possible origin of the soluble RNA here. More complete investigations are required to establish the origin of the RNA of the cytoplasmic fractions.

SUMMARY

1. When protoplasts of *Bacillus megaterium* are incubated with [³H]uridine, RNA with the highest specific radioactivity is found after short periods in the two components of the nuclear fraction. Whereas the radioactivity of the DNA-containing component soon reaches a stage of slow increase, that in the nuclear residue (ribosomal) continues to increase.

2. The specific radioactivity in cytoplasmic ribosomes increases slowly and approximately linearly with time, but the total amount present soon exceeds that in the nuclear material.

3. In all RNA fractions, part of the radioactivity is incorporated terminally, the amount being high during the first few minutes of labelling but falling progressively with time.

4. When the nuclear material is analysed on a caesium chloride gradient, most of the radioactivity is associated with the DNA band.

5. Experiments are given suggesting that the labelled RNA can be displaced from the nuclear material to other cell fractions.

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Physicochemical Studies on Cytochrome b_2

SOME PROPERTIES OF MODIFIED FORMS OF THE ENZYME AND OF THE DEOXYRIBONUCLEIC ACID COMPONENT

By J. McD. ARMSTRONG,* J. H. COATES† AND R. K. MORTON
*Department of Agricultural Chemistry, Waite Agricultural Research Institute,
 University of Adelaide, South Australia*

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Cytochrome b_2 , the L(+)-lactate dehydrogenase of baker's yeast, was first isolated as a crystalline

deoxyribonucleoprotein (Appleby & Morton, 1954, 1959*a*, 1960; Morton, 1955) containing equimolecular proportions of flavin monophosphate and protohaem (Appleby & Morton, 1954, 1959*b*). This material behaves as an apparently homogeneous protein on sedimentation and on electrophoresis (Armstrong, Coates & Morton, 1963). The

* Present address: Department of Physical Biochemistry, Australian National University, Canberra, Australia.

† Present address: Department of Physical and Inorganic Chemistry, University of Adelaide, South Australia.

DNA component is dissociated from the enzyme by salt fractionation and by enzymic digestion (Morton, 1958; Appleby & Morton, 1960; Montague & Morton, 1961; Morton & Shepley, 1961). The resulting polynucleotide-free enzyme is not readily crystallized (Appleby & Morton, 1960), although large crystals can be obtained under appropriate conditions (Morton & Shepley, 1961; Morton, 1961a). Cytochrome b_2 prepared by salt fractionation and by column chromatography (Bach, Dixon & Zervas, 1946; Boeri, Cutolo, Luzzati & Tosi, 1955; Boeri & Rippa, 1961a; Nygaard, 1959, 1961) appears to have lost the specific DNA component.

When ferrocytochrome b_2 is exposed to air or to high or low pH values, or is treated with organic mercurials or with urea, there is considerable loss of enzymic activity. The appearance of typical flavin fluorescence is evidence for a modification of the protein and for the formation of free flavin (Appleby & Morton, 1954, 1959b; Armstrong, 1961).

The present paper describes physicochemical properties of cytochrome b_2 free of the DNA component, and of the DNA isolated from the enzyme. The effects of oxygen, *p*-chloromercuribenzenesulphonate and urea on the physicochemical properties of the enzyme are also described. Brief accounts of some aspects of this work have been published (Armstrong, Coates & Morton, 1960a, b, 1961; Armstrong & Morton, 1961a, b).

MATERIALS AND METHODS

Nomenclature. Type I cytochrome b_2 (containing DNA) and Type II cytochrome b_2 (DNA-free) refer to enzymically active preparations with or without the specific DNA component of the enzyme (see Morton, 1961a). All preparations were enzymically reduced and in the presence of lactate unless otherwise specified.

General. Appleby & Morton (1959a, b) and Armstrong *et al.* (1963) have described the procedures for the preparation of crystalline Type I cytochrome b_2 and for obtaining anaerobic conditions. Unless otherwise stated, solutions of enzyme contained about 50 μ M-cytochrome b_2 (expressed in terms of haem) in 0.3M-sodium lactate-0.05M-tetrasodium pyrophosphate-0.1 mM-EDTA, pH 6.8.

Deoxyribonucleic acid of cytochrome b_2 . The DNA preparation was that described by Montague & Morton (1961), purified by chromatography on ECTEOLA cellulose.

Type II cytochrome b_2 . Solutions containing recrystallized Type I cytochrome b_2 (approx. 80 μ M expressed in terms of haem) in 0.5M-sodium lactate-0.1 mM-EDTA, pH 6.8, were dialysed for 14–24 hr. against a solution of 70% saturated ammonium sulphate in 0.1M-sodium lactate-0.1 mM-EDTA, pH 6.8, under anaerobic conditions until precipitation of the protein was complete. The flocculent pink precipitate was collected by centrifuging at about 10 000g for 15 min., washed with a solution of 70% saturated ammonium sulphate in 0.1M-sodium lactate-

0.1 mM-EDTA, pH 6.8, and dissolved in the appropriate buffer solution. Ammonium sulphate was removed by dialysis against buffer.

Attempts to prepare Type II cytochrome b_2 by the slow addition of powdered ammonium sulphate to Type I cytochrome b_2 were unsuccessful. The precipitate so obtained was oily in appearance, flowing readily at 2° even after prolonged centrifuging, and contained the Type I enzyme.

Physicochemical measurements. Ultracentrifuging and electrophoresis were carried out in similar buffer solutions, and under conditions similar to those described by Armstrong *et al.* (1963), unless stated otherwise. The sedimentation and diffusion coefficients of the DNA were determined in the Spinco analytical centrifuge by using the ultra-violet-absorption optical system (see Schumaker & Schachman, 1957). The diffusion coefficient was determined from the rate of boundary-spreading during centrifuging in a synthetic-boundary cell. The boundary was formed by layering the buffer over the dialysed solution of the DNA at 12 000 rev./min. The speed was then decreased to 3397 rev./min. so that diffusion would occur across the boundary without appreciable sedimentation of the DNA.

Absorption spectra. An Optica CF-4 manual spectrophotometer was used. It was calibrated for wavelength accuracy by standard procedures; the extinction scale was calibrated with reference neutral-density filters supplied by the National Standards Laboratory, Sydney, Australia.

Fluorescence. The yellow fluorescence due to flavin was observed on irradiation with a Hanovia lamp with a maximum emission at about 360 m μ .

Enzymic activity. This was determined as described by Appleby & Morton (1959a, b) and Armstrong *et al.* (1963).

Determination of thiol groups. The extinction changes due to the reaction of *p*-chloromercuribenzenesulphonate with thiol groups were first determined by using GSH as a model compound: measurements were made at pH 7.0 in 0.3M-sodium lactate-0.05M-tetrasodium pyrophosphate. The maximum change of extinction (ΔE) for the reaction of *p*-chloromercuribenzenesulphonate and GSH was measured with *p*-chloromercuribenzenesulphonate in the reference cell, and was corrected for the extinction of GSH; the maximum ΔE was at 235 m μ . However, at 235 m μ some change in extinction also occurred for the reaction of *p*-chloromercuribenzenesulphonate with EDTA. At 240 m μ , ΔE for the reaction of *p*-chloromercuribenzenesulphonate with GSH was still large, but negligible for the reaction of *p*-chloromercuribenzenesulphonate with EDTA. With GSH standardized with *N*-ethylmaleimide (Gregory, 1955), $\Delta \epsilon$ at 240 m μ for the reaction between *p*-chloromercuribenzenesulphonate and GSH was 7700. Because of possible contamination with EDTA, measurements were made at 240 m μ .

The thiol content of freshly recrystallized cytochrome b_2 (Type I) at pH 7.0 in 0.3M-sodium lactate-0.05M-tetrasodium pyrophosphate was determined as follows. Equal portions (0.6 ml.) of the enzyme solution were transferred to matched silica cuvettes (2 mm. light-path). To one (blank) was added 0.025 ml. of buffer (as above) and to the other 0.025 ml. of 0.06M-*p*-chloromercuribenzenesulphonate in the same buffer. The change in transmittance at 240 m μ after the addition of *p*-chloromercuribenzenesulphonate to the enzyme was followed until it became constant. The stray light reaching the detector after passage through the

blank was then estimated as follows. The spectrophotometer was standardized at $240\text{ m}\mu$ and then the wavelength drum was turned to the far stop (less than $182\text{ m}\mu$). At this setting, the monochromator allows almost no light of the indicated wavelength to pass through so that any residual light reaching the detector is stray light. The residual transmittance ($T_r = 7.6\%$) was used to correct the observed transmittance (T_o) for the reaction of enzyme with chloromercuribenzenesulphonate, and the extinction increment was calculated as $\log [(100 - T_r)/(T_o - T_r)]$. This value was then corrected for the change in extinction due to *p*-chloromercuribenzenesulphonate alone, which was determined in a separate experiment. The final concentrations of cytochrome b_2 (expressed in terms of haem) and of *p*-chloromercuribenzenesulphonate were $65\text{ }\mu\text{M}$ and $2400\text{ }\mu\text{M}$ respectively.

Chemicals. *p*-Chloromercuribenzenesulphonate was supplied by Sigma Chemical Co., St Louis, Mo., U.S.A. Other chemicals were of analytical reagent grade.

RESULTS

Absorption spectrum of Type II cytochrome b_2

In Fig. 1 are compared the absorption spectra of lactate-reduced Type I and Type II cytochrome b_2 . As was observed by Appleby & Morton (1959*b*), the removal of the DNA component of the enzyme causes a shift of the maximum extinction at $265\text{ m}\mu$ (Type I) to $268\text{--}269.5\text{ m}\mu$ (Type II). The $E_{265\text{ m}\mu}:E_{423\text{ m}\mu}$ ratio is approx. 0.9 (Type I) and the $E_{269\text{ m}\mu}:E_{423\text{ m}\mu}$ ratio is approx. 0.6 (Type II), the latter value being markedly influenced by traces of contaminant DNA. On the basis of enzyme-bound haem, the rates of lactate oxidation with either ferricyanide or horse-heart cytochrome *c* as electron acceptor were essentially the same for both Type I and Type II cytochrome b_2 .

Sedimentation and electrophoresis of Type II cytochrome b_2

Provided that oxygen was rigorously excluded, Type II cytochrome b_2 sedimented with a single symmetrical boundary (Fig. 2). Fig. 3 shows the variation of sedimentation coefficient with protein concentration. This is linear over the concentration range $5\text{--}125\text{ }\mu\text{M}$ (with respect to haem), and can be expressed by the equation:

$$S_{20,w}^{c_0} = S_{20,w}^0 (1 - kc_0)$$

where $S_{20,w}^{c_0}$ is the sedimentation coefficient at an initial concentration c_0 , $S_{20,w}^0$ is the intrinsic sedimentation coefficient (8.56 s), and k has the value $0.534 \times 10^{-3}\text{ l./}\mu\text{mole}$ of cytochrome b_2 (expressed in terms of haem). Electrophoresis at several pH values within the range pH 5.5–8.5 showed only a single symmetrical boundary, as was observed with Type I cytochrome b_2 (see Fig. 4 of Armstrong *et al.* 1963). However, under similar conditions the mobility of Type II cytochrome b_2 was always less than that of Type I cytochrome b_2 .

Fig. 1. Absorption spectra of Type I (continuous line) and of Type II (broken line) cytochrome b_2 . The preparations were in 0.3 M -sodium lactate– 0.05 M -sodium pyrophosphate– 0.1 mM -EDTA, pH 6.8, at room temp. (approx. 25°); the enzyme concentration was approx. $37\text{ }\mu\text{M}$ (expressed in terms of haem). An Optica CF-4 grating spectrophotometer was used with cuvettes of 1 cm . light-path.

Fig. 2. Sedimentation pattern of Type II cytochrome b_2 . The preparation contained $51\text{ }\mu\text{M}$ -cytochrome b_2 (expressed in terms of haem) in 0.3 M -sodium lactate– 0.05 M -tetrasodium pyrophosphate– 0.1 mM -EDTA, pH 6.8. The photographs were taken (i) 16 min. and (ii) 48 min. after reaching $44\,770\text{ rev./min.}$ at 3.0° . Sedimentation is from left to right.

Fig. 3. Concentration dependence of the sedimentation coefficient of Type II cytochrome b_2 in 0.3 M -sodium lactate– 0.05 M -tetrasodium pyrophosphate– 0.1 mM -EDTA, pH 6.8.

Molecular weight of the deoxyribonucleic acid of cytochrome b_2 from sedimentation and diffusion measurements

At pH 7.0 and I 0.1, the DNA component sedimented with a single boundary and showed no evidence of gross heterogeneity (Fig. 4); $S_{20,w}$ was determined to be 5.8 ± 0.2 s. From measurement of boundary-spreading, $D_{20,w}$ was determined to be $3 \times 10^{-7} \pm 1 \times 10^{-7}$ cm.²/sec. These measurements, together with an assumed value of 0.55 cm.³/g. for $\bar{v}$ (Doty, 1955), give a molecular weight (from the Svedberg equation) of $1.2 \times 10^5 \pm 0.4 \times 10^5$.

For thymus-gland DNA, Oth (1955) established the empirical relationship $M = kS^3$, where M is the molecular weight and S is the sedimentation coefficient. If it is assumed that for thymus-gland DNA the average molecular weight is 7×10^6 and $S_{20,w}$ is 23 s, one obtains a value for k which, in Oth's equation, gives a molecular weight of 1.1×10^5 for the DNA of cytochrome b_2 .

Effects of oxygen, of p-chloromercuribenzenesulphonate and of urea on the physicochemical properties of Type I cytochrome b_2

Effect of oxygen. When no special precautions were taken to exclude air from lactate-containing solutions of Type I cytochrome b_2 , they slowly changed from coral-pink to brownish pink and also developed a strong fluorescence due to flavin. There was also a progressive decline in enzymic activity with ferricyanide and with heart-muscle cytochrome c as hydrogen acceptors. Sedimentation of a preparation exposed to air showed that it contained several separate components (Fig. 5); the pink colour was associated with the sedimenting protein, and the supernatant fluid was yellow and strongly fluorescent owing to the presence of free flavin.

Table 1 shows the sedimentation coefficients of the several groups of components observed in different preparations of Type I and Type II cytochrome b_2 that had been exposed to air. The components have been designated as A (7.9.5 s), B (10–14 s), C (15–17 s) and D (20–22 s). After exposure to air for 15–30 min., the haem group of cytochrome b_2 in lactate-containing buffers remains substantially in the ferrous state, yet one or more of the rapidly sedimenting components is usually detected by sedimentation analysis; under strictly anaerobic conditions, only a single component is observed (see Fig. 1 of Armstrong *et al.* 1963). The rate of formation of the rapidly sedimenting components is influenced somewhat by the type of buffer used, and it appears to be accelerated by sodium chloride and by traces of ferrous salts.

When a solution of Type I cytochrome b_2

(approx. 53 μ M expressed in terms of haem) was exposed to air for about 36 hr. at 2–4°, sedimentation analysis showed that it contained two major components (A and B) in about equal amounts. However, only a single protein component was detected on electrophoresis for 4 hr.; a fast-moving minor component was probably free flavin. The protein had about the same mobility as that of Type I cytochrome b_2 stored under anaerobic conditions at the same pH and ionic strength.

Effect of p-chloromercuribenzenesulphonate. Solutions of Type I cytochrome b_2 (54–73 μ M expressed in terms of haem) were treated with p-chloromercuri-

Fig. 4. Microdensitometer tracings of the sedimentation pattern of DNA from Type I cytochrome b_2 . The DNA was dissociated from the enzyme by treatment with ammonium sulphate and was chromatographed on ECTEOLA cellulose. The DNA concentration was 0.003% in 0.01 M-potassium phosphate–0.078 M-sodium chloride, pH 7.0 and I 0.1. Successive photographs were taken with the ultra-violet-absorption optical system of the centrifuge at 8 min. intervals after reaching 59 780 rev./min. at 16.3°. The Figure shows the microdensitometer tracings of the successive photographs, the tracing of the first photograph being at the left-hand side.

Fig. 5. Sedimentation pattern of Type I cytochrome b_2 after exposure to air. The preparation contained 78 μ M-cytochrome b_2 (expressed in terms of haem) in 0.4 M-sodium lactate–0.15 M-sodium chloride–0.1 mM-EDTA, pH 6.8; it was prepared anaerobically and then exposed to air for about 3 hr. before centrifuging at 7°. The photographs were taken (i) 28 min. and (ii) 44 min. after reaching 59 780 rev./min. Sedimentation is from left to right.

Table 1. *Sedimentation coefficients of components present after exposure of cytochrome b_2 to air*

The solutions of enzyme (for definition of components A–D, see the text) were prepared anaerobically and then exposed to air for periods from 1 to 40 hr. at 2–4°. The preparations were in 0.3M-sodium lactate–0.05M-tetrasodium pyrophosphate–0.1 mM-EDTA, pH 6.8–7.4, unless otherwise shown. Experimental details are given in the text. N.D., Not detected.

Type of cytochrome b_2	Prepn. no.	Concn. of cytochrome b_2 (μ M-haem)	Component			
			A	B	C	D
			$S_{20,w}$ (s)			
I	1*	78	8.6	12.2	15.8	N.D.
I	2	110	7.6	10.7	N.D.	21.1
I	3	50	8.4	12.1	N.D.	N.D.
I	4†	79	8.8	12.4	15.0	N.D.
I	5†	—	9.5	13.1	15.1	N.D.
II	6	51	8.9	12.8	16.8	N.D.

* In 0.4M-sodium lactate–0.15M-sodium chloride–0.1 mM-EDTA, pH 6.8.

† These preparations contained chloromercuribenzenesulphonate (1 mM) added after exposure of the solutions to air.

Fig. 6. Sedimentation pattern of Type I cytochrome b_2 after treatment with chloromercuribenzenesulphonate and exposure to air. The solution contained 54 μ M-cytochrome b_2 (expressed in terms of haem) in 0.3M-sodium lactate–0.05M-tetrasodium pyrophosphate–0.1 mM-EDTA, pH 6.8, and chloromercuribenzenesulphonate (final concn. 0.1 mM). It was stored at 2° aerobically for about 30 hr. before centrifuging. The photographs were taken (i) 20 min. and (ii) 40 min. after reaching 59 780 rev./min. at 2.8°. Sedimentation is from left to right.

benzenesulphonate (final concn. 0.1 mM) anaerobically at about 2° and at pH 6.8. After about 30 min., the solution had changed from coral-pink to brown, and strong flavin fluorescence had developed, although the haem group was substantially in the ferrous form. The protein sedimented with a single symmetrical boundary; the supernatant was yellow and showed an intense flavin fluorescence. On exposure to air there was very rapid oxidation of the haem group; however, for periods up to 48 hr. under aerobic conditions at 2° the sedimentation behaviour of the protein was unchanged and only a single component was observed (Fig. 6). Up to 30 hr. after the addition of approx. 2 μ moles of *p*-chloromercuribenzenesulphonate/ μ mole of enzyme-bound haem to a solution of Type I cytochrome b_2 , there was no detectable

Table 2. *Effect of storage with p-chloromercuribenzenesulphonate on the sedimentation coefficients of components A and B of Type I cytochrome b_2 after exposure to air*

A preparation of Type I cytochrome b_2 in 0.3M-sodium lactate–0.05M-tetrasodium pyrophosphate–0.1 mM-EDTA, pH 6.8, was exposed to air. It contained both components A and B (see the text) as shown by sedimentation analysis. Sedimentation coefficients were determined at various times during storage anaerobically at 2–4° after the addition of *p*-chloromercuribenzenesulphonate; the final concentrations were: cytochrome b_2 , 38 μ M (expressed in terms of haem); *p*-chloromercuribenzenesulphonate, 1 mM. For the control, the sedimentation coefficients, corrected for dilution, were determined immediately before the addition of *p*-chloromercuribenzenesulphonate. Experimental details are given in the text. N.D., Not detected.

Storage period	Component	
	A	B
	$S_{20,w}$ (s)	
Control	8.4	12.0
1 hr.	9.2	13.4
1 day	8.7	15.6
5 days	8.5	N.D.
13 days	7.8	N.D.

disappearance of the rapidly sedimenting components formed by previous exposure of the preparation to air.

When enzyme containing components A and B, induced by exposure to air, was treated with 26 μ moles of *p*-chloromercuribenzenesulphonate/ μ mole of enzyme-bound haem and then stored for up to 14 days, there was a progressive disappearance of the rapidly sedimenting components and a change in the sedimentation coefficient of the component A (Table 2). Table 3 shows that treatment with 12–24 μ moles of *p*-chloromercuribenzenesulphonate/ μ mole of enzyme-bound haem causes

some increase of the sedimentation coefficient of Type I cytochrome *b*₂; at concentrations of 1–2 μmoles of *p*-chloromercuribenzenesulphonate/μmole of enzyme-bound haem, there was little change in the sedimentation coefficient.

Effect of urea. Type I cytochrome *b*₂ in 0.5–5.0 M-urea very rapidly loses lactate-oxidation activity with ferricyanide or with horse-heart cytochrome *c* as hydrogen acceptors. On treatment with 2.7 M- or 5 M-urea, solutions changed extremely rapidly from coral-pink to brownish pink, and became strongly fluorescent, and the haem group gradually was oxidized. These changes were slower under strictly anaerobic conditions and with lower concentrations of urea. When Type I cytochrome *b*₂ was stored aerobically with 5 M-urea at 2°, there was a slow progressive loss of the ferrohaemochrome spectrum, which suggests chemical changes in the haem group of the enzyme.

Table 3. *Effect of chloromercuribenzene-p-sulphonate on the sedimentation coefficient of Type I cytochrome b₂*

Solutions of Type I cytochrome *b*₂ in 0.3 M-sodium lactate–0.05 M-tetrasodium pyrophosphate–0.1 mM-EDTA, pH 6.8, were treated anaerobically with chloromercuribenzene-*p*-sulphonate (final concn. 1 mM). Sedimentation coefficients were determined within 2 hr. of the addition of the mercurial, and compared with those, corrected for dilution, of the untreated control solutions. Experimental details are given in the text. Only a single component was detected in each solution.

Concn. of cytochrome <i>b</i> ₂ (μM-haem)	<i>S</i> _{20,w} (s)	
	Control	With chloro- mercuribenzene- <i>p</i> -sulphonate
79	8.2	8.4
42	8.6	9.2

With Type I cytochrome *b*₂ in 2.7 M-urea at pH 6.8 under anaerobic conditions, only a single sedimenting component was observed (Fig. 7*a*).

Fig. 7. Sedimentation pattern of Type I cytochrome *b*₂ in 2.7 M-urea. The preparation contained about 94 μM-cytochrome *b*₂ (expressed in terms of haem) in 0.3 M-sodium lactate–0.05 M-tetrasodium pyrophosphate–0.1 mM-EDTA. (*a*) Photographs taken before dialysis: the pH was 6.9; the photographs were taken (i) 38 min. and (ii) 79 min. after reaching 44 770 rev./min. at 2.8°. (*b*) Photographs taken after dialysis for 14 hr. against similar buffer but without urea: the pH was 6.7; the photographs were taken at (i) 16 min. and (ii) 88 min. after reaching 44 770 rev./min. at 2.9°. Sedimentation is from left to right.

Table 4. *Effect of urea on the sedimentation coefficient of Type I cytochrome b₂*

Solutions of Type I cytochrome *b*₂ in 0.3 M-sodium lactate–0.05 M-tetrasodium pyrophosphate–0.1 mM-EDTA were separately adjusted under anaerobic conditions to give the final urea concentration shown. Sedimentation coefficients were determined in the presence of urea and after removal of the urea by dialysis for 14–24 hr. at 2° under anaerobic conditions against similar buffer without urea. Values in urea were determined from the relationship:

$$S_{20,w} = S_{20,b} (1 - \bar{v} \rho_{H_2O}) / (1 - \bar{v} \rho_{urea})^\dagger$$

assuming that $\bar{v}$ for native protein, and also after treatment with urea, was 0.71. Experimental details are given in the text.

Expt. no.	pH	Concn. of cytochrome <i>b</i> ₂ (μM-haem)	Concn. of urea (M)	<i>S</i> _{20,w} (s)		
				Control	With urea	Urea removed by dialysis
1	7.3	30–40	2.7	—	5.1	8.1
2	7.4	40	2.7	—	6.6	—
3	7.3	40	2.7	—	7.2	—
4	8.8	50	2.7	8.6	3.5	—
5	6.9	94	2.7	8.3	7.8	8.0
6	7.1	97	5.0	8.3	—	8.0*

* Rapid spreading of the sedimenting boundary was observed.
† *S*_{20,b} is the sedimentation coefficient corrected only for viscosity of the solvent.

Fig. 8. Sedimentation pattern of Type I cytochrome b_2 in 5M-urea. The preparation contained $98 \mu\text{M}$ -cytochrome b_2 (expressed in terms of haem) in 0.3M-sodium lactate-0.05M-tetrasodium pyrophosphate-0.1 mM-EDTA. (a) Photographs taken before dialysis: the pH was 7.1; the photographs were taken (i) 38 min. and (ii) 94 min. after reaching 44 770 rev./min. at 9.5° . (b) Photographs taken after dialysis for 16 hr. against similar buffer without urea: the pH was 6.7; the photographs were taken (i) 12 min. and (ii) 84 min. after reaching 44 770 rev./min. at 5.8° . Sedimentation is from left to right.

After removal of the urea by anaerobic dialysis against similar buffer without urea for 24 hr. at 2° , the sedimentation patterns (Fig. 7b) were essentially the same as before treatment with urea, but there was some decrease in the sedimentation coefficient (Table 4). In 2.7M-urea at pH 8.8, the sedimentation pattern was essentially as obtained in 2.7M-urea at pH 6.8, but there was an apparent decline in the sedimentation coefficient.

With 5M-urea at pH 7.1, there was incomplete resolution of the boundary (Fig. 8a). Similar behaviour was observed on treatment of Type I cytochrome b_2 with 5M-urea at pH 8.8. After removal of the urea by dialysis, the sedimentation coefficient of the protein was substantially the same as that of untreated Type I cytochrome b_2 (Table 4). Only one component was detected, but the boundary of the sedimenting material spread rather rapidly (Fig. 8b).

Reactive thiol groups of Type I cytochrome b_2

After the addition of *p*-chloromercuribenzenesulphonate to the enzyme, the extinction at

240 $m\mu$ increased rapidly and became constant after 50 min. The enzyme solution ($65 \mu\text{M}$ expressed in terms of haem) contained $298 \mu\text{M}$ -thiol, corresponding to 4.6 reactive groups/molecule of enzyme-bound haem. Since the blank solution absorbed about 98 % of the incident radiation at 240 $m\mu$, errors arising from uncertainties in the correction for stray light are large, and the accuracy of this estimate is probably between ± 10 and ± 20 %.

DISCUSSION

Type I and Type II cytochrome b_2

The differences between the two forms of the enzyme in absorption spectra and in electrophoretic and sedimentation behaviour are attributable to the absence of the DNA group from Type II cytochrome b_2 ; the protein appears to be little changed by the mild salting-out procedure used to dissociate the DNA from Type I cytochrome b_2 . Type II cytochrome b_2 retains a distinctive absorption peak near 269 $m\mu$ (Fig. 1) which is probably due to the riboflavin phosphate group, although the haem and the tyrosine and phenylalanine groups may contribute slightly (see Drabkin, 1961; Morton, Armstrong & Appleby, 1961; Morton, 1958, 1961a, b). Like the Type I enzyme, Type II cytochrome b_2 migrates as a single component on moving-boundary electrophoresis in the pH range 5.5–8.5 at I 0.63 but has a somewhat lower mobility. The proportionate change of sedimentation coefficient of Type II cytochrome b_2 with concentration (Fig. 3) is essentially the same as that of bovine serum albumin, whereas the change of that of Type I cytochrome b_2 is about three times as great and this is probably due to intermolecular interaction caused by the DNA component (Armstrong *et al.* 1963).

The intrinsic sedimentation coefficient of Type II cytochrome b_2 is 8.56s, compared with 9.17s for Type I cytochrome b_2 (Armstrong *et al.* 1963). The values previously reported for the Type II enzyme (Armstrong *et al.* 1961; Morton & Shepley, 1961; Armstrong & Morton, 1961b) were not corrected for buoyancy of the buffer (see Armstrong *et al.* 1963). The minimum weight of Type II cytochrome b_2 is 77 000, compared with 82 000 g./mole for Type I cytochrome b_2 (Appleby & Morton, 1960; Morton *et al.* 1961). Since the $S_{20,w}^0$ value for Type II cytochrome b_2 is proportionally less than that of Type I cytochrome b_2 , it is assumed that the molecular weight of Type II cytochrome b_2 is less than that of the Type I enzyme but that, like Type I cytochrome b_2 (Armstrong *et al.* 1963), Type II cytochrome b_2 has 2 flavin and 2 haem groups/molecule.

Reactive thiol groups of cytochrome b_2

Whereas each of Type I and Type II cytochrome b_2 sediment as a single component under strictly anaerobic conditions (Fig. 2; and Fig. 1 of Armstrong *et al.* 1963), even traces of oxygen in the presence of lactate cause appearance of other components (Fig. 5 and Table 1). These are undoubtedly aggregated forms of the protein.

Cytochrome b_2 is an extremely labile enzyme; the formation of aggregates induced by oxygen is related to loss of enzymic activity, development of fluorescence of the protein and dissociation of free flavin from the protein. At about pH 7 and in the presence of lactate, a similar loss of enzymic activity, development of fluorescence of the protein and subsequent displacement of free flavin from the protein is caused by reagents that preferentially react with thiol groups, e.g. *p*-chloromercuribenzoate (Appleby & Morton, 1954, 1959*b*), *p*-chloromercuribenzenesulphonate (Boeri & Tosi, 1956; Armstrong, 1961; Armstrong & Morton, 1961*b*) and *N*-ethylmaleimide (Armstrong *et al.* 1960*a*; Armstrong, 1961). Treatment of Type I cytochrome b_2 with *p*-chloromercuribenzenesulphonate (1–2 μ moles of *p*-chloromercuribenzenesulphonate/ μ mole of enzyme-bound haem) prevents the formation of aggregates induced by oxygen (Fig. 6 and the Results section). Hence it is concluded that aggregate formation and the development of fluorescence are due to reactions involving thiol groups of the enzyme.

Appleby, Morton & Simmonds (1960) found that there are 18 half-cystine residues/molecule of cytochrome b_2 (expressed in terms of haem). From the present results 4–6 of these residues are thiol groups that react readily with *p*-chloromercuribenzenesulphonate. However, an equimolecular proportion of *p*-chloromercuribenzenesulphonate to that of cytochrome-bound haem suffices to prevent the aggregate formation induced by oxygen, and also causes fluorescence of the protein and substantial inhibition of enzyme activity (Armstrong *et al.* 1960*a*). It is therefore concluded that only 1–2 of the reactive 4–6 thiol groups are at the active centre of the enzyme, and that these groups are preferentially involved in aggregation through formation of new intermolecular disulphide bonds. Since the principal molecular species of the enzyme under anaerobic conditions has 2 flavin and 2 haem groups/molecule (Armstrong *et al.* 1963) a portion of the active centre of the enzyme may be represented as shown in Scheme 1. Even with this simplified representation it is apparent that there are several possibilities for the formation of aggregates of different molecular sizes (cf. Fig. 5 and Table 1) via intermolecular disulphide bonds. The formation of these

Scheme 1

bonds probably proceeds via hydrogen peroxide, which causes irreversible inactivation of the enzyme (Appleby & Morton, 1959*b*). Boeri & Rippa (1961*a*) found that reduction of oxygen to water, catalysed by the enzyme in the presence of lactate, is a slow reaction that is inhibited by lactate and is accelerated by Fe^{2+} and other metal ions. No hydrogen peroxide is detected among the reaction products, and the enzyme is about 50 % inactivated after 750 reactions with oxygen/flavin group (Boeri & Rippa, 1961*a*). The rate of inactivation with ferricyanide or cytochrome *c* as oxidant is negligible compared with that with oxygen, and correspondingly aggregates are not readily formed. Moreover, as compared with ferrocyclochrome b_2 in the presence of lactate, aggregates are not readily formed on the exposure of Type I ferricytochrome b_2 to air (R. K. Morton & K. Shepley, unpublished work). From these observations, and those of Appleby & Morton (1959*b*) on the effects of hydrogen peroxide on the enzyme, it is suggested that reactions (1)–(5) may occur in the presence of oxygen and lactate:

where Prot(SH)-flavin represents a portion of the active centre of the enzyme, $\text{flavin}_{(\text{red.})}$ and $\text{flavin}_{(\text{ox.})}$ represent the reduced and oxidized states of flavin respectively; and $\text{Prot}\cdot\text{SH}$ and $\text{Prot}\cdot\text{S}\cdot\text{S}\cdot\text{Prot}$ are the non-aggregated and aggregated forms respectively of the flavin-free haemoprotein. These reactions account for formation of the intermolecular disulphide bonds, but the reaction is probably much more complex. It is not proposed that this is the major pathway for reduction of oxygen in the presence of the enzyme and lactate; the major pathway probably involves intramolecular transfer from the reduced flavin group to the haem group of the enzyme (Morton *et al.* 1961; Morton, 1961*a*).

The prevention of aggregate formation by *p*-chloromercuribenzenesulphonate (Fig. 6) is attributed to the rupture of the weak flavin–thiol bond with consequent inactivation of the enzyme,

and formation of an air-stable derivative between the thiol group and the organic mercurial. There is some alteration in protein structure when an excess of *p*-chloromercuribenzenesulphonate is used, since the derivative (having 4–6 mercuriphenyl groups/molecule of enzyme-bound haem) sediments somewhat more rapidly than does the intact enzyme (Table 3); there is less modification with 1–2 mercuriphenyl groups/molecule of enzyme-bound haem. The disappearance of oxygen-induced aggregates caused by prolonged storage with *p*-chloromercuribenzenesulphonate (Table 2) suggests that chloromercuribenzenesulphonate may slowly react with intermolecular disulphide bonds; a reaction of this type has been described at elevated temperatures (Cunningham, Nuenke & Strayhorn, 1957).

Although a hydrogen bond between the protein thiol group and the imino nitrogen of the isoalloxazine ring of the flavin group was proposed by Appleby & Morton (1959*b*), the bond may be due to electrostatic interaction between the thiol ion and the positively-charged imino dipole of the flavin group. Present evidence does not permit a conclusive choice between these alternatives. The ready dissociation of the flavin group caused by urea (see below) could be due to rupture of a hydrogen bond but it may also be caused by such secondary effects as reversible deformation of the flavin-binding site of the protein. The involvement of the flavin imino nitrogen in binding to the apoprotein of flavoprotein enzymes is not yet firmly established.

Effects of urea on cytochrome b_2

With 2.7*M*- and 5*M*-urea there is inactivation of the enzyme and some modification of the protein as indicated by changes in sedimentation behaviour (Figs. 7 and 8, and Table 4). However, sedimentation analysis provides no evidence of more than a single component after treatment of cytochrome b_2 with 2.7*M*-urea at pH 6.7–6.9 (Fig. 7) or at pH 8.8. This is further evidence for the homogeneity of crystalline Type I cytochrome b_2 (see Armstrong *et al.* 1963; Armstrong & Morton, 1961*b*).

The rapid inactivation of the enzyme at 0.5–5.0*M*-urea is due initially to displacement of the flavin group. At 0.5–2.7*M*-urea, and under strictly anaerobic conditions at about pH 7, some reactivation occurs on subsequent removal of urea followed by incubation with riboflavin phosphate (R. K. Morton & K. Shepley, unpublished work). This is somewhat similar to the reactivation found after displacement of the flavin by salting-out of the protein at pH 3.2 (Morton, 1961*a*). Boeri & Rippa (1961*b*) have also reported reversible inactivation of cytochrome b_2 in concentrations of urea less than

3*M*. The similarity of sedimentation coefficients in buffer and in 2.7*M*-urea, and particularly after removal of urea by dialysis (Table 4), suggests that the protein structure is not irreversibly modified by this concentration of urea. Greater modification occurred in 5*M*-urea at both pH 6.7–7.1 and at pH 8.8, but the modified protein obtained at pH 6.7–7.1 sedimented with an apparently symmetrical boundary after removal of the urea (Fig. 8).

Deoxyribonucleic acid component of Type I cytochrome b_2

Morton (1955) first reported the association of DNA with crystalline cytochrome b_2 , and Appleby (1957), Appleby & Morton (1960), Montague & Morton (1961), Pereira & Mahler (1962) and Mahler & Pereira (1962) have described some properties of the isolated DNA. Despite the lack of precision in the estimate of the diffusion coefficient, the molecular weight ($1.2 \times 10^5 \pm 0.4 \times 10^5$) derived by combination of sedimentation and diffusion coefficients is in good agreement with that (1.1×10^5) calculated from the empirical relationship established by Oth (1955); both of these calculations make assumptions that may not be justified for cytochrome b_2 DNA, which has many unique properties (see Montague & Morton, 1961; Pereira & Mahler, 1962). However, the estimate given here is preferred to that quoted by Montague & Morton (1961). Since preparations of crystalline Type I cytochrome b_2 appear to be homogeneous by several criteria (Armstrong *et al.* 1963), and Type II cytochrome b_2 , devoid of the DNA component, has distinctive spectral, electrophoretic and sedimentation characteristics that enable identification in preparations of Type I cytochrome b_2 , it is concluded that each molecule of the Type I enzyme has a DNA group associated with it. There are approx. 15 molecules of nucleotide bases/molecule of haem in Type I cytochrome b_2 (Appleby & Morton, 1959*b*, 1960). This is an average value but, as yet, no evidence of gross heterogeneity has been detected in preparations of DNA from cytochrome b_2 . Based on the molecular weight of Type I cytochrome b_2 (Armstrong *et al.* 1963), the expected weight of the isolated DNA is approx. 10 000 g./mole. The discrepancy may be due to aggregation, or to some specific intermolecular interaction that occurs when the DNA is dissociated from the enzyme. This would affect the hydrodynamic behaviour without affecting the end-group analysis based on enzymic procedures and which indicated a molecular weight of 10 000–12 000 (Montague & Morton, 1961). End-group analysis, however, is subject to error arising from trace contaminants and from uncertainty of the influence of DNA configuration on the rate of reaction. At

present, therefore, it is concluded that the molecular weight of the isolated DNA is in the range 10^4 – 10^5 . It is clearly very different from that of DNA isolated from thymus-gland nuclei and most other sources, which has a molecular weight of at least 10^6 .

SUMMARY

1. The absorption spectrum, and the sedimentation and electrophoretic behaviour of DNA-free cytochrome b_2 (Type II cytochrome b_2) are described. Only one protein component was found.

2. The concentration-dependence of the sedimentation coefficient is described by the equation $S_{20,w}^0 = S_{20,w}^0 (1 - kc_0)$, where c_0 is the concentration (μM) of cytochrome b_2 (expressed in terms of haem), k is $0.534 \times 10^{-3} \text{ l./}\mu\text{mole}$ of cytochrome b_2 (expressed in terms of haem), and $S_{20,w}^0$ (the intrinsic sedimentation coefficient) is 8.56s. The concentration-dependence of $S_{20,w}$ is about one-third of that found for DNA-containing cytochrome b_2 (Type I cytochrome b_2).

3. Oxygen and p -chloromercuribenzenesulphonate cause dissociation of the enzyme-bound flavin. In the presence of lactate, exposure to air forms aggregates of the protein, apparently through formation of intermolecular disulphide bonds. p -Chloromercuribenzenesulphonate prevents the formation of these aggregates.

4. There are 4–6 thiol groups/molecule of enzyme-bound haem in Type I cytochrome b_2 which react rapidly with p -chloromercuribenzenesulphonate. It is suggested that one of these groups is at the active centre of the enzyme and forms a labile bond with the riboflavin phosphate group.

5. Treatment with urea causes displacement of the flavin group and inactivation of the enzyme; the inactivation can be partly reversed. No new sedimenting components were observed with cytochrome b_2 in 2.7M- or in 5M-urea at pH 6.7, or after subsequent removal of the urea by dialysis. After removal of the urea, the sedimentation coefficient of the protein was substantially the same as that of untreated enzyme.

6. The sedimentation coefficient of DNA isolated from Type I cytochrome b_2 is $5.8 \pm 0.2\text{s}$, and the diffusion coefficient is $3 \times 10^{-7} \pm 1 \times 10^{-7} \text{ cm}^2/\text{sec}$. From these values the molecular weight is calculated to be $1.2 \times 10^5 \pm 0.4 \times 10^5$. The DNA of cytochrome b_2 appears to be aggregated when dissociated from the protein.

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Oxalic Acid Synthesis in Shoots of *Oxalis pes-caprae*

THE PRECURSORS OF GLYCOLLIC ACID AND GLYOXYLIC ACID

By ADELE MILLERD, R. K. MORTON AND J. R. E. WELLS

Department of Agricultural Chemistry, Waite Agricultural Research Institute, University of Adelaide, South Australia

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Oxalis pes-caprae contains large amounts of oxalic acid, which appears to be formed predominantly from glyoxylic acid and glycollic acid; the origin of these latter compounds was not established (Millerd, Morton & Wells, 1963). To account for synthesis of oxalic acid in *Aspergillus niger*, Cleland & Johnson (1956) proposed that two molecules of ribose were converted into five molecules of glycolaldehyde, which were then oxidized to oxalic acid via glyoxylic acid and glycollic acid. Glycollic acid may also be derived from ribose in photosynthetic tissue (Griffith & Byerrum, 1959). It appeared likely, however, that glycollic acid in *Oxalis* arose by reduction of glyoxylic acid, which could be formed by cleavage of isocitric acid catalysed by isocitrate lyase. Although Carpenter & Beevers (1959) had found that this enzyme occurred only in those plant tissues that actively convert fat into carbohydrate, their study did not include plant tissues that contained a high concentration of oxalic acid. Such tissues would clearly be active in the synthesis of C_2 compounds (glyoxylic acid). This paper describes the results of studies to determine the relative contribution of $[2-^{14}C]$ glucose, $[1-^{14}C]$ ribose, $[1,5-^{14}C_2]$ isocitric acid and of sodium $[1-^{14}C]$ glycollate to oxalic acid synthesis in the white emergent shoots of bulbs of *Oxalis pes-caprae*.

MATERIALS

Plants. Bulbs of *Oxalis pes-caprae* were harvested, germinated in the dark and used as described by Millerd *et al.* (1963).

Chemicals. The following ^{14}C -labelled compounds, with the specific activities noted, were used: D- $[2-^{14}C]$ glucose,

13.1 $\mu\text{C}/\text{mg.}$; D- $[1-^{14}C]$ ribose, 13.0 $\mu\text{C}/\text{mg.}$; sodium $[1-^{14}C]$ glycollate, 19.4 $\mu\text{C}/\text{mg.}$ (all from The Radiochemical Centre, Amersham, Bucks.); DL(+)-allo- $[1,5-^{14}C_2]$ isocitric acid lactone, 34.6 $\mu\text{C}/\text{mg.}$ (California Corp. for Biochemical Research). Phenol, toluene, 3-methylpropan-1-ol, pyridine and formic acid (British Drug Houses Ltd.) were A.R. grade. Butan-1-ol (Colonial Sugar Refineries Ltd.) and propionic acid (British Drug Houses Ltd.), both L.R. grade, were redistilled before use. 2,5-Diphenyloxazole and *p*-bis-2,5-phenyloxazylbenzene were A.R. scintillation grade [Nuclear Enterprises (G.B.) Ltd., Scotland].

METHODS

Hydrolysis of DL(+)-allo- $[1,5-^{14}C_2]$ isocitric acid lactone. Three equivalents of KOH were added to the lactone and the solution (final vol., 0.21 ml.) was maintained on a steam bath for 15 min. and then cooled. The final pH of the solution was 5–6 (cf. Kornberg & Beevers, 1957).

Treatment of shoots with radioactive compounds. D- $[2-^{14}C]$ Glucose (50 μC) was dissolved in 0.3 ml. of water and 0.02 ml. was applied to each shoot. D- $[1-^{14}C]$ Ribose (50 μC) was dissolved in 0.18 ml. of water and 0.02 ml. was applied to each shoot. Sodium $[1-^{14}C]$ glycollate (100 μC) was dissolved in 1.0 ml. of water and 0.025 ml. was applied to each shoot. Potassium DL(+)-allo- $[1,5-^{14}C_2]$ isocitrate (0.21 ml., containing approx. 20 μC) was administered immediately after hydrolysis of the lactone; 0.025 ml. was applied to each shoot. Two shoots were used in each experiment. A narrow groove was cut along the length of each shoot (approx. 1 mm. $\times$ 1 mm.), the slice of tissue removed and the appropriate volume of ^{14}C -labelled material was introduced with a micropipette. The shoots were kept in the dark for 1 hr. or 6 hr. at room temperature and then detached and extracted as described by Millerd *et al.* (1963). The combined extracts from the shoots were then divided into non-ionic compounds (mainly sugars), amino acids and organic acids by chromatography with Dowex 50 (H^+ form)