The preparation and some properties of cytochrome \( f \)

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Hill & Scarisbrick (1951) obtained from green leaves two new soluble cytochrome components, called \( b_3 \) and \( f \). The latter appeared to be a constituent of the chloroplast, and its properties have now been examined further with particular reference to a comparison with cytochrome \( c \).

A method is described for preparing the component from the leaves of parsley (Petrolinum sativum). Spectroscopic examination of acetone powders of elder and parsley leaves showed that the total cytochrome present bore a similar ratio to total chlorophyll in both species, and about one-third of the total could be obtained in solution by the method described. A similar component was observed spectroscopically in acetone powders of four species of algae.

In solution at pH 7 ferrocytochrome \( f \) does not combine with oxygen or carbon monoxide. The spectrum resembles that of ferrocytochrome \( c \), but the bands are sharper and are moved towards the red. At pH 7·4 ferricytochrome \( f \) has a parahematin type of spectrum. Using ferrocyanide-ferricyanide mixtures as pH buffers in vacuo, the characteristic potential \( E'_o \) at pH 6 to 7·7 and 30° was 0·365 V. In the alkaline range a basic dissociation of ferrocytochrome (pK = 8·4) occurs to give \( E'_o/pH = -0·06 \) V.

The component is denatured by heating above 58° C or at pH below 4 or above 11 to give an autoxidizable haemochromogen. The haem of cytochrome \( f \) is not removed by acid acetone, and evidence is presented that in addition to iron-protein bonds there are stable linkages between protein and the tetrapyrryl ring resembling those of cytochrome \( c \).

Qualitative solubility observations indicated an isoelectric point at pH 4·7. Sedimentation and diffusion measurements gave \( s_{20} = 6·91 \times 10^{-13} \) and \( D_{20} = 6·1 \times 10^{-7} \) cm²/s. Thus \( M_f = 110,000 \). The possible significance of cytochrome \( f \) in the oxidation-reductions of the chloroplast reaction is discussed.

INTRODUCTION

By the direct spectroscopic examination of living material Keilin (1925) was able to observe the components of the cytochrome system in plant as well as animal tissues. Hill & Bhagvat (1939, 1951) confirmed and extended these observations and correlated the cytochrome oxidase activity of plant tissue preparations with the spectroscopic changes which occurred when oxygen was withheld from the preparations.

The detection of small concentrations of haematin pigments in living plants by direct spectroscopic observation is limited to those tissues which do not contain large amounts of sap or plastid pigments. When leaves are examined this technical difficulty is particularly acute. The only haematin compound which has been detected in this way is the ‘plant haemochromogen’ first observed by Keilin (1925) in the chlorophyll-deficient parts of leaves of Euonymus japonica. Although this was observed in other variegated leaves by Hill & Scarisbrick (1951) they were unable to detect it directly in normal green leaves.
Recently, Rosenberg & Ducet (1949) have shown that isolated spinach chloroplasts possess cytochrome oxidase activity, but it was not established that cytochrome $c$ occurred in leaves until Hill & Scarisbrick (1951) succeeded in extracting the component in soluble form. At the same time they isolated two new soluble cytochrome components from leaves. The first of these, like cytochrome $c$, occurs also in the non-photosynthetic portions of plants. It is autoxidizable, does not combine with carbon monoxide and has the spectroscopic properties of a cytochrome of the $b$ group. It was therefore called $b_3$.

The second new component was called cytochrome $f$. It was initially observed spectroscopically in preparations of leaves from which plastid pigments had been removed by acetone treatment. These preparations from many species of plants showed the $a$-band of the reduced component at $555\,\mu\mu$, but the cytochrome appeared to be insoluble. Soluble preparations were obtained by ethanol extraction of fresh leaves. From the ethanol extract Hill & Scarisbrick obtained concentrated solutions by acetone precipitation followed by ammonium sulphate fractionation of the dissolved precipitate. In the reduced form cytochrome $f$ has a spectrum similar to that of ferrocytochrome $c$, but the absorption bands are distinctly sharper and moved to a longer wave-length. The component is not autoxidizable, does not combine with carbon monoxide and has a more oxidizing potential than cytochrome $c$. Hill & Scarisbrick were unable to detect cytochrome $f$ in non-photosynthetic tissues, and they presented indirect evidence that within the leaf it is confined to the chloroplast. This suggests the possibility that the component may mediate in electron transfers occurring in photosynthesis. But before any function can be ascribed to cytochrome $f$ it would seem necessary to define its properties \textit{in vitro} and to exclude the possibility that it is an artifact arising by the modification of other haematin compounds in the course of the extraction. We shall therefore compare some of the properties of cytochrome $f$ with those of cytochrome $c$.

**Preparation**

In spite of an extensive search for other methods of extraction the procedure we shall describe is based substantially on that of Hill & Scarisbrick (1951). Although cytochrome $f$ may be detected in the acetone powders of most leaves their occur wide specific differences in the ease with which it can be extracted in solution. Hill & Scarisbrick used elder (\textit{Sambucus nigra}) as their starting material and obtained high yields of the component in the ethanol extract. We have now found that subsequent purification of the elder extract is complicated by the presence of large amounts of non-cytochrome protein and brown pigments. Although it gives a smaller initial yield, curled garden parsley (\textit{Petroselinum sativum}) gives extracts which are more readily purified.

In the course of the preparation the concentration of cytochrome $f$ in the various solutions was measured spectroscopically, using the spectrocolorimeter arrangement described by Hill (1936). The cup of the spectrocolorimeter contained a standard purified solution of the ferrocytochrome, and the $a$-band of the standard was matched against that of the unknown solution. The density at the $a$-band
maximum of the standard solution (555 m\l) was measured in the Beckman spectrophotometer. In computing the concentration in terms of haematin it was assumed that the molar extinction coefficient (\(\varepsilon\)) at the \(\alpha\)-band maximum was the same as for ferrocytochrome \(c\). The validity of this assumption will be discussed later. The extinction coefficient is defined as:

\[
\varepsilon = \frac{1}{cl} \log_{10} \frac{I_0}{I}
\]

where \(c\) = concentration of haem as gram atoms Fe/l., \(l\) = thickness of cell in cm, and \(I_0\) and \(I\) the intensities of the incident and transmitted light respectively. For ferrocytochrome \(c\), \(\varepsilon = 2.81 \times 10^4\) (Theorell 1936). The relative purity of preparations was obtained by relating the absorption spectrum measurements to estimations of dry weights or alternatively Kjeldahl nitrogen.

Leaves are picked from parsley plants in their first year of growth. After removal of the petioles the leaves are weighed and soaked in tap water for about 30 min. They are then drained, roughly dried by swinging in a cloth, and reweighed. Freshly picked parsley leaves were found to contain 84\% water, so that the volume of water in the roughly dried leaves can be calculated. Immediately before grinding that volume of cold \((-10^\circ C)\) 97\% ethanol containing 1.5\% v/v ammonia solution (sp.gr. 0.880) which will give 50\% ethanol in the ground material is poured over the leaves. Grinding is then carried out in an electrically driven mill adapted from one originally designed for the manual grinding of small quantities of corn. The essential mechanism of the mill is a worm feed attached to a fluted male cone rotating within a fluted female cone. When feeding the mill care is taken to maintain as constant a proportion ethanol:leaves as possible.

The ground leaves are rapidly squeezed through a cloth, and the green extract centrifuged in a refrigerated centrifuge for 10 min. The green precipitate is rejected and the yellow extract, which shows the \(\alpha\)-band of ferrocytochrome \(f\) when viewed with a Zeiss microspectroscope in a 2 cm layer, is cooled further. When large amounts of leaves are to be treated better yields are obtained if the grinding and centrifuging operations are carried out successively upon small batches of leaves. The ethanol extract is then accumulated in a deep-freeze cabinet at \(-15^\circ C\) until the whole batch has been dealt with. Thus 3000 g fresh leaves after soaking weighed 4250 g. Treatment in batches of 600 g gave 5090 ml. ethanol extract containing \(0.8 \times 10^{-4}\) M-cytochrome \(f\) haematin and having a dry weight of 22.5 mg/ml. To the cooled ethanol extract is added 1.1 vol. cold \((-10^\circ C)\) acetone with continuous stirring. The precipitate is filtered off on a Buchner funnel with 15 g kieselguhr as filter aid. When nearly dry the filter pad is washed with a cold mixture of acetone/ethanol/water in the proportions 2:1:1 containing 0.5\% 0.880 ammonia solution (all v/v). Washing is continued until the drainings are colourless. Without allowing the pad to dry it is washed with a 66\% saturated ammonium sulphate solution, pH 8. This displaces the acetone: ethanol mixture and extracts some non-cytochrome protein together with variable amounts of brown pigments. The filter pad is now scraped from the paper and suspended in 50\% saturated ammonium sulphate at pH 8. It is re-formed into
a filter pad on the smallest possible Buchner funnel and washed with the ammonium sulphate solution. After sucking nearly dry the cytochrome $f$ is eluted from the pad with a minimum volume of 0.06M-Na$_2$HPO$_4$. The yield at this stage is 220 ml. of a solution containing $1.6 \times 10^{-5}$ M-cytochrome $f$ haematin representing a recovery of 86% from the ethanol extract. The solution is filtered with kieselguhr and dialyzed against 3 l. m/500-ammonium carbonate solution.

The cytochrome is now adsorbed from solution on tricalcium phosphate gel. Successive small additions of the gel are added and centrifuged down. The proportion of cytochrome remaining in solution after each addition is estimated spectroscopically. The progress of the adsorption is linear to 90%, and the adsorbed fraction between 10 and 80% is selected for further treatment. The selected fractions are eluted from the gel by three additions of 0.06 M-Na$_2$HPO$_4$. The eluted cytochrome, now brownish pink in colour, is taken by dialysis to 20% saturation with respect to ammonium sulphate (pH 8). The precipitate is centrifuged off and rejected and the supernatant taken to 45% saturation. The pink precipitate is dissolved in a minimum volume of 0.06 M-Na$_2$HPO$_4$, dialyzed against 3 l. m/500-ammonium carbonate and the sequence of adsorption on tricalcium phosphate gel followed by fractionation between 20 and 45% saturated ammonium sulphate repeated twice more.

The final precipitate is dialyzed against m/100-Na$_2$HPO$_4$ to remove ammonium sulphate. The yield is 20 ml. of a solution $2.5 \times 10^{-5}$ M with respect to cytochrome $f$ haematin and representing 13% of that present in the original ethanol extract. The nitrogen concentration is 0.36 mg/ml., and the dry weight, corrected for phosphate, 2.25 mg/ml. This solution, which is 300 times as pure as the original extract, is freeze-dried and kept over phosphorus pentoxide, where it is stable for at least 2 years.

**RATIONALE OF THE METHOD OF PREPARATION**

In earlier preparations great variations in the yield of cytochrome $f$ were encountered from day to day. Careful attention to certain details of the method greatly minimize these variations.

(1) *The physical state of the leaves*

The highest yields are obtained with fresh material in a fully turgid condition. Little cytochrome can be extracted from wilted leaves, but the yield is partly restored by soaking in water until turgidity is regained.

(2) *The method of grinding*

Manual grinding of small quantities of leaves (e.g. 50 g) in a mortar gives the most efficient extraction. The yield decreases as the scale of the operation is increased. This is true to a smaller extent when mechanical methods of grinding are used. The ordinary meat mincer or the Waring blender are very inefficient. A grinding or shearing action in which the leaf is rubbed between two hard surfaces appears to be essential. Moreover, the broken turgid cells must come into
Immediate contact with alkaline ethanol to a final concentration around 50%.
Very little cytochrome is extracted when alkaline ethanol is added to previously
ground leaves. Similarly, if the leaves are ground with ethanol and the mush
subsequently made alkaline with ammonia only traces of the component appear
in the extract. These observations illustrate the difficulty of proving by extraction
procedures the location of the cytochrome within the cell. The sum of the amounts
extracted from leaf fractions always falls short of the amount extracted from whole
leaves under optimum conditions. It appears that rupture of the leaf tissue
initiates a rapid and irreversible change in the protein complex of the cells which
renders the cytochrome component insoluble.

(3) The subsequent treatment of the ground leaves

Removal of the leaf debris after grinding should be rapid. The yield diminishes
rapidly on standing, and even when the material is rapidly squeezed through a
cloth the first runnings from the cloth contain a higher concentration of the
component than the final runnings. Since cytochrome f is adsorbed from ethanol
solution by a variety of added adsorbents a similar mechanism may operate in
the presence of the leaf debris.

The grinding operation is carried out at around room temperature, but the
ethanol is cooled to compensate for heat arising from friction and from the heat
of solution of ethanol. Subsequent operations, until ethanol and acetone are
removed by ammonium sulphate, are carried out at below 0°C. This greatly
increases the yield of soluble cytochrome. If the whole preparation is carried out
at room temperature as much as 50% of the cytochrome in the ethanol extract
may remain in an insoluble form on the filter pad.

(4) The extracting solvent

Aqueous extractants yield no cytochrome f. Ethanol is the most efficient;
propanol, n-butanol and methanol are less so. The only non-alcohol solvent which
was found to extract the cytochrome is dioxane but the extracts were a dark
brown and after precipitation with acetone the component was insoluble.

In their initial attempts to extract cytochrome f in soluble form Hill & Scaris-
brick (1951) tried ethanol on the assumption that the component occurred in the
cell as part of a complex involving lipids. The procedure appeared drastic, but
recently alcohols have been successfully applied to the extraction and fractionation
of a variety of enzymes. Askonas (1951) used ethanol and other solvents in fractionating
the water-soluble proteins of rabbit muscle. Morton (1950) has found
n-butanol a valuable reagent in promoting the solution of a wide range of hitherto
insoluble enzymes, apparently by removing lipids. These demonstrations of the
more general application of alcohols in the manipulation of native proteins would
appear to diminish the possibility that cytochrome f is an artifact arising from
the action of organic solvents on another haematin compound in the leaf, and to
strengthen the view that the component is dissociated by ethanol from a lipid-
protein complex.
Hill & Scarisbrick (1951) measured the concentration of cytochrome \( f \) in the leaves of *Stellaria media*, *Lamium album* and *Triticum vulgare* by matching the \( \alpha \)-band of the component in a known thickness of acetone powder suspensions against that of an optically variable globin haemochromogen standard in the spectrophotocolorimeter cup. On a fresh weight basis the concentration, as protohaematin, was between 0.16 and \( 0.26 \times 10^{-4} \) M. The spectroscopic differences between cytochrome \( f \) and the standard in this comparison may introduce an error which, according to Hill & Scarisbrick, may be as large as 50%. We have therefore carried out similar measurements upon elder and parsley leaves using a purified preparation of cytochrome \( f \) as standard, and have compared the results of these measurements with those derived from estimations of the component in ethanol extracts of similar leaves. In order to obtain the maximum extraction the leaves were ground in small quantities (50 g) in a mortar. Concurrently with these measurements chlorophyll was estimated by the spectrophotometric method of Mackinney (1941). The results are given in table 1, and show that although the concentration of chlorophyll in elder was more than twice that in parsley, the chlorophyll/cytochrome \( f \) ratio was of the same order in both species. Of the total cytochrome \( f \) visible in acetone powder suspensions about one-third could be extracted by ethanol from the fresh leaves of the two species.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>molar conc. referred to fresh weight of leaves</th>
<th>cytochrome ( f ) in acetone powder (total ( f ))</th>
<th>in ethanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>chlorophyll ( a )</td>
<td>( b )</td>
<td>total</td>
</tr>
<tr>
<td>parsley</td>
<td>1.3 x 10^{-3}</td>
<td>5.0 x 10^{-4}</td>
<td>1.8 x 10^{-3}</td>
</tr>
<tr>
<td>elder</td>
<td>2.8 x 10^{-3}</td>
<td>1.2 x 10^{-3}</td>
<td>4.0 x 10^{-3}</td>
</tr>
</tbody>
</table>

### Molar ratios

<table>
<thead>
<tr>
<th></th>
<th>total chlorophyll</th>
<th>extracted cyt. ( f )</th>
</tr>
</thead>
<tbody>
<tr>
<td>total cyt. ( f )</td>
<td>parsley</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>elder</td>
<td>430</td>
</tr>
</tbody>
</table>

### Occurrence of Cytochrome \( f \) in Some Algae

During these measurements acetone powders from three species of algae were examined spectroscopically.

### Description of Plate 26

**Figure 6.** Visible absorption spectra of *A*, cytochrome \( c \); *B*, cytochrome \( f \), pH 8.0; *C*, cytochrome \( f \), pH 11.0; *D*, cytochrome \( f \), heat denatured at pH 12 + Na\( \text{S}_2\text{O}_4 \). Diffraction grating 14941 lines/in.; dimensions 1 x 1 in. Collimator and camera lens focal lengths 5.75 in., diameter 1.9 in.; slit 10Å wide; Kodak plate P300; enlarged x 5, with compensation for colour sensitivity of plate.
Figure 6
Ulva lactuca. An acetone preparation from the fresh young growth of this alga showed a spectrum identical with that of acetone powders from higher plants. Vaucheria sp. The cytochrome component could be extracted from the fresh acetone preparation (in the cold) by dilute Na$_2$HPO$_4$. It was non-autoxidizable, and the spectrum resembled a mixture of cytochrome $f$ with a little cytochrome $c$.

Euglena gracilis and Fucus serratus. Acetone preparations showed a spectrum of a non-autoxidizable component with a broad absorption band in the position of the mixed $a$-bands of cytochromes $f$ and $c$.

It is concluded that cytochrome $f$, or a closely related pigment, is present in these four representative species of algae. The ratio of cytochrome pigments present in the acetone powders to the total chlorophyll was of the same order as higher plants.

**The properties of the extracted pigment**

A strong solution of cytochrome $f$, made by dissolving a dried preparation in water, shows the complex visible spectrum of seven bands described by Hill & Scarisbrick (1951) (figure 6B, plate 26). In addition, if the solution is diluted until the $a$-band, when viewed in the Zeiss microspectroscope, is faint, and a cobalt glass filter is also placed in the light beam, the Soret band of the component can easily be seen at 421 m$\mu$. Figure 1 shows the visible and ultra-violet absorption spectra of ferrocytochromes. Continuous line, ferrocytochrome $f$. Broken line, ferrocytochrome $c$.

![Absorption spectra of ferrocytochromes](http://rspb.royalsocietypublishing.org/)
spectrum of a ferrocytochrome $f$ preparation. It is not yet possible to express the spectrophotometric data in terms of iron content. In order to facilitate comparison with cytochrome $c$ and with the data of Theorell (1936), the concentration of the cytochrome $f$ solution used in determining figure 1 was adjusted so that the density at the $\alpha$-band maximum ($\alpha_{555} = 0.647$) is related in a simple way to the absorption constant ($\beta$) at the corresponding maximum of ferrocytochrome $c$ ($\beta_{550} = 0.647 \times 10^8 = 2303\epsilon$). The most striking feature of the absorption spectrum is the great sharpness and intensity of the Soret band. The ratio of its density to that of the $\alpha$-band is 7.0 compared with 5.1 for ferrocytochrome $c$ (Theorell 1936).

**Figure 2.** Absorption spectrum of ferricytochrome $f$. Concentration as figure 1.

During purification, with favourable material, a stage is reached when the concentration of brown pigments is reduced to negligible levels. Further purification beyond this stage removes colourless protein, and the density of the band at 278$\mu$m relative to the Soret band decreases. The relative densities of the bands at wave-lengths longer than 300$\mu$m are not altered by this treatment. It therefore appears that the band at 330$\mu$m is also characteristic of ferrocytochrome $f$.

Solutions of cytochrome $f$ between pH 6 and 10 or the dried preparations are not significantly autoxidized in air, and it has not been found possible to obtain the ferricytochrome in the absence of an oxidizing agent. Keilin & Hartree (1937) showed that cytochrome $c$ is readily oxidized by potassium ferricyanide, copper salts and hydrogen peroxide. Ferrocytochrome $f$ is not so readily oxidized by these reagents. When sufficient hydrogen peroxide to bring about complete oxidation was added to a preparation of cytochrome $f$, the catalytic activity of the preparation rapidly destroyed the peroxide and the reduced spectrum slowly returned. Copper sulphate at a concentration of 0.001M does not render the
cytochrome autoxidizable. Potassium ferricyanide oxidizes the component but relatively large concentrations are required, as we shall describe when the oxidation-reduction potential is discussed. For the measurement of the spectrum of ferrixytochrome $f$, ferricyanide was used as oxidizing agent in the following way. The ferroxytochrome solution was dialyzed against a large volume of a 0.001 M solution of the ferricyanide. Ferrocyanide arising from the oxidation of the cytochrome is thus dialyzed away. After two changes of the dialyzing solution with increasing ferricyanide concentration the spectrum was measured in 0.0003 M ferricyanide containing 0.05 M-phosphate buffer, pH 7.4. The dialyzing solution was used in the blank cell. The concentration of the ferricytochrome used for the measurements in figure 2 was the same as that for figure 1.

The spectrum of ferricytochrome $f$, like that of ferrocytochrome $c$ at the same pH, is of parahematin type. Oxidation results in a 33% decrease in the density of the Soret band, accompanied by a decrease in sharpness and a shift of 11 m/$\mu$ to shorter wave-length. A band at 350 m/$\mu$ replaces that at 330 m/$\mu$, but the density and position of the band at 278 m/$\mu$ is unaltered.

**The oxidation-reduction potential**

The resistance shown by ferroxytochrome $f$ to reagents which readily oxidize ferrocytochrome $c$ is in agreement with the findings of Hill & Scarisbrick (1951) that cytochrome $f$ has the more oxidizing potential. Hill & Scarisbrick found that when ferricytochrome $c$ and ferroxytochrome $f$ are mixed they react to give an equilibrium mixture of the oxidized and reduced forms of the two components. By measuring the concentrations of the ferrocytochromes in the equilibrium mixture they concluded that cytochrome $f$ is the more oxidizing by between 60 and 76 mV at pH 6.0. They did not claim great accuracy for this measurement since it depended upon a visual estimate of the densities of the ferrocytochrome $\alpha$-bands in the mixture. The band maxima are separated by only 5 m/$\mu$ and thus appear to fuse when mixtures of the ferrocytochromes are viewed with a low dispersion spectroscope.

In the experiments to be described mixtures of potassium ferricyanide and ferrocyanide have been used as oxidation-reduction buffers in solutions of cytochrome $f$. According to Clark, Cohen & Gibbs (1925) $E_0^*$ for the ferrocyanide:ferricyanide system at 30° C is +0.43 V between pH 4 and 10. Potassium ferrocyanide and ferricyanide were twice recrystallized from water. The former was dried over deliquescent calcium chloride and the latter over sulphuric acid to constant weight. Immediately before each experiment solutions were made up by weighing and kept in darkness.

The experiments were carried out in Thunberg tubes of internal diameter 1.89 cm. Into each tube was pipetted 7 ml. of a solution containing 0.01 M-ferricyanide, 0.4 x 10^-4 M-cytochrome $f$ (as haematin) and the appropriate pH buffer. After the tube had been evacuated the tap was closed and the side arm filled with a solution similar in composition except that 0.01 M-ferrocyanide replaced the ferricyanide. To the side arm a 2 ml. graduated pipette containing
the same solution was attached by a short length of pressure tubing. By cautiously
opening the tap small measured volumes of the ferrocyanide solution could be
admitted successively to the evacuated tube. After each addition the tube was
allowed to equilibrate for 5 min in the dark. The concentration of ferrocyanide
f in the tube was then measured by the spectrophotometric method of Hill (1936).
This involves the visual matching of the spectrum of the solution in the tube with

\[
\text{log}_{10} \left( \frac{\text{ferrocyanide}}{\text{ferricyanide}} \right) = \frac{1}{45} \times E - 0.3
\]

that of a variable optical mixture of ferri- and ferrocyanide f of the same
effective concentration in the colorimeter cups. The pH of the solutions was
determined with the glass electrode at the end of each experiment. Sorensen's
phosphate mixtures (0.05 M) covered the range pH 6 to 7.7, and 0.05 M-K2HPO4
adjusted with 0.05 M-NaOH was used in the more alkaline range. The cytochrome
could be recovered at the end of an experiment by dialyzing away the ferro-
cyanide: ferricyanide mixture, and no evidence that it had been altered by the
treatment was obtained.

Some of the results of these measurements are given in figure 3. Since oxidation
of ferrocyanide involves a single electron transfer the 45° slope of the points from
each experiment plotted in this figure with the same logarithmic scale as ordinates

**Figure 3.** Oxidation-reduction potentials of cytochromes f and c. Effect of varying proportions in ferrocyanide-ferricyanide mixtures on the degree of reduction of cytochromes f and c.

- **A,** cytochrome c: × pH 4.7; △ pH 5.9. The remaining points refer to cytochrome f.
  - **B,** pH 9.95;
  - **C,** pH 9.7;
  - **D,** pH 9.86;
  - **E,** pH 8.8;
  - **F,** × pH 6.1; ○ pH 7.7; △ pH 7.0.
and abscissae indicates that \( n = 1 \) for the oxidation of the ferrocytochrome also. In this it resembles other haemochromogens and cytochrome \( c \). From the data in the figure, by extrapolation where necessary, the ratio \( \text{Fe(}CN\text{)}_6^{2+}/\text{Fe(}CN\text{)}_6^{3+} \) corresponding to 50 % reduction of the cytochrome can be obtained. \( E'_{\text{b}} \) at that ratio, derived by the ordinary electrode equation, will then correspond to \( E'_0 \) for the cytochrome at the pH indicated on the figure. The effect of pH on the characteristic potential of cytochrome \( f \), calculated from the data in figure 3 and adjusted to 30° C, is shown in figure 4. Between pH 6 and 8 \( E'_0 \) is constant at -0·365 mV. In the more alkaline range \( E'_0/\text{pH} = -0·06 \text{ V} \), indicating the presence of a ferricytochrome \( f \) of a basic dissociating group. The pK of this dissociation appears to be at 8·5.

![Figure 4](http://rspb.royalsocietypublishing.org/)

**Figure 4.** Relation between characteristic potential \( (E'_0) \) and pH in cytochromes \( f \) and \( c \). A, cytochrome \( f \). B, cytochrome \( c \) (data from Paul 1947). C, cytochrome \( c \) (data from Rodkey & Ball 1947). The additional points for cytochrome \( c \) are from this paper.

Included in figures 3 and 4 are the results of two experiments carried out with cytochrome \( c \) using the ferro-ferricyanide buffers. The derived values for the characteristic potential of cytochrome \( c \) agree well with the recent potentiometric determinations of Paul (1947) and Rodkey & Ball (1947). The findings of these authors differ in detail. Rodkey & Ball found \( E'_0 = +0·251 \text{ V} \) at pH 6·5 with a dissociation pK 7·7. The corresponding values of Paul were +0·266 V and pK 6·85.

Cytochrome \( f \) at pH 7 is thus more oxidizing than cytochrome \( c \) by about 0·1 V, and has a more positive potential than any haem compound hitherto examined. It would be difficult to evaluate the significance of this finding at present, since no system analogous to cytochrome oxidase, which will specifically oxidize cytochrome \( f \) has yet been extracted from leaves.
Stability of cytochrome f in solution

(1) Temperature

Purified or impure preparations of cytochrome f, in contrast to cytochrome c, are irreversibly denatured in 10 min at 58°C. The denatured product is autoxidizable and upon reduction with sodium hyposulphite gives a haemochromogen spectrum $\lambda = 552 \text{m} \mu$. The bands of this haemochromogen (figure 6D, plate 26) in the visible region are more diffuse than those of the native ferrocytochrome, but spectrophotometric measurements showed that the denaturation does not alter the value of the density at the $\alpha$-band maximum.

(2) pH

Compared with cytochrome c, cytochrome f is stable over a comparatively narrow range of pH. Cytochrome c is unchanged after short exposure to 0.1 N mineral acids or alkali. As was shown by Keilin & Hartree (1937) it is rapidly autoxidized in air outside the limits pH 4 to 12. Hill & Scarisbrick (1951) found that their preparations of ferrocytochrome f became autoxidized below pH 5 to give an acid haematin type of spectrum. This would correspond to the acid haematin spectrum of cytochrome c resulting from the dissociation at pH 0.42 (Theorell & Akesson 1941). The more purified parsley preparations do not show this change. Autoxidation occurs very slowly in all solutions, and its rate is increased at mildly acid pH. Thus the density at the $\alpha$-band maximum of a solution at pH 5 diminished by 20% after standing in air for 4 days at 4°C. The original density was restored by adjusting the pH to 7 and reducing and was not then rapidly diminished by aeration.

Below pH 5 the component begins to precipitate at the isoelectric point. The precipitate reaches a maximum at about pH 4.7 and redissolves at more acid pH. With increasing acidity over this range the bands of ferrocytochrome f fade more rapidly, but the $\alpha$-band of a solution $0.2 \times 10^{-4} \text{M}$ was still visible after 1 h at pH 3 and 20°C in spite of aeration. The change is not, however, entirely due to autoxidation of the native cytochrome. In an experiment with a preparation $1.0 \times 10^{-4} \text{M}$ the apparent autoxidation was allowed to proceed almost to completion in 24 h at pH 4. The spectrum was of parahaematin type with diffuse bands at 560 (very faint) and 530 m$\mu$ and resembled ferricytochrome f at pH 7.4 (figure 2). Restoration of the pH to 7.4 followed by reduction with sodium hyposulphite gave the spectrum of denatured cytochrome f haemochromogen. In the presence of carbon monoxide the spectrum changed to that of a carbon monoxide haemochromogen ($\alpha = 565 \text{m} \mu$, $\beta = 530 \text{m} \mu$). Apparent autoxidation of cytochrome f in acid solution could thus be explained as denaturation to the autoxidizable haemochromogen.

In alkaline solution cytochrome f is stable to pH 10.8. In this stable range the $\alpha$-band of the component is asymmetrical. From the absorption maximum at $555 \text{m} \mu$ the density falls away rapidly towards longer wave-lengths. Towards the blue the density falls away less rapidly at first to give a ‘shoulder’ between 555 and $552 \text{m} \mu$ (figure 6B, plate 26). This asymmetry is not resolved by the spectrophotometer but can easily be seen in a low dispersion spectroscope. At pH 10.8...
The form of the $\alpha$-band changes, and it is resolved into a double band with a sharp component at 556 m$\mu$ and an equally sharp but less dense component at 551 m$\mu$ (figure 6C, plate 26). The separation is then sufficiently great for the bands to be solved by the Beckman spectrophotometer. The density at the maximum of the main component is not altered during this change. Neither component of the double $\alpha$-band is affected by carbon monoxide or oxygen. At pH 10-8 this type of spectrum is stable for several days in vacuo at 20° C, and upon decreasing the pH to 8 the normal spectrum is restored. Above pH 10-8 both bands fade slowly, and the apparently autoxidized cytochrome then gives the spectrum of denatured cytochrome $f$ haemochromogen upon reduction. No explanation of this pH phenomenon can as yet be suggested.

From the above account it is clear that cytochrome $f$ is more sensitive than cytochrome $c$ to the denaturing effects of acids and alkalis. So far as we have been able to observe the denaturation is not reversible.

(3) Other denaturants

Within the pH range where cytochrome $f$ is stable it shows a considerable resistance to the action of protein denaturants. In the initial extraction a solution in 50% ethanol shows the same spectrum after 3 h at 15° C. Urea has been widely used as a protein denaturant. A solution of cytochrome $f$ $2.0 \times 10^{-5}$ m with respect to haematin was saturated with urea. Allowing for the volume increase, the spectrum, measured spectrophotometrically, was unaltered. Removal of the urea by dialysis also was without effect upon the spectrum. This does not exclude the possibility that in concentrated urea solutions there may occur a change in the protein molecule less drastic than that induced by heat treatment. Steinhardt (1938) found horse haemoglobin to be dissociated into half-molecules in the presence of urea without accompanying change in the oxygen capacity or absorption spectrum. The dissociation was reversed upon removal of the urea by dialysis.

Anionic detergents have also been widely used in the study of proteins. In particular, Keilin & Hartree (1940) observed some interesting changes in the spectrum of cytochrome $c$ in the presence of sodium dodecyl sulphate (S.D.S.). In neutral solution the para-haematin type spectrum was replaced by that of acid haematin. On reduction the spectrum resembled that of haem with feeble diffuse bands at 563 and 530 m$\mu$. This compound was autoxidizable and combined with carbon monoxide. The effect was reversible, and the spectrum of ferricytochrome $c$ returned when the S.D.S. was dialyzed away. Other components of the cytochrome system in heart-muscle preparations were irreversibly denatured by S.D.S. treatment.

We have repeated the experiments described by Keilin & Hartree using a pure preparation of S.D.S. In sodium veronal buffer at neutral pH the spectrum of a strong solution of cytochrome $c$ was unaffected by 1% S.D.S. in 12 h at 20° C. At pH 5 the spectrum immediately changed to one having a band at 630 m$\mu$ resembling acid haematin. On reduction and with carbon monoxide the sequence of spectroscopic changes recorded by Keilin & Hartree was observed. The reversibility of the action of S.D.S. was confirmed.
With a strong solution of cytochrome $f$ in 0.1 M-veronal buffer pH 7 addition of S.D.S. to 2 % produced no change in the spectrum. At pH 6 the ferrocyanochrome spectrum slowly faded and was replaced by a parahaematin spectrum with diffuse bands at 560 and 530 m$\mu$. No trace of a band in the red could be detected. On reduction the spectrum was that of denatured cytochrome $f$ haemochromogen, and no reversal of the denaturation occurred when S.D.S. was removed by dialysis. The addition of the detergent thus appeared to render the component more sensitive to the acid denaturation we have described.

The nature of the haem-protein link in cytochrome $f$

Cytochrome $f$, denatured in the presence of pyridine and sodium hyposulphite, gives a pyridine haemochromogen spectroscopically similar to that produced from cytochrome $c$. The visible absorption band positions in both compounds are the same ($\alpha = 550 m\mu; \beta = 520 m\mu$). Drabkin (1942) and others have pointed out that cytochrome $c$ pyridine haemochromogen is not pyridine protohaemochromogen ($\alpha = 558 m\mu$), but more closely resembles pyridine mesohaemochromogen ($\alpha = 547 m\mu$). He considered this to be evidence that the porphyrin contains no unsaturated linkages in the side chains in positions 2 and 4 of the tetraptroprolyl ring. This is in agreement with the evidence of Theorell (1937, 1938) derived from a study of the ether-insoluble 'porphyrin $c$' of Hill & Keilin (1930) that the $\alpha$-carbon atoms in these side chains are connected to two $\beta$-cysteine residues of the protein by stable thio-ether linkages. Because of the presence of these linkages the prosthetic group of cytochrome $c$ cannot be removed by treatment with acid acetone as it can from other natural haem-protein compounds.

The spectrum of cytochrome $f$ pyridine haemochromogen suggested the possibility that a similar second link between haem and protein exists here also. Moreover, Hill & Scarisbrick (1951) obtained an ether-insoluble porphyrin similar to porphyrin $c$ by treating cytochrome $f$ with hydrazine hydrate in glacial acetic acid. We have now found that the prosthetic group of cytochrome $f$ is not split off by acid acetone.

Peters & Wakelin (1947) have shown that silver salts under mildly acid conditions readily split many thio-ether linkages. Valuable additional evidence for Theorell's conclusion has come from the application of this method to cytochrome $c$ by Paul (1949, 1950). He obtained quantitative yields of haematoporphyrin in this way. We have repeated the reaction upon cytochrome $f$ with similar results.

One of us (Davenport 1952) has shown that sodium amalgam is capable of bringing about the reductive splitting of the stable porphyrin-protein link of cytochrome $c$. This reagent does not modify the unsaturated vinyl groups of protohaematin and yields protoporphyrin by the removal of iron. With cytochrome $c$, on the other hand, the product is mesoporphyrin. The reaction was interpreted as a simultaneous removal of iron and reductive splitting of the stable linkages with the protein involving the side chains in positions 2 and 4. Under similar conditions cytochrome $f$ also yields mesoporphyrin with no detectable traces of other porphyrin. It appears safe to conclude from this evidence that cytochrome
Cytochrome $f$

shares with cytochrome $c$ the property of being the only naturally occurring haem-protein compounds which have been shown to possess these stable linkages.

**Electrophoresis**

The minimum solubility of cytochrome $f$ at about pH 4.7 suggested that this was the isoelectric point of the component. This would constitute a major difference from cytochrome $c$ where, owing largely to the high lysine content, the protein is basic with the isoelectric point at the unusually high value of pH 10.05 (Theorell & Akesson 1941a, b). Confirmation of the acidic nature of cytochrome $f$ was obtained by electrophoresis at pH 7 in phosphate buffer of ionic strength 0.1. The protein migrated towards the anode and became resolved into two boundaries. The more intense of these had the greatest mobility and could be seen from its colour to be that of the cytochrome component. Quantitative measurements of the mobility were made difficult by a progressive spreading of the boundaries. This occurred only when the current was passing and did not therefore appear to be due to either thermal convection or diffusion. We have not yet determined the explanation of this spreading.

**Sedimentation and Diffusion**

The sedimentation constant of cytochrome $f$ was measured on the ultracentrifuge of the Agricultural Research Council Plant Virus Unit at the Molteno Institute, Cambridge. We have to thank Dr R. Markham for making this measurement. The preparation, in 0.1 M-phosphate, pH 7.1, contained cytochrome $f$ equivalent to $9.04 \times 10^{-5}$ M-haematin. The nitrogen content was 0.98 mg/ml. and the dry weight 6.13 mg/ml. Three boundaries were formed, but that of the cytochrome could be recognized by its colour gradient. From the Schlieren pattern this boundary represented at least 95% of the protein in the solution. The sedimentation constant ($S_{20}$) reduced to water at $20^\circ$ C was $6.91 \times 10^{-13}$. It was possible during the ultracentrifuge run to scan the field of the cell with a hand spectroscope. No difference could be detected in the appearance of the spectrum at different levels of the cell below the cytochrome $f$ boundary.

In order that an estimate could be made of the molecular weight of the component the diffusion constant was measured by the method of Northrop & Anson (1929). The cell constant was determined with ox oxyhaemoglobin prepared by the method of Adair (1925). $D_{20}$ for the haemoglobin was taken as $6.44 \times 10^{-7}$ cm$^2$/s. Cytochrome $f$ under the same conditions gave $D_{20} = 6.1 \times 10^{-7}$ cm$^2$/s. In deriving the molecular weight from the formula

\[
M_s = \frac{RTS}{D(1 - V\rho)}
\]

it was assumed that $V$ (the partial specific volume) was the same for cytochrome $f$ as for a typical protein (0.749). $M_s$ is therefore 110000.

In the preparation used in these measurements 1 mole haematin was equivalent to 68000 dry weight. The molecule of cytochrome $f$, mol.wt. 110000, must therefore
contain more than one haem group. This conclusion depends on the assumption, made throughout this work, that the density at the $\alpha$-band maximum of ferrocytochrome $f$ is the same as that of ferrocytochrome $c$. The spectroscopic similarity of the derivatives of the two components has already been pointed out. In particular, when cytochrome $f$ is converted to its pyridine haemochromogen (25% pyridine, pH 12) there is an 8% increase in the density of the $\alpha$-band. The corresponding increase for cytochrome $c$ was found to be 12%. Drabkin (1942) has compared the molecular extinction coefficients of a number of pyridine haemochromogens including that from cytochrome $c$. The extreme variation in the values for the $\alpha$-band maxima did not exceed 12% and that for the cytochrome $c$ derivative was the same as pyridine protohaemochromogen from blood. Until quantities of purified cytochrome $f$ sufficient for accurate iron estimations can be obtained it appears to be safe to assume that estimates of the haematin concentration obtained from spectroscopic measurements on the ferrocytochrome will not be in error by more than 5%.

**Discussion**

It is concluded from the observations we have described that cytochrome $f$ belongs to a class of haem-protein compounds hitherto represented only by cytochrome $c$. In neutral solution neither component combines with oxygen or carbon monoxide. Theorell (1937) has suggested that this property of cytochrome $c$ is related to the rigid structure imparted to the molecule by the presence of stable linkages between the haem and protein. The presence of similar linkages in cytochrome $f$ favours this interpretation and provides a basis for the common classification of the two components.

In spite of this fundamental resemblance between the cytochromes, there is wide divergence in those properties which are more specifically determined by the nature of the protein moiety of the molecule. In its extreme resistance to denaturation and its basic properties cytochrome $c$ is an unusual protein. Cytochrome $f$, on the other hand, resembles in these properties the majority of proteins.

In haemoglobin the essential property of reversible oxygenation is imparted to the molecule by the combination of haem with the highly specific protein-globin. However, variations in the nature of the globin can give rise to variations in the oxygen affinity of individual haemoglobins and these variations have been shown in many cases to be of adaptive significance. The corresponding essential property of a cytochrome component is its capacity to undergo reversible oxidation and reduction and thus to mediate in electron transfers in the cell. In evaluating the possible adaptive significance of the properties of cytochrome $f$ attention is therefore directed to the extremely oxidizing potential of the component compared with cytochrome $c$. It has already been pointed out, in connexion with the present study (Hill 1951a, b), that the potential of cytochrome $f$ was about half the difference between the oxygen electrode and the potential of the iron oxalate system. So far, the reduction of reagents by the illuminated chloroplast system has not been shown to occur to a significant extent with any artificial reagent of
greater reducing potential than ferrous oxalate, indicating that in the chloroplast under such conditions a system with an \( E'_0h \) of about zero at pH 7 may be concerned. The data obtained with ferric oxalate showed between 95 and 100% reduction at 4 mm Hg pressure of oxygen at pH 7.9 (Hill 1939). The calculated values for the chloroplast reducing potential \( (E'_0h) \) at pH 7, in relation to the oxygen electrode (1 atm), would therefore lie between -0.04 and -0.02 V.

The difference between the measured potential of cytochrome f and the oxygen electrode is \( 0.814 - 0.365 = 0.449 \) V. Taking a preliminary value of the chloroplast reducing reaction as \(-0.03 \) V and subtracting this from the potential of cytochrome f a difference of \(-0.395 \) V is obtained. These two intervals of potential are seen to be each of the order of \( 0.4 \) V. The potentials of cytochrome f and the preliminary value for the chloroplast reaction are shown in figure 5a in relation to the oxygen and hydrogen electrodes at pH 7.

**Figure 5a.** Diagram showing on the left-hand scale potentials at pH 7 of cytochrome f \((4/1)\) and the hydrogen-donating part of the chloroplast reaction in relation to percentage reduction. The position of the two other intervals \((4/3 \) and \(4/4, \text{etc.}\)) (see legend to figure 5b) are indicated by the arrows on the right-hand scale.

**Figure 5b.** Diagram showing the fluorescence band of chlorophyll a in the leaf in relation to the measured potential of cytochrome f \((4/1)\) and to the chloroplast reaction \((4/2)\). The width of the shaded areas corresponds in \( \mu \) to a 10 mV difference in oxidation-reduction potential in the former and a 20 mV difference in the latter. The values for \( n \) in the conversion of potential to equivalent frequencies are given by the fractions, \( n = 4 \) corresponding to the production of 1 mol. of oxygen, \( n = 2 \) corresponding to \( \frac{1}{2} \) mol. of oxygen. The denominators represent integral numbers of light quanta corresponding to the energy per 4H equivalents transferred.

If the reduction of cytochrome f is represented as:

\[
4fOH = 4f + O_2 + 2H_2O, \quad \Delta F_{298}^\circ = 41400 \text{ cal} \ (173 \text{ kJ}) \tag{1}
\]

1 cal. \( (15^\circ) = 0.004182 \) kilojoule. If the chloroplast reaction is represented in the limiting case where \( R = \text{reagent,} \) as follows:

\[
R + H_2O = RH_2 + \frac{1}{2}O_2, \quad \Delta F^\circ = 38950 \text{ cal} \ (163 \text{ kJ}) \tag{2}
\]
The value of the light energy per Einstein for the maximum of the fluorescence band of chlorophyll $a$ in the plant at about $20^\circ$ C is

$$E (= 685\text{ m}\mu) = 41500\text{ cal (174 kJ)}.$$  

The diagram (figure 5b) shows the $\Delta E^o$ of reactions (1) and (2) converted to the corresponding wave-length. The width of the shaded areas corresponds to a range of 10 and 20 mV respectively, in the values of the potentials from which the wave-lengths are calculated. The curve represents the fluorescence spectrum of chlorophyll $a$ as measured by Zchiele & Harris (1943) in ether and adjusted to the wave-length found for the fluorescence maximum in a living leaf by Dhéré (1937). From the results of an investigation of haematin compounds associated with chloroplasts (Hill & Scarisbrick 1951) we are almost obliged to conclude that these energy relations are significant and not fortuitous.

These considerations would therefore lend support to the view that the processes of respiration and photosynthesis consist of a series of similar but not necessarily identical steps in hydrogen transport. This would imply that each drop in hydrogen potential, with reference to oxygen potential in respiration has as its counterpart, when photosynthesis is dominant, a corresponding rise in hydrogen potential. It is therefore possible that the reaction (1) may closely correspond to the most oxidizing step in photosynthesis; the converse process to the oxidation of one of the cytochrome components in respiration. It must of course be assumed that in photosynthesis the steps of potential are coupled in such a way that the final high level of reducing potential can be reached. Supposing, for example, that one quantum is sufficient to bring about reaction (1) it can easily follow that by coupling two such systems there could result half the H per quantum at twice the reducing potential; the extra $2H$ and $\frac{1}{2}O_2$ would have cancelled out simply by electron transfer, in such a case two quanta would correspond to two oxygen liberated. The basic assumption underlying this would be that each effective light absorption corresponds to nearly complete transformation of the energy which involves four equivalents, or a transfer of four electrons. As we have at present no experimental means of determining independently the numbers of effective and ineffective light absorptions with the chloroplast system in vitro, the basic assumption cannot yet be put to direct experimental test. It may be, however, that from present biological knowledge large energy steps, for example the direct production by one quantum of $H + \frac{1}{2}O_2$ ($E'_o h = -0.99\text{ V, pH 7}$), would be improbable. The one hydrogen tending towards this reducing potential might therefore result from a series of four steps, but in this case there is no evidence as to how a coupling could occur. As, however, cytochrome $f$ seems to be part of the chloroplast substance (Hill & Scarisbrick 1951) there is no objection to the coupling of two steps in potential, giving the equivalent of reaction (2).

We have drawn attention to a hypothetical interpretation of these energy relationships with isolated systems mainly to emphasize that the observed properties of cytochrome $f$ are based on a biological adaptation or chemical modification which can be derived from cytochrome $c$ as a model.
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