cytochrome in heart-muscle preparation

Spectroscopic examination of the reduced cytochrome in this preparation shows a remarkably strong band *a* which is more intense than the bands *b* and *c*. There is an obvious deficiency of cytochrome *c*, as its band, instead of being stronger than that of the component *b*, is of approximately the same intensity.

In addition to the bands *a*, *b* and *c*, which are the α-bands of the corre-

* These bands are hardly perceptible in preparations showing reduced cytochrome which may be due either to the reduction of flavoprotein itself or to the changes in the background taking place during the reduction of cytochrome.

sponding components of cytochrome, and the band *d* representing the β-bands of at least the components *b* and *c*, our preparation also shows the three Soret or γ-bands of cytochrome (fig. 1). These bands can easily be seen in a dilute muscle preparation suspended in a 70 % solution of cane sugar or in glycerine and examined with a strong light passing through a suitable colour filter, such as Wratten *C* and *D* filters or a solution of ammoniacal copper sulphate. Bands γ can also be made clearly visible by peptizing muscle preparations with sodium taurocholate or desoxycholate which make the preparation transparent and suitable for spectroscopic examination.*

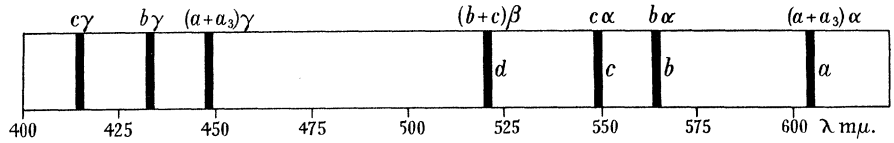

FIG. 1. Diagram showing positions of absorption bands of reduced cytochrome components together with old and new notation of bands.

The γ, like the other bands of cytochrome, can be greatly intensified and sharpened when the suspension is cooled to the temperature of liquid air. For this purpose a very dilute suspension of muscle preparation reduced with sodium succinate is poured into the flattened end of a tube where it forms a layer 2 mm. thick. The tube is cooled in liquid air and rapidly examined spectroscopically.

The γ-bands appear in approximately the same positions as the corresponding bands in the transparent thoracic muscles of bees as described by Warburg and Negelein (1931), namely, 448, 432 and 415 mμ (fig. 1 and I, fig. 2, Plate 1). That the latter two γ-bands belong respectively to the components *b* and *c* of cytochrome we are able to prove now by showing that each of these bands always appears and disappears simultaneously with the corresponding α-band and independently of other bands. The true nature of the first γ-band (448 mμ) will be discussed later.

It is important to remember that the γ-bands in our preparation are only visible when cytochrome is reduced. In the oxidized state these bands are invisible, the first two γ-bands being probably very weak and masked by general absorption in this region, while that of *c* is shifted towards the blue end of the spectrum (405 mμ) and lies outside the region suitable for direct spectroscopic observation.

* The use of bile salts, which is extensively applied to the study of visual purple, was recommended to us by Dr E. L. Smith.

IV. Effects of respiratory inhibitors on cytochrome

We shall now examine the changes in the absorption spectrum of cytochrome brought about by the addition of substances like CO, KCN, NaN_3, H_2S, NH_2OH, NaF and peroxides which are known as reversible inhibitors of cellular respiration, or as substances combining reversibly with such haematin compounds as haemoglobin and methaemoglobin, peroxidase and catalase. Throughout this study, unless otherwise stated, cytochrome in the heart-muscle preparation was reduced with sodium succinate. In the description which follows heart-muscle preparation showing the absorption spectrum of reduced cytochrome will be referred to as "reduced cytochrome", the absorption bands as "bands", and oxidized and reduced components of cytochrome will be designated by the corresponding letters and signs of valency, writing, for instance, $c^{\cdots}$ and $c^{\cdot\cdot}$ instead of oxidized and reduced components c of cytochrome. A convenient notation for the absorption bands of cytochrome which is proposed in the present paper (see Table I, p. 177) consists in the customary use of the letters α, β and γ to denote the three bands of each component together with prefixes a, b and c to differentiate between the cytochrome components. Thus, the α-, β- and γ-bands of the component c will be respectively referred to as $c\alpha$, $c\beta$ and $c\gamma$.

We can mention at this stage that a critical analysis of the experimental results now to be described has led us to the conclusion that there exists in aerobic cells a new cytochrome component which we have denoted as a_3 (Keilin and Hartree, 1938*b*).

Description of Plate 1

Fig. 2. Diagrammatic figure of reconstructed absorption spectra of reduced and oxidized cytochrome in heart-muscle preparation, untreated and treated with different respiratory inhibitors. Bands of each spectrum are represented as seen in three different depths of preparation; their ratio, according to bands, being approximately as follows: 8 for α and β bands; 2·5 for $a\gamma$ and $a_3\gamma$; 1·5 for $b\gamma$ and $c\gamma$. Band $c\gamma$ is seen more distinctly because deficiency of this component in this preparation is compensated by addition of soluble c. Notation of bands given above and below diagram refers only to spectra I and VII respectively. Spectrum II shows $a_3^{\cdot\cdot}$ CO α-band at 590 mμ, while band $a_3CO\gamma$ appears at 432 mμ, where it overlaps and intensifies band $b\gamma$. Band $a\gamma$ is seen at 452 mμ. In spectrum III bands α and γ of a_3 are invisible owing to its oxidation and combination with inhibitors. Spectra IV, V and VI are clearly explained in the figure. Spectrum VII, representing oxidized cytochrome reconstructed from different depths of preparation, shows remains of reduced $a\alpha$-band, two diffuse parahaematin bands ($p\alpha$ and $p\beta$) of compounds b and c, and two diffuse bands (f) of a flavoprotein compound, which become hardly perceptible in preparations showing reduced cytochrome.

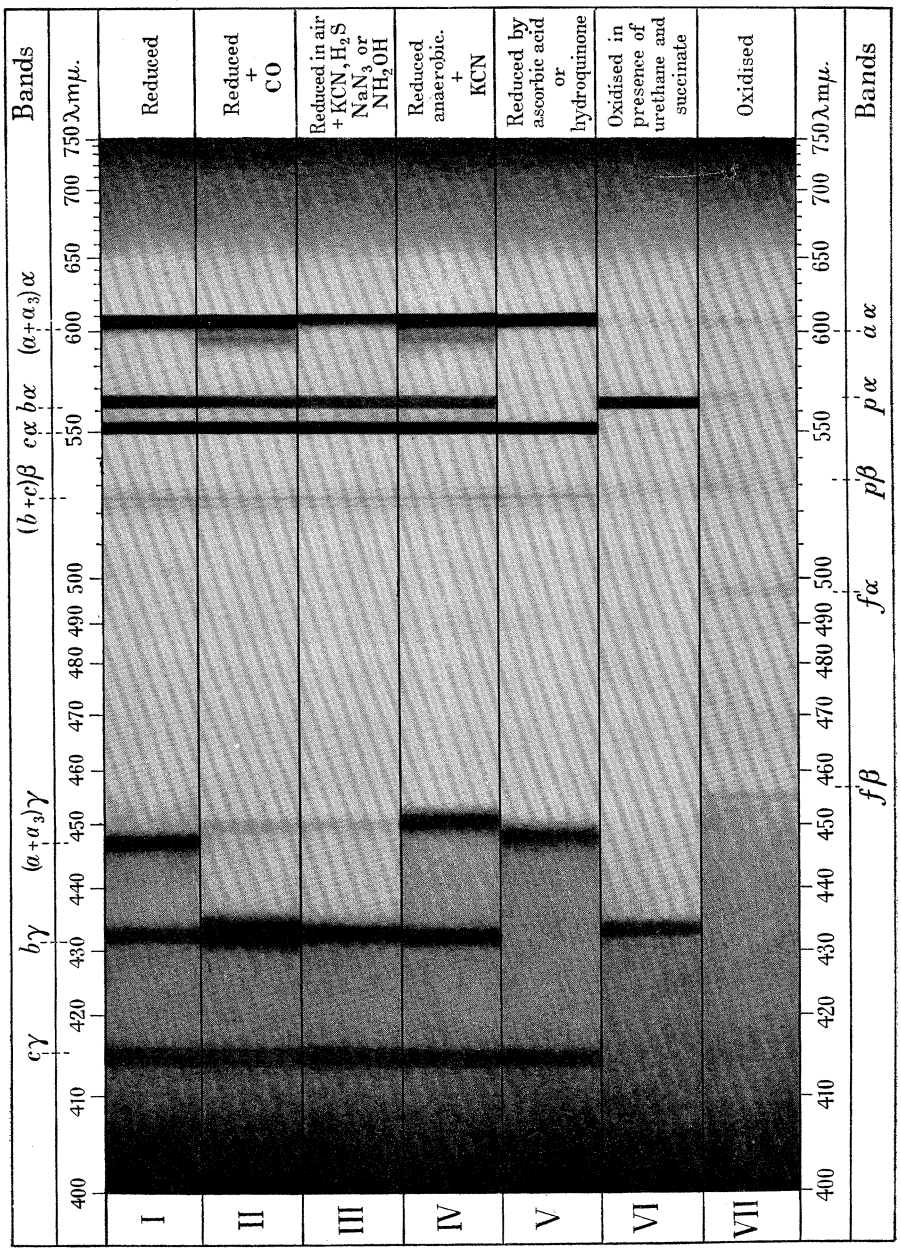

FIG. 2

(*Facing p.* 170)

(1) *Effect of* CO

On treating cytochrome with carbon monoxide, the bands of $b\alpha$, $c\alpha$ and $(b+c)\,\beta$ remain unmodified, while the band $a\alpha$ undergoes a very marked change. It becomes wide, asymmetric and spreads towards the blue end of the spectrum, partly obliterating the space which separates it from the band $b\alpha$. It shows, moreover, an additional reinforcement at about 590 mμ, while its long wave side remains sharply defined. The strong band $a\gamma$ (448 mμ) disappears and is replaced by a very pale band at 452 mμ, while $b\gamma$ becomes much stronger and sharper (II, fig. 2, Plate 1). The correct interpretation of these changes can only be arrived at by studying the effect of other inhibitors on cytochrome.

(2) *Effect of* KCN

The effect of KCN on reduced cytochrome can be studied in two ways: *aerobically* in open tubes and *anaerobically* in Thunberg tubes.

In *aerobic* experiments, a small amount of cyanide is added to the muscle preparation containing sodium succinate and showing reduced cytochrome. Spectroscopic examination of the mixture does not reveal any marked change in the α-bands of all the components. On the other hand, $a\gamma$ disappears and is replaced by a weak band at 452 mμ, while $b\gamma$ and $c\gamma$ remain unchanged (III, fig. 2, Plate 1). On treating this preparation with CO no changes can be noticed in the spectrum. This shows that KCN, which affects only the band $a\gamma$, prevents the formation of the CO compound which is so easily formed in absence of KCN. Moreover, on addition of KCN to the preparation treated previously with CO, the spectrum due to the presence of CO disappears and is replaced by that which is obtained in presence of KCN.

In *anaerobic* experiments, the muscle preparation (4 c.c.) containing sodium succinate and a drop of caprylic alcohol is put into a modified Thunberg tube the hollow stopper of which receives 0·5 mg. of solid KCN. The tube is evacuated and, after standing for 15 min., its contents are mixed. Spectroscopic examination now reveals a very marked change in the appearance of band $a\alpha$ which broadens towards the blue end of the spectrum and has a distinct appearance of being double. Band $a\gamma$ becomes wider and somewhat more diffuse lying now at 450 mμ, while the other bands remain unchanged (IV, fig. 2, Plate 1). On letting air in, the appearance of the spectrum changes at once and reverts to that of reduced cytochrome treated with KCN in an open tube. Similar results can be obtained also in an open tube provided cytochrome is reduced not by the succinic system but by an excess of $Na_2S_2O_4$.

No clear changes can be observed on addition of KCN to oxidized cytochrome which is due to the diffuse appearance of its spectrum.

(3) *Effects of* H_2S, NaN_3 *and* NH_2OH

These substances act on cytochrome in the same way as KCN but only under aerobic conditions. When added to reduced cytochrome a marked change is seen only in the band $a\gamma$ which is replaced by a weak and diffuse band at 452 mμ, while other bands remain unmodified (III, fig. 2, Plate 1). Like KCN these substances prevent the formation of a compound with CO. This inhibition is, however, not as complete as that produced by KCN. In fact, even if allowed to stand in presence of an excess of these substances, the CO compound is gradually formed.

(4) *Effect of* NaF

Sodium fluoride cannot be considered as an inhibitor of the oxidase. On the contrary, in presence of sodium succinate, it inhibits not the oxidation but the reduction of cytochrome. This inhibition, as can be demonstrated spectroscopically and manometrically, is not very marked. Thus, NaF in concentrations of 0·003, 0·01 and 0·02 M inhibits the oxygen uptake by only 15, 42 and 70 % respectively. On treating the preparation with NaF the $a\gamma$-band (448 mμ) is replaced by a weak band lying at 452 mμ, while other bands of cytochrome remain unchanged. On standing, this band very soon reverts to its usual intensity and position which shows that the effect of NaF on cytochrome is not as marked as that of NaN_3 or NH_2OH.

(5) *Effect of peroxides*

The effect of hydrogen peroxide on cytochrome in heart-muscle preparation is very difficult if not impossible to observe owing to the presence in our preparation of a sufficiently active catalase to decompose rapidly all the H_2O_2 added to it. The addition of cyanide or of hydroxylamine to poison the catalase does not help in this case because these inhibitors, as has just been shown, also react with cytochrome. Although we have failed so far to discover a reaction between cytochrome and H_2O_2 the experiments with hydrogen peroxide were not devoid of interest. They have shown, for instance, that a great excess of H_2O_2 added to the muscle preparation containing sodium succinate and 0·003 M cyanide did not oxidize the reduced components *a*, *b* and *c*. This seems to indicate that if

H_2O_2 is formed within living cells as a result of some primary oxidation reactions, it would not oxidize reduced cytochrome. The previously observed oxidation of extracted cytochrome *c* by H_2O_2 may be due to a peroxidatic reaction catalysed either by *c* itself or by some haematin derivatives resulting from their partial destruction by H_2O_2. It is also possible that all the components of cytochrome in heart-muscle preparation, including *c*, are absorbed to, or combined with, some insoluble material, which protects them from oxidation and destruction by H_2O_2.

In order to observe an effect of a peroxide on cytochrome an attempt was made to replace H_2O_2 by ethylhydroperoxide which is not decomposed by catalase. A slight complication in these experiments is the partial oxidation of cytochrome. The experiments properly controlled seem, however, to show that the addition of ethylhydroperoxide causes the fading of the band $a\gamma$ and is responsible for the delay in the formation of a compound with carbon monoxide. In other words the effect of ethylhydroperoxide on cytochrome is somewhat similar to, although less marked than, that of KCN, NaN_3 or NH_2OH.

V. Existence of component a_3

The experiments related in the previous chapter show that the absorption spectrum of reduced cytochrome in the heart-muscle preparation is affected by substances like CO, KCN, NaN_3, H_2S, NH_2OH, NaF and C_2H_5OOH which are known either as respiratory inhibitors or as substances combining with different haematin compounds. According to the effects which they produce on cytochrome these substances can be grouped into three categories:

(1) CO which affects the bands $a\alpha$, $a\gamma$ and $b\gamma$ and modifies them in a similar manner whether the reaction takes place anaerobically or in presence of a small concentration of oxygen.

(2) KCN which affects the bands $a\alpha$ and $a\gamma$ but only under strictly anaerobic conditions.

(3) KCN, NaN_3, H_2S, NaF, NH_2OH and C_2H_5OOH which markedly affect only the band $a\gamma$ under aerobic conditions; the presence of oxygen being essential for this reaction.

In all these experiments only the bands $a\alpha$, $a\gamma$ and $b\gamma$ of cytochrome appear to be affected by the respiratory inhibitors. At first sight these experiments seem to suggest that the changes observed in the absorption spectra of cytochrome are simply due to the formation of compounds

between the components a or b and the substances mentioned above. Careful analysis of these results, however, does not support this view. In fact, it is difficult to understand why the formation of a compound between a and KCN or NaN_3, for instance (III, fig. 2, Plate 1), requires the presence of oxygen and affects only the band $a\gamma$, while the band $a\alpha$, being unchanged, clearly shows that component a remains still in the reduced state. We can hardly ascribe these changes to the combination of a with KCN without postulating the formation of a peculiar compound, one portion of which, responsible for band $a\alpha$, is reduced, while the other, responsible for band $a\gamma$, is oxidized. The existence of such a compound is, moreover, difficult to reconcile with the formation of another KCN compound which only takes place under strictly anaerobic conditions (IV, fig. 2). Finally, it is difficult to explain how CO can affect simultaneously both α- and γ-bands of one component (a) and only the γ-band of another component (b) (II, fig. 2).

These facts make it very difficult if not impossible to accept the view that the observed spectroscopic changes are due to the formation of compounds between the components a or b and the respiratory inhibitors.

The only satisfactory explanation of these changes which takes into account the results of all our experiments consists in postulating the existence of a new component, the absorption bands of which have not been hitherto recognized because they lie too closely to the corresponding bands of component a. We shall designate this new component as a_3 in order to distinguish it from components a_1 (589 mμ) and a_2 (630 mμ) which are known to occur only in a few bacteria devoid of component a.* The absorption bands $a\alpha$ and $a\gamma$, which have hitherto been considered as belonging to component a only, are in fact the fused bands of two components a and a_3. While the band $a_3\alpha$ is weak compared with $a\alpha$ and lies probably at about 600 mμ, the band $a_3\gamma$, lying at 448 mμ, is, on the contrary, much stronger than $a\gamma$, which occupies only the long wave margin of $a_3\gamma$ and lies at 452 mμ (fig. 3).

If the component a_3 now undergoes oxidation, while the component a remains reduced, the band α (605 mμ) will hardly change its intensity but the band γ (448 mμ) will disappear leaving a pale shading at 452 mμ. The component a_3 differs, moreover, from a in several other properties. Thus while a is not autoxidizable and does not react with respiratory inhibitors, a_3 is autoxidizable and forms with these inhibitors definite and more or less stable compounds.

* The nature of these two components (a_1 and a_2), which have certain properties in common with a_3, and their relationship with the latter, will be discussed in a separate paper.

With CO, the component a_3 combines only in the divalent state forming a compound with two bands shifted towards the blue end of the spectrum. Thus, the $a_3\alpha$-band becomes distinctly visible lying at about 590 mμ and remaining still fused with and broadening the $a\alpha$-band. The $a_3\gamma$-band moves from 448 to 432 mμ, where it overlaps and intensifies the $b\gamma$-band (II, fig. 2 and fig. 3). The weak $a\gamma$-band becoming thus unmasked can be seen at about 452 mμ.

With KCN a_3 combines both in the divalent and trivalent states, forming two distinct compounds. The former compound, which can only be obtained under strictly anaerobic conditions, shows a very characteristic absorption spectrum (IV, fig. 2, Plate 1). This derivative is, however, unstable and easily undergoes autoxidation to a trivalent a_3 CN compound.

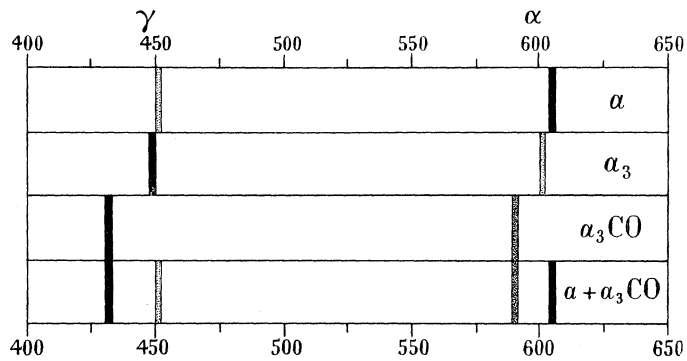

FIG. 3. Diagram showing relationship between bands of components a and a_3 and effect of CO on a_3.

In the trivalent state a_3 has a very great affinity for KCN. When reduced cytochrome exposed to air is treated with cyanide, a_3 undergoes oxidation and readily combines with KCN forming a compound ($a_3^{\cdots}$ CN) which cannot be easily reduced by the succinic system. Cyanide can be considered therefore as an inhibitor stabilizing this component in the oxidized state. The great affinity of $a_3^{\cdots}$ for cyanide and the stability of the $a_3^{\cdots}$ CN compound explain the remarkable property of a_3 to undergo oxidation on the addition of KCN, even in the presence of only traces of oxygen, when all the other components of cytochrome are completely reduced. It must be remembered that trivalent a_3 whether free or combined with cyanide does not show distinct absorption bands in any region of the visible spectrum. The formation of the $a_3^{\cdots}$ CN compound must therefore be accompanied by the disappearance of the $a_3\alpha$- and $a_3\gamma$-bands. The disappearance of the $a_3\alpha$-band would hardly affect the intensity of the band

$a\alpha$ of which it represents only a small fraction. On the contrary, the disappearance of the $a_3\gamma$-band reduces the strong γ-band (448 mμ) to a very weak band lying at 452 mμ and belonging to the component a. The existence of a_3 clearly explains the changes in the absorption spectrum of reduced cytochrome taking place in presence of KCN and traces of oxygen.

Other substances like H_2S, NaN_3, NH_2OH and NaF combine only with trivalent a_3 forming with it a series of compounds analogous to $a_3^{\cdots}$ CN. The affinities of H_2S and NaN_3 for a_3 are, however, somewhat lower than that of KCN but much higher than those of NH_2OH and NaF. The compounds of a_3 with the latter two substances are unstable, and on standing in presence of a reducing system rapidly undergo dissociation followed by the reduction of a_3 as indicated by the reappearance of the band $a_3\gamma$ (448 mμ).

Although normally the components a and a_3 are found in the same state of valency, conditions can be realized when one of the components is oxidized while the other is reduced. Thus, in presence of inhibitors like KCN, H_2S, NaN_3, etc., and traces of oxygen, the component a_3, as we have seen, undergoes oxidation and combines with these inhibitors while component a remains reduced. On the other hand, on shaking the preparation containing CO with air, the component a may undergo oxidation while a_3 remains almost completely reduced and combined with CO. All this strongly supports the view that we are dealing here with two distinct but closely allied and intimately connected compounds a and a_3.

VI. Properties of cytochrome components

Before discussing the possible functional relationships between a_3 and the other components of cytochrome we shall briefly record their properties. The positions of the bands of these components together with those of the two spectroscopically recognizable derivatives of a_3 are given in Table I. This table also shows the new notation for the absorption bands proposed in this paper compared with the notation previously used. It can be mentioned here that so far no β-band could be detected in the components a and a_3 or in derivatives of the latter. This band is probably masked by the α-band of components b or c.

(1) *Component a*

The main properties of this component have already been considered in the section dealing with the effects of inhibitors on cytochrome. We may add here that this component is a haem-protein compound which is in-

soluble or intimately bound to the insoluble material of the cell. It is thermolabile, undergoing destruction above 52° C. It does not combine with KCN, CO and other inhibitors, and is very easily reduced by sodium succinate. Its absorption bands are as follows:

$a\alpha$—605 mμ, $a\beta$—?, and $a\gamma$—452 mμ.

TABLE I

Components of cytochrome	Notation of bands		Position in mμ
	Previously used	Proposed now	
a	a	$a\alpha$	605
	—	$a\beta$	?
	—	$a\gamma$	452
a_3	—	$a_3\alpha$	600
	—	$a_3\beta$	?
	—	$a_3\gamma$	448
b	b	$b\alpha$	564
	d	$b\beta$	530
	—	$b\gamma$	432
c	c	$c\alpha$	550
	d	$c\beta$	521
	—	$c\gamma$	415
a_3·· CO	—	a_3COα	590
	—	a_3COβ	?
	—	a_3COγ	432
a_3·· CN	—	a_3CNα	590
	—	a_3CNβ	?
	—	a_3CNγ	450

(2) *Component* a_3*

This component, as we have seen, is thermolabile and combines with the respiratory inhibitors such as CO, KCN, H_2S, NaN_3, NH_2OH and probably with NaF and C_2H_5OOH. Its absorption bands are:

$a_3\alpha$—600 mμ, $a_3\beta$—?, and $a_3\gamma$—448 mμ.

* For reference to components a_1 and a_2, see footnote, p. 174.

(3) *Component b*

This component is also a thermolabile haem-protein compound showing three absorption bands in the reduced states: $b\alpha$—564 mμ, $b\beta$—530 mμ and $b\gamma$—432 mμ. It is also insoluble or bound to insoluble material of the cell and cannot therefore be isolated in an unmodified or undenatured state. The so-called "extracted cytochrome *b*" of Yakushiji and Mori (1937) is probably only a haematin derivative of *b* mixed with some denatured proteins and cannot therefore be identified with native *b*. Cytochrome *b* differs from components *a* and *c* in being distinctly autoxidizable. In fact, if *b* is reduced by substances like ascorbic acid or adrenaline which do not require to be activated by specific dehydrogenases, it can be reoxidized in air even in presence of a great excess of cyanide. If *b* is reduced by succinic acid, its autoxidation in presence of cyanide can only be observed when the dehydrogenase system is inhibited by the addition of sodium malonate. It is important to remember, however, that cytochrome *b* is much more easily reduced by the succinic dehydrogenase system than by the reducing substances like ascorbic acid, adrenaline, hydroquinone or *p*-phenylenediamine (V, fig. 2, Plate 1). This indicates that component *b* does not play an important role in the catalytic oxidation of these compounds. The integrity of the component *b* in the heart-muscle preparation treated in different ways cannot therefore be recognized from the mere fact that the catalytic oxidation of *p*-phenylenediamine by this preparation proceeds normally.

There are, on the other hand, clear indications that the activity of the component *b* is intimately connected with those of dehydrogenases. The aerobic oxidation of substances like succinic acid depends not only on the integrity of components *a*, a_3 and *c* but also on that of *b*. This is clearly shown by the behaviour of cytochrome *b* in presence of narcotics (Keilin 1925) and can be illustrated by the following experiment: 3 c.c. of heart-muscle preparation, containing a small amount of succinic acid and showing rapid oxidations and reductions of cytochrome, receives 0·3 c.c. of a 30 % solution of urethane. On shaking the suspension with air, components *a*, a_3 and *c* are rapidly oxidized and do not undergo reduction even on standing, while *b* remains completely reduced even on vigorous shaking with air and clearly shows its bands $b\alpha$, $b\beta$ and $b\gamma$ (VI, fig. 2, Plate 1). Urethane, therefore, like all other narcotics, inhibits the reduction of *a*, a_3 and *c* and the oxidation of *b*. According to Tamiya and Ogura (1937), of the three components of cytochrome, *b* reacts directly with the dehydrogenase system, *c* with the oxidase and oxygen, while *a* forms a link between

b and *c*, being reduced by the former and oxidized by the latter. The peculiar effect of urethane on component *b* they ascribe to its inhibition of the activity of *a*, thus breaking the link between *b* and *c*. Considering that narcotics inhibit to a certain degree the anaerobic reduction of methylene blue by the same systems, the supposition of these workers would naturally imply that this reaction also proceeds through at least two components of cytochrome, *a* and *b*. So far, there is no direct evidence supporting this view. In fact, even in the presence of narcotics and sodium succinate *b* can undergo oxidation, when the activity of succinic dehydrogenase is abolished by the addition of oxalacetic acid. Moreover, when *b* is reduced by substances not activated by dehydrogenases, narcotics do not prevent its reoxidation. It is conceivable that the effect of narcotics consists in bringing about the formation of a not easily dissociable complex composed of dehydrogenase, substrate and cytochrome *b* and so making it inaccessible to the portion of the system reacting with oxygen.

It is important to mention here that narcotics, which hardly affect the oxidation of *p*-phenylenediamine, inhibit more strongly the aerobic oxidation of succinic acid by the cytochrome system than its anaerobic oxidation by methylene blue. It appears, therefore, that there is no strict parallelism between the property of the muscle preparations to reduce cytochrome *c* and their property to reduce methylene blue. In fact, from the heart-muscle suspension treated in different ways, preparations can be obtained which in presence of succinic acid hardly reduce cytochrome *c* and are yet capable of rapidly oxidizing the substrate by means of methylene blue.* This seems to indicate that the activated molecules of substrate do not react with cytochrome *c* directly but through another and more labile compound which may be either cytochrome *b* itself or a hitherto unrecognized component of the system.

(4) *Component c*

Cytochrome *c*, unlike the other components, is thermostable and comparatively resistant to denaturation. It is soluble or perhaps more easily detached from the insoluble material of the cell and can therefore be extracted and very easily prepared in a pure state (Keilin and Hartree 1937). Its spectrum is composed of three bands: $c\alpha$—550 mμ, $c\beta$—521 mμ and $c\gamma$—415 mμ. Within the physiological range of pH it is not autoxidizable and does not combine with CO. In the oxidized state it does not combine with KCN, H_2S or NaN_3. It combines very readily, however, with NO.

* The reduction of cytochrome by the succinic and other dehydrogenase systems will be discussed in a special paper dealing with this subject.

At pH below 4 and especially above 12 it becomes autoxidizable and combines with CO forming a compound very sensitive to light. Oxidized *c* is easily reduced both by activated metabolites and by a great variety of reducing substances such as cysteine, adrenaline, ascorbic acid and *p*-phenylenediamine. Reduced *c* is, on the other hand, very rapidly oxidized when brought into contact with even a very dilute preparation of heart muscle.

On applying to the study of cytochrome *c* a greatly improved spectrophotometric technique, Altschul and Hogness (1938) believed to have found that this component combines with CO throughout the entire range of pH. It is, however, difficult to draw such a conclusion from the curves given by these workers, as the slight effect of CO which they reveal at pH 7·96 may be due to its combination either with a small fraction of denatured *c* or with some other haematin impurity in their preparation. That CO does not combine with unmodified cytochrome *c* below pH 13 can be demonstrated by the following manometric experiments.

80 c.c. of strong cytochrome *c* solution obtained from two horse hearts is precipitated by 4 vol. of cold acetone, resuspended in a small volume of water and dialysed against 0·5 % NaCl. A slight precipitate is filtered off giving 20 c.c. of a clear and very strong solution containing 0·0565 mg. of cytochrome Fe per c.c.; 4·4. c.c. of this solution being equivalent to 100 cu. mm. of CO.

The manometric experiments are carried out in Barcroft differential manometers at 19° C. The right-hand flask of one receives 4·4 c.c. cytochrome *c*, 1·6 c.c. 2 M phosphate buffer pH 7, and 5 mg. of dry $Na_2S_2O_4$ in a dangling tube suspended from the central potash tube. The left-hand flask receives 4·4 c.c. water, 1·6 c.c. buffer and 5 mg. $Na_2S_2O_4$ in a dangling tube. The manometer is evacuated, washed three times with pure N_2 and filled with pure CO. After temperature equilibration, the dangling tubes are dislodged and the manometer is read every 5 min.

TABLE II. THEORETICAL ABSORPTION, 100 CU. MM.

Exp. no.	pH	cu. mm. CO absorbed
1	7	11
2	10	21
3	13	104

Two other experiments were carried out in a similar way but in more alkaline solutions, one at pH 10 and the other at pH 13. The results of these experiments, which are given in Table II, show that only at pH 13

does cytochrome c'' combine with CO. The small uptakes at lower pH are probably due to a slight denaturation of cytochrome *c* by acetone.

A significant contribution to our knowledge of the structure of the cytochrome *c* molecule has been made by Theorell (1938) who was able to correlate its stability with a unique double thioether linkage between the protein and the prosthetic group.

VII. Relationship between components *a* and a_3

The invariable association of components *a* and a_3 clearly indicates that they are in some way intimately connected. This is, moreover, supported by the existence of a definite proportionality between these two components; namely, the stronger the absorption bands of *a* in a preparation the more intense are the bands of spectroscopically visible derivatives of a_3. In other words a_3 can only be detected easily in cells showing a high concentration of *a*.

That these two components have an identical haem nucleus is shown by the similarity of their absorption spectra and, more so, by the fact that on denaturation with alkali and treatment with pyridine both components yield only one spectroscopically recognizable haemochromogen. This haem nucleus is probably very similar to that of chlorocruorin.

The proteins of these compounds also show great similarities in their properties. Thus, both are insoluble or bound to insoluble material of the cell, and both exhibit the same degree of fragility when treated with alkali, acids, alcohols and acetone, dried in air or warmed above 52° C. Both compounds are, if anything, less stable than most enzymes.

On denaturation by any treatment the α-band of these compounds moves towards the blue end of the spectrum to occupy a position at about 583 mμ. This derivative (of *a* and a_3) has the properties of an ordinary haemochromogen, namely, it is autoxidizable and combines with CO. The native proteins of *a* and a_3 must, however, differ in some respects so as to form with the same haem nucleus two compounds having distinct properties. The differences may also be of such a nature as not to exclude the interconvertibility of these two components.

VIII. Component a_3 and cytochrome oxidase

(1) *Evidence for identity of a_3 and oxidase*

As to the physiological significance of a_3, the evidence which supports the view of its identity with cytochrome oxidase can be summarized as follows:

(*a*) Like oxidase a_3 is thermolabile being affected by temperatures above 52° C.

(*b*) Its oxidations and reductions can be followed spectroscopically within living cells by examination of band $a_3\gamma$ (448 mμ).

(*c*) It is rapidly and efficiently reduced by biological reducing systems.

(*d*) It combines reversibly with such substances as KCN, H_2S, NaN_3, NH_2OH and CO, which are known as reversible inhibitors of oxidase activity and of the respiration of cells.

(*e*) The two bands of its compound with CO ($a_3^{\cdot\cdot}$ CO) namely, α—590 mμ and γ—432 mμ, occupy approximately the same positions as the corresponding bands in the photochemical absorption spectrum obtained by Warburg and his co-workers (see Warburg 1932).

(*f*) Although KCN combines both with divalent and trivalent a_3, the divalent $a_3^{\cdot\cdot}$ KCN rapidly undergoes autoxidation, while the trivalent $a_3^{\cdot\cdot\cdot}$ KCN is very stable and does not easily undergo reduction. The inhibition of the catalytic properties of this component is therefore due to its stabilization in the ferric state.

(*g*) The component a_3 can be observed not only in a heart-muscle preparation, but also in fresh thoracic muscles of bees and other insects, in baker's yeast and in aerobic bacteria.

The view which identifies the component a_3 with cytochrome oxidase corroborates, therefore, the results previously obtained by Warburg and his co-workers who have shown that a haematin with a definite absorption spectrum plays an essential role in cellular respiration.

(2) *Some difficulties in the identification of a_3 with cytochrome oxidase*

Although the hypothesis identifying the component a_3 with cytochrome oxidase is supported by strong evidence, several observations have been made in the course of this investigation which do not appear to corroborate this view. Before examining these observations in detail we can say at once that some of the difficulties so raised are only apparent and may readily be explained. On the other hand, certain difficulties which still remain require further investigation before the true nature of a_3 and its relationship to cytochrome oxidase can be definitely established.

A. *Oxidation of components a, b, and c in presence of $a_3^{\cdot\cdot}$ CO compound.*

On shaking with air a preparation containing succinic acid and treated with CO the components *a*, *b* and *c* undergo oxidation while the spectrum of $a_3^{\cdot\cdot}$ CO remains still clearly visible. This experiment seems to indicate that the components *a*, *b* and *c* could not possibly be oxidized by a_3 since

it remained combined with CO. A careful analysis of this experiment has convinced us now that this objection is only apparent. The absorption spectrum of $a_3^{\cdot\cdot}$ CO which remains visible during the oxidations of the other components indicates only that the rate of reduction of a_3 and formation of $a_3^{\cdot\cdot}$ CO is faster than the rate of its oxidation. If oxidized $a_3^{\cdot\cdot\cdot}$ is capable of oxidizing the three other components, the small amount of $a_3^{\cdot\cdot\cdot}$, which is being constantly formed under these conditions from the $a_3^{\cdot\cdot}$ CO compound, could easily account for the oxidation of components *a*, *b* and *c*, even though the bands of $a_3^{\cdot\cdot}$ CO remain clearly visible.

B. *Functional relationship between* a_3 *and c.*

If the component a_3 is the oxidase it should be possible to demonstrate spectroscopically its reaction with at least one of the non-autoxidizable cytochrome components. As cytochrome *c* was proved to be essential for the catalytic activity of the oxidase (Keilin and Hartree 1938*a*) a large amount of the divalent $c^{\cdot\cdot}$ added to the muscle preparation should directly or indirectly reduce the component $a_3^{\cdot\cdot\cdot}$. Experimentally this problem can only be approached by bringing together under strictly anaerobic conditions a heart-muscle preparation and a solution of reduced cytochrome *c*, both completely free from reducing substances and metabolites. In order to reduce cytochrome *c* for this purpose a strong solution of it in 0·1 M Na_2HPO_4 is mixed with platinum black and treated with a current of hydrogen. When cytochrome *c* is completely reduced the platinum black is filtered off and the solution kept in a Thunberg tube free from oxygen.

The experiment is carried out in a modified Thunberg tube provided with a hollow stopper and an additional rotating side bulb. The tube receives 3 c.c. of fresh heart-muscle preparation, the hollow stopper receives 0·5 c.c. of reduced *c* (10^{-3} M) and about 1 c.c. of chromous chloride solution is placed in the side bulb. The tube is evacuated, washed out with purified nitrogen, and shaken in order to complete the removal of oxygen. On spectroscopic examination the preparation shows only the faint bands of oxidized cytochrome. On mixing the contents of the tube with that of the hollow stopper, a certain portion of cytochrome *c* becomes oxidized by some traces of oxygen still present in the muscle preparation but neither the band $a\alpha$ nor the band γ of $a+a_3$ can be seen. The same result was obtained with a dilute preparation of heart muscle suspended in cane sugar and therefore more suitable for the study of the γ-bands. Here also no traces of the band $a\gamma+a_3\gamma$ could be detected while a portion of *c* remained in the

reduced state. These experiments gave therefore no evidence supporting the view that cytochrome $c^{\cdot\cdot}$ can be directly or indirectly oxidized by $a_3^{\cdot\cdot\cdot}$.

If, on the other hand, the Thunberg tube is evacuated and filled with pure CO the addition of reduced c to the muscle preparation induces the formation of the $a_3^{\cdot\cdot}$ CO compound. One of the difficulties connected with this experiment is the tendency of $a_3^{\cdot\cdot\cdot}$, in presence of CO, to undergo gradual reduction with the formation of $a_3^{\cdot\cdot}$ CO even without the addition of the reduced c. This reduction is, however, accelerated when reduced c is added to the preparation.

In view of certain technical difficulties connected with such experiments we cannot yet definitely exclude the possibility that divalent $c^{\cdot\cdot}$ may yet be oxidized by trivalent $a_3^{\cdot\cdot\cdot}$, though so far the experiments have not supported such a possibility.

C. *Effect of light on the* $a_3^{\cdot\cdot}$ CO *compound.*

Considering that the CO inhibition of respiration is light sensitive, the effect of light on $a_3^{\cdot\cdot}$ CO is of special interest. In our experiments the spectrum of $a_3^{\cdot\cdot}$ CO is not affected even when a thin and almost transparent layer of our preparation is exposed to a strong source of light. In this respect $a_3^{\cdot\cdot}$ CO behaves very differently from alkaline cytochrome c or from pyridine haemochromogen, the CO compounds of which, when exposed to light, liberate the CO and revert to the corresponding haemochromogens. In the case of the haemochromogen, however, the CO competes not with oxygen but with pyridine:

$$\text{Haem}\begin{matrix}\text{Pyridine}\\ \text{Pyridine}\end{matrix} + \text{CO} \underset{\text{light}}{\overset{\text{dark}}{\rightleftharpoons}} \text{Haem}\begin{matrix}\text{Pyridine}\\ \text{CO}\end{matrix} + \text{Pyridine}$$

The effect of light on $a_3^{\cdot\cdot}$ CO probably becomes apparent only during the catalytic activity of a_3 in presence of oxygen which rapidly oxidizes the reduced a_3 thus preventing its reaction with CO.

D. *Respiratory mechanism in absence of cytochrome a.*

Another interesting point to be considered is the respiratory mechanism of some cells devoid of cytochrome a and yet showing a strong inhibition by KCN as well as a light-insensitive inhibition by CO. In these cells it is conceivable that one of the other components of cytochrome such as b or c may have an easily autoxidizable derivative with the properties and functions of a_3 and capable therefore of replacing it. Alternatively, the true cytochrome oxidase may be replaced in these cells, as in cells com-

pletely devoid of cytochrome (spores of *Bacillus subtilis*), by an entirely different enzyme, e.g. a copper-protein compound. This would explain the KCN and light-insensitive CO inhibition of their respiration.

IX. Other suppositions as to the nature of cytochrome oxidase

Before we discuss the results of the experiments described in previous chapters, a few other suggestions as to the nature of cytochrome oxidase may be briefly considered. Thus, the oxidase might be: (1) a compound very similar to components a and a_3 but present in a concentration too low to be seen spectroscopically, (2) a compound of iron and protein devoid of porphyrin and therefore spectroscopically invisible, (3) an active protein devoid of prosthetic group and functioning as cytochrome oxidase, (4) a compound of copper and protein, or (5) identical with catalase.

The first supposition is inaccessible to experimental test and should be seriously considered only if all other explanations are disproved.

The second supposition, when tested experimentally, gave very inconsistent results. The iron present in the heart-muscle preparation was found to belong mainly to haematin compounds showing different degrees of stability.

The third supposition would imply the existence of an active protein capable of combining with at least one cytochrome component and activating it. On combining with component a, such a protein may form a complex within which this component reacts with oxygen, carbon monoxide and other respiratory inhibitors forming a series of derivatives spectroscopically recognizable. This supposition is, however, not easily accessible to experimental text.

The two remaining possibilities (4 and 5) should, however, be considered in more detail.

A. *Oxidase and intracellular copper-protein*

The supposition that the enzyme may be a copper-protein compound is supported by the following considerations:

(*a*) The very wide if not universal distribution of intracellular copper.

(*b*) The facility with which copper salts oxidize reduced cytochrome.

(*c*) The necessity of copper for development of "indophenol oxidase" and of cytochrome a demonstrated by culture experiments on yeast and feeding experiments with mice (Elvehjem 1931; Cohen and Elvehjem 1934; Yoshikawa 1937).

(*d*) A certain similarity in properties between cytochrome oxidase and

polyphenol oxidase which, as has recently been shown, is a copper-protein compound (Kubowitz 1937; Keilin and Mann 1938).

Owing to its insolubility, attempts to fractionate and purify cytochrome oxidase have met with little success. So far, the highest activity reached for the oxidation of *p*-phenylenediamine in presence of an excess of cytochrome *c* at 38° C corresponds to $Q_{O_2} = 1400$. This preparation contained 0·0129 % of non-dialysable copper which can be considered as a fairly high copper content for an insoluble biological material. However, neither the activity nor the copper content have been sufficiently increased to permit the drawing of any definite conclusions as to the relation of the oxidase to a copper-protein compound.

All the above considerations show that the possibility of the copper nature of cytochrome oxidase is based only on indirect evidence. Against this view, moreover, is the fact that the CO compound of copper-protein enzymes is not light sensitive, while the CO poisoning of cells containing cytochrome *a* is sensitive to light.

That copper plays an active part in the formation of cytochrome oxidase has already been mentioned although the mechanism of such activity is not yet understood.

B. *Oxidase and catalase*

Considering that catalase is always found in cells containing cytochrome and that it is present in the heart-muscle preparations it may be asked whether this enzyme has any connexion with cytochrome oxidase.

At first sight such a supposition seems to be plausible, especially as it is well known that catalase, which is a haematin compound, is affected by and combines with all the inhibitors which affect the oxidase reaction of the cell. This supposition was not, however, confirmed by further study of the problem and is shown to be untenable from the following considerations:

(*a*) Catalase may be present in a high concentration in cells, e.g. red blood corpuscles of mammals, which are completely devoid of cytochrome oxidase.

(*b*) Catalase, unlike the oxidase, can be obtained in clear solution.

(*c*) Unlike the oxidase, it shows great resistance to organic solvents such as alcohol and chloroform which are used for its purification.

(*d*) Catalase, which is a methaemoglobin-like compound, cannot be reduced by biological systems such as succinic dehydrogenase. It remains in the ferric state even in the presence of strong reducers such as $Na_2S_2O_4$,

although it is easily reduced by H_2O_2 and reoxidized by molecular oxygen (Keilin and Hartree 1938*c*).

(*e*) It does not oxidize reduced cytochrome *c* which is at once oxidized by the muscle preparation.

(*f*) While 10^{-4} M hydroxylamine inhibits the catalase activity of the muscle preparation by 95 %, it has no effect on the oxidase activity of the same preparation.

All this clearly shows that catalase and cytochrome oxidase are two distinct intracellular catalysts.

X. Discussion

The study of heart-muscle preparations reveals the existence of a new haematin compound, a_3,* in addition to the components *a*, *b* and *c* of cytochrome. The existence of this compound was not previously recognized because in the oxidized state its absorption bands are invisible while in the reduced state they coincide with the corresponding bands of the component *a*. The evidence for its existence is obtained mainly from the study of the effects of certain respiratory inhibitors on the absorption spectrum of cytochrome. As these substances produce definite modification in the bands of component *a* it appears at first sight that they react directly with *a*. Careful analysis of these reactions reveals, however, that the absorption bands $a\alpha$ and $a\gamma$ do not belong to the component *a* only but to a mixture of *a* and a_3. The effects of the respiratory inhibitors on the appearance of the bands of cytochrome *a* is not due to their reaction with *a* but to the compounds they form with a_3.

One of the main properties of a_3 is its marked autoxidizability, and in this respect it differs from other components of cytochrome which are considered as non-autoxidizable haematin compounds. The term "non-autoxidizable" should not, however, be taken in too strict a sense because cytochrome *b*, as we have seen, is to a great extent autoxidizable, although the rate of its oxidation by molecular oxygen is not as rapid as that of a_3 or of an ordinary haem or haemochromogen. The components *a* and *c* can also undergo a slow autoxidation even in presence of cyanide. Furthermore, a solution of pure cytochrome *c* cannot be preserved in the reduced state unless it is protected from oxygen. The autoxidation of *a* and *c* is, however, too slow to have any biological significance, while the autoxidation of *b*, although slower than that of a_3, may play a certain role in biological oxidation reactions.

* For reference to components a_1 and a_2 see footnote, p. 174.

It may be mentioned here that the property of "non-autoxidizability" is very rare among haematin compounds. It is known only in a few natural compounds such as haemoglobin, helicorubin (> pH 7) and cytochrome, and so far has never been obtained artificially. In fact, haem and haemochromogen compounds prepared from every type of porphyrin or combined with any nitrogenous substance, as well as the compounds obtained by denaturation of haemoglobin and of cytochrome, are invariably autoxidizable and combine with KCN and with CO.

The mere observation that an intracellular haematin compound is autoxidizable is, therefore, not sufficient to identify it with cytochrome oxidase. It must at the same time react with all the specific inhibitors and be influenced by all the factors which affect the oxidase reaction of the cell. It must also react with at least one of the non-autoxidizable components of cytochrome, of which *c* has already been found essential for the catalytic activity of the oxidase (Keilin and Hartree 1938*a*).

So far, the component a_3 seems to be the only intracellular substance which answers most, although apparently not all, of these requirements.

In fact, the component a_3 is thermolabile and is affected by all treatments such as drying, freezing, acetone, alcohol, acids, alkali, etc., in the same way and to the same degree as is the oxidase activity of the preparation. It is autoxidizable and can be seen to undergo oxidations and reductions during the catalytic oxidation of metabolities. It forms two compounds with KCN: a divalent compound which is easily autoxidizable, and a trivalent compound which does not easily undergo reduction. In the trivalent state it combines with H_2S, NaN_3 and NH_2OH which, like KCN, stabilize it and prevent its reduction. In the divalent state it combines with CO forming a compound with bands occupying the same positions (590 and 432 mμ) as the corresponding bands in the photochemical absorption spectrum obtained by Warburg and his co-workers. The component a_3 is therefore the only intracellular haematin compound which may be responsible for this photochemical absorption spectrum.

All this strongly supports the view of the identity of the component a_3 with cytochrome oxidase. This conclusion is, moreover, in agreement with the main results obtained by Warburg who has demonstrated that a haematin compound, which with CO gives an absorption spectrum showing bands at about 590 and 432 mμ plays an essential role in cellular respiration. Component a_3 can therefore be identified with Warburg's respiratory or oxygen transporting enzyme.

There are still, however, a few points which require further consideration before the view identifying a_3 and oxidase can be definitely accepted.

Outstanding among them is a direct and convincing demonstration of the reaction between $c^{\cdot\cdot}$ and $a_3^{\cdot\cdot\cdot}$. Our failure to demonstrate this reaction may be due either to some unrecognized technical difficulties or perhaps to the fact that this reaction is more complicated than it appears and takes place only in presence of molecular oxygen which would reoxidize a_3 as rapidly as it is reduced by $c^{\cdot\cdot}$. Hence the reduced $a_3^{\cdot\cdot}$ could hardly be expected to be visible spectroscopically.

Two other difficulties (see p. 184) which have not yet received a satisfactory explanation are (1) that strong light has no effect on the spectrum of $a_3^{\cdot\cdot}$ CO, and (2) that there exist cells, the respiration of which is poisoned by KCN and by CO, and yet they are devoid of cytochrome or of its component a.

In spite of these few points which still remain open for further investigation, the view of the identity of cytochrome oxidase with a_3 seems to be sufficiently well founded to be accepted as the best explanation of all the observed facts discussed in this paper.

As to the relationship between components a and a_3, the invariable coexistence and proportionality of these two compounds, the identity of their haem nuclei, a certain similarity in their absorption spectra and in the properties of their proteins suggest that they must be intimately connected if not interconvertible. It is only by a careful study of the effects of various factors on what was always considered as the absorption spectrum of cytochrome a that the existence of a_3 was revealed. The fact that the main portion of the band lying at 448 mμ belongs to the reduced component a_3 shows that the concentration of this component within the cell is of the same order of magnitude as that of other components of cytochrome.

If we accept now the identity of a_3 with cytochrome oxidase, it is immaterial whether a_3 is considered as an enzyme and a, b and c as carriers, or whether a_3 is considered as one of the cytochrome components working in a catalytic chain with the other components. In fact, every component of cytochrome can be defined as a catalytically active conjugated protein with a haematin as its active or prosthetic group. Each of these components can, therefore, be considered as an oxidizing enzyme in the same sense as the yellow enzyme.

It must be remembered that all the oxidizing properties of the heart-muscle preparations which can be measured, including the ordinary indophenol reaction, do not belong to one substance, such as a_3, but are the results of the catalytic activity of the whole cytochrome system.

Cytochrome components form within the cell a highly active catalytic

system which by utilizing molecular oxygen can easily oxidize to water certain hydrogen atoms in the substrate molecules activated by specific dehydrogenase systems.

It is conceivable, however, that in addition to this function some of the cytochrome components may also act as carriers between different dehydrogenase systems, thus playing a part in some anaerobic oxido-reduction reactions taking place in aerobic cells.

XI. Summary and conclusions

1. Heart-muscle preparation can be obtained, exhibiting strong cytochrome oxidase and succinic dehydrogenase activities and showing not only the usual α- and β-bands of components *a*, *b* and *c*, but also the three Soret or γ-bands lying at 448, 432 and 415 mμ.

2. In addition to components *a*, *b* and *c* of cytochrome this preparation reveals the existence of a component a_3, the bands of which are fused with those of *a*. While the main portion of the α-band (605 mμ) belongs to component *a*, most of the γ-band (448 mμ) belongs to component a_3. The bands at 432 and 415 mμ belong to components *b* and *c* respectively.

3. The existence of a_3 can be demonstrated also in fresh untreated thoracic muscles of insects, in baker's yeast and in strictly aerobic bacteria.

4. Component a_3 undergoes reduction under the same conditions as the other components of cytochrome.

5. Like components *a* and *b*, a_3 is thermolabile and very sensitive to organic solvents, alkali and acids. However, unlike the other components of cytochrome, a_3 is very easily autoxidizable, and in the reduced state combines with CO forming a compound which shows two absorption bands: α—590 mμ and γ—432 mμ.

6. Component a_3 combines with KCN both in the divalent and trivalent states forming two different compounds, of which the former is very easily autoxidizable, while the latter is stabilized in the oxidized state and cannot be easily reduced.

7. In the trivalent state a_3 combines with H_2S, NaN_3, NH_2OH and possibly with NaF and C_2H_5OOH, exhibiting in this respect a close analogy with methaemoglobin, catalase and peroxidase.

8. The main properties of component a_3, such as its thermolability, autoxidation, reduction by biological systems, formation of reversible compounds with KCN, H_2S, NaN_3 and NH_2OH, which stabilize it in the trivalent state, and with CO, which stabilizes it in the divalent state, strongly support the view identifying this component with cytochrome

oxidase. The catalytic activity of this enzyme depends, however, entirely on co-operation with components *a*, *b* and *c* of cytochrome.

9. Cytochrome oxidase or component a_3 may therefore be identified with the respiratory or oxygen-transporting enzyme of Warburg and his co-workers.

10. There still remain, however, a few weak points in the theory identifying a_3 with cytochrome oxidase. Outstanding among them is the failure to demonstrate a direct or indirect reduction of the oxidized component a_3 by addition of reduced *c* under strictly anaerobic conditions and in complete absence of other reducing substances.

11. The co-existence of *a* and a_3, their proportionality, the identity of their haem nuclei, the great similarity in some properties of their protein suggest that these two components are very intimately connected if not interconvertible.

12. The concentration of a_3 within the cell is of the same order of magnitude as that of any other component of cytochrome.

13. Component *b* is autoxidizable but not as efficiently as a_3. It does not combine, however, with CO or other respiratory inhibitors.

14. Narcotics, which in presence of biological reducing systems inhibit the reduction of *a*, a_3 and *c*, inhibit, on the contrary, the oxidation of *b*.

15. Contrary to the statement by other workers it has been demonstrated manometrically that cytochrome *c* does not combine with CO, within the physiological range of pH.

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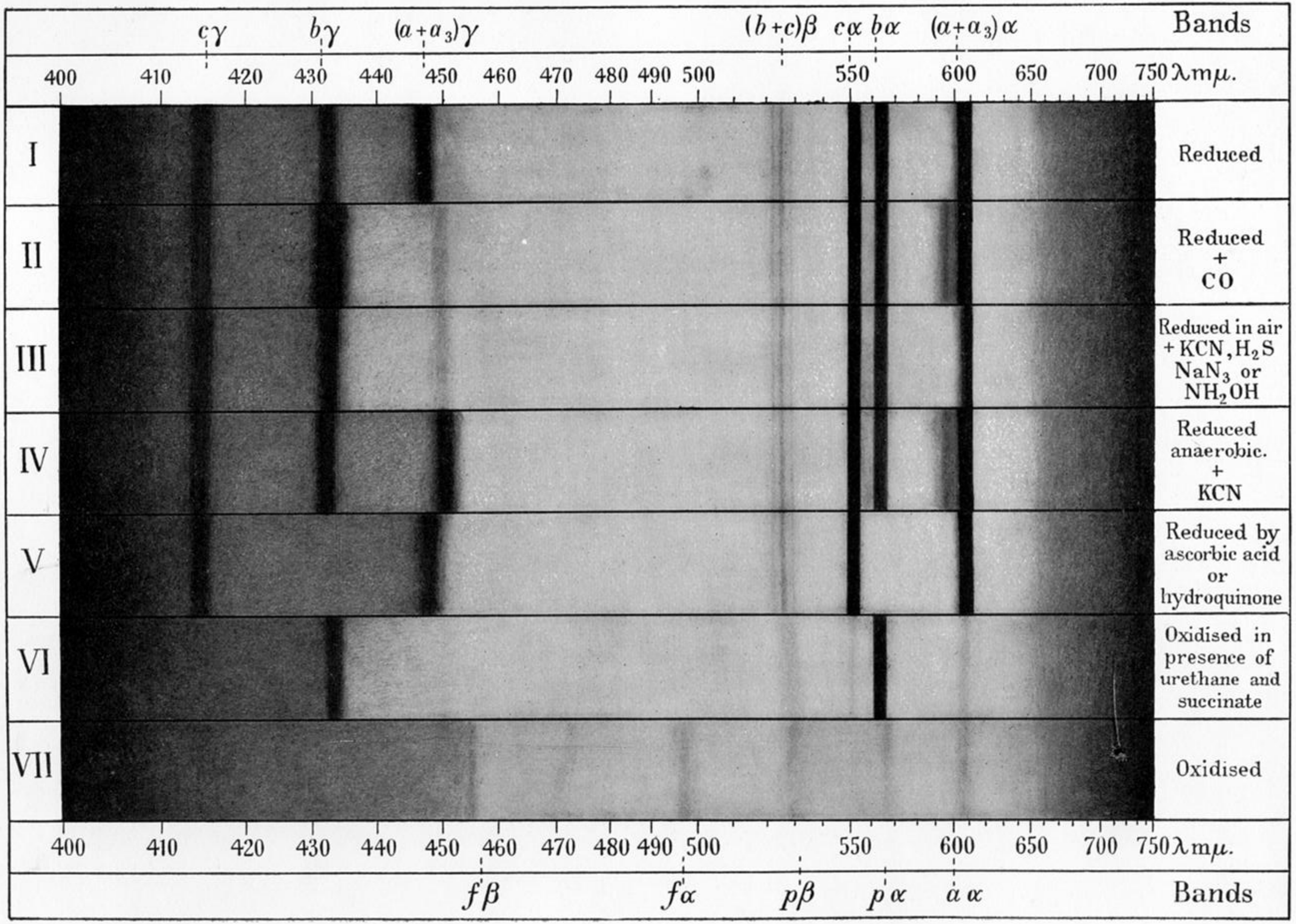

FIG. 2

(Facing p. 170)