

Cytochrome c_3 and Desulphoviridin; Pigments of the Anaerobe *Desulphovibrio desulphuricans*

BY J. R. POSTGATE

Chemical Research Laboratory, Teddington

SUMMARY: Suspensions of various mesophilic strains of *Desulphovibrio desulphuricans* show absorption bands attributable to a cytochrome and a green protein; there are small differences in the position of absorption maxima depending on the strain and culture medium. Both pigments have been extracted, together with flavins rich in flavinadenine dinucleotide; an electrophoretically and chromatographically pure preparation of the cytochrome has been obtained and is designated c_3 . The green protein has been termed 'desulphoviridin'.

Cytochrome c_3 is a soluble autoxidizable thermostable haemoprotein (reduced bands at 553, 525 and 419 $m\mu$.) of low redox potential (-205 mV.), high iso-electric point ($\text{pH} > 10$) and containing 0.9% Fe. Degradation studies indicate that it is a bifunctional haemato-haematin with the thio-ether haem-apoprotein links also found in cytochromes c and f ; its m.w. is approx. 13,000 ($S_{20, w} = 1.93 \times 10^{-13}$). Spectroscopic data for various derivatives including haemin c_3 and a porphyrin derivative are recorded. Material purified to at least 94% by cellulose and ion-exchange chromatography acts as carrier in the reduction in hydrogen of sulphite, thiosulphate, tetrathionate or dithionite by detergent-treated bacterial preparations; a similar role has been demonstrated with cell-free systems which reduce sulphite, thiosulphate and tetrathionate. Benzylviologen would replace cytochrome c_3 . No preparation has been obtained showing c_3 -linked sulphate reduction; the evidence for this depends on difference spectra and competition by known sulphate antagonists.

Oxidation of H_2 or organic compounds with O_2 has been demonstrated with these bacteria; the H_2/O_2 reaction takes place fastest in an atmosphere containing 4% O_2 , when oxygen is frequently reduced faster than sulphate. The reaction requires the mediation of cytochrome c_3 and is probably a consequence of the autoxidizibility of c_3 .

Desulphoviridin is a thermolabile, soluble, acidic porphyrin protein absorbing at 630, 585 and 411 $m\mu$.; no metabolic function has been detected. It is stable over a limited pH range and decomposes readily, yielding a chromophoric group which fluoresces red in ultraviolet light, absorbs at 595 $m\mu$. in neutral and alkaline solution (solution red) and at 612 $m\mu$. in acid solution (solution blue-green). This material can be purified by chromatography on 'Florisil' or paper. It is very photo-sensitive and water-soluble. Its character is obscure; it may be a highly carboxylated chlorin. Spectroscopic data are recorded.

The cytochromes have for long been regarded as pigments characteristic only of aerobic or facultatively anaerobic bacteria. For many years they were believed to be absent from obligate anaerobes (see Keilin, 1933; Keilin & Slater, 1953), a view which was supported by a recent examination of seven anaerobes, which allowed for the possibility of adaptive cytochrome formation in air (Schaeffer & Nisman, 1952). However, a cytochrome has now been observed in the sulphate-reducing bacterium *Desulphovibrio desulphuricans* (Butlin & Postgate, 1953; Ishimoto, Koyama & Nagai, 1954*a, b*; Postgate,

1954*a, b*), though this bacterium is an exacting anaerobe (Grossman & Postgate 1953*a, b*); cytochromes have also been demonstrated in species of the obligately anaerobic photo-autotrophs *Chromatium* and *Chlorobium* (Kamen & Vernon, 1954*a, b*; Vernon & Kamen, 1954; Gibson & Larsen, 1955). The present paper describes the spectroscopic properties of a strain of sulphate-reducing bacteria, the extraction of the components responsible for the spectroscopic absorption bands, the purification of the cytochrome component, and some of its chemical and biological properties.

METHODS

Spectroscopy. The behaviour of suspensions of bacteria was observed in a Hartridge reversion spectroscope modified for use with relatively opaque material and calibrated with the emission lines of neon; quoted readings were taken at limiting dilution as recommended by Lemberg & Legge (1949). Spectrophotometric measurements were made in the Hilger 'Uvispek' instrument. Measurements on intact organisms were made by the procedure of Barer (1955), in which scattering was reduced to a minimum by suspending the organisms in a medium of refractive index similar to that of themselves. The refractive index of the bacteria used in this work was shown by phase-contrast microscopy to be 1.383 ± 0.001 ; therefore a solution of bovine serum albumin (Armour Laboratories, Fraction V) of *c.* 34% (w/w; refractive index, measured in the hand refractometer, equivalent to 31.5% sucrose) was the most suitable suspending medium.

Manometry. Conventional Warburg manometers were used for experiments involving gas exchanges; details of procedure were given by Grossman & Postgate (1955).

Organisms used and their cultivation. *Desulphovibrio desulphuricans* strain Hildenborough (National Collection of Industrial Bacteria NCIB 8303), purified according to Postgate (1953) was used, except where otherwise mentioned; its origin, maintenance and methods of subculture were described by Postgate (1951*a*). Large quantities of organisms for fractionation were obtained from continuous culture experiments being conducted elsewhere in this laboratory (Report, 1953); the conditions of culture changed in detail from time to time, but normally the bacteria were grown in a medium containing Na₂SO₄ equivalent to 0.4% (w/v) S, yeast extract (Difco) 0.4% (w/v) and small amounts of NH₄⁺, K⁺ and Mg⁺⁺ (Report, 1954, p. 53). Effluent containing 0.4–0.8 mg. air-dry wt. organisms/ml. was harvested during several days, centrifuged in 100 l. lots in a Sharples centrifuge, the organisms washed with distilled water and dried by addition to 10 vol. cold acetone (4°), followed by washing with acetone and then ether. Yield: 30–35 g. dried material/100 l. culture medium. Organisms freshly harvested during the logarithmic phase of growth were used for metabolic experiments; a few experiments were done with bacteria from batch cultures.

Chromatography. Cellulose columns were packed with Whatman cellulose powder (standard grade) and washed with 4 vol. of distilled water. Ammonium

'Amberlite' columns were prepared as follows: a finely divided form of Amberlite IRC 50 (XE 97, Chas. Lennig and Co. Ltd.) was obtained and the fraction which sedimented between 3 and 20 min. in water was collected. This was converted to the ammonium form by adding excess 2 N-NH₄OH, packed and washed with distilled water (c. 8 vol.) until the effluent contained less than 0.0025 N-HN₄OH (pH 9.5 to 10). Mean flow rate: 75 ml./hr. The resin XE 97 was also used for chromatography with sodium phosphate buffers (0.34 g.-ion Na⁺/l.) following the procedure described by Boardman & Partridge (1955). It was washed successively with 2 N-NaOH, 2 N-HCl, then water and buffer; the columns (12 × 0.9 cm. diam.) were then packed and run at room temperature; mean flow rate 0.8–1.1 ml./hr. Florisil (30/60 mesh; Floridin Co., Florida, U.S.A.) was packed in water and washed with 20 vol. of 0.1 N-HCl followed by 20 vol. distilled water. Cross-linked polymin P columns were prepared following the advice of Mr D. K. Hale of this Laboratory. Polymin P (Badische Anilin und Soda Fabrik, Germany; a soluble polyethylenimine; 40 g.) was mixed with 40 ml. methanol, 4 ml. epichlorhydrin and 36 g. powdered cellulose (above) in that order; this mixture was allowed to polymerize at 60° for 3 days, and the product broken up in 2 N-HCl and washed successively with water, 2 N-NaOH, water, 2 N-acetic acid and 0.02 N-sodium acetate buffer (pH 5.0). The column, after packing, was washed with distilled water. Paper chromatography was conducted at room temperature on Whatman no. 1 filter-paper sheets, using the ascending method.

Electrophoresis. Electrophoresis on strips of Whatman no. 1 paper was carried out in the EEL instrument (Evans Electro Selenium Ltd., Harlow, Great Britain).

Potentiometry. The Cambridge pH meter was used for fine pH measurements with a glass electrode, and for potentiometric titrations with a bright Pt electrode and a calomel reference electrode. Redox potentials were studied by titrating the test material in KH₂PO₄ (1%, w/v, adjusted to pH 7 ± 0.02 with 2 N-NaOH) against Na₂S₂O₄ (30 mg./ml. buffer) in a current of O₂-free N₂ in a thermostat at 30 ± 0.05°.

Units of cytochrome. The millimolar extinction coefficient of the cytochrome studied here is not known. Evidence is presented in this paper that the cytochrome has a similar molecular weight to cytochrome *c* but two haemin groups per molecule. Hence ϵ_{mM} should theoretically be 54 (compare 27 for cytochrome *c*); the units used in this paper are derived from spectrophotometric measurements at the α -peak in the reduced form: 1 mUnit (mU.) = 1 mMole if $\epsilon_{\text{mM}} = 54$.

Abbreviations. The following abbreviations are used in this paper: CTAB for cetyltrimethylammonium bromide, FAD for flavin adenine dinucleotide, FMN for flavin mononucleotide, DPN for diphosphopyridine nucleotide, TPN for triphosphopyridine nucleotide, ATP for adenosine triphosphate, TCA for trichloroacetic acid.

RESULTS

Observations with whole bacteria

Suspensions of the Hildenborough strain heavier than 10 mg. dry wt./ml. KH_2PO_4 (0.5%, w/v; pH 6.9) showed strong adsorption bands at 553 and at 630 $m\mu$. when viewed through a depth of 0.5 cm. A weaker band at 525 $m\mu$. was seen, and old suspensions showed a shading about 595 $m\mu$. This last band was later shown to be due to a chromophore liberated by partial decomposition of the 630 $m\mu$.-component. The bands at 525 and 553 $m\mu$. disappeared on

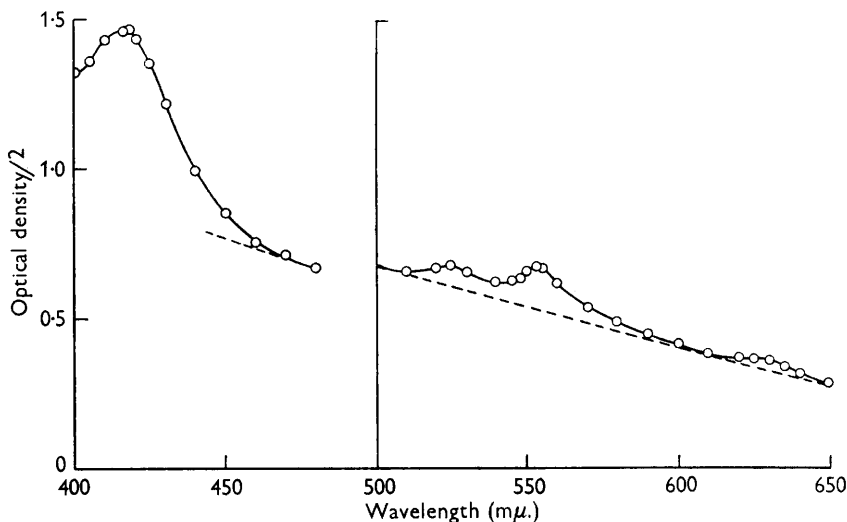


Fig. 1. Absorption spectra of *Desulphovibrio desulphuricans* (Hildenborough). Organisms were suspended in strong bovine plasma albumin and examined spectrophotometrically through a depth of 0.5 cm. after addition of $\text{Na}_2\text{S}_2\text{O}_4$. Visible range: 65 mg. dry wt./ml.; Soret range: 20 mg./ml. Dotted lines represent hypothetical scatter curve of a suspension of bacteria devoid of pigments.

shaking in air and returned on (a) standing, (b) passing in H_2 , (c) adding $\text{Na}_2\text{S}_2\text{O}_4$. The band at 630 $m\mu$., and the 595 $m\mu$. band when present, were not affected by these procedures, nor by addition of H_2O_2 , $\text{K}_3\text{Fe}(\text{CN})_6$, or on passing in pure O_2 . The bands at 525, 553 and 630 $m\mu$. intensified and shifted to shorter wavelengths on freezing and de-vitrifying a suspension in 50% glycerol with liquid N_2 , but remained single; hence they represented single compounds (Keilin & Hartree, 1955). Inspection of the suspension through a blue filter (dilute methylene blue) showed that the limit of visibility in the violet shifted from about 420 $m\mu$. to about 410 $m\mu$. with aerated suspensions. The absorption bands were recorded spectrophotometrically by Barer's procedure (Fig. 1).

This sulphate-reducing organism thus contained a reversibly oxidizable pigment resembling the cytochrome *c* of muscle, responsible for absorption peaks at 553 and 525 $m\mu$. as well as at about 420 $m\mu$. in the reduced condition. The

absorption peak at 630 $m\mu$., though similar in position to cytochrome a_2 , had none of the properties of a conventional cytochrome. The cytochrome probably represented the sole intracellular haematin, because treatment of a suspension of bacteria with $\text{Na}_2\text{S}_2\text{O}_4$ in the presence of alkali and pyridine led to a shift of the cytochrome band to about 550 $m\mu$., but no increase in its intensity; hence no additional haemochromogens were formed.

The presence of cytochrome and the 630 $m\mu$.-component was confirmed with five other mesophilic strains of *Desulphovibrio desulphuricans* examined in washed suspension at 8–12 mg. dry wt. organisms/ml. (Table 1). Small differences in the position of the bands occurred between strains, wider variation occurred with bacteria derived from different media: El Agheila Z had its strongest band at 555 $m\mu$. when harvested from lactate media and at 559 $m\mu$. from malate media (for media see Grossman & Postgate, 1955). This was not due to the presence of another or a different cytochrome, since: (a) cytochrome extracted from malate-grown organisms by the procedure described below had its α -peak at the usual 553 $m\mu$.; (b) the α -band in malate-grown organisms was homogeneous in liquid N_2 .

Table 1. *Visible absorption spectra of suspensions of various strains of Desulphovibrio desulphuricans*

Bacteria were harvested from lactate media except in the case indicated when El Agheila Z was grown in a malate medium. All strains halophilic except those marked *.

Strain	NCIB no.	Absorption peaks ($m\mu$.)		
		525	553·8	630·0
Hildenborough*	8303	525	553·8	630·0
California 43:63	8364	525	555·2	630·0
El Agheila Z	8380	525	555·2	630·8
El Agheila Z (malate)	—	525	559·8	629·0
Canet 41	8393	525	553·6	630·0
Wandle*	8305	525	555·7	630·0
Venice 2	8323	525	554·0	628·5

Oxidation by sulphate

It was possible that the cytochrome was concerned as a carrier in the reduction of sulphate and other reducible anions (see Postgate, 1951 *b*), but addition of sulphate or sulphite to washed suspensions in anaerobic conditions led to no obvious change in the visible absorption bands. This was not surprising, however, since the end product of reduction of these compounds—sulphide—is a powerful reducing agent and might well mask any oxidation. To avoid this difficulty, suspensions of bacteria were incubated *in vacuo* in Thunberg tubes containing CdCl_2 (10 %, w/v) in the side arm to remove sulphide continuously, and, after 30 min. at room temperature at pH 7·0, the visible cytochrome bands were clearly less intense, by some 40 %, in the presence of excess sodium sulphate, sulphite, thiosulphate or tetrathionate, than in a control suspension without these. The peak at 630 $m\mu$. did not alter in these conditions, except that, after prolonged exposure to sulphite, evaporation of SO_2 produced conditions sufficiently alkaline to cause decomposition, as a result of which

a band appeared at 595 $m\mu$. and the suspension fluoresced red in ultraviolet light. Ishimoto, Koyama & Nagai (1954*b*) reported observation of cytochrome oxidation by certain sulphur-containing anions without taking these precautions; the discrepancy between our results may be attributable either to the use of different strains or to difference in the amount of sulphide present at the moment of adding the oxidizing agent.

The oxidation of cytochrome by sulphate was recorded by difference spectra (Fig. 2). Since the modified spectrophotometric apparatus recommended by Chance (1954) was not available, Fig. 2 was obtained with suspensions of organisms rendered translucent by Barer's procedure. The introduction of sulphate caused a slight change in the refractive index of the suspending medium as compared with the control; in consequence the difference spectrum in Fig. 2 did not lie exactly about the abscissa.

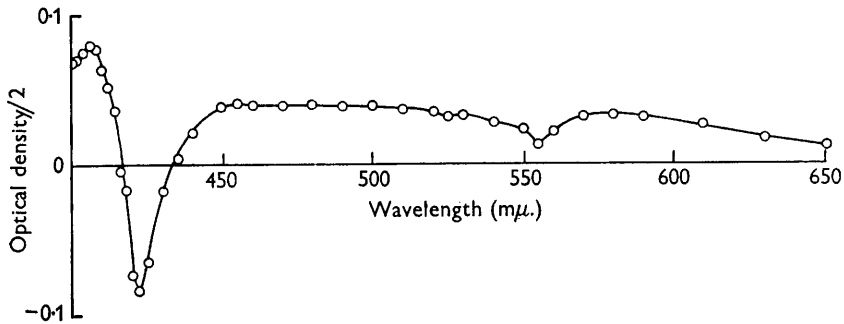


Fig. 2. Difference spectrum of *Desulphovibrio desulphuricans* (Hildenborough). The absorption of a bacterial suspension (7.2 mg. dry wt./ml. plasma albumin) containing excess sulphate was measured with a reference cell containing a similar suspension without sulphate.

Effect of inhibitors

Selenate or monofluorophosphate, which are competitive inhibitors of sulphate reduction (Postgate, 1949, 1952), inhibited the oxidation of the cytochrome by sulphate for several hours, though they did not affect oxidation by sulphite. The absorption bands were unaffected by passing in pure CO, adding KCN (10^{-2} M), Na_2S (5×10^{-3} M), or NH_2NH_2 (10^{-2} M); their reaction with air took place normally in the presence of these compounds. CTAB (recrystallized from acetone; 100 $\mu\text{g.}/\text{mg.}$ dry wt. organisms) inhibited the reduction of the oxidized cytochrome on standing, but did not prevent its reduction in H_2 .

Mode of action of CTAB

Salton (1951) showed that CTAB rendered Gram-positive aerobes permeable to substances of low molecular weight. Suspensions of *Desulphovibrio desulphuricans* treated with CTAB (100 $\mu\text{g.}/\text{mg.}$ dry wt.) were killed: the viable count by the method of Grossman & Postgate (1953*b*) decreased from $1.7 \times 10^9/\text{ml.}$ to $2.4 \times 10^3/\text{ml.}$ Material absorbing at 265 $m\mu$. was demonstrated in the supernatant liquid after centrifugation together with free cytochrome,

though hydrogenase and the 630 m μ -component were absent. Thus CTAB rendered the organisms permeable to the cytochrome; this conclusion was supported later by the observation that untreated bacteria reduced added purified cytochrome only slowly in H_2 , though CTAB-treated organisms reduced it practically instantaneously. CTAB-treated organisms did not reduce sulphate in H_2 , though they reduced methylene blue at a normal rate; they also did not reduce methylene blue with lactate or pyruvate.

Extraction of the pigments and purification of the cytochrome

The routine procedure given below for extraction of the cell pigments was devised to give maximum yields, as well as preparations of hydrogenase and of denatured cytochrome; it is therefore more complicated than if cytochrome only had been required and if yield had been unimportant. A rapid procedure by which purified cytochrome alone may be obtained in about 24 hr. is also given.

Routine procedure. Acetone-dried organisms were extracted at 4° with KH_2PO_4 (0.5 %, w/v; pH 7.0 \pm 0.1) yielding a green-brown solution containing cytochrome, 630 m μ -component, hydrogenase, free flavins and flavoproteins. The latter were removed by addition of H_2SO_4 (2 N) to pH 5.0 \pm 0.2 and the precipitate extracted with NH_4HCO_3 , dialysed in H_2 and freeze-dried to yield the hydrogenase fraction. The supernatant fluid from the precipitation at pH 5 was half saturated with $(NH_4)_2SO_4$ and the precipitate, containing the 630 m μ -component and some cytochrome, was extracted with NH_4HCO_3 (c. 1 %, w/v), dialysed, passed through a column of ammonium Amberlite-XE 97 to remove as much cytochrome as possible, dialysed and freeze-dried as the 630 m μ -component. Some purified cytochrome was eluted from the Amberlite column with NH_4OH (0.25 N) and added to the major fraction obtained later. The supernatant from the $(NH_4)_2SO_4$ precipitation was brought to pH 2.6 \pm 0.2 with 2 N- H_2SO_4 and the precipitate, whose cytochrome content increased the longer it was left in contact with the supernatant, was dissolved in $NaHCO_3$ solution and dialysed. The supernatant fluid was passed through a column of Whatman 'standard grade' cellulose powder, the effluent (containing flavins) discarded and the adsorbed cytochrome eluted, together with some flavin, with NH_4OH (0.25 N) and dialysed. The combined dialysed products from the last two steps were adsorbed on to a column of ammonium Amberlite-XE 97 and washed with water. The coloured effluent contained denatured cytochrome which was collected and freeze-dried; it had no enzymic activity and reacted with CO. The native protein was eluted with NH_4OH (0.25 N), dialysed and freeze-dried. Fig. 3 records the steps in a typical preparation. Sulphuric acid was used in preference to TCA for pH adjustment because some earlier fractionations with TCA had yielded wholly denatured products—consistent with Lewis's (1954) observation that TCA has a more drastic effect than H_2SO_4 on haemoproteins. Verhoeven & Takeda (1956) encountered similar difficulties during the extraction of bacterial cytochrome c from nitrate-reducing bacteria, and finally chose citric acid for their fractionation procedure. Filtrations were

avoided at all stages since the cytochrome was readily absorbed on cellulose; Keilin & Hartree (1945) reported that salt-free solutions of cytochrome *c* behaved similarly. Ishimoto & Koyama (1955) used an essentially similar procedure based on acetone precipitation in place of acid fractionation.

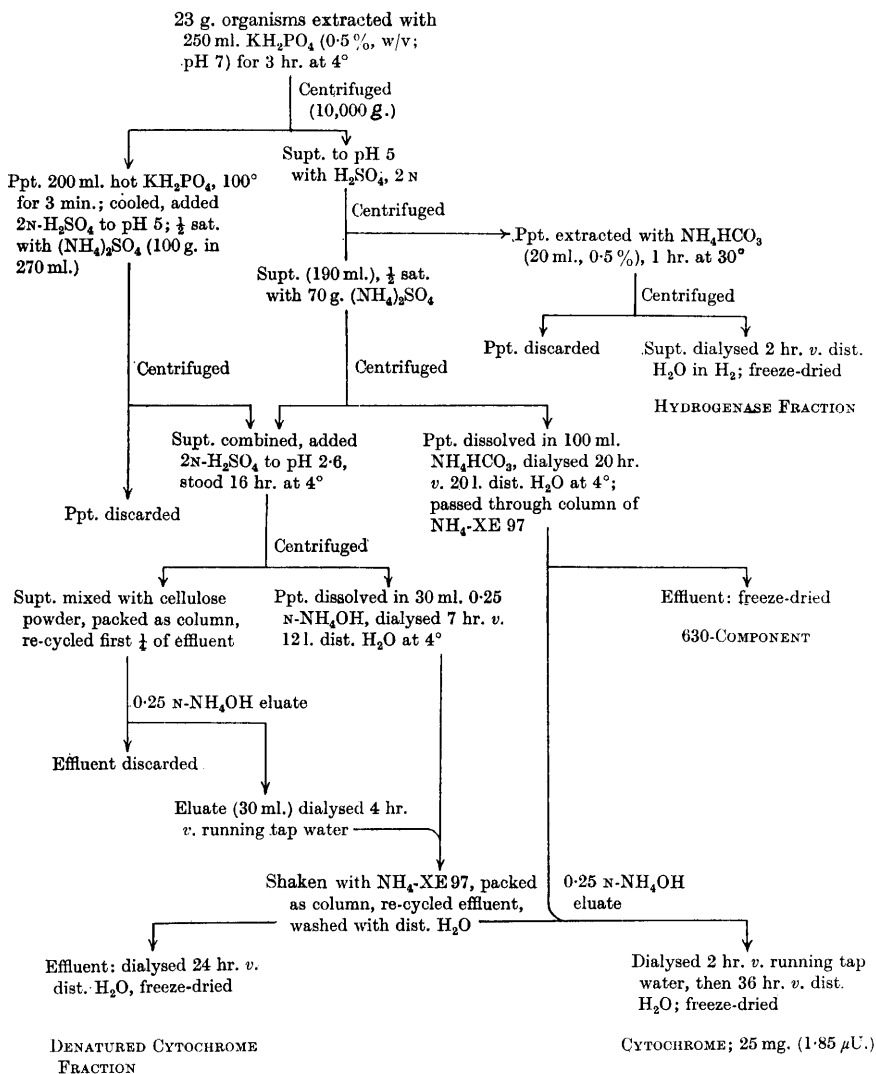


Fig. 3. Fractionation of acetone-dried *Desulphovibrio desulphuricans* (Hildenborough). The bacteria contained *c.* 0.22 μU . cytochrome c_3 /g. air-dry mass; assuming their acetone-dried mass was similar, the yield was *c.* 28% of theory.

Rapid procedure. Rapid procedures based on the release of cytochrome by CTAB or by heat were sometimes used; a record of a typical preparation follows: wet centrifuged bacteria (28 g. dry wt./100 ml. distilled water) were added slowly to 1 l. boiling and stirred KH_2PO_4 solution (0.5%, w/v;

pH 7.0 ± 0.05), boiled 3 min. and allowed to cool at 4° . After 4 hr. the mixture was centrifuged, the supernatant fluid dialysed for 16 hr. against 12 vol. distilled water and shaken with 50 ml. ammonium Amberlite XE 97, packed as a column, and the first portion of effluent re-cycled to ensure complete absorption of cytochrome. As in the earlier procedure, a portion of 'denatured' material, able to react with CO, was not held by the resin. The pink column was washed with distilled water, the cytochrome eluted with $0.25\text{ N-NH}_4\text{OH}$, and the product dialysed against 100 vol. distilled water and freeze-dried. The yield was 16.6 mg. purified powder (c. 19% theory).

THE PROPERTIES OF THE CYTOCHROME OF
DESULPHOVIBRIO DESULPHURICANS

A preliminary account of the chemical and physical properties of the bacterial cytochrome has appeared elsewhere (Postgate, 1955*b*).

Spectrum. The cytochrome prepared by the procedure above had typical haematin spectra (Fig. 4). The analogy of the ferricytochrome spectrum at pH 7.0 to that of ferricytochrome *c* type III (Theorell & Akesson, 1941) is

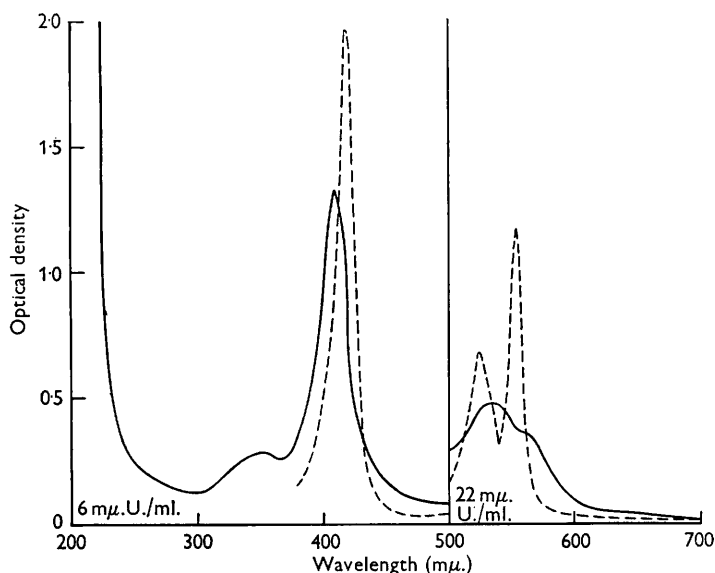


Fig. 4. Spectrum of purified cytochrome c_3 from *Desulphovibrio desulphuricans* (Hildenborough). Readings were taken at $1\text{ m}\mu$. intervals at absorption peaks, $5\text{ m}\mu$. intervals elsewhere; pH 7.00 ± 0.02 ; $t = 20^\circ$; $22\text{ m}\mu\text{U.}$ cytochrome/ml. in visible range, $6\text{ m}\mu\text{U.}$ in ultraviolet; - - -, reduced form; —, oxidized form.

marked, even to the inflexion about $565\text{ m}\mu$., but the band at $280\text{ m}\mu$. was missing and no evidence for a band about $685\text{ m}\mu$. (Theorell, 1948) was found. The ferrocytochrome spectrum was not examined in the ultraviolet region since it was not stable in the absence of dithionite, which absorbs in this region, but the visible and Soret peaks again resembled those of cytochrome *c*

except that they were shifted a few $m\mu$. towards the red. The visible peaks were measured with greater precision in the optical spectroscope and were $\alpha = 553.2 m\mu$. and $\beta = 525.2 m\mu$. at $\text{pH } 7.00 \pm 0.02$ and 19° . Some ratios of the intensities of various peaks are given in Table 2.

Table 2. *Ratios of heights of absorption peaks in cytochrome c_3*

$\alpha = 553 m\mu$., $\beta = 525 m\mu$., $\gamma = 419 m\mu$., $\delta = 350 m\mu$..

Peaks	Ratio
$\gamma : \alpha$ (reduced)	5.75
$\gamma : \alpha$ (oxidized)	12.6
γ (reduced) : (oxidized)	1.51
$\gamma : \delta$ (oxidized)	6.6
$\alpha : \beta$ (reduced)	1.52

The specific extinction coefficient was obtained by dissolving known amounts of well-dialysed, freshly chromatographed cytochrome in buffer; at 20° and $\text{pH } 7.0$ $\epsilon_{\text{sp.}} = 4.20 \pm 0.06$ in the reduced condition at $553 m\mu$., and 1.60 in the oxidized condition at $535 m\mu$.

Purity. Electrophoresis on paper in KH_2PO_4 (0.5%, w/v, $\text{pH } 7.0$) showed only the single cytochrome zone even after staining with bromothymol blue. Chromatography in buffer on a cation exchange resin (Boardman & Partridge, 1955) indicated small amounts of a colourless impurity which absorbed light at $280 m\mu$.; the pH value of the buffer used was less than $\text{pH } 7.0$ recommended by Boardman & Partridge for cytochrome c , since at the latter pH value the band due to the bacterial cytochrome spread badly. A typical experiment showing the presence of impurity absorbing at $280 m\mu$. is recorded in Fig. 5; the R_f value of the impurity was 0.9–1.0 (that of the cytochrome was 0.38), and a rough integration indicated that it amounted to less than 6% of the total protein. Since purification by chromatography in buffer was laborious and conveniently applicable only to small amounts of material, the work reported in this paper was done with material containing this impurity.

Reactions in solution. The freeze-dried powder had a deep red colour. It dissolved completely in phosphate buffer or 0.25 N- NH_4OH to give a clear solution; it also dissolved in distilled water but much more slowly, and salt-free solutions were best prepared by dialysis of salt-containing solutions. Preparations stored for over 1 month at -12° sometimes contained insoluble brown material, but the supernatant fluid was enzymically active. Exposure of solutions to preparations of organisms caused some denaturation, since cytochrome recovered from metabolic experiments (below) always contained a portion of material which was not retained by ammonium Amberlite XE 97, and which reacted with CO.

No spectroscopic change occurred in any of the following conditions: heating at $\text{pH } 7.0$ to 100° for 5 min.; passing in pure CO; adding KCN up to 10^{-2} M; adding excess ascorbic acid, NaBH_4 , $\text{K}_4\text{Fe}(\text{CN})_6$ or FeSO_4 . With cysteine the reduced form of the cytochrome appeared slowly and incompletely, with Na_2S it appeared slowly but completely, and with $\text{Na}_2\text{S}_2\text{O}_4$ it appeared at once. Sodium nitrite and diluted acetic acid gave a nitroso-derivative of the

oxidized form (visible bands of equal intensity at 532.5 and 563.5 $m\mu$.; compare 531.1 and 563.4 $m\mu$. for muscle cytochrome c); treatment with 2 N -NaOH denatured the preparation, since it then reacted with CO (absorption peaks: 415, 530, 565 $m\mu$.; compare 415, 531.5, 564.5 $m\mu$. for muscle cytochrome c) or with pyridine + dithionite (absorption peaks of 'pyridine haemochromogen':

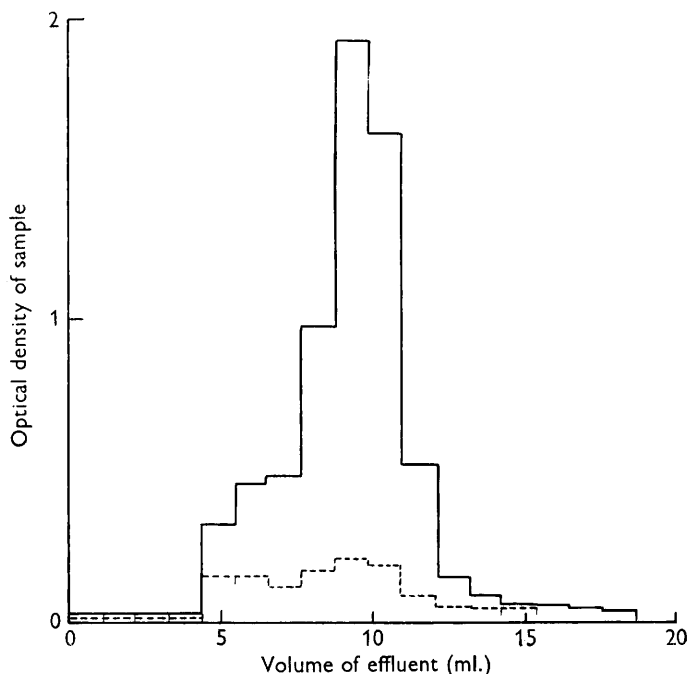


Fig. 5. Chromatography of purified cytochrome from *Desulphovibrio desulphuricans* (Hildenborough). 2 mg. cytochrome c_3 was chromatographed in phosphate buffer (pH 6.40 ± 0.02) on Amberlite XE 97 (see text), flow rate 1.1 ml./hr.; room temperature; retention volume of column 3.8 ml. Absorptions of 1.1 ml. samples at 410 and 280 $m\mu$. (dotted lines) were plotted; R_f of cytochrome c_3 (410) = 0.38.

418, 521.5, 551.8 $m\mu$.; compare 415, 521.8, 550.6 $m\mu$. for muscle cytochrome c). The denatured form had no enzyme activity, and its reduced absorption spectrum at pH 7.0 had bands at 418, 552.6 and 524.9 $m\mu$. which were indistinguishable from the native protein without special care.

Autoxidizibility. The cytochrome was apparently autoxidizable, but it was desirable to prove that this was a property of the native protein since apparent autoxidizibility might also be due to denaturation or contamination with an oxidase. The following evidence is in favour of the view that the native protein is autoxidizable: (i) enzymically active preparations were fully autoxidizable but did not react with CO; (ii) the oxidation was not affected by KCN (10^{-2} M), a powerful inhibitor of cytochrome c oxidase; (iii) no sign of a cytochrome c oxidase was detected at any stage in the purification of the cytochrome; (iv) the standard redox potential (below) was such that, on physico-chemical grounds, the reduced form was unlikely to be stable in air.

Iso-electric point. The fact that the cytochrome could be purified by ion-exchange with an ammonium Amberlite XE 97 suggested a basic iso-electric point; this was confirmed by paper electrophoresis in KH_2PO_4 buffers (0.5%, w/v) adjusted to various pH values with 20% (w/v) NaOH, on paper strips of 5 cm. width with the instrument setting at 2 mA./strip (potential between buffer compartments about 380 V.). At pH 6.99, 8.10, 9.23 (borate buffer) and 10.15 the protein migrated towards the cathode rather more slowly than chromatographically purified cytochrome *c* on the same strips. At pH values in the region of ten paper strips took up CO_2 with a decrease in pH value, so that a precise determination of the iso-electric point was not attempted. However, during a 2 hr. run starting at pH 10.66 (the iso-electric point of cytochrome *c*) and ending at pH 10.30 the protein migrated slowly towards the cathode together with a control spot of cytochrome *c*. Hence the iso-electric point probably lies between these values.

Redox potential. Careful reduction with $\text{Na}_2\text{S}_2\text{O}_4$ in the presence of redox dyes indicated a standard potential between that of sodium indigo disulphonate ($E'_0 = -125$ mV.) and benzylviologen ($E'_0 = -395$ mV.) and lying in the range of Janus green ($E'_0 = -225$ mV.); Ishimoto *et al.* (1954*b*) obtained a similar value with their preparation. This strongly reducing potential accounted for the failure of ascorbic acid, $\text{K}_4\text{Fe}(\text{CN})_6$, etc., to reduce the protein, and also made impracticable the measurement of the standard redox potential by spectrophotometric procedures (e.g. Davenport & Hill, 1952) since there is no stable redox system in this range. The potential was therefore determined by potentiometric titration in O_2 -free nitrogen with excess anthraquinone-2-sulphonic acid ($E'_0 = -250$ mV.), which did not absorb significantly in visible light and which poised in the appropriate potential range. Three titrations of 0.15 μU . cytochrome + 1.5 mg. anthraquinone-2-sulphonic acid in 29 ml. sodium phosphate buffer (0.34 g. ion Na^+/l .; pH 7.0 ± 0.02) at $30 \pm 0.3^\circ$ against $\text{Na}_2\text{S}_2\text{O}_4$ indicated a standard potential of $E'_0 = -205 \pm 4$ mV.

Molecular weight. The sedimentation coefficient of a preparation of the cytochrome was determined by Dr A. G. Ogston at the Department of Biochemistry, University of Oxford. A solution of *c.* 0.5% cytochrome in NH_4OH (0.25 M), dialysed overnight against 1000 vol. of a solution containing NaCl (0.1 M) + Na_2HPO_4 (0.0627 M) + KH_2PO_4 (0.0133 M) had a sedimentation coefficient $S_{20, w} = 1.93 \times 10^{-13} \pm 2\%$ (compare 1.83×10^{-13} quoted for cytochrome *c* of 0.43% Fe content; Paul, 1952). The sedimenting boundary seemed symmetrical, no obvious sign of heterogeneity was observed, and all the light-absorbing material appeared to be associated with the sedimenting material. This sedimentation coefficient implies a minimum possible value of 10,200 for the molecular weight, on the assumption that $f/f_0 = 1$, the particles being spherical, unhydrated and with a hydrodynamic specific volume equal to the partial specific volume taken as 0.71, the value given by Theorell (1936) for cytochrome *c*. Since most globular proteins have values of f/f_0 considerably greater than 1, the sedimentation coefficient above would be consistent with a molecular weight in the region of 13,000 (see Discussion).

Iron content. Analysis of some residues recovered from metabolic experi-

ments indicated a high iron content (0.75%), and a single analysis of 8.1 mg freshly chromatographed, well-dialysed cytochrome, by the *o*-phenanthroline procedure (Sandell, 1944) indicated an iron content of 0.92%.

Linkage of prosthetic groups. The haemin of cytochrome *c* is linked to the apoprotein by thio-ether links to the porphyrin ring, as well as by co-ordination to the iron atom; as a result the molecule shows considerable thermo-stability and resistance to cleavage of its prosthetic group from the apoprotein by acids. Moreover, the molecule readily yields, with mineral acids, an ether-insoluble porphyrin in which the sulphur-containing amino acid groups remain attached to the porphyrin residue.

The bacterial cytochrome showed similar thermal and acid stability. Porphyrin was released from the oxidized form only by concentrated H_2SO_4 (bands at 404, 550.8, 593 $m\mu$. in 2 *N*-HCl; HCl number, 0.05 ± 0.01 %); the reduced form was less stable and 3.3 *N*-HCl in the presence of $Na_2S_2O_4$, released mainly a porphyrin which was insoluble in ether + glacial acetic acid (15%, v/v), and which had its main band at 554 $m\mu$. (compare 553 $m\mu$. quoted by Keilin (1933) for 'porphyrin *c*').

Ice-cold acetone containing 10% (v/v) glacial acetic acid, which removes the haemin from haemoglobin, catalase, etc. (see Lewis, 1954), precipitated the bacterial cytochrome unchanged. Ice-cold acetone containing 1 ml. 5 *N*- H_2SO_4 /50 ml. acetone did not yield an ether-soluble haemin, although the protein was converted to a brown water-soluble material from which ether-soluble haemin could be obtained by Paul's (1950) procedure. These properties all suggest that thio-ether links participate in the binding of prosthetic groups and protein in the bacterial cytochrome.

Nature of prosthetic groups. A haemin was released from the cytochrome by Paul's (1950) procedure. A typical experiment was as follows: 1 ml. chromatographed cytochrome solution (0.7 μ U/ml.) was treated with 0.2 ml. glacial acetic acid and 1 ml. $AgSO_4$ (8 mg./ml.) for 30 min. at 75–80°, cooled and the haemin extracted three times into ether containing 25% (v/v) acetic acid, dried, dissolved in KH_2PO_4 (0.5% w/v, pH 7.0) and examined spectrophotometrically. The product had a Soret band at 390 $m\mu$. as compared with 391 $m\mu$. found in a control preparation of haematohaemin from cytochrome *c*. Pyridine haemochromes prepared from these haemins were spectroscopically closely similar: pyridine haematohaemochrome had peaks at 409, 518.5 and 546.0 $m\mu$.; the pyridine haemochrome found from the isolated haemin of the bacterial cytochrome absorbed at 408, 517.2 and 546.0 $m\mu$. It is interesting that, as with cytochrome *c*, the spectrum of the 'pyridine haemochromogen' formed directly from the protein (quoted earlier, p. 555) differed from that obtained from the separated haemin.

These observations suggested that the prosthetic groups of cytochrome *c* and the bacterial cytochrome were closely similar, and further evidence for this was obtained by reductive fission of the protein to porphyrin. Davenport (1952) obtained mesoporphyrin and a chlorin from cytochrome *c* by reduction *in vacuo* with sodium amalgam; degradation of the bacterial cytochrome in this manner gave a porphyrin spectroscopically resembling mesoporphyrin as

well as a chlorin (α -band at 643 $m\mu$. in ether, identical with a control preparation from cytochrome *c*). The wavelengths in $m\mu$. of the absorption peaks of the porphyrin, freed of chlorin, in dioxane and in 2 *N*-HCl are given below:

In dioxane	Mesoporphyrin from cytochrome <i>c</i>	402, 496.5, 529.8, 566.8, 621.5
	Porphyrin from bacterial cytochrome	402, 497, 530.2, 566.5 621.5
In 2 <i>N</i> -HCl	Mesoporphyrin from cytochrome <i>c</i>	402, 547.5, 570.5, 590.5
	Porphyrin from bacterial cytochrome	402, 549.2, 571, 591

These observations permit the tentative conclusion that the prosthetic groups of the bacterial cytochrome are OH-substituted haematohaemins linked to the protein by thio-ether bridges as in cytochrome *c*, a view further supported by the close spectroscopic similarities of the pyridine, carboxy- and nitroso-derivatives of the two proteins (p. 555).

Metabolic function of the bacterial cytochrome

The probability that the cytochrome acted as electron carrier during the biological reduction of sulphate and related ions was investigated further by studying preparations of bacteria able to oxidize the cytochrome anaerobically with these substrates and by measuring the effect of the cytochrome on the rates of substrate reduction in hydrogen by these preparations. Owing to shortage of cytochrome the quantitative aspects of its effect on these reactions could not be studied thoroughly, but some qualitative impressions of its relative activity are recorded. A preliminary account of this work was given elsewhere (Postgate, 1955*a*).

Anaerobic cytochrome oxidation

Bacteria treated with CTAB (50 $\mu\text{g.}/\text{mg.}$ dry wt.) oxidized the cytochrome anaerobically with Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3$ or $\text{Na}_2\text{S}_4\text{O}_6$, but not with Na_2SO_4 , provided precautions were taken to remove sulphide continuously as in the earlier experiments with living organisms (above). In a typical experiment a suspension of 0.2 mg. CTAB-treated cells/ml. at pH 7.0 was placed in a double side-arm Thunberg tube under H_2 until all added cytochrome (5 $\mu\text{U.}/\text{ml.}$) was reduced. One side arm contained CdCl_2 (10 %, w/v) on filter-paper to absorb sulphide, the other contained substrate. The hydrogen was then pumped out, the substrates added *in vacuo* and the suspension examined spectroscopically. The intensities of the cytochrome bands, compared with controls without substrate or with Na_2SO_4 , diminished markedly during 5 min., though the bands did not disappear entirely even after several hours.

While this work was in progress, Millet (1955) obtained a cell-free sulphite reductase from another strain of *Desulphovibrio desulphuricans*, and kindly provided instructions on how to prepare active extracts. Such preparations, made from the Hildenborough strain, contained, in addition to a sulphite reductase and hydrogenase, thiosulphate and tetrathionate reductases. Being

transparent they permitted quantitative measurement of the extent of cytochrome oxidation by sulphite, thiosulphate and tetrathionate (Table 3). A constant percentage oxidation was reached much more slowly with Na_2SO_3 than with $\text{Na}_2\text{S}_2\text{O}_3$ or $\text{Na}_2\text{S}_4\text{O}_6$; quoted values in Table 3 are corrected for a slow oxidation which occurred in the control tubes without substrate.

Table 3. *Anaerobic oxidation of cytochrome c_3 by cell-free extracts of Desulphovibrio desulphuricans (Hildenborough)*

Vacuum-dried organisms were shaken with distilled water (25 mg./ml.) for 1 hr. at 37° under N_2 and the debris removed at 15,000 g (15 min.). The supernatant fluids were diluted 1/3 with KH_2PO_4 (0.5%, w/v; pH 7.0 \pm 0.05) containing 25 $m\mu\text{U}$. cytochrome/ml., added to double side-arm Thunberg tubes containing substrate in one arm and CdCl_2 (10%, w/v) on filter-paper in the other. After reducing the cytochrome in H_2 the substrates were added *in vacuo* and the optical density at 554 $m\mu$. was measured at intervals until a constant percentage oxidation was reached.

Substrate	expt:	Percentage oxidation of cytochrome	
		1	2
Na_2SO_3		19	14
$\text{Na}_2\text{S}_2\text{O}_3$		70.5	76
$\text{Na}_2\text{S}_4\text{O}_6$		82	84

Substrate reduction in hydrogen

CTAB released cytochrome from the bacteria. Consequently, cytochrome-linked reactions would be expected to be inhibited by CTAB owing to dilution of the co-factor, and the inhibition should be overcome by adding large amounts of purified cytochrome. This approach was used to investigate the effect of cytochrome on the reduction of substrates in hydrogen.

Thiosulphate reduction. CTAB (50 $\mu\text{g.}/\text{mg.}$ dry wt. organisms) prevented the reduction of sulphate in H_2 by bacterial suspensions, but permitted slow reduction of sodium thiosulphate. Addition of the cytochrome augmented the reaction rate (Fig. 6), thus indicating a carrier action. FAD, DPN, TPN and ATP had no carrier action.

Tetrathionate reduction. Similar experiments, using sodium tetrathionate in place of thiosulphate, showed that the cytochrome acted as a carrier for the tetrathionate reductase system (Fig. 6).

Sulphite reduction. Similar experiments, with sulphite in place of thiosulphate, showed that the cytochrome acted as a carrier in this system, but its effect was much less marked than with thiosulphate and tetrathionate (Fig. 6). This may be due to: (a) a greater requirement for cytochrome in this system; (b) need for additional co-factors; (c) greater damage to the sulphite reductase caused by CTAB. The latter explanation was favoured by the further observations that doubling the CTAB concentration totally inhibited the sulphite reductase, though the thiosulphate reductase remained active. A mixture of FAD, DPN, TPN and ATP did not augment the effect of the cytochrome.

Dithionite reduction. Sodium dithionite hydrolyses in anaerobic solution to a mixture of thiosulphate and metabisulphite, hence the ability of the

cytochrome to act as carrier in its reduction by CTAB-treated bacteria (Fig. 6) was not unexpected. The experiment is of interest however, since it demonstrated that cytochrome augmented the reaction rate in an environment having, at least initially, a redox potential of about -350 mV. (that of a weak dithionite solution), some 150 mV. more reducing than the standard potential of the cytochrome itself.

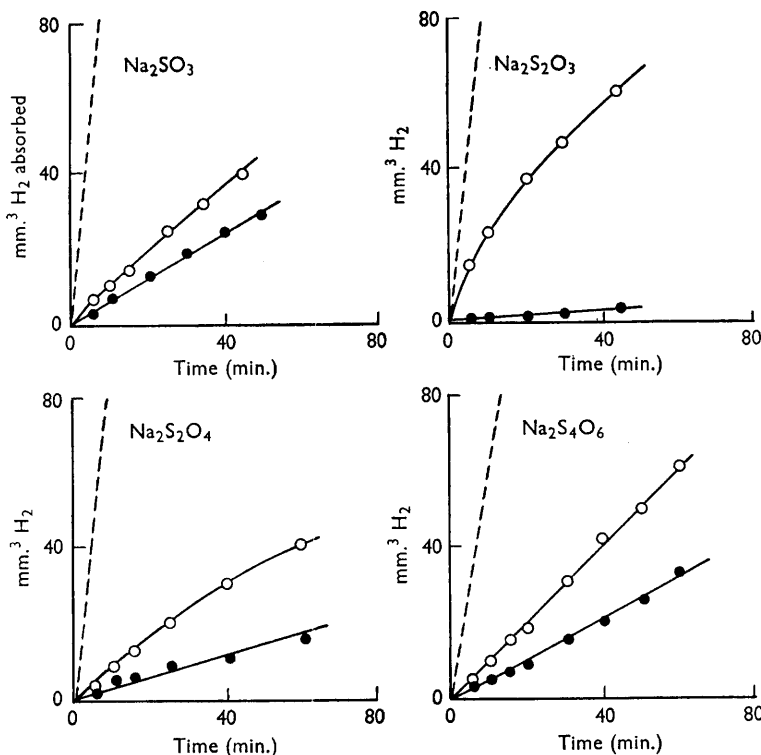


Fig. 6. Cytochrome-linked reductases in CTAB-treated *Desulphovibrio desulphuricans* (Hildenborough). Organisms (1.5 mg./vessel) were treated CTAB (50 μ g./mg. dry wt. bacteria) and their ability to reduce substrates in H_2 with and without cytochrome c_3 was measured manometrically. Cytochrome: 50 $m\mu$ U./ml.; vessels preincubated 1 hr. before adding *c.* 1 mg. substrate (dithionite added as solid); centre well; $CdCl_2$ (0.25 ml., 10%, w/v); reaction fluid 1.25 ml. final; pH 6.9 ± 0.05 ; 37° , H_2 gas phase; dotted lines indicate average rates of substrate reduction without CTAB, O, cytochrome; ●, without added cytochrome.

Sulphate reduction. The cytochrome did not influence the rate of reduction of sulphate in H_2 by intact bacteria. None of the following preparations reduced sulphate in hydrogen: organisms treated with CTAB, acetone-dried or vacuum-dried bacteria and soluble extracts therefrom; extracts of cells ground with Al_2O_3 ; hence experiments analogous to those recorded above were not undertaken. The failure of acetone-dried bacteria to reduce sulphate conflicts with a report by Sadana & Jagannathan (1954), who stated that sulphate acted as hydrogen acceptor for crude preparations of acetone-dried organisms. Indirect

evidence pointing to a function in sulphate reduction is summarized later (see 'Discussion').

Oxygen reduction. The fact that the cytochrome is autoxidizable gave theoretical reasons for suspecting that the sulphate-reducing bacteria could reduce oxygen in spite of their anaerobic habit; synthesis of water from H_2/O_2 mixtures was detected and reported earlier (Postgate, 1954*b*). The reaction took place at a maximum rate with H_2 /air mixtures containing 4% (v/v) O_2

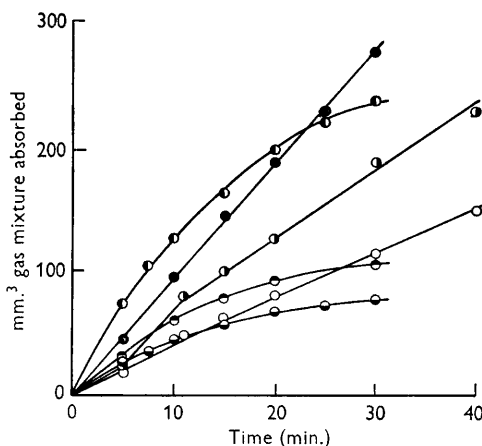


Fig. 7

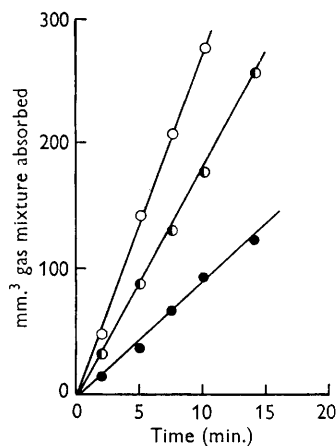


Fig. 8

Fig. 7. Hydrogen-oxygen reaction in *Desulphovibrio desulphuricans* (Hildenborough). Bacteria were harvested from a lactate yeast extract + sulphate static culture and the rates of gas uptake in hydrogen-air mixtures were determined manometrically. 9.25 mg. dry wt. organisms/vessel; total fluid volume 3 ml.; buffer: KH_2PO_4 (0.5%, w/v; pH 6.3 ± 0.05); 37°, gas phase concentrations of O_2 (v/v): ○, 1%; ●, 2%; ●, 4%; ●, 8%; ●, 12%; ●, 16%.

Fig. 8. Effect of cytochrome c_3 on hydrogen-oxygen reaction by CTAB treated *Desulphovibrio desulphuricans* (Hildenborough). Bacteria were treated with CTAB (100 $\mu g./mg.$ dry wt.) and the rate of gas uptake measured manometrically. 4.3 mg. dry wt. organisms/vessel; fluid volume 1.5 ml.; buffer: KH_2PO_4 (0.5%, w/v; pH 6.9 ± 0.05). ○, without CTAB; ●, with CTAB; ●, with CTAB + cytochrome c_3 (250 $\mu U./ml.$).

(Fig. 7); at lower pO_2 values availability of oxygen presumably limited the reaction velocity, and at higher pO_2 values oxidation of hydrogenase inhibited the reaction. This situation is familiar in the biological aerobic oxidation of hydrogen, but the system in *Desulphovibrio desulphuricans* appears to be more sensitive to oxygen inhibition than most (compare optimal pO_2 values of 8% for *Hydrogenomonas flavus*, Kluver & Manten, 1942; 4–10% for *Azotobacter vinelandii*, Wilson, Lee & Wilson, 1942; about 9% for *Escherichia coli*, Lascelles & Still, 1946; 5–15% for an unspecified *Hydrogenomonas* sp., Schlegel, 1953).

A remarkable feature of the H_2/O_2 reaction was that, in the optimum atmosphere of 4% (v/v) O_2 , oxygen was frequently reduced faster than sulphate. The Q_{O_2} of the control curve in Fig. 8 is $-129 \text{ mm.}^3/\text{mg. dry wt. organisms/hr.}$, corresponding to an oxygen reduction rate of $5.8 \mu\text{mole/mg./hr.}$

The mean Q_{H_2} value in several experiments with sulphate as hydrogen acceptor was $-440 \text{ mm.}^3/\text{mg./hr.}$, corresponding to a sulphate reduction rate of $4.9 \mu\text{mole/mg./hr.}$

CTAB ($100 \mu\text{g./mg.}$ dry wt. organisms) decreased the reaction velocity to a low value, and addition of cytochrome increased the reaction rate again (Fig. 8). Oxidation of lactate, pyruvate, fumarate and malate with O_2 as terminal H-acceptor ($N_2 + 4\%$ (v/v) O_2) was demonstrated with strain Hildenborough (Postgate, 1954*b*) and strain El Agheila Z (Grossman & Postgate, 1955), but was not studied further.

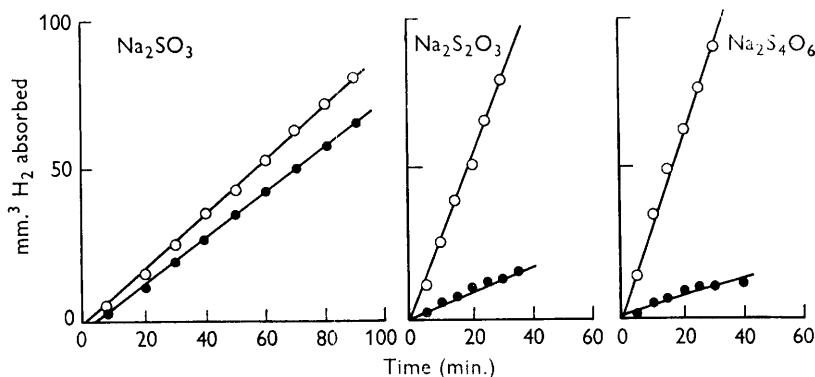


Fig. 9. Influence of cytochrome c_3 on hydrogen uptake by cell-free reductase preparations from *Desulphovibrio desulphuricans* (Hildenborough). Vacuum-dried bacteria were extracted under N_2 at 37° with distilled water (1 ml./25 mg.; 1 ml./50 mg. for sulphite reductase) and the supernatant fluids (0.5 ml.) used in the manometers. Fluid volume 1.5 ml.; buffer: KH_2PO_4 (0.5%, w/v; pH 6.3 ± 0.05); centre well: $CdCl_2$ (0.25 ml., 10%, w/v); substrates: $5 \mu\text{mole}$; H_2 gas phase; 37° . O, with cytochrome c_3 (560 $m\mu\text{U./ml.}$; 680 $m\mu\text{U./ml.}$ with sulphite reductase); ●, control without cytochrome.

Reduction by cell-free preparations. The demonstration of cytochrome-linked reductases by the use of CTAB was open to the criticism that the cytochrome may act, not as an electron carrier, but merely by reversing chemically an inhibitory effect of CTAB. It was thus desirable to demonstrate carrier action by a technique not involving CTAB.

Extracts of vacuum-dried bacteria prepared following Millet's advice reduced thiosulphate, tetrathionate or sulphite in hydrogen, unlike extracts of acetone-dried cells, which reduced only thiosulphate in these conditions. The reduction rate was augmented by addition of the bacterial cytochrome (Fig. 9). As in the experiments with CTAB-treated organisms, the effect of the cytochrome on $Na_2S_2O_3$ and $Na_2S_4O_6$ reduction was much more marked than on Na_2SO_3 reduction. The reason for this seemed most likely to be that the total sulphite-reductase content of the extracts was low since benzylviologen, which also has a cytochrome-like effect on the sulphite reductase system, did not much augment the activity of these preparations.

Cytochrome-like effect of benzylviologen. Ishimoto, Koyama & Nagai (1955) extracted a soluble thiosulphate-reductase system from their strain of *Desulphovibrio desulphuricans* which conducted the reaction: $S_2O_3^{2-} + H_2 = SO_3^{2-} + H_2S$,

and whose reaction rate was augmented by benzyl- or methyl-viologen. Later Ishimoto & Koyama (1955) showed that their bacterial cytochrome had a similar effect on these preparations. It was of interest to see whether benzylviologen had a cytochrome-like effect on the major systems examined in the present work. Suspensions of CTAB-treated bacteria reduced benzylviologen with $\text{Na}_2\text{S}_2\text{O}_3$, $\text{Na}_2\text{S}_4\text{O}_6$ or Na_2SO_3 , but not with Na_2SO_4 . In a typical experiment a suspension of 0.33 mg. CTAB-treated bacteria/ml. at pH 7 was treated as in the comparable experiments with the bacterial cytochrome except that benzyl viologen (2 $\mu\text{mole/ml.}$) replaced the added cytochrome. The time taken to decolorize the reduced benzylviologen at 37° was noted. In contrast to

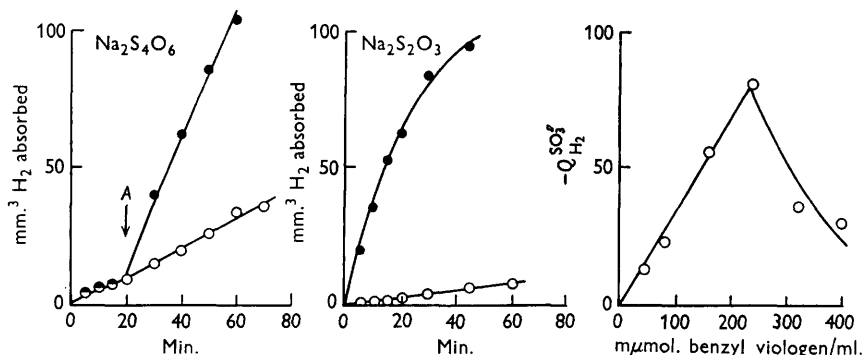


Fig. 10. Cytochrome-like effect of benzyl viologen. Organisms of *Desulphovibrio desulphuricans* (Hildenborough) were treated with CTAB (50 $\mu\text{g./mg.}$ dry wt.) and the rates of hydrogen uptake in the presence of reducible substrate measured manometrically. Cell contents as for Fig. 6 except that benzylviologen replaced cytochrome c_3 . With sulphite as substrate a curve illustrating the effect of benzylviologen concentration on substrate reduction rate is given; with tetrathionate the benzylviologen was tipped in after the start of the experiment (A). ●, with 200 μmol benzylviologen/ml.; ○, control.

their behaviour with cytochrome, the suspensions re-oxidized reduced benzyl viologen completely with the substrates mentioned; re-oxidation with Na_2SO_3 was markedly slower (70 min.) than with thiosulphate (15 min.) or tetrathionate (8 min.); without substrate or with Na_2SO_4 the dye remained reduced for more than 170 min. Jebb (1949) used a somewhat similar technique to show that the 'tetrathionase' of a coliform organism re-oxidized reduced Nile blue. Benzylviologen augmented the rate of reduction of thiosulphate, tetrathionate or sulphite in hydrogen by CTAB-treated bacteria and by cell-free reductase preparations. A selection of curves illustrating this is given in Fig. 10; the findings confirm and extend those of the Japanese workers.

THE COMPOUND ABSORBING AT 630 $\mu\text{m.}$

Concentrates of the material prepared as described always contained variable amounts of cytochrome and material insoluble in water. The material was purified further by extracting into distilled water and passing the solution through a column of cellulose coated with an aliphatic polyimine resin in the

acetate form (see 'Methods'). The effluent contained cytochrome and flavo-protein, and the 630 m μ -component remained on the column as an emerald green zone. It was eluted with sodium acetate + acetic acid buffer (5 M, pH 5.0) and dialysed against distilled water.

Properties. After prolonged dialysis, preparations of the 630 m μ -component precipitated in the dialysis sac, but the precipitate re-dissolved, on adding traces of phosphate buffer or NaHCO₃, to give an emerald-green solution. This solution had the spectrum shown in Fig. 11; the Soret peak at 411 m μ . did not change in height or position on adding dithionite, indicating that the material was free from cytochrome; there was an inflexion at *c.* 390 m μ ., a minor peak at 585 m μ . and a strong peak at 632.5 \pm 0.2 m μ . Paper electro-

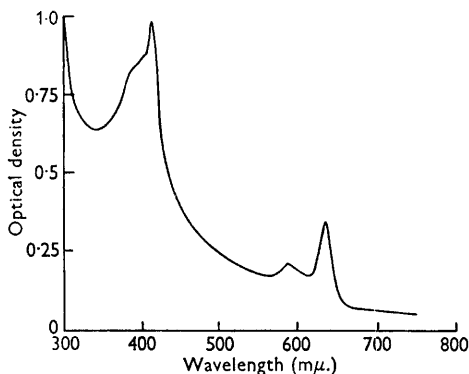


Fig. 11

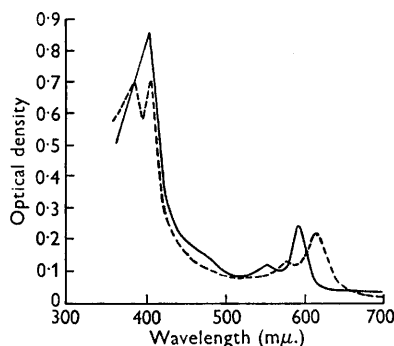


Fig. 12

Fig. 11. Spectrum of chromatographed '630 m μ -component'. Readings taken at 1 m μ . intervals at the Soret peak, 2.5 m μ . at α -peak, 5 m μ . at β -peak, 10 m μ . elsewhere. Protein in KH₂PO₄ (0.5 %, w/v; pH 7.0 \pm 0.05); 20°.

Fig. 12. Spectra of chromophore from '630 m μ -component'. Spectra obtained after chromatography on 'Florisil' (see text). —, in acetic acid (0.1 N); - - -, in HCl (N). Readings every 1 m μ . at Soret peaks, 2.5 m μ . at visible peaks, 10 m μ . elsewhere.

phoresis showed an iso-electric point on the acid side of pH 7.0. On heating above 70° or treatment with acid to pH < 4 or alkali to pH > 9 the 630 m μ -band shifted towards the green and the solution fluoresced red in ultraviolet light (365 m μ). No evidence for formation of a pyridine haemochrome was obtained; the main visible band moved to 595 m μ . with alkali with or without pyridine and/or dithionite.

Chromophoric group. The red fluorescent material was readily photo-oxidized with loss of fluorescence, and had to be handled in the dark, where it survived 3 min. in contact with warm conc. H₂SO₄. It was slightly soluble in ether containing 15 % (v/v) glacial acetic acid, but was not extracted quantitatively from aqueous solution with this mixture; it returned to the aqueous phase on shaking with distilled water. It was chromatographed in the dark on paper in 5 % (w/v) Na₂HPO₄, running as a single spot of *R_f* = 0.74 (20°). The chromophore was also adsorbed as a blue-green band from acid solution by 'Florisil' and was eluted with aqueous pyridine (1 % v/v) as a pink-brown solution with

an intense red fluorescence; with dilute mineral acids the colour changed to blue-green and the fluorescence became more purple. The spectra of preparations obtained in this fashion depended on the pH value of the solution (Fig. 12). Both acid and neutral forms showed their major visible absorption peak in the red, unlike a simple aetioporphyrin; the absorption peaks lay at 404, 594.5 and 551.0 $m\mu$. (alkaline or neutral) and 385, 404, 575.0 and 613.5 (N -HCl). The position and height of the double Soret peak of the acid form were unchanged in 2 N -HCl. A neutral spectrum of this material was not successfully obtained owing to its insolubility in organic solvents.

The chromophore was also observed by its fluorescence in old cultures that had become alkaline owing to loss of H_2S from the medium. Red fluorescence in u.v. light after adding NaOH to a culture was a sensitive test for the presence of the 630 $m\mu$.-component in various strains. The chromophore was undoubtedly responsible for the shading at 595 $m\mu$. sometimes observed in the spectrum of washed bacterial suspensions.

OTHER PIGMENTS

The spectrum of crude extracts of acetone-dried organisms showed a hump at about 450 $m\mu$. which disappeared on adding dithionite or on passing in hydrogen; simultaneously the extracts ceased to fluoresce yellow in ultraviolet light, a change characteristic of flavins. Protein was removed from such extracts with TCA (4%, w/v), the TCA was removed by extraction of the tri-*n*-octylamine salt into chloroform (Hughes & Williamson, 1951), and the extracts concentrated and chromatographed in the dark on paper in *n*-butanol/acetic acid/water (4 : 1 : 5) or aqueous Na_2HPO_4 (5%, w/v). The chromatograms indicated the presence of FAD, FMN and traces of riboflavin. An examination of freshly harvested *Desulphovibrio desulphuricans* was undertaken by Dr J. L. Peel, who showed that the Hildenborough strain has a low flavin content compared with most other anaerobes he has examined, but an unusually high ratio of FAD to FMN; the riboflavin observed was probably an artefact of the acetone-drying procedure; Dr Peel's results are reported elsewhere (Peel, 1955). As would be expected, these flavins exist in the normal cell as conjugates, since CTAB at concentrations able to release cytochrome into the medium released no flavin. No evidence was obtained for association of flavin with the cytochrome, unlike the cytochrome b_2 -lactic dehydrogenase system of Appleby & Morton (1954).

DISCUSSION

The cytochrome. Table 4 lists the properties of a number of cytochromes which have an α -peak within 1 $m\mu$. of that of the cytochrome described here, and shows that it is not identical with any of them. It may be identical with a degradation product of heart muscle cytochrome *b* (Hübscher, Kiese & Nicolas, 1954), but insufficient published data are available to judge. Hence the systematic name '*Desulphovibrio desulphuricans* cytochrome 553' should be applied to the material studied here (see Scarisbrick, 1947), but since this

name is cumbersome and does not bring out its relation to muscle cytochrome *c*, the trivial name of cytochrome c_3 has been adopted.

The relationship to cytochrome *c* may be summarized: c_3 is thermostable, soluble, has firm chemical linkings between the haemin and apoprotein and is strongly basic. It differs mainly in (a) its low redox potential and consequent autoxidizibility; (b) its different metabolic function; (c) details of its spectrum.

Table 4. *Some properties which distinguish various cytochromes from that present in Desulphovibrio desulphuricans ('cytochrome c_3 ')*

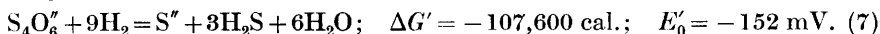
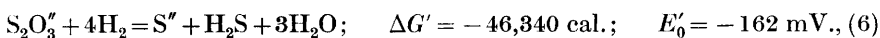
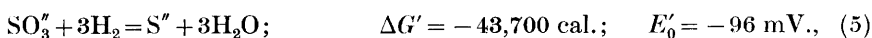
Pigment	Reference	Source	Major distinctive properties
Cytochrome c_1 (<i>e</i>)	1	Heart muscle	Thermolabile, not autoxidizable
Cytochrome <i>f</i>	2	Green leaves	Not autoxidizable, positive E'_0
Cytochrome b_4	3	Halotolerant bacterium	Not autoxidizable, acid iso-electric point
' <i>Chlorobium limicola</i> cytochrome 553'	4, 5	<i>C. limicola</i>	Not autoxidizable, positive E'_0
' <i>Acetobacter suboxydans</i> cytochrome 554'	6	<i>A. suboxydans</i>	Insoluble, not autoxidizable
'Hemoprotein 554'	7	Heart muscle	Positive redox potential
' <i>Chromatium</i> ' cytochrome	8	<i>Chromatium</i> 'D'	High m.w., limited pH stability
Cytochrome c_3	—	<i>D. desulphuricans</i>	Thermostable, low m.w., soluble, autoxidizable, negative E'_0 , basic iso-electric point

References: 1, Keilin & Hartree (1955); 2, Davenport & Hill (1952); 3, Egami *et al.* (1953); 4, Kamen & Vernon (1954*a*); 5, Gibson & Larsen (1955); 6, Smith (1954), also personal communication; 7, Widmer *et al.* (1954); 8, Newton & Kamen (1955).

A further difference from cytochrome *c* may be noted. The specific extinction coefficient of ferrocycytochrome *c* at 550 $m\mu$. is about 2.1, whereas ϵ_{sp} . for cytochrome c_3 at 553 is about double this (4.2). Since the molecular weights of cytochromes *c* and c_3 are of a similar order, it follows that c_3 has two haemin groups/molecule. This view is confirmed by the high iron content (0.92 %) of c_3 , more than twice that of the purest *c* recorded (see Paul, 1952).

Though c_3 can act as carrier in reactions in which oxygen is terminal hydrogen acceptor, the strictly anaerobic character of these bacteria emphasized by Grossman & Postgate (1953*a*) leaves little doubt that these reactions are of limited metabolic significance and merely reflect the low redox potential of c_3 . Such reactions might, however, provide a mechanism by which the organism could remove traces of O_2 from its environment since O_2 , while not lethal, prevents growth and sulphate reduction. The absence of inhibition by KCN, CO, etc. indicates that no ordinary cytochrome *c* oxidase is present, and the presence of a cytochrome *c* oxidase insensitive to cyanide like that of *Myrothecium verrucaria* (Darby & Goddard, 1950) is excluded since neither suspensions nor preparations of the strain studied here oxidized reduced cytochrome *c*. However, though no preparation has been obtained in which a linkage between cytochrome c_3 and sulphate reduction could be demonstrated directly,

The failure to observe complete oxidation of c_3 by sulphate and other ions, in contrast to benzy viologen, could be because sulphide was incompletely removed from solution in the test conditions, with the result that sufficient remained in solution to hold some cytochrome reduced. This explanation is unlikely, however, since the percentage oxidations quoted in Table 3 should then be independent of the substrate, which they are not. The phenomenon might be accounted for if the E'_0 values of the reductions of sulphite, thio-sulphate, etc., were of an order similar to that of c_3 ; the ions would then come to redox equilibrium with c_3 rather than oxidize it completely. If $\Delta G'$ values at pH 7 are calculated for the reductions of sulphite, thiosulphate and tetrathionate (ΔG° for $S_2O_3^{2-}$ obtained from Mel, 1954), in the manner used above for sulphate, and the values are converted to potentials, the quantities below are obtained:



These potentials do not lie in the order necessary to account for the results in Table 3; hence no simple thermodynamic formulation of the reactions involved will account for the phenomenon.

The low E'_0 values of cytochrome c_3 and of the reduction of sulphate, sulphite, etc., are consistent with the strictly anaerobic habit of these bacteria. Starkey & Wight (1945) showed that the initiation of growth of sulphate-reducing bacteria was accompanied by a decrease in redox potential of the environment to below -200 mV.; ZoBell & Rittenberg (1948) quoted an E_h value of -100 to -300 mV. as being most favourable to growth of marine strains; Stárka (1951) showed that the ripening of medicinal muds, attributed to thermophilic strains of *Desulphovibrio*, was associated with a decline of E'_0 value to between -200 and -300 mV.; Grossman & Postgate (1953*a, b*) showed that small inocula did not multiply unless the medium was supplemented with Na_2S or cysteine, which would bring the redox potential into this range. Clearly, then, one can regard *D. desulphuricans* as an organism which conducts oxidative reactions at the strongly reducing potential of about -200 mV.

The presence of c_3 in *Desulphovibrio desulphuricans* accounts, at least partly, for the iron requirement first demonstrated by Butlin, Adams & Thomas (1949). The fact that cytochrome c_3 is present in relatively large amounts (a typical content of $0.22 \mu\text{U. cytochrome } c_3/\text{g. dry wt. organisms}$, observed spectroscopically by Barer's procedure, corresponds to a c_3 content of about 3 mg./g. ; i.e. 0.3% of the air-dry bacterial mass) and is very easily extracted and purified, makes it surprising that c_3 was not observed earlier. The explanation must lie in (a) the convention of growing *D. desulphuricans* in the presence of excess ferrous salts, thus producing a spectroscopically impenetrable black mass of bacteria and FeS ; (b) the relative difficulty of obtaining and maintaining pure cultures of these bacteria; (c) the low yields obtained in even the best batch cultures; (d) the belief, supported by earlier studies among the clostridia, that looking for cytochromes in obligate anaerobes was a waste of time.

The 630 m μ -component. This pigment, though a protein, is clearly not a cytochrome in the conventional sense of the word, and the spectroscopic resemblance to cytochrome a_2 is misleading. The present work provides no clue to its metabolic function. Oxidation and reduction lead to no spectroscopic change; there is no obvious reaction with CO, KCN or NaN_3 and no compound that can be definitely classed as a haemochrome was observed, in contrast to the report of Ishimoto *et al.* (1954*b*). The name 'desulphoviridin' is proposed for this pigment. It appears to be a simple porphyrin-protein since heat, acid or alkali treatment all yield the same product: the fluorescent photo-oxidizable chromophoric group. Verhoeven & Takeda (1956) obtained a blue protein which absorbed at 600–630 m μ . from *Pseudomonas aeruginosa* during the isolation of the cytochrome concerned in nitrate reduction; but Verhoeven's pigment is clearly different from desulphoviridin since it has little absorption in the region of 411 m μ ., where desulphoviridin absorbs strongly. The chromophoric group of desulphoviridin is clearly a porphyrin-like compound since it survives contact with conc. H_2SO_4 ; a fluorescent metallo-porphyrin such as chlorophyll, or a metallo-bile pigment such as the red fluorescent zinc derivatives, would lose their metal in these conditions. The character of the chromophore has not been established; the type of spectrum in aqueous media was reminiscent of that of a chlorin, but the high solubility in water, which prevented a neutral spectrum being obtained, is quite uncharacteristic, because the HCl numbers of chlorin lie in the region of 15–20%. The properties so far established would not be inconsistent with a highly carboxylated chlorin structure but are insufficient to allow a definite conclusion.

The author wishes to record his indebtedness to the very large number of authorities who have discussed this work with him and made helpful suggestions. Particularly is he grateful to Professor D. D. Woods; Dr June Lascelles (who first observed cytochrome c_3); Professor Keilin and Dr Hartree; Dr N. K. Boardman; Dr J. E. Falk; Dr K. G. Paul; Dr R. Barer; Dr Lucile Smith; Dr A. G. Ogston, all of whom spared him considerable time to discuss various points. Errors and misinterpretations are, however, the author's own. He is also indebted to Dr M. Ishimoto, Dr Jacqueline Millet and Dr W. Verhoeven for giving him access to unpublished material, and to Mr P. S. S. Dawson and his colleagues in the author's laboratory for supplying large quantities of centrifuged bacteria. This paper is published by permission of the Director, Chemical Research Laboratory.

REFERENCES

- ALLEN, M. B. & VAN NIEL, C. B. (1952). Experiments on bacterial denitrification. *J. Bact.* **64**, 397.
- APPLEBY, C. A. & MORTON, R. K. (1954). Crystalline cytochrome b_2 and lactic dehydrogenase of yeast. *Nature, Lond.* **175**, 749.
- BAALSRUD, K. & BAALSRUD, K. S. (1954). Studies on *Thiobacillus denitrificans*. *Arch. Mikrobiol.* **20**, 41.
- BARER, R. (1955). Spectrophotometry of clarified cell suspensions. *Science*, **121**, 709.
- BOARDMAN, N. K. & PARTRIDGE, S. M. (1955). Separation of neutral proteins on ion-exchange resins. *Biochem. J.* **59**, 543.
- BUTLIN, K. R., ADAMS, M. E. & THOMAS, M. (1949). The isolation and cultivation of sulphate-reducing bacteria. *J. gen. Microbiol.* **3**, 46.

- BUTLIN, K. R. & POSTGATE, J. R. (1953). Microbial formation of sulphide and sulphur. *Microbial Metabolism, Symp. 6th Congr. int. Microbiol.* p. 126.
- CHANCE, B. (1954). Spectrophotometry of intercellular respiratory pigments. *Science*, **120**, 767.
- DARBY, R. T. & GODDARD, D. R. (1950). The effects of cytochrome oxidase inhibition on the cytochrome oxidase and respiration of the fungus *Myrothecium verrucaria*. *Physiol. Plant.* **3**, 453.
- DAVENPORT, H. E. (1952). Reductive cleavage of cytochrome c. *Nature, Lond.* **169**, 75.
- DAVENPORT, H. E. & HILL, R. (1952). The preparation and some properties of cytochrome f. *Proc. roy. Soc. B*, **139**, 327.
- EGAMI, F., ITAHASHI, M., SATO, R. & MORI, T. (1953). A cytochrome from halo-tolerant bacteria. *J. Biochem., Tokyo*, **40**, 527.
- GIBSON, J. & LARSEN, H. (1955). Cytochromes from *Chlorobium thiosulphatophilum*. *Biochem. J.* **60**, 27.
- GROSSMAN, J. P. & POSTGATE, J. R. (1953a). The cultivation of sulphate-reducing bacteria. *Nature, Lond.* **171**, 600.
- GROSSMAN, J. P. & POSTGATE, J. R. (1953b). The estimation of sulphate-reducing bacteria. *Proc. Soc. appl. Bact.* **16**, 1.
- GROSSMAN, J. P. & POSTGATE, J. R. (1955). The metabolism of malate and certain other compounds by *Desulphovibrio desulphuricans*. *J. gen. Microbiol.* **12**, 429.
- HODGMAN, C. D. (1949). *Handbook of Chemistry and Physics*. Chemical Rubber Publishing Co., Cleveland, Ohio, U.S.A.
- HÜBSCHER, G., KIESE, M., NICOLAS, R. (1954). Untersuchungen über Cytochrome. III. Cytochrom b aus Rinderherzen. *Biochem. Z.* **325**, 223.
- HUGHES, D. E. & WILLIAMSON, D. H. (1951). Removal of acids by trioctylamine from samples for microbiology assay. *Biochem. J.* **48**, 487.
- ISHIMOTO, M. & KOYAMA, J. (1955). On the role of a cytochrome in thiosulfate reduction by sulphate-reducing bacteria. *Bull. chem. Soc. Japan*, **28**, 231.
- ISHIMOTO, M., KOYAMA, J. & NAGAI, Y. (1954a). A cytochrome and a green pigment of sulfate-reducing bacteria. *Bull. chem. Soc. Japan*, **27**, 565.
- ISHIMOTO, M., KOYAMA, J. & NAGAI, Y. (1954b). Biochemical studies on sulfate-reducing bacteria. IV. *J. Biochem., Tokyo*, **41**, 763.
- ISHIMOTO, M., KOYAMA, J. & NAGAI, Y. (1955). Biochemical studies on sulfate-reducing bacteria. V. *J. Biochem., Tokyo*, **42**, 41.
- JEBB, W. H. H. (1949). The use of Nile blue in the study of tetrathionase activity. *J. gen. Microbiol.* **3**, 112.
- KAMEN, M. D. & VERNON, L. P. (1954a). Existence of haem compounds in a photosynthetic obligate anaerobe. *J. Bact.* **67**, 617.
- KAMEN, M. D. & VERNON, L. P. (1954b). Enzymatic activities affecting cytochromes in photosynthetic bacteria. *J. biol. Chem.* **211**, 663.
- KEILIN, D. (1933). Cytochrome and intracellular respiratory enzymes. *Ergebn. Enzymforsch.* **2**, 239.
- KEILIN, D. & HARTREE, E. F. (1945). The purification and properties of cytochrome c. *Biochem. J.* **39**, 289.
- KEILIN, D. & HARTREE, E. F. (1955). Relationship between certain components of the cytochrome system. *Nature, Lond.* **176**, 200.
- KEILIN, D. & SLATER, E. C. (1953). Cytochrome. *Brit. med. Bull.* **9**, 89.
- KLUYVER, A. J. & MANTEN, A. A. (1942). Some observations on the metabolism of bacteria oxidizing molecular hydrogen. *Leeuwenhoek J. Microbiol. Serol.* **8**, 71.
- LASCELLES, J. & STILL, J. L. (1946). Utilization of molecular hydrogen by bacteria. *Aust. J. exp. Biol. med. Sci.* **24**, 37.
- LEES, H. & SIMPSON, J. R. (1955). The use of cyanate and chlorate in studies on the relation between the nitrite oxidation and the reduction of a cytochrome system in *Nitrobacter*. *Biochem. J.* **59**, 16.

- LEMBERG, R. & LEGGE, J. W. (1949). *Hematin Compounds and Bile Pigments*. New York, U.S.A.: Interscience Publishers Inc.
- LEWIS, H. J. (1954). Acid cleavage of heme proteins. *J. biol. Chem.* **206**, 109.
- MEL, H. C. (1954). Chemical thermodynamics of aqueous thiosulfate and bromate ions. *Chem. Abstr.* **48**, 6228.
- MILLET, J. (1955). Le sulphite comme intermédiaire dans la réduction du sulfate par *Desulphovibrio desulphuricans*. *C.R. Acad. Sci., Paris*, **240**, 253.
- NEWTON, J. W. & KAMEN, M. (1955). *Chromatium* cytochrome. *Arch. Biochem. Biophys.* **58**, 247.
- PAUL, K.-G. (1950). The splitting with silver salts of the cysteine and porphyrin bonds in cytochrome *c*. *Acta chem. scand.* **4**, 239.
- PAUL, K.-G. (1952). Iron-containing enzymes. A. Cytochromes. In *The Enzymes* (ed. Summer, J. B. & Myrbäck, K.) **2**, 357. New York: Academic Press.
- PEEL, J. L. (1955). The flavins of some micro-organisms. *J. gen. Microbiol.* **12**, ii.
- POSTGATE, J. R. (1949). Competitive inhibition of sulphate reduction by selenate. *Nature, Lond.* **164**, 670.
- POSTGATE, J. R. (1951*a*). On the nutrition of *Desulphovibrio desulphuricans*. *J. gen. Microbiol.* **5**, 714.
- POSTGATE, J. R. (1951*b*). The reduction of sulphur compounds by *Desulphovibrio desulphuricans*. *J. gen. Microbiol.* **5**, 725.
- POSTGATE, J. R. (1952). Competitive and non-competitive inhibitors of bacterial sulphate reduction. *J. gen. Microbiol.* **6**, 128.
- POSTGATE, J. R. (1953). On the nutrition of *Desulphovibrio desulphuricans*: a correction. *J. gen. Microbiol.* **9**, 440.
- POSTGATE, J. R. (1954*a*). Presence of cytochrome in an obligate anaerobe. *Biochem. J.* **56**, xi.
- POSTGATE, J. R. (1954*b*). Dependence of sulphate reduction and oxygen utilization on a cytochrome in *Desulphovibrio*. *Biochem. J.* **58**, ix.
- POSTGATE, J. R. (1955*a*). Cytochrome-linked bacterial reduction of sulphite and related ions. *Abs. 3rd Int. Congr. Biochem.* (10-39), p. 94.
- POSTGATE, J. R. (1955*b*). Cytochrome c_3 , a bifunctional haematohaematin. *Biochim. biophys. Acta*, **18**, 427.
- REPORT (1953). *Chemistry Research 1952. Rep. Chem. Res. Bd. Lond.*
- REPORT (1954). *Chemistry Research 1953. Rep. Chem. Res. Bd. Lond.*
- ROSSINI, F. D., WAGMAN, D. D., EVANS, W. H., LEVINE, S. & JAFFE, I. (1952). Selected values of chemical thermodynamic properties. *Circ. U.S. Bur. Stand.* no. 500.
- SADANA, J. C. & JAGANNATHAN, V. (1954). Purification of hydrogenase from *Desulphovibrio desulphuricans*. *Biochim. biophys. Acta*, **14**, 287.
- SALTON, M. R. J. (1951). The adsorption of cetyltrimethylammonium bromide by bacteria, its action in releasing cellular constituents and its bactericidal effects. *J. gen. Microbiol.* **5**, 391.
- SANDELL, E. B. (1944). *Colorimetric Determination of Traces of Metals*. New York, U.S.A.: Interscience Publishers, Inc.
- SATO, R. & EGAMI, F. (1949). Nitrate reductase, III. *Bull. chem. Soc., Japan*, **22**, 137.
- SCARISBRICK, R. (1947). Haematin compounds in plants. *Rep. Progr. Chem.* **44**, 226.
- SCHAEFFER, P. & NISMAN, B. (1952). Recherches sur le métabolisme des cytochromes et des porphyrines. Cas des bactéries anaérobies strictes. *Ann. Inst. Pasteur*, **82**, 109.
- SCHLEGEL, H.-G. (1953). Physiologische Untersuchungen an Wasserstoffoxydierenden Bakterien. *Arch. Mikrobiol.* **18**, 362.
- SMITH, L. (1954). Bacterial cytochromes. Difference Spectra. *Arch. Biochem. Biophys.* **50**, 299.

- STÁRKA, J. (1951). Nové poznatky o mikrobiální redukcii sulfátů při vzniku léčivého bahna. *Biol. listy*, **32**, 108.
- STARKEY, R. L. & WIGHT, K. M. (1945). *Anaerobic Corrosion of Iron in Soil*. New York, U.S.A.: Amer. Gas Assoc.
- TAGER, J. M. & RAUTANEN, N. (1955). Sulphite oxidation by a plant mitochondrial system. *Biochim. biophys. Acta*, **18**, 111.
- THEORELL, H. (1936). Reines Cytochrom c. II. *Biochem. Z.* **285**, 207.
- THEORELL, H. (1948). A comment on the absorption bands of ferricytochrome c. *Arch. Biochem.* **17**, 359.
- THEORELL, H. & ÅKESSON, A. (1941). Studies on cytochrome c. II. The optical properties of pure cytochrome c and some of its derivatives. *J. Amer. chem. Soc.* **63**, 1812.
- VERHOEVEN, W. & TAKEDA, Y. (1956). Bacterial cytochrome c and denitrification. *J. Bact.* (in the Press).
- VERNON, D. & KAMEN, M. D. (1954). Hematin compounds in photosynthetic bacteria. *J. biol. Chem.* **211**, 643.
- WIDMER, C., CLARK, H. W., NEUFELD, H. A. & STOTZ, E. (1954). Components of the soluble SC factor preparation. *J. biol. Chem.* **210**, 861.
- WILSON, J. P., LEE, S. B. & WILSON, P. W. (1942). Mechanism of biological nitrogen fixation. IX. *J. biol. Chem.* **144**, 265.
- ZOBELL, C. E. & RITTENBERG, S. C. (1948). Sulphate-reducing bacteria in marine sediments. *J. mar. Res.* **7**, 602.

(Received 18 October 1955)