Spectrophotometric Observations Relating to the Oxidation–Reduction Potential of Cytochrome *b* in Non-phosphorylating Heart-muscle Particles*

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The oxidation–reduction potential of cytochrome *b* of heart muscle was estimated by Ball (1938) to be −40 mv at pH 7·4 and 25°. Since then it has become increasingly evident from the work of Chance (1952*a*, *b*) on the kinetics of the respiratory chain and from measurements by Hill (1954, and personal communication) that the true value of the potential is likely to be considerably more positive. In the work reported here we have reinvestigated the equilibrium between the bound cytochrome *b* of heart-muscle particles and the succinate–fumarate system, by using spectrophotometric methods which make small changes in the oxidation state of particle-bound pigments readily measurable (Holton, 1957) and employing a method of calculation which does not require experimental data relating to complete reduction of cytochrome *b*. Our results show that that portion of the cytochrome *b* which is functional in the succinic-oxidase system of the heart-muscle particles (see Colpa-Boonstra, 1959) possesses an oxidation–reduction potential which at pH 7 is some 120 mv more positive than the value estimated by Ball at pH 7·4. These results have been briefly reported elsewhere (Colpa-Boonstra & Holton, 1959).

The experiments of this paper consist largely of measurements of changes of extinction which are attributed wholly or in part to one or other of the respiratory pigments present in the heart-muscle particles. Interpretation of the data depends upon a knowledge of details of the difference spectra of the individual pigments (reduced state minus oxidized state for the respiratory-chain pigments, deoxygenated state minus oxygenated state for myoglobin). For convenience, Fig. 1 summarizes such data compiled from various sources. They form the basis for much of the discussion which follows.

* Part of this work has appeared in a preliminary form (Colpa-Boonstra & Holton, 1959).

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EXPERIMENTAL

Heart-muscle particles

These were prepared by the method of Keilin & Hartree (1947) from fresh horse or pig heart. The particles were stored at 0° as a concentrated suspension (20–30 mg. of protein/ml.) in 0·2M-potassium phosphate buffer, pH 7·4, and were used within a week of preparation. The presence of myoglobin, bound to the particles or adsorbed on to them, was readily detected by studying the kinetics of extinction changes associated with deoxygenation of respiring suspensions of the particles. Since extinction changes caused by deoxygenation of oxymyoglobin would have interfered seriously with observations of cytochrome *b* reduction (see Fig. 1), the contribution of myoglobin to the spectrophotometric measurements was eliminated by treating the preparations with nitrite, as suggested by Chance (1952*b*), following the method described recently by Colpa-Boonstra & Minnaert (1959). Nitrite oxidizes oxymyoglobin to metmyoglobin, which undergoes no chemical or spectral change when oxygen is removed from solutions or suspensions containing it. The effectiveness of this procedure is illustrated in Fig. 2, which compares the kinetics of extinction changes accompanying deoxygenation in an untreated preparation with those observed in the same preparation after nitrite treatment. Fig. 2 shows that as the suspension of untreated particles became deoxygenated, the increase of E at 434 mμ began some 5 sec. before any change was apparent either at 451·5 or at 479 mμ. This points to the presence in the untreated particles of a pigment with an oxygen affinity lower than that of the respiratory chain and which possesses spectral properties similar to those of myoglobin (see Fig. 1). From the detailed study by Colpa-Boonstra & Minnaert (1959) this effect may be confidently ascribed to the deoxygenation of oxymyoglobin. Fig. 2 shows that myoglobin no longer contributes to extinction changes in nitrite-treated preparations. Fig. 2 also shows that in experiments of the type which form the basis of this paper, in which the oxidation of succinate is partially inhibited by fumarate, the presence of oxymyoglobin is readily detected. All the preparations used were treated with nitrite, and no evidence of residual oxymyoglobin was observed in the resulting material. The kinetics of extinction changes at 451·5 mμ were similarly examined for evidence of residual nitrite, which, in this spectral region, causes a characteristic decrease of E which begins as the reduction of the

cytochromes nears completion (Colpa-Boonstra & Minnaert, 1959). Residual nitrite was always found to be absent.

Protein was determined by the biuret method of Cleland & Slater (1953).

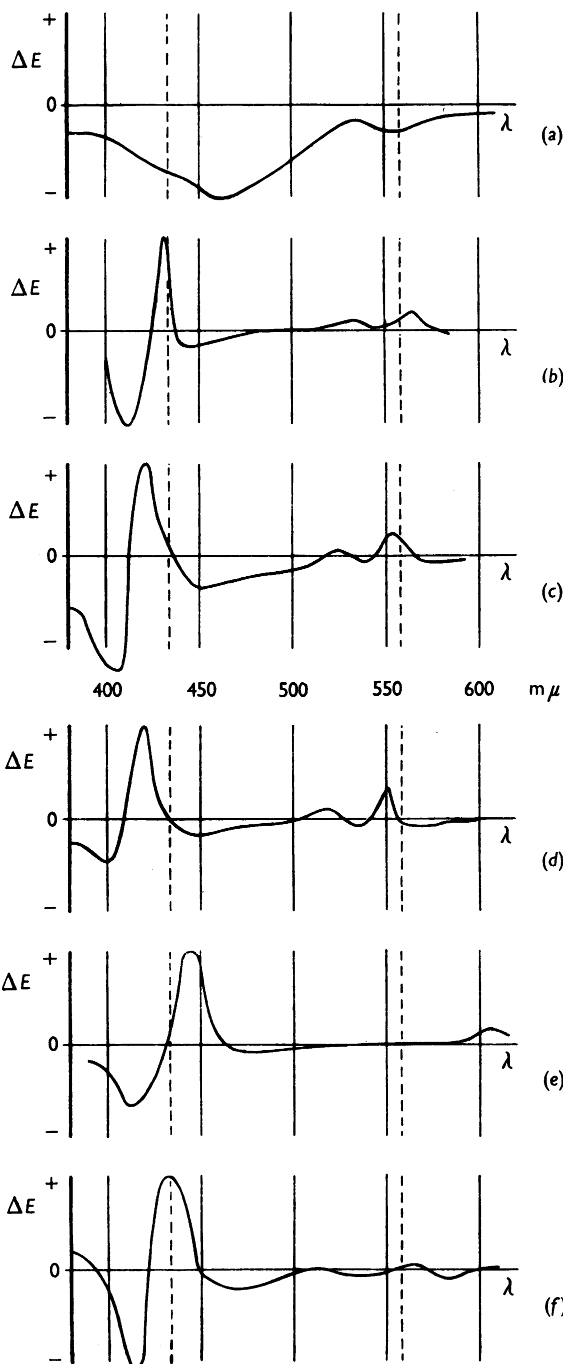

Fig. 1. For legend see foot of next column.

Measurements of succinic-oxidase and fumarase activities

The succinic-oxidase activity of the preparations was determined by the spectrophotometric method of Slater & Bonner (1952). Final concentrations of reagents were: dipotassium succinate, pH 7, 25 mM; disodium potassium ethylenediaminetetra-acetate, 1 mM; potassium phosphate buffer, pH 7·0 or 7·4, 87 mM. A measured volume of diluted

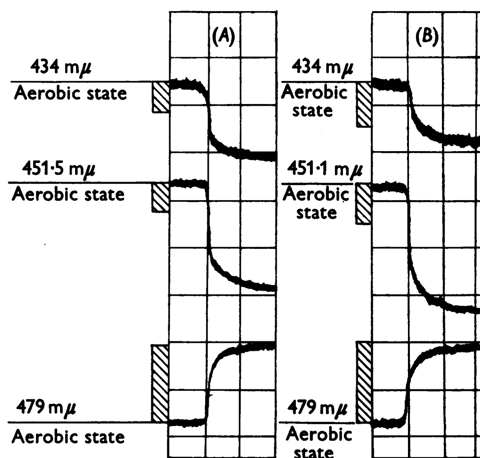

Fig. 2. Kinetics of extinction changes associated with deoxygenation of a suspension of heart-muscle particles before nitrite treatment (*A*) and after nitrite treatment (*B*). A downward deflexion equal to the height of the rectangle shown on the left of each trace corresponds to an increase in *E* of 0·05. The time-scale runs from left to right and is indicated by the vertical lines, which are drawn at 30-sec. intervals. Concentrations of particles and of reagents were the same in experiments (*A*) and (*B*). Heart-muscle particles, 7·6 mg. of protein/ml.; succinate, 14·8 mM; fumarate 0·148 M; phosphate buffer, pH 7·4, 0·148 M; ethylenediaminetetra-acetate, 1 mM. Temperature, 23·2°. Optical depth, 0·5 cm. Expt. 8.

Fig. 1. Difference spectra of individual purified respiratory pigments. Curves show the relation between wavelength and the change of extinction occurring upon reduction (succinic dehydrogenase and cytochromes) or deoxygenation (myoglobin). The ordinate scale has been chosen arbitrarily for each pigment to make the major absorption bands of equal height. Broken lines indicate the two wavelengths chosen for the study of cytochrome *b* in heart-muscle particles in the present work. (*a*) Ox-heart succinic dehydrogenase, from data of Singer, Kearney & Bernath (1956). This enzyme is one of the flavoproteins of the succinic-oxidase system. (*b*) Ox-heart cytochrome *b*, from data of Hübscher, Kiese & Nicolas (1954) and of Sekuzu & Okunuki (1956). (*c*) Ox-heart cytochrome c_1, from data of Okunuki, Hagihara, Sekuzu & Horio (1958). (*d*) Horse-heart cytochrome *c*, from data of Margoliash & Frohwirt (1959). (*e*) Pig-heart cytochromes *a* and a_3, from data of Smith (1955). (*f*) Horse-heart myoglobin, compiled from data of Sidwell, Munch, Barron & Hogness (1938), Beznák (1948) and Bowen (1949).

heart-muscle preparation containing 0·1–0·5 mg. of protein was stirred into the reaction mixture and the rate of increase of E at 230 mμ in a reaction volume of 3 ml. was observed in a cuvette of 1 cm. optical depth. The reaction rate at 25° was calculated from the rate observed at room temperature by use of the equation

$$\frac{d(\ln v)}{d(1/T)} = -E_a/\boldsymbol{R},$$

where v represents the reaction rate observed at an absolute temperature T, $\boldsymbol{R}$ is the gas constant and E_a = 11 250 cal. between 20° and 37° (Thorn, 1951). Spectrophotometric data were converted into manometric units by using the value $\epsilon = 4{\cdot}38 \times 10^3$ for the molar extinction coefficient of fumarate at 230 mμ (Colpa-Boonstra, 1959). Fumarase activity was measured in two of the preparations by following the rate of decrease of extinction at 230 mμ in a reaction mixture similar to that given above, but in which succinate was replaced by 0·1 mM-dipotassium fumarate, pH 7. Fumarase activity was found to be negligible in both instances. Manometric estimations of succinic-oxidase activity were also carried out on two of the preparations, the same reaction mixture being used as in the spectrophotometric determinations. They indicated activities about 20% higher than those determined spectrophotometrically (Table 1). The reason for this discrepancy is not known.

The measurements of succinic-oxidase activity described in the preceding paragraph were carried out primarily in order to examine whether the oxidation–reduction potential of cytochrome *b* was different in preparations of high and low activity, and secondly in order to provide a common criterion by which the material studied in this work might be compared with that of other workers. In addition, knowledge of the succinic-oxidase activity enabled us to estimate whether, during the spectrophotometric measurements of the oxidation state of the endogenous pigments described in the next section, oxygen diffusion into the open top of the cuvette would compete successfully with oxygen removal by respiration, and hence hinder the attainment of anaerobic equilibrium in the lower part of the cell. For this purpose, the oxidase activity in concentrated particle suspensions (3·3–9·9 mg. of protein/ml.) was calculated from the time which elapsed between the moment of succinate addition (see below) and the moment at which the final rapid shift of the oxidation state of the respiratory chain towards anaerobic equilibrium was half-completed (cf. Chance, 1952*b*). This time was measured from the spectrophotometric record of extinction at 451·5 mμ. For this calculation the oxygen concentration at the moment of adding succinate was calculated from oxygen-solubility data (Hodgman, Weast & Selby, 1955), by use of the activity coefficient given by Randall & Failey (1927) for oxygen dissolved in 0·15M-NaCl. Since competition between diffusion and respiration becomes more important with decreasing respiration rate (see the next section), it was necessary to know the oxidase activities of suspensions containing high concentrations of added fumarate, in which respiration was strongly inhibited. For the most strongly inhibited suspensions the deoxygenation time (5–10 min.) could be measured accurately from the spectrophotometric record (chart speed 1 in./min.). A rough check of the validity of the calculation was provided by estimating the oxidase activity of uninhibited suspensions in the same manner (deoxygenation time 15–30 sec.), correcting the calculated activity to 25° and comparing the result with the more accurate manometric and spectrophotometric measurements described in the preceding paragraph. This comparison is shown in Table 1. Since the particles were respiring under somewhat different conditions during the measurement of deoxygenation time, with particle concentrations 10–20 times as great as, and succinate concentrations one-fifth to one-eighth of, those in the more accurate estimations, the degree of agreement with the other methods was considered sufficiently good to validate the diffusion calculation described in the next section.

Spectrophotometric measurements of respiratory-chain reactions

Measurements of extinction changes associated with changes in the state of oxidation of the bound respiratory pigments of the heart-muscle particles were carried out in the three-channel spectrophotometer described previously (Holton, 1957, 1960 in preparation). A cuvette of 1 cm. optical depth was used for measurements in the region of the α bands of the cytochromes, and a 0·5 cm. cuvette for γ-band measurements. Calibration of the pen-recorder traces in terms of extinction changes was carried out as

Table 1. *Succinic-oxidase activities of the four preparations of heart-muscle particles*

See text for factor employed for converting spectrophotometric data into manometric units.

Expt. no.	Particle preparation		pH	Succinic-oxidase activity (μl. of O_2 consumed/mg. of protein/hr. at 25°)		
	Designation	Source		Manometric	Spectro-photometric	From deoxygenation time
1, 2, 3	A	Horse	7·0	484	386	275
4, 5	B	Pig	7·0	—	84	74
6, 7	C	Pig	7·0	—	55	50
8, 9, 10	D	Horse	7·4	384	355	204

described (Holton, 1960 in preparation); the calibration procedure was carried out during each experiment at some stage of every deoxygenation cycle. The relation between pen-recorder deflexion and extinction change was nearly linear (Fig. 3).

The following procedure applies to a typical experiment involving measurements in the γ-band region, in which the reaction volume was 2 ml. Volumes were increased appropriately for α-band experiments, in which the reaction volume was 3 ml.

The concentrated suspension of heart-muscle particles was diluted two- to five-fold in a mixture containing 0·16 M-potassium phosphate buffer, pH 6·83, and 2 mM-disodium potassium ethylenediaminetetra-acetate. This mixture had been previously aerated at room temperature. This diluted material was allowed to stand at room temperature throughout the experiment. A measured sample (1·5 ml.) was added to 0·5 ml. of an aerated solution of known fumarate concentration (0–1 M-dipotassium fumarate, pH 7·0) and the whole of the mixture transferred by syringe to the cuvette, which was held permanently before the entry slit of the polychromator. The final protein concentration varied from 3·3 to 9·9 mg./ml. The white-light beam was then passed through the suspension and the amplified photo-currents were recorded for 1–2 min. at the three wavelengths available. During this period, when the extinction of the suspension at all wavelengths remained constant, a calibration signal was usually recorded (cf. Fig. 4). A small volume (0·007–0·03 ml.) of dipotassium succinate solution (1 M, pH 7) was then placed on a polythene rod and stirred rapidly into the suspension. The moment of mixing was recorded by irregular deflexions of the pen records as the light path was disturbed by the rod. After a period of from 0·5 to 10 min., depending on the oxidase activity of the preparation and the degree of its inhibition by the fumarate present, rapid changes of extinction were observed which were characteristic of reduction of the respiratory chain. They indicated exhaustion of the dissolved oxygen. Steady values of amplified photo-current were slowly re-established at a new level some 3–5 min. later, and were taken to indicate the complete absence of oxygen.

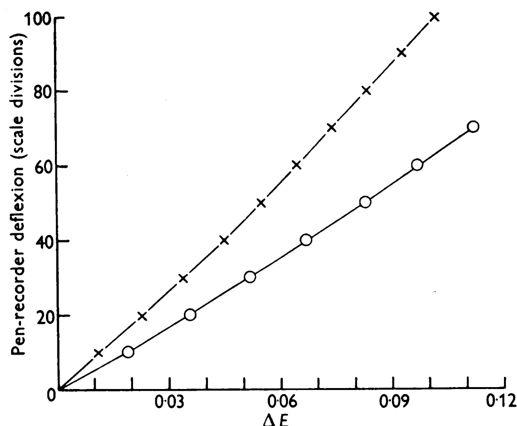

Fig. 3. Relation between pen-recorder deflexion and change of extinction in two typical experiments. ×, Relation for recordings at 558 mμ in Expt. 2; ○, relation for recordings at 434 mμ in Expt. 3.

This assumption rested on the likelihood that, in the final steady state, oxygen did not penetrate into the region occupied by the light beam either by diffusion or by convective transfer from the open top of the cuvette. This likelihood was supported by the following considerations.

(*a*) The greatest depth to which oxygen penetrates by diffusion into respiring material at equilibrium is inversely related to the square root of its respiratory activity (Hill, 1928). This distance was calculated to be 0·28 cm. for the suspension of lowest oxidase activity studied in this work, by using the equation given by Hill (1928), the diffusion coefficient of oxygen in water (Hüfner, 1897) and the lowest value of oxidase activity obtained by calculation from deoxygenation times (see preceding section). Thus 0·28 cm. represents the greatest depth to which oxygen would have penetrated by diffusion at equilibrium in any of the experiments described here. The light beam occupied a rectangle 1·3 cm. high and 0·4 cm. wide in the lower half of the cuvette, with its highest point 1·5 cm. below the lowest point of the meniscus. It is thus unlikely that a significant fraction of the beam emerging from the cell and passing through the entry slit had traversed the upper layers of incomplete anaerobiosis during the course of multiple scattering within the suspension, so long as diffusion was the major mechanism of oxygen transport downwards through the suspension.

(*b*) In a control experiment under typical conditions, neither the time taken to reach the final steady state nor the observed change of extinction was affected by filling the cell completely and sealing it with a glass plate after adding succinate.

(*c*) In control experiments in which the light beam passed close to the surface of a suspension of extremely low oxidase activity the amplified photo-currents corresponding to the anaerobic state were unsteady. This was probably caused by convection and diffusion of oxygen into the suspension. This unsteadiness was never observed in the experiments reported below.

For these reasons it seems justifiable to assume that the final state attained at the end of the deoxygenation cycle represented truly anaerobic conditions.

When the final position of the three traces had been recorded, a volume of phosphate–ethylenediaminetetra-acetate mixture equal to the volume of succinate added earlier was stirred into the cell, and the recording continued until the oxygen introduced by the stirring had been utilized and anaerobic conditions were re-established. This was done in order to measure the optical effect of a simple dilution equal in magnitude to that caused by succinate addition. After washing and draining of the cuvette, the procedure was repeated with the same or a different concentration of added fumarate. An experiment consisted of from 7 to 13 consecutive cycles of this nature. A typical cycle of measurements in the γ-band region is illustrated in Fig. 4. Very similar traces were obtained in the region of the α-bands.

At the end of each cycle the reaction mixtures were collected and the pH of the pooled mixtures was measured by glass electrode. In one experiment the pH values of individual mixtures were measured. They agreed to within $\pm 0{\cdot}06$ pH unit and showed no regular drift with increasing concentrations of fumarate. This showed that

the changes of ionic strength which occurred in succeeding experimental runs did not cause an accompanying change of pH.

For each deoxygenation cycle, the pen deflexion corresponding to the transition from the oxidized to the anaerobic state of the respiratory chain was corrected for the effect of dilution and converted into the corresponding change of extinction ($\Delta E^{an.}_{ox.}$) by reference to the appropriate calibration line (Fig. 3). Values of $\Delta E^{an.}_{ox.}$ in the absence of added fumarate were usually of the order of 0·15 at 434mμ and 0·04 at 558 mμ, and were highly reproducible. For example, four repeated measurements of $\Delta E^{an.}_{ox.}$ at 434 mμ, fresh samples of particle suspension being used each time, gave the value 0·1568 ± 0·0013 (mean ± S.D.).

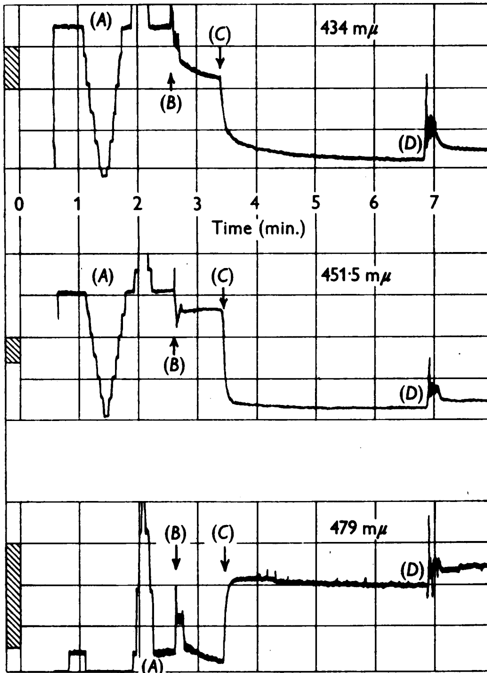

Fig. 4. Tracing of changes of extinction in a suspension of heart-muscle particles upon addition of succinate and upon subsequent exhaustion of dissolved oxygen. A downward deflexion equal to the height of the rectangle shown on the left of each trace corresponds to an increase in E of 0·05. A suspension of particles (2 ml.) containing 2·47 mM-fumarate was placed in the cuvette at time 0. The amplified photo-current corresponding to the oxidized state of the respiratory chain was recorded from 0·6 to 2·6 min., with a calibration signal (A) interposed from 1·1 to 2·2 min. At 2·6 min. 0·028 ml. of M-succinate was rapidly stirred into the suspension (B), which remained aerobic until 3·4 min (C). The rapid changes of extinction which then occurred were characteristic of the approach of the respiratory chain towards anaerobic equilibrium (D), which was attained at about 6·2 min. At 6·85 min., 0·028 ml. of buffer was stirred into the suspension. Top record, 434 mμ, band width 0·6 mμ; middle record, 451·5 mμ, band width 0·7 mμ; bottom record, 479 mμ, band width 0·9 mμ. Optical depth, 0·5 cm.; heart-muscle particles, 6·3 mg. of protein/ml.; temperature, 24·2°. Expt. 5.

In two experiments it was observed that the amplified photo-currents did not attain steady values either in the oxidized or in the anaerobic state, but decreased at a slow steady rate both before the addition of succinate and after completion of the rapid extinction changes due to pigment reduction. This slow drift of extinction was ascribed to settling or aggregation of the particles, and in these two experiments values of $\Delta E^{an.}_{ox.}$ were estimated by extrapolation.

Selection of wavelengths for studying cytochrome b

The purpose of the observations described in this section was to determine at which wavelengths extinction changes observed in suspensions of heart-muscle particles could be attributed mainly or wholly to cytochrome *b*. The method followed that of Holton (1955*a*), who reported that the kinetics of changes of E at between 431 and 434 mμ in suspensions of heart-muscle particles undergoing deoxygenation are diphasic in character. In the present work this was also true of changes of E at 430 mμ (Fig. 5). The earlier observations have been confirmed, and by the use of three-channel recordings it has been possible to show explicitly what was earlier concluded on less sure evidence, namely that the rapid initial fall of extinction observed at these

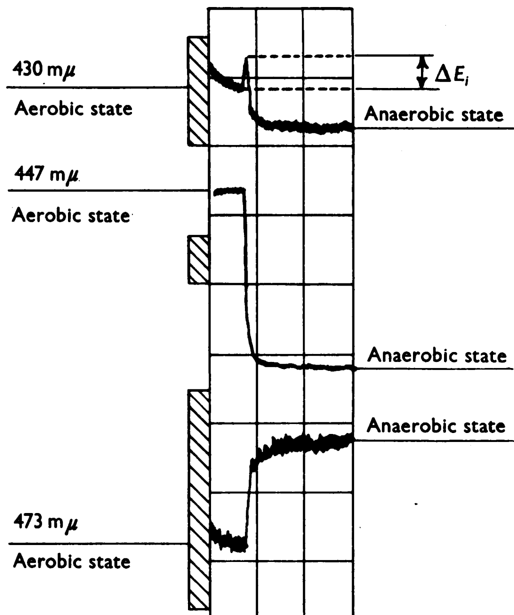

Fig. 5. Diphasic kinetics of changes of E at 430 mμ accompanying deoxygenation of a suspension of heart-muscle particles. A downward deflexion equal to the height of the rectangle shown on the left of each trace corresponds to an increase in E of 0·05. The vertical lines are drawn at 30 sec. intervals. Heart-muscle particles, 3·4 mg. of protein/ml.; succinate, 5 mM; phosphate, 0·1 M; ethylenediaminetetra-acetate, 1 mM; pH 7·0; temperature, 27·1°; optical depth, 0·5 cm. Expt. 2.

wavelengths (ΔE_i, Fig. 5) is synchronous with the beginning of the reduction of cytochrome a_3, which may be measured simultaneously at 445–450 mμ without appreciable interference from other pigments (Fig. 5). The traces in Fig. 5 also suggest that the accompanying decrease of E at 473 mμ is not synchronous with reduction of cytochrome a_3, but follows more closely the kinetics of the slower positive change observed at 430 mμ, and this has been confirmed by similar observations recorded at a faster chart speed. Reference to the difference spectra of the separate pigments (Fig. 1) suggests that these data may be interpreted as showing that the time course of flavoprotein reduction (dominating changes observed at 473 mμ) and of cytochrome b reduction (contributing the increase of E at 430 mμ) lags behind the progress of cytochrome a_3 reduction, which is mainly responsible for the increase of E at 447 mμ.

Similar diphasic kinetics are observed at wavelengths between 564 and 558 mμ, both in single-channel recordings of reactions in heart-muscle sarcosomes (Holton, 1955*b*) and in the non-phosphorylating particles used in the present work (Fig. 6). At these wavelengths also, a rapid initial fall precedes a slower rise of extinction. The rapid initial phase is synchronous with the reduction of cytochrome a (recorded at 605 mμ, Fig. 6). The difference spectra of purified cytochromes (Fig. 1) make it clear that the initial rapid fall of E observed at wavelengths between 564 and 558 mμ cannot be due to the reduction of cytochrome a or a_3, but is due rather to the reduction of cytochrome c. From the above results of observations at both α and γ bands, it may be concluded that as the oxygen concentration approaches zero, the reduction of cytochromes a_3, a and c begins appreciably before that of cytochrome b and of flavoprotein (for a fuller discussion of this phenomenon, see Colpa-Boonstra, 1959). Since the rapid initial fall of E (ΔE_i of Figs. 5 and 6) is due to cytochromes a_3, a and c, their contribution to the observed extinction change will disappear at the wavelengths where this initial drop becomes zero. Fig. 7 shows plots of values of ΔE_i against wavelength for the two spectral regions explored in this way. With this method, the two wavelengths at which the 'slow' components (cytochrome b and flavoprotein) could be studied with minimum interference from the 'fast' components (cytochromes a, a_3 and c) were found to be 434 and 558 mμ. It was considered that observations at these two wavelengths were most likely to reflect cytochrome b reactions and they were employed for this purpose in the experiments described below. The contribution of flavoprotein reactions could not be separated from those due to cytochrome b. It may be noted that the wavelength of 558 mμ arrived at by this study is the same as that suggested by Chance (1952*c*) for the study of cytochrome b in single-channel measurements.

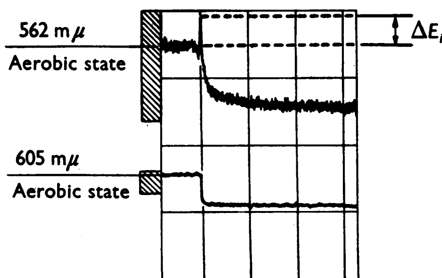

Fig. 6. Diphasic kinetics of changes of E at 562 mμ accompanying deoxygenation of a suspension of heart-muscle particles. A downward deflexion equal to the height of the rectangle shown on the left of each trace corresponds to an increase in E of 0·05. The vertical lines are drawn at 30 sec. intervals. Heart-muscle particles, 5·1 mg. of protein/ml., untreated with nitrite. A small increase of extinction occurring before reduction of the cytochromes can be detected in the upper record. This indicates a slight contribution from deoxygenation of oxymyoglobin. Succinate, 3·3 mM; phosphate, 87 mM; ethylenediaminetetra-acetate, 1 mM; pH 7·0; room temperature; optical depth, 1·0 cm. Expt. 1.

Calculation of the fumarate/succinate ratio at the anaerobic equilibrium

The succinate concentration at the anaerobic equilibrium was calculated from the amount of succinate added, less the amount oxidized to fumarate by the oxygen initially present in the reaction mixture. Since the particles lacked fumarase activity, the final fumarate concentration was

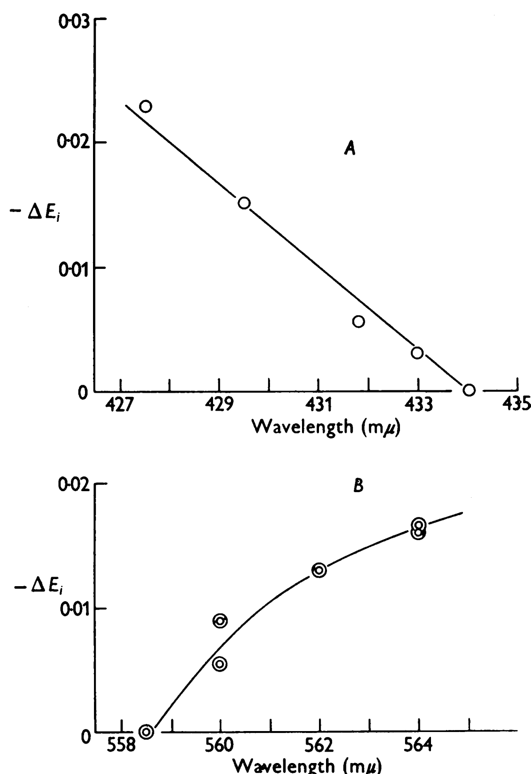

Fig. 7. Variations with wavelength of the magnitude of the rapid initial decrease of extinction, ΔE_i, observed around 430 mμ (*A*, Expt. 2) and around 562 mμ (*B*, Expt. 1). Conditions for *A* were as described in Fig. 5 and for *B* as in Fig. 6.

calculated on the same basis, from the amount added plus the amount formed by aerobic oxidation of succinate. The error which is likely to be involved in using oxygen-solubility data in this way is an overestimation of the initial oxygen concentration, because the presence of particles and of salts is likely to decrease gas solubility, and also because the undiluted particle suspension could not be bubbled in order to ensure gaseous equilibrium since the oxidase activity was rapidly destroyed by such treatment. However, Table 1 shows no evidence for an error in this direction, which would have caused the activities quoted in column 7 to exceed those given in columns 5 and 6. It was concluded that no great errors were introduced into estimates of final fumarate and succinate concentrations by the use of oxygen-solubility data.

Calculation of results

The reaction whose equilibrium was under investigation was assumed to be:

$$\text{Succinate}^{2-} + 2b^{3+} \rightleftharpoons \text{Fumarate}^{2-} + 2b^{2+} + 2\text{H}^{+}. \quad (1)$$

The equation for the equilibrium constant is

$$\bar{K} = \frac{F[b^{2+}]^2\,[\text{H}^+]^2}{S[b^{3+}]^2}$$

and for the apparent equilibrium constant for experiments conducted at constant pH:

$$K = \frac{\bar{K}}{[\text{H}^+]^2} = \frac{F[b^{2+}]^2}{S[b^{3+}]^2}, \quad (2)$$

where F and S represent the equilibrium concentrations of fumarate and succinate respectively. This equation may be rearranged to give:

$$\frac{1}{[b^{2+}]} = \frac{1}{[b]} + \frac{1}{[b]\sqrt{K}}\sqrt{(F/S)}, \quad (3)$$

where $[b] = [b^{2+}] + [b^{3+}]$.

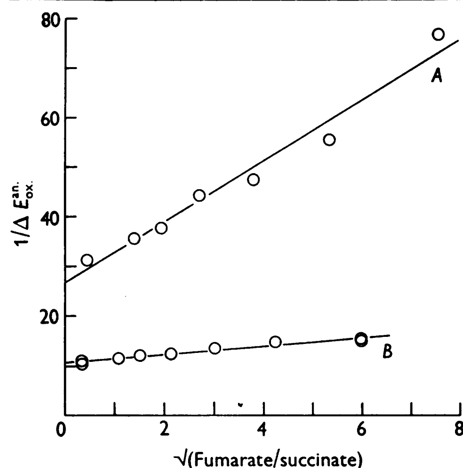

Fig. 8. Relation between the square root of the ratio of concentrations of fumarate to succinate in suspensions of heart-muscle particles and the reciprocal of the change of extinction (anaerobic state minus oxidized state). Extinction changes were observed at wavelengths chosen to reflect the state of oxidation of cytochrome b. A, Expt. 2, 558 mμ; curve B, Expt. 3, 434 mμ. Straight lines were drawn according to the method of Fisher (1950).

The concentration of reduced cytochrome b was assumed to be zero in the presence of oxygen and the absence of substrate, and its concentration at equilibrium was taken to be proportional to $\Delta E^{\text{an.}}_{\text{ox.}}$ measured either at 434 or 558 mμ (see preceding section). Thus

$$[b^{2+}] = \text{constant} \times \Delta E^{\text{an.}}_{\text{ox.}},$$

and hence:

$$\frac{1}{\Delta E^{\text{an.}}_{\text{ox.}}} = \frac{\text{constant}}{[b]} + \frac{\text{constant}}{[b]\sqrt{K}}\sqrt{(F/S)}. \quad (4)$$

Equation (4) shows that values of $1/\Delta E^{\text{an.}}_{\text{ox.}}$ should bear a linear relationship to corresponding values of $\sqrt{(F/S)}$, if the assumptions of the preceding paragraph are correct. Fig. 8 shows that the experimental data yield the expected relationship for both of the wavelengths chosen. Equation (4) also shows that for a graph of $1/\Delta E^{\text{an.}}_{\text{ox.}}$ against $\sqrt{(F/S)}$, the value of K may be obtained from equation (5):

$$\frac{\text{Intercept on } y\text{-axis}}{\text{Slope}} = \sqrt{K}. \quad (5)$$

The regression of values of $1/\Delta E^{\text{an.}}_{\text{ox.}}$ on values of $\sqrt{(F/S)}$ was calculated for each experiment by the method given by Fisher (1950), and values of K were obtained from the calculated values of the regression coefficient and the y-axis intercept.

Values of the oxidation–reduction potential of cytochrome b at the pH of the experiment and at 25° [$E_0'(b)$] were calculated from values of K so obtained and the potential of the succinate–fumarate system at the same pH and temperature [$E_0'(s)$], from equation (6):

$$E_0'(b) = E_0'(s) + 0{\cdot}0295 \log K. \quad (6)$$

The potential of the succinate–fumarate system was calculated from the data of Borsook & Schott (1931).

RESULTS

Evidence for the establishment of an anaerobic equilibrium between cytochrome b *and the succinate–fumarate system*

Experimental evidence has been given above which supports the assumption that the particle suspensions became completely anaerobic at the end of each deoxygenation cycle. The accompanying changes of amplified photo-current finally attained a constant value (Fig. 4). The value of $\Delta E^{\text{an.}}_{\text{ox.}}$ obtained from these progress curves was not affected by the rate of approach towards anaerobic conditions. Table 2 shows that when the oxidase activity of the suspension was strongly inhibited by malonate there was no change in $\Delta E^{\text{an.}}_{\text{ox.}}$ (434 mμ), and hence that the final oxidation state of cytochrome b was unaffected by the rate at which that state was reached. This is the result to be expected if the final state represented a true equilibrium.

It may be noted that the respiratory inhibitions found with both malonate and fumarate in the experiment quoted in Table 2 were similar in magnitude to the inhibitions of oxygen uptake caused by fumarate in the experiments in which the influence of fumarate/succinate ratio on the oxidation

state of cytochrome *b* was investigated. The direct comparison of inhibitory and optical effects of malonate and fumarate given in Table 2 shows that the influence of fumarate on $\Delta E^{an.}_{ox.}$ was not a consequence of the accompanying inhibition of respiration (Laki, 1935), but was due wholly to its effect on the oxidation state of cytochrome *b* at the anaerobic equilibrium.

The addition of fumarate to the anaerobic suspension shifted the oxidation state of cytochrome *b* in the direction expected from the equilibrium equation, and the magnitude of the shift was of the order calculated from the value of the equilibrium constant obtained by the procedure described above. Thus in an experiment conducted at pH 7·4, 0·03 ml. of dipotassium fumarate was stirred into an anaerobic suspension to which fumarate had not been added previously, raising the fumarate/succinate ratio from 0·04 to 1·06. When anaerobic equilibrium had been re-established, $\Delta E^{an.}_{ox.}$, corrected for dilution effects, had decreased by 10·6 % of its original value, as compared with the expected decrease of 14·1 % calculated from the equilibrium constant observed in the subsequent experiment carried out by the more usual procedure. This showed that the final oxidation state of cytochrome *b* could be approached from either direction. A more extensive quantitative demonstration of this important observation was not possible under our experimental conditions, since to shift the position of the anaerobic equilibrium appreciably required the addition of relatively large volumes of concentrated fumarate solution, and the optical effect of the resulting dilution of the suspension was many times greater than the magnitude of the extinction change expected from re-oxidation of reduced cytochrome *b*.

Quantitative data relating to the equilibrium between the succinate–fumarate system and particle-bound cytochrome b

Table 3 summarizes data from all the experiments carried out so far in which values of $\Delta E^{an.}_{ox.}$ were measured for a range of values of the fumarate/succinate ratio, following the procedure described under 'Spectrophotometric measurements of respiratory-chain reactions' (Experimental section). The variation of the calculated values of the oxidation–reduction potential of cytochrome *b* shows no consistent relationship to the oxidase activity of the particles (Table 1), nor to the wavelength at which the observations were made, nor

Table 2. *Effect of fumarate on cytochrome* b

Heart-muscle particles, 7·6 mg. of protein/ml.; potassium phosphate buffer, 0·15 M; ethylenediaminetetra-acetate, 1 mM; succinate, final concentration, 14·3 mM; malonate, where added, 1·32 mM; fumarate, where none was added, final concentration 0·53 mM; fumarate, where added, final concentration 0·241 M; pH 7·38; temperature, 22·8°. Expt. 9.

Inhibitor	Time required for deoxygenation (sec.)	Inhibition of oxidase (%)	$\Delta E^{an.}_{ox.}$ (434 mμ)
None	46		0·156
None	46	0	0·158
None	42		0·158
Malonate	251	82	0·156
Fumarate	165	73	0·089

Table 3. *Values of the equilibrium constant K for the reaction between succinate–fumarate and particle-bound cytochrome* b *of heart muscle*

Values of K were calculated from the regression of values of $1/\Delta E^{an.}_{ox.}$ on values of $\sqrt{(F/S)}$ as described in the Experimental section. Oxidation–reduction potentials of the succinate–fumarate system $E_0'(s)$ were calculated from data of Borsook & Schott (1931) assuming dE_0'/dpH to be −59 mv/pH unit between pH 6·9 and 7·33. The oxidation–reduction potential of cytochrome *b*, $E_0'(b)$, was calculated from values of K and of $E_0'(s)$ as described in the Experimental section.

Particle preparation ...	A		B		C		D	
Source of particles ...	Horse		Pig		Pig		Horse	
Experiment	1	2	3	4	5	6	7	8
Absorption band studied	α	γ	α	γ	α	γ	γ	α
Mean temperature	24·3	25·5	23·6	23·5	26·0	26·4	22·2	23·9
Mean pH	7·02	6·98	6·90	6·90	6·98	6·98	7·33	7·33
No. of observations	7	11	10	9	13	13	10	13
K	18·6	153·4	94·2	22·7	28·3	91·8	24·2	10·9
$E_0'(s)$ (mv)	+23	+25	+30	+30	+25	+25	+5	+5
$E_0'(b)$ (mv)	+60	+90	+88	+70	+68	+83	+46	+36

to the species employed, nor to the pH of the experiment when this was between 6·90 and 7·33. However, the scatter of the data and their small number does not permit the firm conclusion that these factors are without effect on the oxidation–reduction potential. More extensive data would be necessary to determine how the potential varies with pH and other factors. For the six experiments carried out at pH values between 6·90 and 7·02, the mean value of the potential was +76·5 mv ±12·2 (S.D.).

Relationship between the amounts of cytochrome b *reducible by succinate and by dithionite*

The y-axis intercept of the linear plots of $1/\Delta E^{\text{an.}}_{\text{ox.}}$ against $\sqrt{(F/S)}$ yielded the values of $\Delta E^{\text{an.}}_{\text{ox.}}$ which would have been observed in the total absence of fumarate. In any given experiment, this value should be proportional to the total amount of cytochrome *b* reducible by succinate. It was found to be consistently smaller than that observed when the preparation was reduced by the addition of a small quantity of solid sodium dithionite. It was concluded that dithionite reduced a pigment absorbing in the same spectral region as cytochrome *b* which was not reduced by succinate. Its spectral characteristics were not explored further. These observations are consistent with those of Chance (1958) and others whose work has explored this phenomenon more extensively (see Discussion).

DISCUSSION

The data of this paper may be considered to contribute to our knowledge of the oxidation–reduction potential of particle-bound heart-muscle cytochrome *b* at pH 7, and to provide a comment on the accepted value at pH 7·4. Detailed measurements near pH 7 have not been reported before. Our results suggest that the potential at this pH lies between +64 mv and +89 mv. We propose that a reasonable value to assume until better data become available is +77 mv at pH 7 and 25°.

Although our results do not embrace measurements at pH 7·4, two measurements at pH 7·33 yielded a mean value of +41 mv. Together with our measurements at pH 7·0 this value indicates considerable disagreement between our results and Ball's (1938) value of −40 mv at pH 7·4. The chief reason for the discrepancy appears to be that Ball based his oxidation–reduction calculation on an end point obtained with dithionite, and it now appears that this is not justified for the cytochrome *b* which is functional in the succinic-oxidase system of heart-muscle particles. This point has been noted several times before, and there have been a number of recent comments suggesting that Ball's value is too low. Thus Hill (1954) noted that an oxidation–reduction titration with the ferri–ferro-oxalate system yielded a value close to zero for the potential at pH 7 of the cytochrome *b* of the Keilin & Hartree heart-muscle particle (see also Slater, 1953). Stotz, Morrison & Marinetti (1956) quoted unpublished data of Estabrook & Stotz showing that in a solubilized cytochrome *b*–cytochrome c_1 preparation dithionite reduced an amount of cytochrome *b* which was not reducible by succinate. They concluded that Ball's value for the oxidation–reduction potential may be 'somewhat too low'. The cytochrome *b* component of the reduced diphosphopyridine nucleotide (DPNH)–cytochrome *c* reductase of Mackler & Green (1956*a*, *b*) reacts with DPNH and with dithionite in a similar fashion (Estabrook, 1957). Chance (1958) showed that in the Keilin & Hartree heart-particle succinate and dithionite reduced different amounts of cytochrome *b*, and commented that Ball's value for the potential of cytochrome *b* 'is probably much lower than the true value'. We also find that the amount of cytochrome *b* reduced by succinate in the total absence of fumarate is less than the amount reduced by dithionite. If certain assumptions are made, it is possible to show that this well-established observation accounts satisfactorily for the disagreement between our results and Ball's (1938) value for the cytochrome *b* potential. Our observations showed that if no fumarate was added cytochrome *b* was about 98% reduced (Fig. 8), and not 75% as estimated by Ball. This change would raise his value of the potential from −40 mv to +32 mv at pH 7·4. Assuming that the potential of cytochrome *b* itself is the same at pH 7·0 as at pH 7·4, and allowing for the fact that at pH 7·0 the potential of the succinate–fumarate system is some 24 mv more positive than at pH 7·4, Ball's data correspond to a potential of +56 mv at pH 7·0. Finally, our calculations are based on the data of Borsook & Schott (1931) for the potential of the succinate–fumarate system (+24 mv at pH 7·0), whereas Ball used the value +4 mv at pH 7·0. Thus, calculated on the same basis, and allowing for the end-point error in Ball's work, his observations correspond to a value of about +76 mv at pH 7·0, in good agreement with our own estimate of +77 mv.

Our mean value of +77 mv, obtained from measurements on both α and γ bands of cytochrome *b*, is also in good agreement with the results obtained by Chance (1958) from α-band measurements with cyanide-treated particles. His titration data for the equilibrium constant of reaction (1) yield a potential of +73 to +76 mv. However, Chance considers that in his system equilibrium was not established since his value for the equilibrium constant (K 42) did not agree with the ratio of apparent second-order velocity constants for the

reduction and oxidation reactions (k_1/k_2 10). He concluded that his data could not be used to calculate an oxidation–reduction potential. Although our own experimental conditions differ from those of Chance (1958) and we offer no evidence bearing on the relative magnitudes of the second-order velocity constants, it may be profitable to comment on Chance's conclusion since his equilibrium data and ours coincide, and because we consider that equilibrium conditions were established in our experiments. We believe that the agreement between the equilibrium data indicates that Chance's use of his kinetic data is at fault. The following considerations suggest why the ratio of apparent second-order velocity constants may not equal the equilibrium constant for the reaction between succinate and cytochrome *b*. Apparent second-order constants are derived from reaction-progress curves and equations (7) and (8):

Rate of reduction of cytochrome *b* by succinate
$$= k_1 S[b^{3+}]. \quad (7)$$

Rate of oxidation of cytochrome *b* by fumarate
$$= k_2 F[b^{2+}]. \quad (8)$$

The apparent equilibrium constant K derived from these kinetic measurements is given by equation (9):

$$K = \frac{k_1}{k_2} = \frac{F[b^{2+}]}{S[b^{3+}]}. \quad (9)$$

Following the development employed in reactions (2)–(4), equation (9) predicts a linear relationship between $1/\Delta E^{\mathrm{an}}_{\mathrm{ox}}$ and F/S, and implies that the oxidation of one molecule of succinate results in the reduction of one molecule of cytochrome *b*. In the absence of contradicting data, this is a reasonable hypothesis, although Chance (1958) does not propose it explicitly. A number of reaction schemes may be proposed for the interaction of succinate with the respiratory chain in which in addition to cytochrome *b* a second acceptor participates in the oxidation of succinate. Equation (10) shows such a scheme.

$$\text{Succinate}^{2-} + b^{3+} + c_1^{3+} \rightleftharpoons \text{Fumarate}^{2-} + b^{2+} + c_1^{2} + 2\text{H}^+. \quad (10)$$

However, our results show clearly that under our experimental conditions this stoicheiometry is not obeyed. On the contrary, our data provide strong evidence that one molecule of succinate reduces two molecules of cytochrome *b* (Fig. 8). If the same stoicheiometry governed the reactions occurring under the conditions of Chance's (1958) experiments, the discrepancy which he noted between the ratio of second-order velocity constants and the value of the equilibrium constant is understandable, and illustrates that kinetic measurements may only be used to derive an equilibrium constant if the order with which each reaction component appears in the rate equations equals the coefficient of the same component in the stoicheiometric-reaction equation. This interpretation of Chance's work also removes any reason for doubting that equilibrium was attained in his experiments. Our own observation that the final oxidation state of cytochrome *b* was not affected by the rate at which it was attained (Table 2) suggests that at least in our experiments the attainment of a true equilibrium was not impeded by kinetic hindrances.

To summarize, our estimate of + 77 mv for the oxidation–reduction potential of cytochrome *b* at pH 7 gives quantitative expression to the generally held opinion that the value accepted at present is too low. It seems likely that the new value describes the enzymic properties of the bound pigment of the succinic-oxidase system of heart muscle more accurately than does Ball's (1938) value of − 40 mv at pH 7·4. We intend to explore further the variation of the potential with pH.

SUMMARY

1. The equilibrium between the succinate–fumarate system and the bound cytochrome *b* of the Keilin & Hartree heart-muscle preparation has been studied by a spectrophotometric technique. Methods are described by which measurements of extinction include no interference from myoglobin and little contribution from pigments other than cytochrome *b*.

2. The observations were confined to that portion of the particle-bound cytochrome *b* which is reducible by succinate.

3. It is suggested that the oxidation–reduction properties of the cytochrome *b* of the succinic-oxidase system of heart muscle are best represented by our mean value for the oxidation–reduction potential, namely + 77 mv at pH 7 and 25°.

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The Isolation of Coproporphyrin III from *Mycobacterium tuberculosis avium*

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After the demonstration that the addition of traces of certain metal ions to synthetic media had the effect of stimulating the growth of mycobacteria (Paterson, Wright & Patterson, 1958) it was observed during extraction of *Mycobacterium tuberculosis avium* with cold acetone that there was a difference in the pigment content of organisms grown on unsupplemented synthetic media and those grown on media containing added trace elements (Patterson, 1960). A pink pigment which fluoresced red in ultraviolet light was extracted in much greater quantity from the latter organisms and was thought to be a coproporphyrin, since the presence of free porphyrins in mycobacteria has been shown by Fink & Fischer (1925), and because Todd (1949) had isolated coproporphyrin III from *Mycobacterium karlinski*.

The pigment present in *M. tuberculosis avium* is now shown to be identical with that present in the saprophyte *M. karlinski*.

MATERIALS AND METHODS

Organisms. Mycobacterium tuberculosis avium strain D4R was grown on synthetic media which were modifications of that used by Dorset (1934). In the first experiment, coproporphyrin was isolated from bulked extracts of the partially purified pigment obtained from 12 batches of organisms which had been grown for 33–49 days at 37° on 1 l. of the basic medium to which additions of cobalt (0·27 p.p.m.), copper (1·07 p.p.m.) and zinc (6·04 p.p.m.) had been made either separately or in combination. Organisms used in the second experiment were grown for 39–60 days on 1 l. batches of experimental media which differed from the medium of Dorset in iron content (2–64 p.p.m. instead of about 50 p.p.m.) and in zinc supplementation (2–64 p.p.m.). Coproporphyrin was isolated from bulked extracts from 70 cultures.

Extraction of live organisms with acetone. The organisms from 1 l. of medium were harvested by filtering through fine muslin. The filter cake was washed with 1 l. of water, and pressed dry. Pigments were extracted from the organisms with 250 ml. of acetone for 15 hr. at room