urine, particularly the kynurenine derivatives. Bladder-cancer patients dosed with tryptophan excreted larger amounts of anthranilic acid, 3-hydroxyanthranilic acid and 3-hydroxykynurenine.

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Purification, some Properties and the Specific Biological Activity of Cytochromes c_4 and c_5 from *Azotobacter vinelandii*

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The cytochrome system in bacterial cells varies from species to species, as shown by the positions of the absorption bands and by other properties of the individual components (Smith, 1954*a*). Thus cell-free preparations from certain bacteria lack cytochrome *c* oxidase activity (Keilin & Harpley, 1941), even when they are made from organisms in which the absorption bands of cytochrome lie at the same position as those found for heart muscle (Smith, 1954*b*).

The earlier work was based upon direct spectroscopy of intact cells (Smith, 1954*a*) and only recently have some cytochrome components from bacteria been extracted and purified. Egami, Itahashi, Sato & Mori (1953) obtained a soluble cytochrome from halotolerant bacteria, and in the same year Vernon (1953) and Elsden, Kamen & Vernon (1953) isolated from the photosynthetic bacterium *Rhodospirillum rubrum* a pigment similar in several ways to cytochrome *c*. The pigment was, however, not oxidized by cytochrome *c* oxidase preparations. Postgate (1954, 1955, 1956) described a soluble cytochrome from the sulphate-reducing bacterium *Desulphovibrio desulphuricans*, and Gibson & Larsen (1955) have isolated two cytochromes from *Chlorobium thiosulphatophilum*. Kamen & Vernon (1955) have studied the catalytic activity of purified cytochromes of the '*c*' type from facultative photoheterotrophs as well as from denitrifiers and found that in most cases the bacterial pigments failed to react with cytochrome *c* oxidase from heart muscle. They also reported (Kamen & Vernon, 1954) that a '*c*'-type cytochrome and its oxidase from *Azotobacter vinelandii* reacted only with each other and could not be linked to a cytochrome *c*–cytochrome oxidase system from animal tissues.

The present paper deals with the extraction, purification and properties of two cytochromes from *A. vinelandii*, and their specific activity in the cytochrome and succinic oxidase systems in particulate preparations from the same bacterium. The soluble pigments are called here cytochromes c_4 and c_5 because in some essential properties they closely resemble cytochrome *c*. They were extracted from the cells by means of *n*-butanol, by a procedure similar to that of Morton (1950). Cytochrome c_5 has so far been isolated only in small amounts and has thus been less completely characterized than cytochrome c_4. A preliminary account of this work has already been published (Tissières & Burris, 1956).

MATERIAL AND METHODS

Strains and methods of culture

A. vinelandii, Wisconsin strain O, was grown under forced aeration for 48 hr. at 35° in Burk's nitrogen-free mineral salts medium (Burk & Lineweaver, 1930) with the addition of 2% of sucrose. *Escherichia coli*, strain 15 from the Molteno Institute, Cambridge, and *Bacillus megatherium*, strain K.M. (Northrop, 1951), were grown for 24 hr. at 30° under forced aeration, the first one in nutrient broth, the second in 2% Difco peptone. The strain of *Acetobacter peroxidans* was obtained from Professor A. J. Kluyver. It was grown for 72 hr. at 30° on the solid medium described by Chin (1952).

Extraction and purification of cytochromes c_4 and c_5

n-Butanol (250 ml.) was added to 250 g. (wet wt.) of well-packed cells and the mixture blended for 2 min. in a Waring Blendor. The butanol, which formed an upper layer on standing, was decanted off, 300 ml. of water was added to the cell material and the mixture was blended again as before. It was then centrifuged for 30 min. at 12000 rev./min. in a Servall SSI centrifuge, and formed four layers: a large precipitate of cell debris at the bottom of the tube, a clear pink layer, a thin layer of cell debris and a small top layer of butanol. The pink layer, which contained the cytochromes in solution, was pipetted off. The residue was extracted once more with the same amount of water and the pink solutions from both extractions were mixed and dialysed, first against running water for 6 hr., then overnight against tap water at 5°. The solution, now free from butanol, was centrifuged again at 12 000 rev./min. for 10 min. and gave 650 ml. of clear orange-pink solution. The pH was brought to 8 with 0·1 N-NaOH, and 25% (w/v) basic lead acetate was added until no further precipitate was formed. The precipitate was centrifuged off and discarded, and the excess of lead was removed by acidification to pH 6·5 with dilute acetic acid followed by addition of an excess of Na_2SO_4. The precipitate was centrifuged off and discarded. After neutralization with dilute NH_3 soln., two vol. of saturated $(NH_4)_2SO_4$ adjusted to pH 7·0 with NH_3 soln. was added. A pink precipitate was formed, which was collected by centrifuging, dissolved in the minimum amount of 0·02 M phosphate buffer, pH 7·5 (a mixture of 84% of Na_2HPO_4 and 16% of KH_2PO_4) and dialysed first against the same buffer, then against water. An orange-red solution (71 ml.) was obtained which showed strong absorption bands at about 552 and 523 mμ. The extinction was measured at the peak of the α-absorption band; with 23·8 as millimolar extinction coefficient (see below) it was calculated that this solution, derived from 250 g. of cells, contained 14 mg. of pure cytochrome, in terms of cytochrome c_4. It was freeze-dried, giving 82 mg. of dry material, which was kept in a desiccator over P_2O_5. After treatment with lead acetate and sodium sulphate, the position of the α-band, which, as will be shown later, is that of a mixture of cytochromes c_4 and c_5, was found to vary from one preparation to another within the limits 552–551·3 mμ. Thus the position of the α-band of cytochrome ($c_4 + c_5$) shifted slightly towards the blue end of the spectrum during purification, which suggested that the ratios cytochrome c_4: cytochrome c_5 had changed and that some of the cytochrome c_5 had been lost. This was confirmed by the fact that when the α-band of the crude cytochrome extract was near 551 mμ., only traces of cytochrome c_5 were recovered after paper electrophoresis. This is probably why cytochrome c_5 was not isolated in amounts corresponding to the likely ratio of the two pigments in the intact cells.

Paper electrophoresis. This was performed on Whatman no. 3 MM paper with 0·05 M aminotrishydroxymethylmethane buffer, pH 8. A sample (40–50 mg.) of pink dried material was dissolved in the minimum amount of buffer and applied as a thin band across the strip of wet paper 20 cm. wide and 38 cm. long. The application stopped about 1·5 cm. from the edges of the paper. Between the two large electrode vessels into which it dipped the paper was laid across a shallow glass dish covered with a glass plate. The paper was so arranged that it hung clear of the glass plate. A potential of 200 v was applied for 15 hr. The pigment moved towards the positive electrode and separated into two red bands. The faster-moving one, cytochrome c_4, which always comprised more than three-quarters of the total pigment, migrated about 10 cm., and the second one, composed mostly of cytochrome c_5, moved only 5–6 cm. They were eluted separately. The eluate from the slower-moving band showed an absorption maximum at 554 mμ. and still contained a small amount of cytochrome c_4. It was therefore dialysed, freeze-dried and electrophoresis was repeated; a fraction with an α-band at 555 mμ., cytochrome c_5, was then isolated, as well as a fraction containing a small amount of cytochrome c_4. Paper electrophoresis was also performed at pH 7, 6 and 5·5, with 0·05 M phosphate buffer, to obtain some information on the mobility of the pigments at those pH values.

Spectrophotometric methods. A Hilger Uvispek spectrophotometer was used to determine the spectrum of the purified cytochromes, of the cell-free preparations and of the intact cells. The instrument was fitted with a glass prism for measurements in the visible region of the spectrum, and with the high-intensity tungsten lamp for work with cell-free extracts and intact cells. Cells usually employed with turbid suspensions were 1 mm., and in such cases the reference cell contained a piece of Whatman no. 1 filter paper soaked in medicinal paraffin, which gave an extinction of about 1 (Keilin & Hartree, 1955). A Zeiss microspectroscope fitted to a microscope, as described by Keilin & Hartree (1946), was used to study the oxidation and reduction of the cytochrome components with bacterial or heart-muscle preparations.

Iron. This was estimated by the αα′-dipyridyl method of Hill & Keilin (1933). The quantities of the various reagents were reduced in proportion to give a final volume of 0·6 ml. The reaction was performed in a Pyrex tube with an internal diameter of 6 mm. and graduated at 0·6 ml. Two blanks were prepared, the extinctions of which were subtracted from that given by the reaction with dipyridyl and cytochrome: one with the cytochrome and all the reagents except dipyridyl, the second without the cytochrome but with all the reagents including dipyridyl. The content of each tube was transferred to a 0·5 ml. cell, with a light path of 1 cm. Use was made of the extinction coefficient for ferrous iron–dipyridyl given by Keilin & Hartree (1945). It was thus possible to estimate 0·5–3·0 μg. of iron with an error of less than 1%.

Nitrogen. The micro-Kjeldahl method with Markham's (1942) apparatus was used to estimate nitrogen.

Protein. This was determined in cell-free preparations by the biuret method (Weichselbaum, 1946).

Oxidation–reduction potentials. The method of Davenport & Hill (1952) was chosen, mixtures of ferro- and ferricyanide being used as oxidation–reduction buffers. A preliminary series of measurements was made with the microspectroscope as described by the above authors, and it was found that the same results were obtained whether the experiments were done under aerobic or anaerobic conditions. Thus none of the components of the system reacts with oxygen at an appreciable rate. Subsequent measurements were made in the spectrophotometer in the presence of air with 2 cm. cells. The reaction mixture (4 ml.) contained 0·01 M ferrocyanide, 0·05 M phosphate buffer of the appropriate pH and sufficient purified cytochrome to give an extinction of 0·3–0·5 at the peak of the α-band when completely reduced. To this were added 0·02–0·04 ml. of a mixture containing 0·01 M ferricyanide, and the same concentrations of cytochrome and buffer as before. After equilibrium had been reached (2–3 min.) extinctions were read at 551 mμ. for cytochrome c_4 and at 555 mμ. for cytochrome c_5.

Particulate preparations from bacteria. The bacteria were washed three times with at least 50 vol., of water, and the well-packed cells ground by hand in a cold mortar for 2–3 min. with 3 parts (w/v) of very fine Pyrex-glass powder (passed through a 200-mesh sieve) according to the method of Wiggert, Silverman, Utter & Werkman (1940) and extracted with 2 parts (w/v) of ice-cold water. The extract was kept at 0–4° throughout the preparation. A first centrifuging at 7000 rev./min. (3500 *g* at the bottom of the tube) for 15 min. removed the glass powder, intact cells and larger cell debris. The supernatant was centrifuged at 20000 rev./min. (23000 *g* at the bottom of the tube) for 30 min. and a sediment of particles, fraction *L*, was given, then at 40000 rev./min. (145000 *g* at the bottom of the tube) for 60 min. in the no. 40 head of a model L Spinco centrifuge, to give a small red gelatinous pellet, fraction *SP*, and a clear supernatant, fraction *S*. The particulate fractions *L* and *SP* were washed by resuspending in about 50 vol. of 0·05 M phosphate buffer, pH 7, and centrifuging respectively as above. Each particulate fraction was finally resuspended in one-quarter of the original volume of the extract of 0·05 M phosphate buffer, pH 7.

Succinic oxidase and cytochrome oxidase activities. These were measured in microdifferential manometers at 30°, in the presence of air and KOH. The manometric flask contained the enzyme in 0·05 M phosphate buffer, pH 7 (a mixture of 61 % of Na_2HPO_4 and 39 % of KH_2PO_4) and the substrate (final concentration 0·02 M of succinate or of ascorbate in case of the estimation of cytochrome oxidase activity) was tipped in from the side arm at zero time. The volume of the total reaction mixture was 0·5 ml. It contained 0·001 M ethylenediaminetetraacetic acid when ascorbate was used.

Heart-muscle preparations deficient in cytochrome *c* were kindly supplied by Dr E. F. Hartree. They had been prepared according to Tsou (1952). Cytochrome *c* was prepared by the method of Keilin & Hartree (1945) and further purified according to Margoliash (1954). *Rhodospirillum rubrum* cytochrome *c*, prepared by the method of Vernon (1953), was kindly supplied by Dr L. Smith. A sample of cytochrome *f* was obtained through the generosity of Dr R. Hill. Reduced diphosphopyridine nucleotide (DPNH) was from the Sigma Chemical Co.

RESULTS

Absorption spectrum of cytochrome c_4. The absorption spectrum of purified cytochrome c_4 is given in Figs. 1 and 2. It resembles closely that of pure cytochrome *c*, as obtained by Margoliash (see Keilin & Slater, 1953). The absorption bands of the reduced compound have the following positions: α, 551 mμ.; β, 522 mμ.; γ, 416 mμ.; δ, 315 mμ. The γ-band of ferricytochrome c_4 lies at 411 mμ. The millimolar extinction coefficients of the various absorption bands, calculated on the basis of the iron content (see below), are: α, 23·8; β, 17·6; γ (reduced), 157·2; γ (oxidized), 115·8; protein band (270 mμ.), 20·5. Thus the extinction coefficient of the α-band is lower, and that of the γ-band is higher than the corresponding values for cytochrome *c*. The

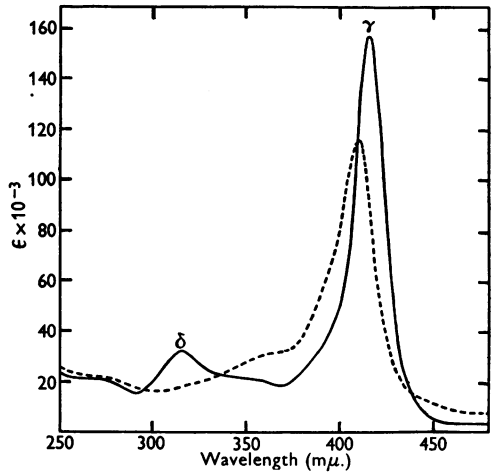

Fig. 1. Absorption spectra of reduced and oxidized cytochrome c_4 containing 0·46 % of iron in the region of the γ- and δ-bands. Unbroken line: reduced cytochrome; broken line: oxidized cytochrome.

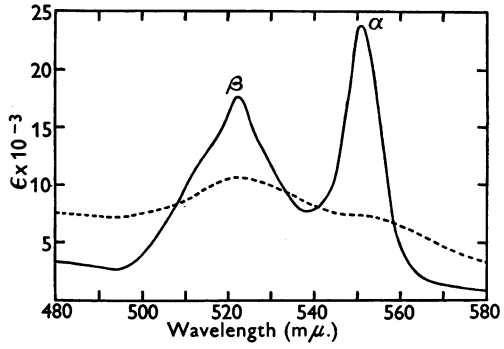

Fig. 2. Absorption spectra in the visible region of cytochrome c_4 containing 0·46 % of iron. Unbroken line: reduced pigment; broken line: oxidized pigment.

extinction coefficient of the protein band is lower than that of cytochrome *c* containing 0·45 % of iron (Keilin & Slater, 1953).

Iron content and molecular weight of cytochrome c_4. The same sample of cytochrome c_4 was dialysed for 48 hr. against glass-distilled water and freeze-dried, and gave 7 mg. of dried material. One sample (1·5 mg.) was used for iron estimations, done in triplicate, and 0·5 mg. was used for nitrogen determinations. The iron content was found to be 0·46 % of either the dry wt. or of the protein calculated as nitrogen × 6·25. Therefore the molecular weight, calculated on the basis of one atom of iron per molecule, is about 12000.

Absorption spectrum of cytochrome c_5. The spectrum of cytochrome c_5 is very similar to that of c_4, the whole spectrum being shifted towards the longer wavelengths by 4 mμ. Cytochrome c_5 has not so far been isolated in sufficient amounts for iron estimations and thus no information on the molecular weight and the molar extinction coefficients is available.

α-Absorption band of a mixture of purified cytochrome c_4 *and* c_5 *in the ratio* 4:1. The mixture was made on the assumption that the extinction coefficient for the α-band of c_5 is the same as that for c_4. The α-peak of the mixture, measured with the spectrophotometer, was symmetrical with a maximum at 552–552·5 mμ., which is the position of the α-band in intact cells, cell-free preparations (see below) and in crude cytochrome extracts before treatment with lead acetate.

Low-temperature spectrum of intact cells, crude cytochrome (c_4+c_5) *extracts and purified* c_4 *and* c_5. When *Azotobacter vinelandii* cells are examined at liquid-air temperature according to the technique of Keilin & Hartree (1949, 1950), the α-absorption band of cytochromes (c_4+c_5) splits into a sharp band nearer the blue side of the spectrum and a wider, asymmetric band, decreasing in intensity towards the red end of the spectrum. Crude cytochromes (c_4+c_5) showed the same picture. Samples of pure cytochromes c_4 and c_5 both show the double α-band characteristic of cytochrome *c* under those conditions. When the spectrum of cytochrome c_4 is compared in the same field of the microspectroscope with that of cytochrome c_5 it is seen that one α-band of the former overlaps with one α-band of the latter, and that a mixture of the two purified pigments in the ratio of one part of c_5 to four parts of c_4 gives a picture identical with that shown by intact cells or by crude (c_4+c_5) extracts.

General properties of cytochromes c_4 *and* c_5. The following findings apply to both cytochromes c_4 and c_5. The pigments in solution remain partly reduced after extraction from the cells. They are not autoxidizable, nor do they combine with carbon monoxide between pH 4·5 and 13. They are not affected by heating for 10 min. in a boiling-water bath. They are remarkably stable in alkali, no change being observed after the addition of 1 vol. of N-NaOH; if pyridine is added to this alkaline solution, the α-band of either c_4 or c_5 shifts to 550 mμ. The pigments are denatured when kept below pH 4·5 for several hours. At pH 3, in the absence of a reducer, the red solutions turn brown and the typical spectra of cytochrome are replaced by those of methaemoglobin type with a wide absorption band at 630–640 mμ. If the solutions are neutralized before denaturation occurs and sodium dithionite is added the brown pigments turn red and the spectra revert to those of the reduced cytochromes. This effect is similar to that which is obtained on acidification of a mammalian cytochrome *c* (Theorell & Åkeson, 1941). Unlike cytochrome *c*, cytochromes c_4 and c_5 are not adsorbed on Amberlite IRC-50.

Isoelectric point. Cytochromes c_4 and c_5 move towards the positive electrode on paper electrophoresis between pH 8 and 5·5; they thus have isoelectric points in the acid range, in contrast to cytochrome *c* where the isoelectric point is above 10. Cytochrome c_5 migrated more slowly than cytochrome c_4, which suggests that its isoelectric point is somewhat higher than that of c_4.

Oxidation–reduction potentials. It was found that the potential of half reduction, E'_0, was +0·30 v for cytochrome c_4 and +0·32 v for cytochrome c_5 between pH 6·0 and 7·5.

Succinic oxidase and cytochrome (c_4+c_5) *oxidase systems in particulate preparations from* Azotobacter vinelandii. In the washed particulate fractions, the cytochrome components were all in the oxidized state and the absorption bands were almost invisible. On addition of succinate, DPNH, ascorbate or sodium dithionite, fraction *L* showed a spectrum very similar to that of intact cells, with a strong (c_4+c_5) band at 552·5 mμ. and a weaker b_1 band at 560 mμ., but the band of reduced cytochrome a_2 at 630 mμ., which is rather weak in intact cells, was not visible. In the fraction *SP*, consisting of the smallest particles, the oxidized band of cytochrome a_2 at 645 mμ. was present before the addition of reducer. This band moved to 630 mμ. on reduction and to 635 mμ. on subsequent treatment with carbon monoxide. Fig. 3 shows the absorption spectra of the fraction *SP*. The intact cells contain relatively more cytochrome (c_4+c_5) and less cytochrome b_1 than the fraction *SP*. This is due to loss of some cytochrome (c_4+c_5) in the soluble fraction *S*, whereas the components b_1 and a_2 remain entirely attached to the particles. The α-band of cytochrome b_1, which forms a shoulder on the longer wavelength side of the cytochrome (c_4+c_5) α-band, appears as a single peak when the difference spectrum is plotted, reduced minus oxidized, as shown in Fig. 4. For this type of experiment the two cells contained the

particulate preparation, oxidized in the reference cell and reduced with dithionite in the other. The difference spectrum [(reduced + carbon monoxide) minus reduced] shows in the 400–430 mμ. region the presence of the 'carbon monoxide-binding pigment' described by Chance, Smith & Castor (1953), with a peak at 416 mμ. With either of the particulate fractions, all the components of cytochrome could be reduced by the addition of small amounts of succinate or DPNH and oxidized on shaking in air.

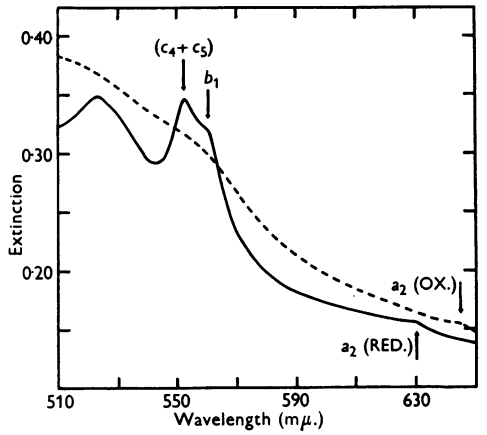

Fig. 3. Absorption spectra of reduced (unbroken line) and oxidized (broken line) fraction *SP*, consisting of small particles. The α-band of reduced cytochrome ($c_4 + c_5$) has its peak at 552·5 mμ. Reduced cytochrome b_1 appears as a shoulder on the longer wavelength side of the cytochrome peak ($c_4 + c_5$). The absorption band of cytochrome a_2 can be seen both in its oxidized and reduced forms.

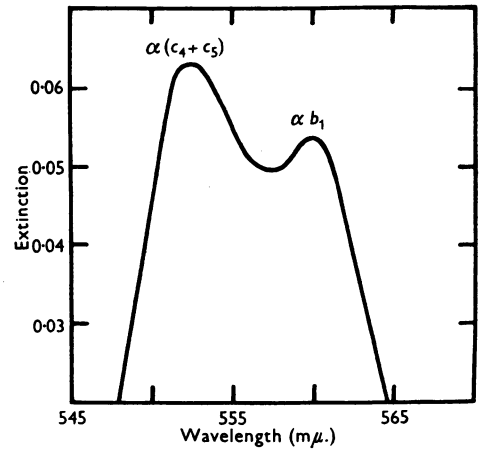

Fig. 4. Difference spectrum of the fraction *SP*, reduced minus oxidized, showing the α-bands of cytochrome ($c_4 + c_5$) and of cytochrome b_1. Both cells contained the particulate preparation, oxidized in the reference cell and reduced with dithionite in the other.

The following substrates were tested: α-oxoglutarate, fumarate, malate, citrate, pyruvate and lactate. None had any effect.

When reduced cytochrome ($c_4 + c_5$), c_4 or c_5 was added to either of the particulate fractions in the absence of succinate or DPNH, the cytochromes became oxidized at once. If then succinate or DPNH was added, the exogenous pigments became reduced and could undergo oxidation on shaking in air and reduction on standing.

When the succinic oxidase activity was measured manometrically there was a lag period which was inversely proportional to the concentration of enzyme preparation. A typical experiment, represented in Fig. 5, shows that when the same amount of oxidase preparation was suspended in a reaction mixture of 1 ml. instead of 0·5 ml. the initial lag period was more pronounced. The subsequent rate of oxygen uptake was, however, equal in both manometers. There was a progressive decrease in the rate of oxygen uptake after 15 min. in the flask with the smaller reaction volume, and in the other the decrease became noticeable only after 20 min. This decrease, which takes place earlier when the reaction mixture contains a higher concentration of enzyme preparation, suggests that an inhibitor is produced during the oxidation of succinate. Although washed particulate preparations do not oxidize any of the intermediates of the citric acid cycle other than succinate at appreciable rates, it is likely that this inhibition is due to the formation of a small amount of oxaloacetate which can com-

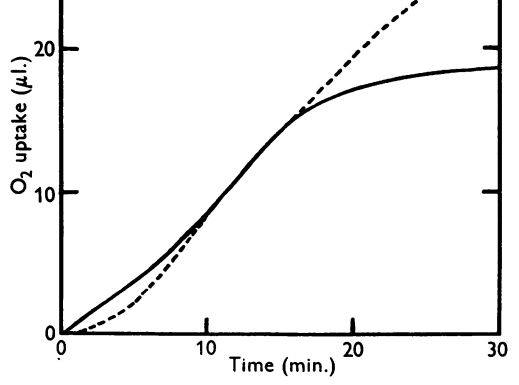

Fig. 5. Succinic oxidase activity as measured manometrically. Broken line: 0·02 M succinate, 0·05 M phosphate buffer and 0·05 ml. of enzyme preparation in a total reaction mixture of 1 ml. Unbroken line: the same amount of enzyme in 0·5 ml. of a reaction mixture of the same composition.

petitively inhibit the oxidation of succinate. The addition of exogenous cytochrome (c_4+c_5), c_4 or c_5 had no effect on the rates of oxygen uptake.

When cytochrome oxidase activity was measured manometrically with ascorbate as substrate, the system did not take up any oxygen unless exogenous cytochrome (c_4+c_5) or c_4 or c_5 was added. Fig. 6 shows the catalytic activities of various amounts of cytochrome c_4. Preliminary experiments with pure cytochrome c_5 suggest that this component has approximately the same catalytic effect as cytochrome c_4. The activities of the succinic oxidase and cytochrome c_4 oxidase systems are shown in Table 1.

The effect of ageing on fractions *L* and *SP* was studied over a period of 48 hr. at 4°. Normally, both the succinic and the bacterial cytochrome oxidases were found to be stable over this period, but in some cases fraction *L* lost all activity within 24 hr. This may be due to the presence of proteolytic enzymes in the larger particles.

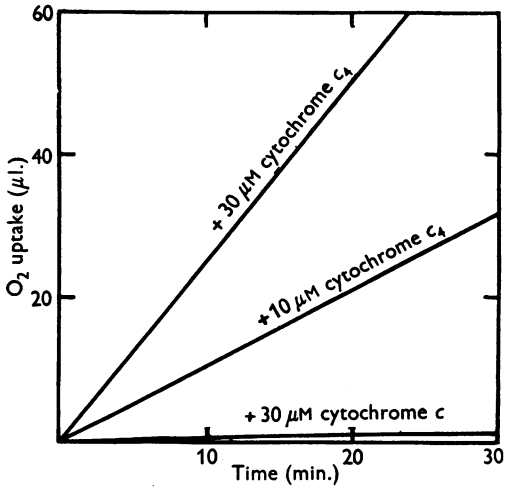

Fig. 6. Specific cytochrome c_4 oxidase activity of a particulate preparation from *Azotobacter vinelandii*. The manometric flask contained the enzyme, 0·02 M ascorbate, 0·05 M phosphate buffer, pH 7·0, and various amounts of cytochrome c_4 in a total reaction mixture of 0·5 ml. Cytochrome *c* (30 μM) had no effect.

Table 1. *Activities of the succinic and cytochrome c_4 oxidase systems in the particulate fractions L and SP from* Azotobacter vinelandii

Results are expressed as $-Q_{O_2}$ (protein). Fraction *L* was collected by centrifuging at 22000 *g* for 30 min., fraction *SP* was collected at 145000 *g* for 60 min. Measurements were made at 30°.

	Fractions	
	L	*SP*
Succinic oxidase	295	1000
Cytochrome c_4 oxidase (with 5×10^{-4} M cytochrome c_4)	460	305

Specific activity of cytochromes c_4 and c_5 and of the cytochrome (c_4+c_5) oxidase. Cytochrome c_4 and cytochrome (c_4+c_5) were tested manometrically in the succinic and cytochrome oxidase systems of heart-muscle preparations deficient in cytochrome *c* (Tsou, 1952). They had no effect on either system, although cytochrome *c* gave rise to a large increase in oxygen uptake. In spectroscopic experiments a slow oxidation of the bacterial cytochromes was observed in the presence of a large excess of heart-muscle preparation, but only after bubbling air through the mixture for several minutes. The speed of this slow reaction is, however, of a quite different order of magnitude from those of cytochromes c_4 and c_5 with their specific oxidase. Cytochrome c_4 was also tested spectroscopically and manometrically (in the presence of ascorbic acid) with particulate preparations from *Acetobacter peroxidans*, *Escherichia coli* and *Bacillus megatherium*, and it was found to have no catalytic activity.

The specificity of cytochrome (c_4+c_5) oxidase was tested in spectroscopic and manometric experiments with cytochrome *c*, *Rhodospirillum* cytochrome *c* and cytochrome *f*. None of these pigments showed any activity.

DISCUSSION

It has been shown that cytochrome *c* and cytochrome c_1, the α-bands of which differ in position by only 4 mμ., show in heart-muscle preparations a symmetrical α-band lying at an intermediate position (Keilin & Hartree, 1955). The α-bands of cytochromes c_4 and c_5 differ also by 4 mμ., and a mixture of these pigments in the ratio 4:1 gives the same picture as that observed in intact cells, with a symmetrical α-band. It is therefore not surprising that the presence of the two cytochromes cannot be detected in *Azotobacter* cells by direct spectroscopic examination.

During the purification procedure there is a substantial loss of the cytochromes, particularly of cytochrome c_5. This is shown by the shift of the α-band from about 552·5 mμ. to 551·3–552 mμ., and by the minute amount of cytochrome c_5 which can subsequently be isolated. Thus, although the method described is convenient for the preparation of cytochrome c_4 in quantities of 5–20 mg., it is unsuited for preparing similar amounts of cytochrome c_5.

In recent years several cytochrome components have been described with α-absorption bands which lie at a position intermediate between those of cytochrome *b* and cytochrome *c*, and the components have generally been attributed to the '*b*' or the '*c*' group, when some of their properties resemble those of cytochrome *b* or of cytochrome *c*. The spectrum of

the pyridine haemochromogen formed with each cytochrome is characteristic of its prosthetic group and therefore can be used as a criterion of classification. Thus the pyridine haemochromogen formed with cytochrome *b* (protohaematin) has an α-absorption band at about 556 mμ. (Hartree, 1955), and the α-band of the pyridine haemochromogen of cytochrome *c* or cytochrome c_1 lies at about 550 mμ. (Keilin & Hartree, 1955). It was upon this basis that the two pigments from *A. vinelandii* were assigned to the cytochrome *c* group, and we have therefore called them cytochrome c_4 and cytochrome c_5. Elsden *et al.* (1953) have used the term cytochrome c_2 for the pigment extracted from *Rhodospirillum rubrum* and Postgate (1955) has called the pigment from the anaerobe *Desulphovibrio desulphuricans* cytochrome c_3.

The particulate fractions made up either of large particles, fraction *L*, or of small particles, fraction *SP*, contain a complete oxidase system for succinate, DPNH and cytochromes c_4 and c_5. The succinic oxidase activity of these fractions is not increased by addition of exogenous bacterial cytochromes, which is not surprising if one considers the high activity of this system: the small particles *SP*, which are two or three times more active than the larger one, *L*, have the highest Q_{O_2} reported so far for a system able to oxidize succinic acid. They thus probably contain enough cytochromes c_4+c_5 for maximum activity. Alexander & Wilson (1955) also reported that in *A. vinelandii* extracts the smallest particles, corresponding to those of our fraction *SP*, were more active in the oxidation of succinate than the larger particles, comparable to those of fraction *L*.

No attempt has yet been made to study in detail the properties of the cytochrome (c_4+c_5) oxidase system in the particulate fractions. It is, however, probable that both cytochrome a_2 and the 'carbon monoxide-binding pigment' of Chance *et al.* (1953) are concerned.

The question of the specificity of cytochrome c_4 and c_5 and of their oxidase will be discussed elsewhere.

SUMMARY

1. Two cytochromes of the '*c*' type, cytochromes c_4 and c_5, have been extracted from *Azotobacter vinelandii* and purified.

2. The absorption spectra of cytochrome c_4 and cytochrome c_5 resemble that of cytochrome *c*. The α-, β- and γ-bands of the reduced cytochrome c_4 lie at 551, 522 and 416 mμ. respectively. Those of cytochrome c_5 lie 4 mμ. nearer the red end of the spectrum.

3. Pure cytochrome c_4 contains 0·46% of iron. Its molecular weight, calculated on the basis of one atom of iron per molecule, is about 12000.

4. The α-absorption bands of the pyridine haemochromogens derived from both cytochromes c_4 and c_5 lie at about 550 mμ.

5. The isoelectric points of both pigments are in the acid range.

6. The oxidation–reduction potentials, E'_0, were found to be +0·30v for cytochrome c_4 and +0·32v for cytochrome c_5, between pH 6·0 and 7·5.

7. Two particulate fractions were isolated from cell-free extracts: fraction *L*, composed of large particles, and fraction *SP*, consisting of the smallest particles. Fraction *SP* had the highest activity in the oxidation of succinate.

8. Both particulate fractions contain the same cytochrome pigments as are found in the intact cells.

9. Cytochromes c_4 and c_5 were found to react only with the oxidase from *A. vinelandii*; these bacterial oxidase preparations did not oxidize heart-muscle cytochrome *c*, *Rhodospirillum rubrum* cytochrome *c*, or cytochrome *f*.

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The Effects of Salicylate on Creatine Phosphate and Adenosine Triphosphate in the Isolated Rat Diaphragm

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Salicylate produces a number of effects on carbohydrate metabolism in isolated tissues, such as an increased oxygen consumption in mouse-liver slices (Sproull, 1954) and in rat-brain preparations (Fishgold, Field & Hall, 1951), and a decreased glycogen synthesis in rat-liver slices (Smith, 1955*a*). The effects of salicylate on the rat diaphragm *in vitro* include a rapid breakdown of glycogen, an accumulation of lactic acid and an increased oxygen uptake and respiratory quotient (Smith & Jeffrey, 1956). It was suggested that these changes were indications of increased substrate breakdown resulting from an interference with the formation of energy-rich phosphate bonds. The present paper is concerned with a study of the effects of salicylate on the levels of creatine phosphate, adenosine triphosphate (ATP) and inorganic phosphate in the isolated rat diaphragm.

METHODS

Preparation of the diaphragms. Male rats of the Wistar strain (wt. 100–120 g.) were used. They were kept without food for 24 hr. before being killed by decapitation. The diaphragms were removed and treated as described previously (Smith & Jeffrey, 1956). Two hemidiaphragms from the same animal were used in each Warburg flask and were incubated at 37° for 15–60 min. under an atmosphere of O_2.

Incubation media. The phosphate–saline solution of Stadie & Zapp (1947) was used initially, because it had been employed in the earlier experiments (Smith & Jeffrey,1956) concerned with the effects of salicylate on glycogen breakdown and oxygen uptake. However, after incubation in this medium the diaphragms were frequently observed to contract during transference to the freezing mixture before extraction of the phosphate compounds. The salt solution of Sacks & Sinex (1952), in which such contractions did not occur, was therefore used in the later experiments. Glucose was added to both media to give a final concentration of 0·01 M, and the sodium salicylate was added from the side arm of the flasks after temperature equilibration to give final salicylate concentrations ranging from 0·1 to 5 mM. The pH was 7·4 for the salt solution of Stadie & Zapp and 7·0 for the Sacks & Sinex solution, and it did not alter significantly during the course of the experiment.

Methods of analysis. After incubation the diaphragms were removed, blotted on filter paper, and immediately frozen in a mixture of ether and solid CO_2 in a stainless-steel crucible. The subsequent extraction procedures were performed in a room maintained at 2–5°. The frozen tissue was powdered with a stainless-steel pestle and mortar, previously cooled with the freezing mixture, and transferred to a centrifuge tube containing 2 ml. of 10% (w/v) trichloroacetic acid. The mixture was thoroughly stirred for 1 min., centrifuged, the supernatant removed, and the residue re-extracted with 2 ml. of 5% (w/v) trichloroacetic acid. The combined supernatants were neutralized to phenolphthalein with 5N-NaOH and the volume was made up to 10 ml. with water. The total extraction time for each diaphragm was 5–6 min. Inorganic phosphate and creatine phosphate (as acid-molybdate-labile phosphate) were estimated in the trichloroacetic acid extract by the method of Ennor & Stocken (1948). This method is not specific for creatine phosphate and may include small amounts of hexose phosphates and other unidentified compounds (Ennor & Rosenberg, 1952). Total acid-labile phosphate was estimated in the trichloroacetic acid extract after hydrolysis in N-HCl for 10 min. and was assumed to consist mainly of creatine phosphate and ATP. The values observed for the phosphate compounds in the control diaphragms showed good agreement with those reported by other workers using more precise analytical techniques (Sacks & Sinex, 1952).

RESULTS

The results in Table 1 show the effect of 5 mM salicylate on the levels of inorganic phosphate, creatine phosphate and ATP in rat diaphragms incubated aerobically for 15, 30 and 60 min. at 37° in the medium of Stadie & Zapp (1947), and Table 2 gives the results of similar experiments in the medium of Sacks & Sinex (1952). In all the following tables the significances of the differences between the means of the control and salicylate groups have