Electron Transport in a Park-Williams Strain of *Corynebacterium diphtheriae*

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1. The electron-transport mechanism was examined in the 'particulate' and 'supernatant' fractions of disintegrated cells of a Park-Williams strain of *Corynebacterium diphtheriae*. 2. Succinate-oxidase activity was found mainly in the 'particulate' fraction, and NADH₂ oxidase mainly in the 'supernatant', which was devoid of cytochromes and menaquinone. 3. The sum of the activities of particles and supernatant fractions, with respect to both succinate oxidase and NADH₂ oxidase, was substantially less than that of the crude cell extract from which they were obtained. Full activity was restored on recombining 'particles' and 'supernatant'. The characteristics of this reassembled system were investigated. 4. The strain of organism (CN 2000) examined contained cytochromes corresponding spectroscopically to 'a', 'b' and 'c' types. All three were reduced by succinate, lactate or NADH₂; but a portion of the cytochrome b, susceptible to reduction by dithionite, could not be reduced by the substrates. 5. Triton X-100 inhibits oxidation of succinate by particulate fraction; on adding succinate, the reduction of cytochrome b is not affected but that of cytochromes a and c is delayed. 6. Irradiation at 360 mμ completely destroys menaquinone in the particle fraction. Succinate oxidation is severely decreased; succinate dehydrogenase and NADH₂ oxidation are little affected. Certain menaquinones will restore succinate oxidation in the irradiated material. 7. On adding succinate to irradiated particulate material cytochrome b is partially reduced at once, but reduction of cytochromes a and c is much delayed. A portion of the cytochrome b remains not reduced, but reduction occurs rapidly on the addition of menaquinone (MK-2).

The respiratory system of *Corynebacterium diphtheriae* has been studied in some detail since Fujita & Kodama (1934) detected a-, b- and c-type cytochromes. Pappenheimer & Hendee (1947, 1949) found no evidence for cytochromes other than b, and proposed a relationship between this pigment and the toxin that is produced in high yield by the classical Park-Williams no. 8 strain. Rawlinson & Hale (1949) found that the major pigment of their strain was a b cytochrome, though they were also able to detect an a type. The α-peak of their b cytochrome was unsymmetrical in a manner suggesting the presence of a c cytochrome as well. While the present work was in progress Pappenheimer, Howland & Miller (1962) published a detailed account of the electron-transport system of several strains. They found that the reduced-minus-oxidized difference spectrum of one strain (PW8-Pd) showed strong peaks at 430 and 558 mμ (cytochrome b-558) accompanied by much weaker peaks at 445 and 605 mμ (cytochromes 'a + a₃'). Other strains contained c, b (564 mμ) and a + a₃

cytochromes. The PW8-Pd strain was also noteworthy in that oxidation of NADH₂ proceeded mainly through a soluble autooxidizable flavoprotein, whereas succinate oxidation followed a cytochrome pathway.

The present studies were performed with a strain, CN 2000, commercially used for producing high yields of toxin. This strain is also of interest in that it contains relatively large amounts of a menaquinone of unusual structure (Scholes & King, 1964). A preliminary account of the present work has been published (Scholes & King, 1963).

Note on nomenclature. The group of compounds formerly known as 'vitamin K₂' are referred to in this paper as 'menaquinones', with the abbreviation MK, followed by a number indicating the number of isoprenoid units attached to position 3 on the nucleus; thus 'vitamin K₂₍₄₀₎' is referred to as MK-8, it being understood that each isoprene unit contains one double bond. Where this is not the case, the number of additional hydrogen atoms is indicated; thus MK-8(2H) indicates a mena-

quinone with eight isoprenoid units in a side chain containing only seven double bonds.

METHODS

Organism. *C. diphtheriae* CN2000 was supplied by Burroughs Wellcome Ltd., The Wellcome Research Laboratories, Beckenham, Kent. It was maintained on blood-agar slopes and grown for experimental purposes in 250 ml. conical flasks shaken at 200 cyc./min. at 35° and containing 100 ml. of medium of the following composition (per l.): 18 g. of peptone (Oxoid Ltd., L46); 3 g. of yeast extract (Difco Ltd.); 5 ml. of acetic acid, neutralized to pH 7 with NaOH; 24 g. of maltose; 450 mg. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1 mg. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.8 mg. of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.3 mg. of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; 20 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; the pH was adjusted to 7.8. Cultures were harvested by centrifugation after 48 hr. growth at 35° (maximum growth was reached at 60–70 hr.), washed twice on the centrifuge with deionized water and made up into heavy suspensions (30–40 mg. dry wt./ml.) with glass-distilled water or 50 mM-phosphate buffer, pH 7.4.

Disintegration and fractionation. Suspensions were disintegrated for 2.5 min. in 5 ml. batches at below 3° in a 20 kcyc./sec. 60 W ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London). This treatment disintegrated over 95% of the cells. Unbroken cells and coarse debris were removed by centrifugation (in a Spinco refrigerated ultracentrifuge) for 15 min. at 20000g. The clear red supernatant, the 'crude extract' (S-1), was separated by decantation from the residue (R); it contained about half of the total nitrogen of the cells. Fraction S-1 was then spun at 140000g for 150 min. to give a 'particle fraction' (P) and a 'supernatant' (S-2). This procedure is an improvement on that described by Scholes & King (1963) since it yields a supernatant fraction containing at most only traces of cytochrome or menaquinone.

Cytochrome content. The test material was placed in both test and reference cuvettes of a spectrophotometer, and the cytochrome in the test cuvette was reduced by the addition of sodium dithionite. The difference $E_{430} - E_{455}$ was taken as a measure of the cytochrome content.

Total nitrogen. This was determined by a micro-Kjeldahl method.

Succinate oxidase. This activity was determined by conventional Warburg manometry at 37°. Flasks contained: cell preparation, 0.1–1.0 mg. of N; phosphate buffer, pH 7.4, 200 μ moles; (centre well) 0.2 ml. of 2N-KOH; (side arm) sodium succinate, 150 μ moles. The total volume was 3 ml. Substrate was added after 7 min. equilibration. Oxygen uptake was corrected for endogenous respiration, which was low in most cases.

Succinate-phenazine methosulphate dehydrogenase (Singer & Kearney, 1957). Warburg manometer flasks contained: cell preparation, 0.1–1.0 mg. of N; phosphate buffer, pH 7.4, 200 μ moles; KCN (added last), 3 μ moles; (centre well) 0.2 ml. of M-KCN; (side arm) sodium succinate, 150 μ moles; freshly prepared phenazine methosulphate, 6.8 μ moles. The final volume was 3 ml. Flasks were protected from light. Oxygen uptake was measured at 37° and corrected for the small endogenous uptake.

Succinate-methylene blue dehydrogenase. The procedure was as above but with 6.6 μ moles of methylene blue in place of the phenazine methosulphate.

NADH₂ oxidase. The test cuvette of a spectrophotometer contained (in 3 ml.): phosphate buffer, pH 7.4, 200 μ moles; NADH₂, 0.3 μ mole. The reference cell contained buffer only. Cell preparation (approx. 0.2 mg. of N) was added to both cells. The decline in E_{340} was followed over a period of 5 min. at room temperature (20°) and oxidase activity was estimated from the initial rate. The molar extinction coefficient of NADH₂ was taken as 6220 (Horecker & Kornberg, 1948).

Reduction of cytochromes by substrates. A spectrophotometer cuvette was fitted with a hollow stopper and an outlet for evacuation after the manner of a Thunberg tube. Cell preparations, with buffer and other additions, were placed in the cuvette and the substrate (succinate, lactate, NADH₂ etc.) in the hollow stopper. The apparatus was rendered anaerobic by repeated evacuation and flushing with N₂ (O₂-free grade). The reaction was started by tipping the substrate from the hollow stopper into the cuvette, which was immediately placed in the recording spectrophotometer (Unicam SP.700), and the absorption spectrum was recorded over the range 400–700 m μ . When interpreting these complete spectra, it should be noted that the instrument reached the α -bands about 2 min. after scanning the Soret region. Where relatively rapid reactions were being followed distorted spectra could result and results were confirmed by repeated scanning of narrower regions at shorter intervals, and by continuous measurement of the extinction at fixed wavelengths.

Estimation of naphthaquinone in cell fractions. A 1 ml. sample of the cell preparation was placed in a 15 ml. stoppered tube and 4 ml. of methanol, at –20°, was added from a spring-loaded syringe to ensure rapid mixing. Next, 5 ml. of light petroleum (b.p. 40–60°) was added, the mixture was shaken for 1 min. and then centrifuged, and the petroleum layer was decanted off. The residue was re-extracted with 3 ml. and 2 ml. of petroleum. The combined extracts were washed for 30 sec. with 2.5 ml. of 95% (v/v) methanol. The petroleum layer was removed and evaporated to dryness in a stream of O₂-free N₂ (cf. Pumphrey & Redfearn, 1960). The extract showed the characteristic four-banded 'vitamin K' spectrum, and the naphthaquinone was estimated by the difference $E_{270} - E_{280}$ (cf. Bishop, Pandya & King, 1962). The accuracy of this method will depend on the relation between the absorption due to vitamin K in this region and the irrelevant 'end-absorption'. *C. diphtheriae* CN2000 has a high 'vitamin K' content and, when the method is applied to the crude lipid extract of the naphthaquinone-carrying fractions of the cell, it yielded values that differed by less than 10% from those obtained by the more precise method of separating the naphthaquinone fraction by chromatography on alumina. In less favourable cases, e.g. preparations of the organism in which the naphthaquinone had been destroyed by ultraviolet irradiation, quantitative estimation was not possible; but, even where the characteristic four-banded 'vitamin K' spectrum had disappeared completely, it was still possible to cite an upper limit for the naphthaquinone content.

Irradiation of cell preparations. An 8 ml. sample of preparation (approx. 2 mg. of N/ml.) in a 6 cm. Petri dish was placed 15 cm. from a Hanovia type 5BC lamp with main emissions at 360 m μ . The material was kept at 4° and agitated with a magnetic stirrer. Controls were treated similarly but covered with an opaque plate during irradiation.

RESULTS

Ultrasonic treatment of bacteria leads to progressive breakdown of cell structure and release of increasing amounts of 'insoluble' cell components (e.g. cytochrome, naphthaquinone, succinate oxidase) into the fraction (S-1) not sedimented at 20000g (cf. Bishop & King, 1962), but this breakdown may be accompanied by considerable loss of enzymic activities. The disintegration time selected (2.5 min.) decreases the viable count to less than 5% of the initial value without causing appreciable destruction of cytochrome, naphthaquinone or NADH₂ oxidase. Succinate oxidase and the succinate-phenazine methosulphate and succinate-methylene blue dehydrogenases suffer about 5-15% inactivation, but lactate-oxidase activity is severely decreased (cf. White, 1964).

Composition of centrifugally separated fractions. This is set out in Table 1. Cytochrome and menaquinone are essentially sedimentable components. The behaviour of succinate oxidase and NADH₂ oxidase is less straightforward. High-speed centrifugation of the crude extract (S-1) yielded a supernatant that was always very low in succinate-oxidase activity and that, in some runs, contained no measurable amounts. Any succinate oxidase remaining in the high-speed supernatant (S-2) showed the same degree of sensitivity to cyanide as the sedimented oxidase. The particulate fraction (P) contained succinate oxidase, but at a variable and much lower activity than the parent S-1 fraction. The recovery of succinate oxidase in the P and S-2 fractions is usually only about 40-50% of the activity of the parent S-1 material. The full activity of fraction S-1 is recovered, however, when fractions P and S-2 are recombined.

Some NADH₂-oxidase activity is found in the

supernatant (S-2). However, the oxidase in the S-2 fraction is not inhibited by 20 mM-cyanide; the P fraction suffers 43% inhibition under similar conditions. The sum of the activities of fractions P and S-2 is rather less than that of the parent crude extract and activity can be fully recovered by mixing the P and S-2 fractions; the effect is less striking than with succinate oxidase. The fractionation and recombination of the oxidase activities of the crude bacterial extract is considered in detail below.

Reduction of cytochrome in the particulate fraction. Reduction of the particulate fraction (P) with dithionite yielded the difference spectrum shown in Fig. 1. The major component is a *b*-type cytochrome whose α -band at 564 m μ almost obscures cytochrome *c*, whose presence is here suggested only by the asymmetry of the band. A band at 606 m μ and a barely perceptible shoulder on the Soret band at 445 m μ have been attributed to cytochromes *a* + *a*₃ by Pappenheimer *et al.* (1962). We accept their interpretation but will refer to this component for convenience as cytochrome *a*.

The anaerobic addition of succinate, lactate or NADH₂, or both succinate and lactate, leads to the same final result; but the spectrum obtained with these substrates differs markedly from that obtained with dithionite. The α -band of cytochrome *c* is now well-defined at 554 m μ and the cytochrome *a* shoulder at 445 m μ on the Soret band is seen more clearly. The particulate fraction thus contains a pigment that is not reduced (or at least not fully reduced) by the substrates, but is reduced by dithionite. The spectral characteristics of this pigment were determined by means of a difference spectrum with dithionite in the experimental cuvette and with lactate plus succinate in the reference cuvette (Fig. 2). The absorption maxima at 430 and 559 m μ

Table 1. *Distribution of constituents of respiratory system among cell components*

Values are percentages of the amounts in whole cells. Absolute values for whole cells were: menaquinone, 6.8 μ moles/g. dry wt.; cytochrome, 0.3-0.4 $E_{430} - E_{455}$ /cm./mg. of N/ml. Enzyme activities (μ moles of substrate oxidized/min./mg. of N): succinate oxidase, 0.51; succinate-methylene blue dehydrogenase, 0.71; succinate-phenazine methosulphate dehydrogenase, 1.34; NADH₂ oxidase, 0.041.

	Nitrogen	Menaquinone	Cytochrome	Succinate-oxidase activity	Succinate-dehydrogenase activity		NADH ₂ -oxidase activity
					Methylene blue	Phenazine methosulphate	
Whole cells	100	100	100	100	100	100	100
Disintegrated cells				94	88	91	100
Residue (R)	52	79	71	47	26	17	39
Crude extract (S-1)	48	21	29	44	30	41	58
Particles (P)	11	21	29	17	21	19	7
Supernatant (S-2)	37	*	*	1.9	13	26	32

* The characteristic spectra were not detectable.

Fig. 1. Difference spectra of particulate fraction (P) reduced with either dithionite (----), or succinate ($60\mu\text{moles}$) (- - - - -), immediately after addition; —, 30 min. later. Both cuvettes contained (in 3 ml.): phosphate buffer, pH 7.4, $200\mu\text{moles}$; P fraction, 2.8 mg. of N. The reductant was added to the experimental cuvette but not to the reference cuvette.

Fig. 2. Component of particulate (P) fraction reducible by dithionite but not by substrates. Cuvettes contained (in 3 ml.): phosphate buffer, pH 7.4, $200\mu\text{moles}$; P fraction, 2.8 mg. of N. Difference spectra: —, succinate ($60\mu\text{moles}$) in experimental cuvette, no reductant in reference cuvette; ----, dithionite in experimental cuvette, succinate and lactate in reference cuvette. Spectra were scanned 30 min. after the addition of reductants.

correspond to a *b* cytochrome and are coincident with those reported for the dominant cytochrome of the PW8-Pd strain by Pappenheimer *et al.* (1962).

Although succinate gave the same final reduced spectrum as lactate or NADH₂ its action was much more rapid. Fig. 1 shows that reduction by succinate is almost complete in the spectrum in which scanning commences only 30 sec. after the addition of substrate. In corresponding experiments with either lactate or NADH₂ the bands at 430 and 564 m μ were only just appearing at this stage. It will be appreciated that in the curve scanned 'immediately' after the addition of succinate (Fig. 1) the trace reached the Soret region about 30 sec. after the addition of the substrate, but that the α -bands were reached about 2 min. later. To examine the relatively rapid reduction of the cytochromes it is necessary to set up experiments in which, instead of scanning the whole spectrum, only the region of the α -peaks is examined. This allows traces to be obtained within 20 sec. of the addition of the substrate, and repeat tracings can be performed at intervals of 20–30 sec. When this is done only the cytochrome *b* peak (564 m μ) is detectable up to 45 sec. after the addition of succinate: by this time it has reached nearly full development. The cytochrome *c* and *a* peaks then appear together and reach full development rapidly. The overlapping of the cytochrome *b* and *c* bands, and the limited sensitivity of our instrument, make it difficult to say whether slight reduction of the *a* and *c* cytochromes has taken place at an earlier stage; but it is clear that, whereas reduction of cytochrome *b* commences immediately on the addition of succinate, reduction of at least the major portion of cytochromes *a* and *c* takes place only after a lag period, and not until maximum reduction of cytochrome *b* is almost accomplished. This was confirmed in another experiment in which reduction was allowed to proceed to completion and the cell then opened and shaken with air. The cell was then returned to the spectrophotometer and the α -band region scanned within 20 sec. A weak cytochrome *b* band was seen with no sign of cytochrome *a* or *c*. After about 1.5 min. (approximately the time required for exhaustion of the oxygen in the solution) the cytochrome *b* band increased in intensity and the cytochrome *c* and *a* bands made their appearance and rapidly reached full intensity.

When lactate was used as reductant the final spectrum was identical with that obtained with succinate. The reduction was very much slower and, particularly with particulate preparations that had been washed in phosphate buffer, the sequence of events was somewhat different. This is shown by the tracings of the Soret region in Fig. 3. At first only the cytochrome *b* band is seen. The cyto-

Fig. 3. Reduction of particulate fraction by lactate. Cuvettes contained (in 3 ml.): phosphate buffer, pH 7.4, 200 μ moles; P fraction (washed with phosphate buffer), 3 mg. of N. Lactate (60 μ moles) was added to the experimental cuvette and the difference spectra were taken after 30 sec. (—), 10 min. (----) and 20 min. (-·-·-).

chrome *a* band comes up later (as does also the cytochrome *c* band, as shown by the inflexion at 420 m μ), but it is fully developed (see 10 min. tracing) at a stage when the cytochrome *b* band has reached only half its final intensity. We must emphasize, however, the relatively slow oxidation of lactate by this system; any traces of oxygen remaining in the system would thus have a more marked effect than in the experiments with succinate.

Inhibition of succinate and NADH₂ oxidation by Triton X-100 and by cyanide. Succinate oxidation by the crude extract (S-1) is inhibited 70–80% by 1 mM-cyanide or by 0.01% Triton (the inhibition is not increased further if the Triton X-100 concentration is raised to 0.25%). Succinate-methylene blue-dehydrogenase activity in fraction S-1 is not affected by Triton X-100, but in the particulate fraction the dehydrogenase activity is slightly enhanced (15–20%), possibly because the detergent renders the system more accessible to the reagents. In the crude extract (S-1) even a relatively high concentration of Triton X-100 (0.14%) has no immediate effect on NADH₂ oxidation, though after a few minutes up to 25% inhibition may be established. NADH₂ oxidation is also not appreciably inhibited by 0.8 mM-cyanide, though only about 20% inhibition is attained even at 10 mM-cyanide.

Fig. 4. Reduction of cytochromes by succinate in the presence of Triton X-100. Cuvettes contained (in 3 ml.): phosphate buffer, pH 7.4, 200 μ moles; fraction P, 2.8 mg. of N; Triton X-100, 0.2%. Difference spectra were obtained on adding to the experimental cell either dithionite (----) or succinate (-.-.-), immediately after addition; —, 30 min. later).

If, however, fraction S-1 is centrifugally separated into the particulate (P) and high-speed supernatant (S-2) fractions, the NADH₂-oxidase activity of the former shows 43% inhibition at 20 mM-cyanide, whereas in the latter NADH₂ oxidation is not affected by cyanide.

Effect of Triton X-100 on cytochrome reduction in particulate fractions. The difference spectra obtained on adding succinate to Triton-treated P fraction are shown in Fig. 4, and should be compared with the spectra obtained with the same sample in absence of Triton X-100 (Fig. 1). The first spectrum (scanning commenced 30 sec. after the addition of succinate) shows a distinct band of reduced cytochrome *b* (564 $m\mu$); but neither the α -band of cytochrome *a* at 606 $m\mu$ nor the Soret band inflexion at 445 $m\mu$ are visible; nor is the band of cytochrome *c* at 554 $m\mu$. After 30 min., however, reduced bands of all three cytochromes (*a*, *b* and *c*) are well developed. The delayed reduction of cytochromes *a* and *c* can be seen more clearly in Fig. 5, which shows the Soret region of a difference spectrum between the initial and final stages of reduction. Here the peaks of all three cytochromes are well-defined; the *b* cytochrome is not fully

Fig. 5. Effect of Triton X-100 on the reduction of cytochrome by succinate. Experimental arrangements were as given for Fig. 4, except that cuvettes contained fraction P, 0.8 mg. of N. Spectra were taken immediately after the addition of succinate (----) and 30 min. later (-.-.-); the difference between the 'immediate' and '30 min.' spectra (—) is also shown.

reduced in the spectrum taken at 30sec., so that its peak appears between those of cytochromes *c* and *a*, which are still predominantly in the oxidized state. When the time-sequence is studied by taking frequent scanings of the α -band region it is found that reduction of most of the *b* cytochrome has occurred even before the first reading can be taken (about 20sec.) but there is a further lapse of 3min.

Fig. 6. Effect of irradiation (360 mμ) on the menaquinone content of the particulate fraction (P). (a) Spectra of whole lipids of the fraction; (b) spectra of the 'menaquinone fraction' obtained by chromatography on alumina. Spectra: —, lipid from P fraction (6.9 mg. of N) in 6 ml. of cyclohexane (non-irradiated control); ----, lipid from irradiated fraction P (13.8 mg. of N) in 6 ml. of cyclohexane.

before the cytochrome *a* and *c* bands appear. The effect of Triton X-100 on the reduction of cytochromes by lactate is less dramatic but here, too, the lag that occurs before reduction of cytochromes *a* and *c* is prolonged. Triton X-100 does not, however, affect the reduction of the cytochromes by NADH₂.

Effect of ultraviolet irradiation on electron-transport activity. The particle fraction (P) was irradiated for 2hr. at 360 mμ and the lipid extracted as described in the Methods section. The absorption spectrum (Fig. 6a) still shows signs of specific absorption in the region of the menaquinone absorption bands, but this is due to material that separated from the menaquinone when the lipid was fractionated on activated alumina (Bishop *et al.* 1962). The fraction eluted with ether-light petroleum (1:49, v/v), which normally carries the menaquinone, shows no sign of specific absorption (Fig. 6b). Irradiation must have destroyed not less than 99% of the menaquinone originally present.

Table 2 shows that the loss of succinate-oxidase activity in crude extract (S-1) ran roughly parallel with the destruction of menaquinone, save that some succinate-oxidase activity survived when menaquinone destruction was virtually complete. A similar relationship was found after irradiation of the particulate fraction (P). Succinate-phenazine methosulphate and succinate-methylene blue dehydrogenases and NADH₂ oxidase are much less sensitive to irradiation. The small amount of cyanide-sensitive NADH₂-oxidase activity is, however, completely lost after 90min. irradiation. About one-third of the succinate-oxidase activity in particulate fraction survives complete destruction of the menaquinone. This residual activity can be enhanced by the addition of the supernatant (S-2) fraction but shows no sensitivity to 1mm-cyanide, which produces 70-80% inhibition in the non-irradiated material.

Attempts were made to reactivate the succinate system in the irradiated material by the addition of various quinones (Table 3). The particulate

Table 2. Losses of menaquinone and enzyme activity on ultraviolet irradiation

Values are percentages of initial values. —, Not determined.

Time (min.)	Mena- quinone	Succinate- oxidase activity	Succinate-dehydro- genase activity		NADH ₂ - oxidase activity
			Methylene blue	Phenazine metho- sulphate	
0	100	100	100	100	100
30	46	52	94	87	95
60	Trace	35	—	—	81
90	None	24	84	86	77

Table 3. Restoration of succinate oxidation in ultra-violet-irradiated material by the addition of quinones

Fraction P was irradiated (2hr.) and then mixed with an equivalent amount of (non-irradiated) fraction S-2; the latter is, by itself, devoid of succinate-oxidase activity. The quinones were added as dispersions in ethanol.

	Succinate-oxidase activity (μ moles of succinate oxidized/min./mg. of N of fraction P)	
	Without cyanide	With 1 M- cyanide
Non-irradiated control	2.32	0.5-0.7
Irradiated	0.80	0.80
Irradiated + MK-0 (8mm)	3.68	3.61
Irradiated + MK-2 (9mm)	2.26	1.55
Irradiated + MK-2 (1.8mm)	2.16	
Irradiated + MK-2 (0.36mm)	1.18	
Irradiated + MK-2 (0.072mm)	0.94	
Irradiated + MK-8 (2H)* (4.7mm)	0.93	

* From *C. diphtheriae*.

fraction (P) was irradiated and then an equivalent amount of the supernatant (S-2) added; the latter contains no menaquinone and has negligible succinate-oxidase activity. Most of the quinones used were almost insoluble in water and were dispersed either by dissolving in a small volume of ethanol (Redfearn, 1961) and adding to the preparations, or by mixing with the S-2 fraction (before the latter was added to the particulate suspension) and treating in the ultrasonic disintegrator for 15sec.

(Brodie & Ballantine, 1960). As both methods gave similar results, the more convenient ethanol procedure was employed for the experiments recorded in Table 3. Dispersal in the presence of lecithin was also tried (Fleischer & Brierley, 1961), but this yielded no advantage over the other methods. Significant restoration of activity was achieved only with the menaquinones MK-0 and MK-2. The menaquinone occurring naturally in *C. diphtheriae*, namely MK-8(2H), was inactive; possibly the long side chain prevented access to the enzyme centres. The succinate oxidation in the presence of MK-0 was insensitive to cyanide. Here MK-0 might perhaps be regarded as merely providing an artificial shunt to the whole electron-transport chain. The system reactivated by MK-2, however, showed some degree of sensitivity to cyanide, suggesting that here we were truly replacing the menaquinone destroyed by irradiation. MK-1 and MK-3 also reactivated the system, with some degree of sensitivity to cyanide; MK-4, MK-5 and MK-6 did not restore succinate-oxidase activity.

Reduction of cytochromes in the irradiated particulate fraction. When succinate is added to the irradiated particulate fraction the bands of reduced cytochrome *b* appear almost as rapidly as in the untreated material, but there is a delay of about 12min. before the cytochrome *c* and *a* bands become recognizable; these become fully reduced within the next 3min. and after that no further change occurs (cf. Figs. 1 and 7). The final spectrum differs from that obtained with non-irradiated material in that a portion of the cytochrome *b* remains oxidized. This is shown in the difference spectra obtained when succinate is added simultaneously to normal particulate fraction (in the

Fig. 7. Reduction of cytochromes by succinate in irradiated particulate fraction (P). Cuvettes contained (in 3ml.): phosphate buffer, pH 7.4, 200 μ moles; P fraction, 2.5 mg. of N. Succinate (60 μ moles) was added to the experimental cuvette and difference spectra were taken immediately (----) and after 30 min. (—).

Fig. 8. Inhibition of cytochrome reduction in irradiated particulate fraction (P) and restoration by MK-2. All cuvettes contained (in 3 ml.): phosphate buffer, pH 7.4, 200 μ moles; succinate, 60 μ moles (added last). The reference cuvette contained particulate fraction, 1.8 mg. of N; the experimental cuvette contained the corresponding amount of irradiated material. Difference spectra were taken immediately after the addition of succinate (---) and 30 min. later (—), and also after the addition of 0.55 μ mole of MK-2 (-·-·-·).

reference cell) and irradiated material (in the test cell) (Fig. 8). At first the inverse peaks of cytochromes *a* and *c* appear, showing that these are being reduced by the control but not by the irradiated material. Later these disappear (i.e., they are reduced in the irradiated as well as in the control cuvettes) and the only differential absorption remaining is a weak cytochrome *b* spectrum; this rapidly disappears when MK-2 is added to the irradiated cuvette. Irradiation thus: (i) has no effect on the reduction of the major cytochrome *b* component; (ii) much delays the reduction of cytochromes *c* and *a*; (iii) blocks the reduction of a portion of cytochrome *b*, though this can be reversed by MK-2.

The picture obtained when lactate is added to irradiated material is different. There is no rapid initial reduction of cytochrome *b*. The reduction of cytochromes *a* and *c* is delayed slightly less; these two cytochromes begin to show their reduced bands before the major portion of cytochrome *b* is reduced. The main impression obtained, however, is a drastic retardation of the reduction of all three cytochrome components.

The reduction of particulate fraction by NADH_2 follows the same course in irradiated material as in normal controls.

Fractionation and reconstitution of oxidase activities. NADH_2 -oxidase activity is concentrated

Fig. 9. Reconstitution of the NADH_2 -oxidase system by the recombination of the particulate (P) and supernatant (S-2) fractions. The crude extract (S-1) was separated into the particulate (P) and supernatant (S-2) fractions by centrifugation; the former was suspended in buffer to give the same volume as fraction S-2. To 0.2 ml. of fraction S-2 (derived from 1.5 mg. dry wt. of organisms) were added different volumes of a suspension of fraction P, as indicated on the abscissa. ●—●, NADH_2 -oxidase activity determined experimentally; ----, arithmetical sum of NADH_2 -oxidase activities in the amounts of fractions P and S-2 used. The arrow indicates the point at which fractions P and S-2 were being mixed in the proportions in which they were present in the original extract (S-1).

mainly in the supernatant (S-2). If different amounts of particulate material (P) are added there is an enhancement of activity greater than can be accounted for by the activity of the added P fraction. This is brought out in Fig. 9, where the continuous line gives the NADH₂-oxidase activity of a constant amount of S-2 fraction to which increasing amounts of particulate (P) fraction had been added. The broken line represents the arithmetical sum of the S-2 and P activities. The addition of fraction P to fraction S-2 brings about a maximum increment of activity when the two fractions are in the proportion in which they occur in the crude extract (S-1) from which they were derived.

A tentative interpretation of these results is as follows. Particulate fraction contains an orthodox NADH₂-oxidase system consisting of a flavoprotein reductase coupled to a cytochrome system. The overall activity is limited by the flavoprotein. The supernatant contains an autoxidizable flavoprotein (cf. Pappenheimer *et al.* 1962); this may occur as such in the cell, or may be released from an 'insoluble' complex during disintegration. This flavoprotein may transfer electrons directly to oxygen, or pass them through a cytochrome system, the latter being the more rapid reaction. The supernatant fraction itself would thus have a substantial NADH₂-oxidase activity, but the addition of the P fraction would enhance this further by offering a more efficient pathway for electron transfer to oxygen.

The distribution of succinate oxidase is different. Here the P fraction shows one-half to one-third of the activity of the crude extract S-1, whereas the supernatant (S-2) is almost inactive. The activity of the P fraction increases, however, on the addition of S-2 (see Fig. 10) even beyond the point at which fractions P and S-2 are being mixed in the proportions in which they are present in the parent S-1 material. Could these findings be explained on the basis of separation of the succinate-oxidase system into a soluble dehydrogenase and a particulate terminal oxidase system? This seems *prima facie* unlikely, since (1) the succinate-dehydrogenase activity of fraction S-1 is about equally distributed between fractions P and S-2, and (2) the dehydrogenase activity of fraction P is about twice the overall oxidase activity. It is true that the reduction of cytochrome *b* by succinate is slow relative to the subsequent reduction of the *c* and *a* components, but this is in an anaerobic system in which the terminal oxidase is inoperative. The following experiments seem to rule out simple separation into dehydrogenase and terminal oxidase: (1) Samples of fraction S-2 were heated for 15 min. at pH 7 at various temperatures. About 75% of the succinate-phenazine methosulphate-dehydrogenase activity

Fig. 10. Reconstitution of succinate oxidase by the recombination of the particulate (P) and supernatant (S-2) fractions. The crude extract (S-1) was separated into fractions P and S-2 by centrifugation. To fraction P (1 mg. of N) were added different amounts of fraction S-2 (which itself had no succinate-oxidase activity), and the oxygen uptake in the presence of succinate was measured; the no-succinate blanks were negligible. The three curves represent three separate P and S-2 preparations; the arrows indicate the points at which the two components were being mixed in the proportions in which they occurred in the parent S-1 preparations.

survived at 60°, but inactivation was complete at 100°. The succinate-oxidation factor suffered about 65% destruction at 60° but the remaining activity survived heating at 100°. Both heat-stable and heat-labile factors are thus involved, and the latter is more sensitive to thermal inactivation than the dehydrogenase. (2) The S-2 fractions from *Corynebacterium xerosis* and *C. ovis* showed the same stimulation of succinate oxidation as 'S-2' from *C. diphtheriae*, but the corresponding fraction from *Bacillus megaterium* showed only slight activity and that from *Proteus vulgaris* none at all. In all four organisms the succinate-phenazine methosulphate-dehydrogenase activity of the S-2 fraction was only 10-20% of that obtained with *C. diphtheriae*. (3) The S-2 material was passed through a column of Sephadex G-100 (a dextran gel that is not penetrated by substances with mol.wt. above 100 000).

The peak of succinate-phenazine methosulphate-dehydrogenase activity preceded the peak of succinate-oxidase reactivation, though a complete separation was not achieved.

DISCUSSION

Bacterial respiratory systems are normally localized in the particulate material obtained on disintegration (Marr, 1960). In a number of cases, however, maximum oxidative capacity for some substrates has required a combination of both 'particle' and 'supernatant' fractions, e.g. in *Alcaligenes faecalis* (Pinchot, 1953), *Mycobacterium phlei* (Brodie, 1959) and *Escherichia coli* (Kashket & Brodie, 1963). The situation is broadly similar in the strain of *C. diphtheriae* that we have studied. With succinate, the activity of the particulate fraction is restored by the addition of a supernatant fraction that, by itself, is almost devoid of activity. An unidentified factor present in the supernatant must be involved; the results cannot be explained on the hypothesis of separation into 'dehydrogenase' (soluble) and 'oxidase' (insoluble). Moreover, both a heat-stable and a heat-labile factor seem to be involved.

The 'succinate-methylene blue'- and 'succinate-phenazine methosulphate'-dehydrogenase activities show some differences in distribution, the former showing a greater tendency to be associated with the sedimentable material. It is generally believed that phenazine methosulphate couples directly with the flavoprotein of the dehydrogenase (Singer, Kearney & Massey, 1957), whereas methylene blue reacts further along the chain, probably with one of the cytochrome pigments. Pappenheimer & Hendee (1949) isolated a succinate-methylene blue dehydrogenase complex containing cytochrome *b*. Our results are consistent with the idea that a 'sedimentable' electron carrier is situated between phenazine methosulphate and methylene blue.

The behaviour of the NADH₂-oxidase system has already been discussed above; our results are in accordance with the finding that some strains of this organism can actively oxidize NADH₂ through a cyanide-stable soluble oxidase not involving the cytochrome system (Pappenheimer *et al.* 1962). The CN2000 strain, however, appears to be able also to oxidize NADH₂ through a terminal cytochrome system, and to be able, in addition, to 'shunt' the soluble oxidase through this system to some extent.

In the CN2000 strain reduction with either succinate, lactate or NADH₂ leads to the same final spectrum, showing bands attributable to *a*, *b* and *c* cytochromes; their relative amounts cannot be determined owing to lack of data on their molar

extinction coefficients. The absorption due to the *b* type, in both Soret and α -band regions, is at least twice as intense as that attributable to the *a* or *c* components. A substantial portion of the cytochrome *b*, however, is reducible only by dithionite and not by these substrates. A similar situation has been reported in mammalian tissues by Chance (1958) and Holton & Colpa-Boonstra (1960).

It is difficult to formulate a scheme for electron transport that will cover the facts we have presented, since few of the results are clear-cut. In some cases (such as the 'soluble' and 'insoluble' NADH₂-oxidase systems) we have alternative pathways that can couple with each other at undefined points. It is also uncertain whether externally added and 'bound' NADH₂ will be oxidized by particulate fraction in the same way. Triton X-100 appears to intercept the electron-transport chain somewhere between *b* and *c* cytochromes, but at a point beyond that at which the system couples with methylene blue; the reduction of the latter by succinate is unaffected. There appears to be an alternative pathway for succinate oxidation, since 20–30% of the activity of the particulate fraction is stable even to quite high concentrations of Triton. Inhibition of succinate oxidation by cell membranes of *Micrococcus lysodeikticus* by deoxycholate and Tween 80 has been attributed to destruction of the membrane organization through dispersal of the phospholipid (Lukyanova, Gelman & Biryuzova, 1961), but in our organisms these agents inhibited succinate oxidation only at concentrations tenfold greater than the effective concentration of Triton X-100.

The action of ultraviolet irradiation is somewhat more complicated. A substantial part of the activity is insensitive to irradiation and also to cyanide, indicating the presence of an alternative pathway involving neither menaquinone nor the 'cytochrome *a*+*a*₃' system. Irradiation acts at a different site from that blocked by Triton X-100, however, since, although there is a rapid initial reduction of cytochrome *b*, a substantial portion remains oxidized even when reduction of the *a* and *c* components has gone to completion. Apparently there are two *b* components, one directly reducible by succinate, the other only through menaquinone. We cannot say whether these are two distinct chemical entities or the same substance incorporated at different sites in a supermolecular structure. In Scheme 1 we refer to these as '*b*₂' and '*b*₁'.

In common with other bacterial respiratory systems, our particulate fraction was not inhibited by 3 μ M-antimycin A, though at 0.1 μ M mammalian and yeast systems are completely inhibited (Gottlieb & Ramachandram (1961).

It is necessary to assume that cytochrome *b*₂ (or the succinate dehydrogenase itself) is slowly autoxi-

Scheme 1. Oxidation of succinate.

dizable to account for the part of the succinate-oxidase activity that survives cyanide, Triton X-100 or irradiation. The slow by-pass from cytochrome b_x to cytochrome c is required to account for the eventual reduction of the a and c components in irradiated material; this cannot be explained on the grounds of incomplete destruction of the MK because (apart from the chemical evidence to the contrary) this would not explain why cytochrome b_y remains in the oxidized state when cytochromes a and c are reduced.

NADH_2 may be oxidized either by a soluble cyanide-insensitive system (presumably the autooxidizable flavoprotein described by Pappenheimer *et al.* 1962) or by a particulate system that is cyanide-sensitive, and cross-coupling between the two systems seems likely. In the particulate material NADH_2 brought about a reduction of all three cytochrome components, and this was only slightly affected by either Triton X-100 or irradiation. The behaviour of externally added NADH_2 may well be different from its action *in vivo*, and it is possible that *in vitro* NADH_2 (perhaps aided by the flavine oxidase) can react at several points on the electron-transport chain and thus reduce the cytochrome components individually rather than sequentially.

Lactate was tried as a substrate in the hope that it would throw some light on the pathway of oxidation of substrates that (presumably) employ NADH_2 . The results need treating with considerable caution, as the rate of lactate oxidation in disintegrated cells is only about 10% of that obtained with intact organisms. The final spectrum obtained with lactate is the same as that yielded by succinate, but the cytochrome b bands develop much more slowly in relation to the cytochrome a and c bands. Irradiation, which hardly affects the reduction of cytochrome b by succinate, severely retards its reduction by lactate. The facts would be best explained by assuming that lactate enters the chain, either directly or through NADH_2 , at the MK stage.

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REFERENCES

- Bishop, D. H. L. & King, H. K. (1962). *Biochem. J.* **85**, 550.
 Bishop, D. H. L., Pandya, K. P. & King, H. K. (1962). *Biochem. J.* **83**, 606.
 Brodie, A. F. (1959). *J. biol. Chem.* **234**, 398.
 Brodie, A. F. & Ballantine, J. (1960). *J. biol. Chem.* **235**, 226.
 Chance, B. (1958). *J. biol. Chem.* **233**, 1223.
 Fleischer, S. & Brierley, G. (1961). *Biochem. biophys. Res. Commun.* **5**, 367.
 Fujita, A. & Kodama, T. (1934). *Biochem. Z.* **273**, 186.
 Gottlieb, D. & Ramachandram, J. (1961). *Biochim. biophys. Acta*, **53**, 396.
 Holton, F. A. & Colpa-Boonstra, J. (1960). *Biochem. J.* **76**, 179.
 Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
 Kashket, E. & Brodie, A. F. (1963). *Biochim. biophys. Acta*, **78**, 52.
 Lukoyanova, M. A., Gelman, N. S. & Biryuzova, V. I. (1961). *Biokhimiya*, **26**, 786.
 Marr, A. G. (1960). *Annu. Rev. Microbiol.* **14**, 242.
 Pappenheimer, A. M., jun. & Hendee, E. D. (1947). *J. biol. Chem.* **171**, 701.
 Pappenheimer, A. M., jun. & Hendee, E. D. (1949). *J. biol. Chem.* **180**, 597.
 Pappenheimer, A. M., jun., Howland, J. L. & Miller, P. A. (1962). *Biochim. biophys. Acta*, **64**, 229.
 Pinchot, G. B. (1953). *J. biol. Chem.* **205**, 65.
 Pumphrey, A. M. & Redfearn, E. R. (1960). *Biochem. J.* **76**, 61.
 Rawlinson, W. A. & Hale, J. H. (1949). *Biochem. J.* **45**, 247.
 Redfearn, E. R. (1961). *Biochim. biophys. Acta*, **53**, 581.
 Scholes, P. B. & King, H. K. (1963). *Biochem. J.* **87**, 10P.
 Scholes, P. B. & King, H. K. (1964). *Biochem. J.* **91**, 9P.
 Singer, T. P. & Kearney, E. B. (1957). *Meth. biochem. Anal.* **4**, 307.
 Singer, T. B., Kearney, E. B. & Massey, J. (1957). *Advanc. Enzymol.* **18**, 65.
 White, D. C. (1964). *J. biol. Chem.* **239**, 2055.