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Cytochrome Pigments from the Green Photosynthetic Bacterium *Chlorobium thiosulphatophilum*

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(Received 17 October 1960)

The early notion that the cytochrome pigments of living tissues are associated closely with the reduction of molecular oxygen has had to be modified because of two main findings. Hill & Scarisbrick (1951) demonstrated that green plants contain cytochrome *f*, a pigment specifically associated with photosynthetic tissues and not found in other parts of the plant. Postgate (1954) showed that the strictly anaerobic bacterium *Desulphovibrio desulphuricans* is rich in cytochrome *c*₃.

Elsden, Kamen & Vernon (1953) isolated cytochrome from the purple non-sulphur bacterium *Rhodospirillum rubrum*, which is both photosynthetic and predominantly anaerobic in its mode of life. Vernon & Kamen (1954) subsequently demonstrated the presence of cytochrome pigments in extracts of the strictly anaerobic photosynthetic sulphur bacteria *Chromatium* and *Chlorobium*. In these organisms the simplest type of photosynthetic cytochrome system might be expected, uncomplicated by any possibility of oxygen metabolism, and it therefore appeared to be of interest to examine the cytochromes in these organisms more closely. The pigment from *Chromatium* has been prepared, and its properties investigated, by Newton & Kamen (1956).

The present paper describes the isolation and properties of two cytochromes, resembling each other in many of their properties, from the green sulphur bacterium *Chlorobium thiosulphatophilum*.

A preliminary account of part of this work has appeared (Gibson & Larsen, 1955).

METHODS

Organisms and culture methods. A strain of *Chlorobium thiosulphatophilum* kindly supplied by Dr J. Lascelles of Oxford was used for the main part of the work. Small-scale experiments with a second strain, obtained from Dr C. B. van Niel, showed that the two component pigments were again present.

The maintenance and culture of the organisms has been fully described by Larsen (1952). For large-scale growth Larsen's medium, modified so as to contain 0.2% of Na₂S₂O₃ and 0.4% of NaHCO₃ (both w/w), was used to fill completely 25 l. carboys, whose necks were sealed with rubber bungs. Five vessels were illuminated with 12 60 w incandescent bulbs, and the circulation of air round them was limited so as to produce a temperature of about 25° in the medium. The vessels were filled the day before they were inoculated with 200 ml. of a 2-day culture of the organisms grown in a medium containing 0.1% of Na₂S₂O₃ and 0.2% of NaHCO₃ (w/w). Growth was complete in 4–5 days, when the cells were harvested with a Sharples supercentrifuge. The yield ranged from 40 to 60 g. wet wt. equivalent to 14–20 g. of dry matter. The bacterial paste was stored as such at –20°, or spread thinly on glass plates and dried *in vacuo* over H₂SO₄ at room temperature or freeze-dried. The frozen cells and the dried preparations were both stable over a period of many months.

Phosphate buffers. These were prepared from KH₂PO₄ solutions adjusted to the required pH with NaOH, a Pye pH meter being used.

Estimation of cytochrome concentration. A hand spectroscopy was used to follow roughly the distribution of cytochrome during purification. A quantitative estimate of the cytochrome content of the different fractions was obtained by measuring the extinction difference between the fully oxidized and reduced preparations at the absorption peaks of the α and Soret bands. A suitable dilution was made in 0.05M-phosphate buffer, pH 7.0, and approx. 5 mg. of solid sodium dithionite added to 3 ml. in the spectrophotometer cuvette. The extinction of the solution at 417 and 554 $m\mu$ was measured with a Unicam SP. 500 or SP. 600 spectrophotometer with cells of 1 cm. light-path. A second portion was diluted to the same extent in a solution containing the same phosphate concentration and $K_3Fe(CN)_6$ to a final concentration of 0.02M. In this case the blank cell of the spectrophotometer contained the same concentration of $K_3Fe(CN)_6$, and extinction measurements were made at the same wavelengths. In practice it was found that the pigments were entirely in the oxidized state after the first two steps of the purification had been carried out, and it was then possible to measure the extinction difference in the same solution, before and after the addition of a trace of dithionite. The value of the estimates of cytochrome content of the bacterial extracts themselves is uncertain, since such extracts are highly coloured, and it could be seen that there was a slight bleaching of these pigments on addition of dithionite. Cytochrome content of fractions was calculated in the latter preparations by appropriate factors obtained from a highly purified sample of cytochrome 554 whose extinctions and dry weight had been determined.

Absorption spectra. The positions of the absorption bands of the cytochromes and their derivatives were found with a Hartridge reversion spectroscopy. Absorption spectra were obtained with a Unicam SP. 500 spectrophotometer, and with an Optica model CF4 recording spectrophotometer.

Protein estimation and determination of purity of cytochrome. A measure of total protein in extracts and fractions was obtained by measuring the extinction of suitably diluted samples at 280 $m\mu$. Purification of the cytochromes was followed by using the ratio of extinction (E) at 280 $m\mu$ to that at the Soret maximum in the reduced form.

Haemin estimation. Keilin & Hartree's (1951) method was used, modified as follows so as to use less material. The solution to be analysed was added to 0.1 ml. of N-NaOH contained in a 5 ml. graduated cylinder. Pyridine (0.4 ml.), followed by a trace of dithionite, was added, and the whole made up to 2 ml. with water and mixed. The pyridine haemochromogen was measured after exactly 30 min. in a Hilger Spekker absorptiometer in 1 cm. microcells and with a 605 filter. A standard curve was prepared with crystalline haemin.

Iron estimation. The iron content of purified cytochrome solutions was determined by a modification of Coombs's (1936) method for the determination of total iron in blood.

Pyrex incineration tubes (4 in. by $\frac{1}{8}$ in. internal diam.) were calibrated at 2 ml. The samples for estimation were pipetted into the tubes and, if of greater volume than 0.2 ml., were evaporated to about 0.2 ml. Hydrogen peroxide (1 drop of 100 vol.) was added, and the solution evaporated to dryness. If charring occurred, a further drop of peroxide was added and the evaporation repeated. Glass-distilled water (approx. 0.5 ml.) was added and evaporated; this step was repeated once more. The follow-

ing solutions were then added in the order given and mixed: glass-distilled water (1.0 ml.); 40% (w/v) sodium acetate 0.5 ml.; 20% (w/v) glucose 0.2 ml.; $\alpha\alpha'$ -dipyridyl solution 0.1 ml.

The $\alpha\alpha'$ -dipyridyl solution was prepared by dissolving 0.117 g. of the solid in 1.5 ml. of N-HCl, and diluting the solution to 25 ml. with glass-distilled water. The incineration tubes were placed in a boiling-water bath for 10 min., allowed to cool and the contents made up to the mark. The intensity of the red colour was measured in the Hilger Spekker absorptiometer, in microcells of 1 cm. light-path and with 604 filters. A standard curve was prepared by using ferrous ammonium sulphate.

Hydrogen peroxide was found to be a more suitable oxidant than HNO_3 , since it did not lead to the formation of a crystalline precipitate when dried whole bacteria were used for estimation of iron. The step of evaporating the incinerated material repeatedly from water prevented the subsequent formation of a yellow colour which was presumably due to oxidation of glucose by residual peroxide.

Measurement of oxidation-reduction potential. The potential of both cytochromes was found to lie between the ranges covered by the ferri-ferro-cyanide and the ferri-ferro-oxalate couples. Measurements of potential were therefore made by titration of the reduced cytochromes with ferricyanide, the change in potential being measured directly against a standard calomel electrode.

The cytochrome solutions (5 ml., extinction at 554 $m\mu$ approx. 0.5 in 0.1M-phosphate buffer, pH 7.0) were placed in small weighing bottles, of about 10 ml. capacity. A fine stream of O_2 -free N_2 was passed into the solution through a fine capillary to make and maintain anaerobic conditions. A small platinum electrode was placed in the solution and connected to a Pye pH meter used as a potentiometer. The meter was also connected to a standard calomel electrode immersed in a saturated solution of KCl, and the circuit was completed by means of an agar bridge. Potassium ferricyanide (0.1M) was added in 1 μ l. amounts from an Agla syringe (Burroughs Wellcome and Co., London) and the titration curve plotted. The solution was viewed through a hand spectroscopy to confirm the range in which the cytochrome was being oxidized, and the E'_0 obtained from the centre of the plateau in the titration curve.

RESULTS

Influence of growth conditions

Cytochromes were detected in all cultures examined, but the yield appeared to be somewhat greater from cultures that had completed their growth. Cultures in which the colour of the cells varied from a deep green to almost yellow also appeared to contain roughly equal amounts of cytochrome.

Extraction of cytochromes

Acetone-dried powders. The cytochrome bands were first recognized in suspensions of acetone-dried powders to which a trace of dithionite had been added. The cytochromes could be brought partially into solution by extraction with buffers of

pH ranging from 6.0 to 9.0, but the bands were still strong in the residual cellular material.

Dried cells. Extracts prepared from cells which had been dried *in vacuo* at room temperature or from the frozen state all contained cytochromes. At pH 9 the extracts were deep green, and had an absorption spectrum resembling that of fresh cells, with strong bands at 745 and 810 m μ . At lower pH (6.0–7.5) extracts were brownish in colour and showed in addition two sharp bands at 610 and 560 m μ , which were probably due to porphyrin. The yield of cytochromes appeared to be a little higher at high pH, but the accuracy of the estimations in crude extracts is somewhat suspect.

Wet cells. The cells of *Chlorobium* are relatively fragile and cytochromes could be extracted from freshly harvested cells by a variety of techniques, such as incubation with solutions of cetyltrimethylammonium bromide, grinding the cells with sand or powdered glass, breaking the cells in the Hughes press, or sonically with a 1 kw Mullard oscillator (Mullard Ltd., London) or even by freezing and thawing. This last technique was generally used both because of its simplicity and its comparative gentleness which, it was thought, was unlikely to cause denaturative changes in the pigments. The influence of pH on extraction was the same as when dried cells were used, and extraction at pH 9.2 in 5% (w/v) sodium borate was adopted as routine procedure.

Separation of cytochrome components

Batches of about 50 g. dry wt. of cells were used for large-scale preparation of cytochromes. A typical preparation is illustrated in Table 1.

Extraction. The cells were suspended in five times their (dry) weight of 5% (w/v) sodium borate solution, and frozen at -20° and thawed at $+30^{\circ}$; the freezing and thawing were repeated once. The suspension was then centrifuged at 20 000g for 15 min. and the supernatant collected. The cell paste was washed twice with 2 vol. of borate, resuspended in a similar volume and frozen and thawed again, and centrifuged and washed once. Further washing yielded little more cytochromes, and the cell residue was discarded.

First ammonium sulphate precipitation. The dark-green extract was treated with 20 g. of solid ammonium sulphate/100 ml.; the heavy green precipitate was removed by centrifuging or filtration and discarded. The light-brown filtrate was dialysed against running tap water for 48 hr., or until a small sticky precipitate, which was discarded, was formed.

Separation of cytochromes 553 and 554 with cation-exchange resin. Treatment of the dialysed brown solution with the ammonia form of Amberlite IRC50 (Margoliash, 1954) brought about the adsorption of part of the cytochrome. The pigment remaining in solution was not taken up even if

Table 1. Separation and purification of cytochromes from borate-extracted cells

	Total volume	E_{280}^*	E_{417}^*		E_{554}^*		Total cytochrome† (mg.)			Recovery‡ (%)
			ox.	red.	ox.	red.	From $\Delta 417$	From $\Delta 554$	E_{280}/E_{417} red.	
(1) Borate extract	500	56	31	32.6	3.51	3.7	352	148	14.9	100
(2) 20% (w/v) $(\text{NH}_4)_2\text{SO}_4$ supernatant	485	37.2	3.84	4.11	0.4	0.56	57.7	121	9.1	82
(3) (2), dialysed	735	16.8	2.01	2.31	0.198	0.312	98.2	131	7.3	89
Cytochrome 553										
(4) Amberlite column effluent	720	16.2	1.62	1.77	0.294	0.342	47.5	54	9.2	36.5
(5) $(\text{NH}_4)_2\text{SO}_4$ ppt., dialysed	37.5	148	13.8	17.1	1.38	2.22	56	57	8.7	32.4
(6) Butan-1-ol subnatant, dialysed	44	47.7	6.18	7.89	1.62	2.1	33.2	33	6.05	22
Cytochrome 554										
On Amberlite column, by difference	—	—	—	—	—	—	45.5	77	—	52.5
Ammonia eluate	6.2	16.5	48.5	65	3.9	9.8	45	57	0.254	39

* Dilutions were made in 1 mM-phosphate buffer of pH 7.0 so that the extinctions measured lay between 0.05 and 1.0 with cells of 1 cm. optical depth. Numbers given represent the extinctions of the undiluted solutions.

† Calculated from the values ($E_{\text{red.}} - \text{ox.}$ 0.1 $\equiv$ 44 $\mu\text{g.}$ of cytochrome at 417 m μ ; $E_{\text{red.}} - \text{ox.}$ 0.1 $\equiv$ 156 $\mu\text{g.}$ of cytochrome at 554 m μ) obtained from a sample of cytochrome 554, E_{280}/E_{417} red. 0.21, whose dry weight had been determined after freeze-drying.

‡ Based on extinction differences at 554 m μ : borate-extract value was taken as 100%.

further portions of resin were added, and this treatment was therefore used to separate the basic cytochrome 554 from the acidic cytochrome 553. Amberlite was added to the solution (0.5 g./100 ml.) and the suspension stirred for 24 hr. at 4°. The resin was then pale pink. The solution was run through an Amberlite column (10 cm. $\times$ 1 cm.) to remove any residual traces of basic cytochrome. The resin from the batch treatment was added to the top of the column, and the whole washed with 20 ml. of water. The washing was added to the main bulk of the Amberlite effluent.

Further purification of cytochrome 553

Second ammonium sulphate treatment. The effluent from the Amberlite column still contained about half the total cytochrome, as measured by the difference in extinction at 554 m μ between the oxidized and reduced forms. Further ammonium sulphate fractionation did not effect substantial purification, and the effluent was therefore concentrated by total precipitation in saturated ammonium sulphate solution. The precipitate was collected by centrifuging, and the slurry obtained by stirring the sediment with a glass rod was transferred to a dialysis sac and dialysed against running tap water. In one preparation this dialysis was dispensed with, but the losses in the next step appeared in consequence to be greater than usual.

Butan-1-ol treatment. On shaking the dialysed solution from the previous step with 0.2 vol. of butan-1-ol in the cold a heavy precipitate was formed. Three layers separated on centrifuging: a small top layer of deep-green solvent, separated from a cloudy brownish pink aqueous layer by a thick layer of denatured protein. The supernatant was collected and the top layers were washed twice with 10 ml. of 0.01M-phosphate buffer, pH 7.2. The combined supernatants were dialysed against running tap water until the smell of solvent had

gone. Losses of cytochrome at this stage were variable and usually of the order of 50 % in spite of care being taken to carry out the step as rapidly as possible and in the cold. None the less it represented the best treatment devised, and fractionation with other solvents, such as ethanol and acetone, resulted in as great if not greater losses, and a smaller degree of purification.

Fractionation on columns of diethylaminoethyl-cellulose. The solution obtained from the last step was brownish pink, and contained other coloured material as well as cytochrome. The ratio of extinction at 280 m μ to that at the height of the Soret band (416 m μ) in the reduced form varied from 3.6 to 7. Columns of diethylaminoethyl-cellulose, prepared as described by Sober & Peterson (1956), were used to effect further purification. The phosphate concentration of the buffer flowing through the column had to be raised from 2 mM to 0.5M at pH 7, or to 0.15M at pH 6.5, before much movement of the cytochrome took place; in most runs, the cytochrome solution (2 ml. containing 15–20 mg. of protein) was run into the column with 10 ml. of buffer (2 mM), pH 7, the column was washed with two 50 ml. portions of buffer at 0.02M and 0.2M, pH 7, and then developed with 0.5M-buffer, again at pH 7. When good separation of the cytochrome band from the green and brownish bands which preceded it had been obtained, the columns were extruded, cut and the sections eluted with M-phosphate buffer, pH 7. About 50 % of the pigment was usually recovered in the best fractions; both leading and tailing fractions of the main band were contaminated with other material absorbing at 280 m μ . The use of calcium phosphate-gel columns (Swingle & Tiselius, 1951) supported either on Celite or cellulose was investigated, but although considerable purification was achieved much of the pigment became permanently adsorbed and could not be eluted even

Table 2. *Further purification of cytochrome components*

Best values are given.

Treatment	Cytochrome 554		Cytochrome 553	
	$E_{280}/E_{417 \text{ red.}}$	$E_{417}/E_{554 \text{ red.}}$	$E_{280}/E_{416 \text{ red.}}$	$E_{416}/E_{553 \text{ red.}}$
Amberlite: two treatments	0.25	6.44	—	—
	0.24	7.16	—	—
Butan-1-ol	—	—	6.4	4.2
	—	—	3.6	3.3
Calcium phosphate-gel columns	0.234	6.26	1.83	5.98
	—	—	1.71	4.84
	—	—	1.88	6.55
Zone electrophoresis	0.22	6.76	1.18	5.3
	0.171	7.27	—	—
Carboxymethylcellulose	0.199	7.02	—	—
	0.18	6.8	—	—
Diethylaminoethylcellulose	—	—	0.74	5.22
	—	—	0.75	5.5

with m-phosphate. Zone electrophoresis in a cellulose column (Flodin & Porath, 1954) likewise brought about approximately fourfold purification in the best fractions, but the total recoveries were in all cases below 50 %. Results are summarized in Table 2.

Further purification of cytochrome 554

The Amberlite column on which the basic cytochrome had been collected was washed with a further 200 ml. of water and the pigment eluted with 1 % (v/v) ammonia (sp.gr. 0.880). The sharp band of cytochrome was collected in a small tube and the ammonia removed *in vacuo* over sulphuric acid.

The ratio E_{280}/E_{417} for the reduced form of the cytochrome at this stage was usually about 0.26, and repetition of the Amberlite treatment as described by Margoliash (1954) for cytochrome *c* gave products in which this ratio was never lower than 0.24. A sample of this degree of purity run in the ultracentrifuge showed the presence of about 25 % of a colourless component, which separated from the lighter-coloured material remaining at the top of the cell.

Further purification was effected on columns of carboxymethylcellulose prepared from 2 g. of the powder suspended in 2 mm-phosphate buffer as described by Sober & Peterson (1956). The pigment solution (2 ml., containing not more than 15 mg.) was washed into the column with 20 ml. of the same buffer. The column was then washed successively with 50 ml. portions of 5 mM-, 0.02 and 0.05 M-phosphate buffer, and the cytochrome was finally brought off with 0.1 M-buffer, all at pH 7. The red cytochrome band, which showed some tendency to separate into a main, leading band of the scarlet oxidized pigment, and a tailing cherry-red reduced portion, was both preceded and followed by some colourless protein, as judged by the $E_{280}/E_{417 \text{ red.}}$ ratio. In the best runs, 65–75 % of the pigment was recovered in the purest fractions. Other purification procedures were investigated, but produced poorer results. Chromatography on a column of calcium phosphate gel (Swingle & Tiselius, 1951) supported on Celite or cellulose powder produced little decrease in the ratio E_{280}/E_{417} , and recovery of the pigment was

poor. Zone electrophoresis in a cellulose column (Flodin & Porath, 1954) in one instance gave a fraction in which the ratio was as low as 0.171, but results were very variable, and this could not be repeated. Recoveries from the columns were very poor, as much as 70 % of the pigment remaining on the cellulose. Results are summarized in Table 2.

Properties of the cytochromes

Stability. The cytochromes from *Chlorobium* resemble cytochrome *c* in being extremely resistant to denaturation. They are stable in the range pH 3.5–10.0, and can also be placed in a boiling-water bath for 5 min. without permanent changes being caused; the absorption bands of the reduced compounds disappear on heating but are restored when the solution cools.

Reaction with other compounds. Neither of the isolated cytochrome pigments reacts with carbon monoxide or cyanide. The absence of reaction with carbon monoxide has been used as a test for denaturation during purification. Compounds are formed between nitric oxide and the oxidized compounds. While in the crude state the pigments are somewhat auto-oxidizable; this property disappears on purification and the purest preparations were stable in the reduced form in air.

Isoelectric points. These have not been determined accurately, but from the method of preparation they are known to be widely different for the two pigments. Cytochrome 554 is rather more firmly held on Amberlite than is cytochrome *c*, and its isoelectric point is therefore probably rather higher than that of cytochrome *c* (10). The isoelectric point of cytochrome 553 lies well to the acidic side of neutrality, judging by its behaviour on carboxymethylcellulose.

Absorption bands. The positions of the centres of the absorption bands of both components in the reduced and oxidized states and of the nitric oxide compounds are given in Table 3, together with those of heart-muscle cytochrome *c* for comparison. In Fig. 1, the α - and β -bands of the purest preparations are shown, as measured in the recording spectrophotometer at a resolution of 1 Å. The sample of cytochrome 554 had $E_{280}/E_{417 \text{ red.}}$ 0.18, and was electrophoretically homogeneous; the

Table 3. Wavelengths of absorption bands of cytochrome 553 and 554, with those of cytochrome *c* for comparison

Form	Wavelengths (mμ)								
	Cytochrome 554			Cytochrome 553			Cytochrome <i>c</i>		
Oxidized	412	535	—	408	—	533	407	530	—
Reduced	417	523	554	416	522	553	415	520	550
Nitric oxide compound	414	530	570	413	530	568	411	—	565

cytochrome 553 ($E_{280}/E_{416 \text{ red.}}$ 1.06) contained about 27% of colourless impurity, as estimated by ultracentrifuging. A pure sample of cytochrome *c* is included for comparison. The Soret bands of all these preparations were symmetrical.

Nature of the prosthetic group. Paul's (1950) procedure was applied to solutions (1 ml.; E_{417} about 1) of the two components in order to determine the nature of the prosthetic group, a solution of cytochrome *c* being used for comparison. The cytochrome 554 solution required rather longer heating in the silver sulphate-acetic acid mixture in order to obtain good extinction of haemin than did the others. The absorption maxima in the blue were at 390 m μ for both cytochromes whereas that for cytochrome *c* haemin was at 388 m μ . In all probability therefore the prosthetic groups of the *Chlorobium* cytochromes are identical with that of heart-muscle cytochrome *c*.

Molecular size and homogeneity. An impure preparation of cytochrome 554 with $E_{280}/E_{417 \text{ red.}}$ 0.22 was run in the ultracentrifuge in 0.05M-phosphate buffer, pH 7.2. A colourless component was easily separated from the coloured material, which barely moved from the meniscus during 4 hr. at 58 500 rev./min. Such behaviour indicates that this cytochrome is a small molecule, probably of size similar to cytochrome *c*. Another sample, $E_{280}/E_{417 \text{ red.}}$ 0.18, was examined in the Kern electrophoresis apparatus (Kern A.G. Aarau, Switzerland) in 0.05M-phosphate buffer, pH 6.5

(potential gradient 6 v/cm., current 3.4 mA), and showed only one component.

A sample of cytochrome 553 ($E_{280}/E_{416 \text{ red.}}$ 1.06) was examined in the Kern electrophoresis apparatus and also in the ultracentrifuge. Both experiments showed the presence of about 27% of colourless protein. This impurity had a higher mobility than the coloured material in the electrophoresis experiment, which was carried out in 0.05M-phosphate buffer, pH 8.0, with a potential gradient of 6 v/cm. and a current of 3.4 mA. When the cytochrome 553, in 0.05M-phosphate buffer, pH 6.9, was ultracentrifuged at 59 780 rev./min. the impurity moved more slowly than the pigment. The main fraction, with which all the colour was associated, gave a value S_{20} 3.90 at a protein concentration of 3.5 mg./ml., and, assuming a partial specific volume of 0.735, it would appear that the molecular weight is considerably greater than that of the other cytochrome and probably of the order of 45 000–75 000.

Haemin and iron contents. The determination of haemin and iron contents was carried out in duplicate on a sample of cytochrome 554, $E_{280}/E_{417 \text{ red.}}$ 0.22, and gave mean values of 3.1 and 0.37% respectively. If these values are corrected for the impurity present, on the assumption that it contained neither iron nor haemin, and that the pure cytochrome has $E_{280}/E_{417 \text{ red.}}$ 0.18, these values become 3.79 and 0.452%, which agree closely with those for cytochrome *c*.

A sample of cytochrome 553 with $E_{280}/E_{416 \text{ red.}}$ 1.06 was first dialysed against three changes of distilled water, and its dry weight was obtained after freeze-drying. The dried material was then used for determining the iron content, and gave a value of 0.072% of iron. This preparation was not pure and if the value is corrected for the percentage purity estimated in the ultracentrifuge (73%), the iron content of the cytochrome is 0.113%.

Redox potential. The oxidation-reduction potentials of the two cytochromes as obtained by the titration method were: cytochrome 554, $E'_0 + 140$ mv; cytochrome 553, $E'_0 + 163$ mv. A sample of heart-muscle cytochrome *c* gave $E'_0 + 262$ mv.

Other haem compounds in Chlorobium. Elsdén, Kamen & Vernon (1953) found that the photoheterotrophic organism *Rhodospirillum rubrum* contained in addition to cytochrome c_2 a cytochrome of the *b* type, with absorption bands at 560, 525 and 430 m μ . In the course of the present investigation many extracts of *Chlorobium* have been examined with the hand spectroscope, but in no case have any absorption bands appeared on reduction other than the fused bands of the cytochromes described above. Acetone-dried powders have been prepared from cells which had already been exhaustively extracted with borate to remove

Fig. 1. Absorption spectrum of cytochromes 554, 553 and *c* in the visible region. Solutions in 0.01M- KH_2PO_4 -NaOH buffer at pH 7 were reduced with 5 mg. of dithionite, and their concentrations adjusted so as to give identical readings at the α -band maximum. The Optica recording spectrophotometer at a resolution of 1 Å was used to draw the curves. ---, Cytochrome *c* (iron content 0.43%); ..., cytochrome 553 ($E_{280}/E_{416 \text{ red.}}$ 1.06; 73% pure from ultracentrifuging); —, cytochrome 554 ($E_{280}/E_{417 \text{ red.}}$ 0.18; electrophoretically homogeneous).

cytochrome 554 and 553, and thick suspensions were prepared in 50% sucrose solution. In these preparations also no bands appeared on addition of dithionite other than faint ones at 554 and 523 m μ , due to traces of unextracted cytochromes of the known types. Vernon & Kamen (1954) have also described a haemoprotein of novel type (RHP) which is present in considerable quantities both in *Rhodospirillum* and *Chromatium*. This pigment should be easily recognizable in extracts since it reacts with carbon monoxide, and thus the sensitive test of a carbon monoxide difference spectrum can be applied. A number of preparations were made by citric acid extraction (Bartsch & Kamen, 1958) and by disruption in the Mullard sonic oscillator, and subsequently centrifuged in a Spinco preparative ultracentrifuge. The supernatant from the sonically disrupted cells was separated into two layers, an upper pale-green and a lower more deeply coloured one, and an acetone-dried powder was prepared from the almost black pellet at the bottom of the centrifuge tube. A faint indication of some material reacting with carbon monoxide to give a difference maximum at 424 m μ was obtained in suspensions of the acetone-extracted material suspended in 50% sucrose, but not in the other fractions, and in all the preparations examined, at most traces of carbon monoxide-combining material have been found. It must therefore be concluded that a RHP-like pigment is either absent from *Chlorobium* or present only in insignificant amounts.

DISCUSSION

The nomenclature of the cytochromes has become increasingly confused as more pigments have been described, with the result that pigments from widely differing sources have been consecutively numbered. According to the system suggested by Scarisbrick (1947) the full names of the two pigments described above would be *Chlorobium thiosulphatophilum* cytochrome 554 and *Chlorobium thiosulphatophilum* cytochrome 553. For the sake of brevity these have been referred to as cytochromes 554 and 553.

The two pigments resemble each other in many of their properties. The possession of the same prosthetic group would account for the closely similar position of the absorption bands, but Fig. 1 shows the considerable differences in fine structure of the purest preparations obtained which are also visible with the hand spectroscope. The α -band of cytochrome 554 is markedly asymmetrical, whereas those of cytochrome 553 and cytochrome *c* are less obviously so. In all three pigments the β -band is composite, and the relative intensities of the α - and β -bands are quite different for the three compounds.

In addition, as shown in Table 2, the ratio of the intensities of absorption at the Soret maximum and at the α -band maximum is much higher for cytochrome 554 than for cytochrome 553, which has a ratio close to that for cytochrome *c*.

In other properties the pigments show well-marked differences. Whereas the protein of cytochrome 554 is basic in character, that of cytochrome 553 is acidic, and the molecules also differ considerably in size. The basic cytochrome 554 is small, and would appear from the ultracentrifuge run to be about the same size as cytochrome *c*. The iron content of the preparation indicates a minimum molecular weight of about 12 400. Similar calculations for cytochrome 553 indicate a molecular weight of about 50 000 based upon one haem iron per molecule or 45 000–75 000 on the basis of sedimentation rate. The molecule of cytochrome 553 is therefore among the largest cytochrome molecules reported, having a molecular weight of about half that of cytochrome *f* from green plants, which contains two haem groups per molecule (Davenport & Hill, 1952). Among photosynthetic bacteria, the cytochrome from *Chromatium* is assigned a molecular weight of about 30 000 (Newton & Kamen, 1956); the pigment from *Rhodospirillum rubrum*, cytochrome *c*₂, is about 13 000 (Kamen & Takeda, 1956). Both these pigments, like the *Chlorobium* cytochromes, appear to contain one haem group per molecule.

The two pigments may be inter-related in that one is derived from the other, but it is unlikely that such a conversion could be the result of the isolation procedures. Extractions of the pigments have been carried out by a wide range of techniques, both mild and rather drastic, and over a range pH 6.0–9.0, and in every case the two pigments were found to be present in approximately equal amounts. *Chlorobium* is not alone in possessing more than one cytochrome of similar absorption spectrum. Thus Olson & Chance (1958) have obtained evidence for the existence of a number of components in *Chromatium*, Trudinger (1958) has separated five cytochromes with α -band positions from 550 to 557 m μ from extracts of *Thiobacillus* and in animal tissues cytochrome *c*₁ resembles cytochrome *c* closely in its absorption spectrum (Keilin & Hartree, 1955).

The redox potentials of the *Chlorobium* cytochromes are lower than that of cytochrome *c*, but higher than the value of -10 mv given by Newton & Kamen (1956) for the pigment from *Chromatium*. All these cytochromes, and in addition that isolated by Postgate (1956) from *Desulphovibrio*, with the remarkably low E'_0 of -205 mv, and cytochromes *c*₂ from *Rhodospirillum* and *f* from green plants, with E'_0 at 340 and 360 mv respectively, have α -bands lying between 550 and 555 m μ and

appear to contain identical prosthetic groups. It has become increasingly clear over recent years that the group of *c*-type cytochromes is functionally a remarkably heterogeneous one, and that the possession of absorption bands in closely similar positions does not imply any close similarity in other properties.

The cytochromes of *Chlorobium* are present in considerable amounts in the cells, and up to 0.5% of the dry weight of the cells has been extracted as cytochrome, though the exact measurement of cytochrome content of extracts is difficult because of the presence of other pigments affected by reducing agents. Acetone-dried powders prepared from borate-extracted cells still showed the absorption bands at 554 and 523 m μ , and the total cytochrome content forms a substantial part of the cell. On the other hand, as far as could be ascertained, these two components are the only haemoproteins present in the cells in significant amounts.

It is now clear that the possession of cytochrome pigments peculiar to themselves is characteristic of photosynthetic tissues and organisms, and the work of Duysens (1953) and Olson & Chance (1958) has made it clear that changes in absorption on illumination correspond to changes in the state of oxidation of cytochrome-like pigments. These changes do not appear to be very rapid, and at what stage in the complex series of changes initiated by illumination the cytochrome functions remains obscure. It may be significant for the photosynthetic sulphur bacteria that Trudinger (1958) has isolated cytochromes from a *Thiobacillus*, and shown that one of these, with an α -band maximum at 553.5 m μ and $E'_0 + 210$ mv, is reduced by an enzyme which can oxidize thiosulphate, either this cytochrome or ferricyanide being used as acceptor. Some indications have been obtained that the rate of reduction of 2:6-dichlorophenol-indophenol by thiosulphate in the presence of a crude preparation from *Chlorobium thiosulphatophilum* is increased when cytochrome 554 is added; a clarification of this effect must, however, await a separation and identification of the enzymic steps involved.

SUMMARY

1. Cytochrome pigments have been detected in extracts of *Chlorobium thiosulphatophilum* prepared by different techniques.

2. Two pigments have been separated with widely different isoelectric points.

3. The basic pigment, which has been obtained in a homogeneous state, has absorption maxima in the reduced form at 554, 523 and 417 m μ , is not

auto-oxidizable and forms compounds with nitric oxide, when in the oxidized state, but not with cyanide or carbon monoxide when oxidized or reduced. The E'_0 value is 140 mv. The prosthetic group is closely related to that of cytochrome *c*. The molecular weight is about 12 000 and there is one haem group per molecule.

4. The acidic pigment, the best preparation of which still contained about 27% of colourless impurity, has absorption maxima in the reduced form at 553, 522 and 417 m μ , and resembles the basic pigment in general properties. The E'_0 value is 163 mv. The molecular weight is 45 000–75 000 from the ultracentrifuge study and the maximum molecular weight is about 50 000 from the haem content.

5. These pigments together account for up to 0.5% of the dry weight of the bacteria.

6. No other haem compounds have been detected in significant amounts.

My thanks are due to Mr Bowen of the Biochemistry Department, Leeds, and to Dr P. Johnson of the Department of Colloid Science, Cambridge, for the ultracentrifuge studies, and also to Dr T. Hofmann of the Department of Biochemistry in Sheffield, for carrying out the electrophoresis runs.

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