oxidative enzyme systems in the lipid-rich fractions but they appear to be absent from nucleoli.

5. It is concluded that the lipid-rich material may be the heterochromatin associated with the nucleoli and that this is the active site for the incorporation of amino acids by rat-liver nuclei.

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Physicochemical Studies on Cytochrome b_2

SEDIMENTATION, DIFFUSION AND ELECTROPHORESIS OF THE CRYSTALLINE DEOXYRIBONUCLEOPROTEIN

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Cytochrome b_2 is the L(+)-lactate–cytochrome *c* oxidoreductase [L(+)-lactate dehydrogenase] of baker's yeast (Bach, Dixon & Zerfas, 1946; Appleby & Morton, 1954; Boeri, Cutolo, Luzzati & Tosi, 1955). The enzyme was obtained as a crystalline, apparently homogeneous, deoxyribonucleoprotein containing equimolecular amounts of riboflavin phosphate and of protohaem (Appleby & Morton, 1954, 1959*a*, *b*, 1960). Both of these prosthetic groups are reduced in the presence of lactate (Appleby & Morton, 1954; Hasegawa & Ogura, 1961).

Further physicochemical studies of the crystalline deoxyribonucleoprotein (now known as Type I cytochrome b_2; Morton, 1961*a*) are described in this paper. The molecular weight of the enzyme in solution has been determined by a number of methods.

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MATERIALS AND METHODS

General

Cytochrome b_2 (*Type I*). This was prepared from dried baker's yeast essentially as described by Appleby & Morton (1959*a*). Solutions were stored under nitrogen at −15°. The enzyme was recrystallized before use, and experiments with any one sample were carried out within 3 days of recrystallization. The enzyme was dissolved in buffer composed of (final concentrations) 0·3M-sodium lactate, 0·05M-tetrasodium pyrophosphate and 0·1 mM-EDTA (disodium salt), adjusted to pH 6·8 with hydrochloric acid. This buffer (*I* 0·63) was used to obtain the high ionic strength necessary to give adequate concentrations of cytochrome b_2 (see Appleby & Morton, 1959*a*) and because it was found that the enzymic activity was retained for long periods in pyrophosphate buffer.

Nitrogen gas. Commercial oxygen-free nitrogen, stated to contain less than 10 p.p.m. of oxygen, was used.

Dialysis and other procedures. All sedimentation and diffusion experiments were carried out in 0·3M-sodium lactate–0·05M-tetrasodium pyrophosphate–0·1 mM-EDTA buffer, adjusted to pH 6·8 with hydrochloric acid. For electrophoresis, the same buffer was used except that it was adjusted to the pH required with hydrochloric acid.

All experiments were carried out anaerobically. From the initial crystallization onwards, solutions used with the enzyme were degassed (either by boiling or by reduced pressure), saturated with nitrogen and tightly stoppered to prevent contact with air. Cells for sedimentation, diffusion and electrophoresis were flushed out with nitrogen and filled in an atmosphere of nitrogen. The exposed solution surfaces in diffusion and electrophoresis cells were covered with a layer of medicinal paraffin oil.

For dialysis, the Visking cellulose sac containing the enzyme solution was secured to a glass rod in a stoppered container (4 l.) on a platform which was rocked mechanically so that both the enzyme and buffer solutions were continually stirred by the flow of gas bubbles between the ends of the containers.

Dialysis and other procedures were carried out in a cold room at 2°.

Iron and haem analyses. These were carried out on the three-times-crystallized cytochrome b_2 as described by Appleby & Morton (1959*b*), and gave estimates of the minimum molecular weight of 74 400 (iron analysis) and 80 100 (haem analysis): the molecular proportion of iron to haem was 1·1.

Enzymic activities. These were estimated with ferricyanide or with horse-heart cytochrome *c* as the acceptor essentially as described by Appleby & Morton (1959*a*). Activities are here expressed as μmoles of ferricyanide reduced/hr./mg. of enzyme at 20°. The amount of enzyme protein was estimated from the extinctions at 423 mμ (γ-band) or at 556·5 mμ (α-band), by using the extinction coefficients of Appleby & Morton (1959*b*), which were determined for a minimum weight of 80 000 g./mole (see Morton, 1958).

Sedimentation analysis

General. A Spinco model E analytical ultracentrifuge was used. It was equipped with a unit for measuring and controlling the temperature and with a phase plate, and a Wratten no. 16 filter was inserted immediately in front of the light-source, which was aligned as described by Trautman (1958). The cells had an optical path of 12 mm. and had centre sections of aluminium or of aluminium-filled epoxy resin, or, in some of the equilibrium experiments, centre sections of fluorocarb (Kel-F).

Most experiments were carried out between 1° and 5°. Sedimentation coefficients were corrected to 20° in water for both viscosity and buoyancy, by using the value of 0·71 calculated for $\bar{v}$ (see below).

Sedimentation-velocity experiments. These were carried out at 59 780 and at 44 770 rev./min. Sedimentation coefficients were normally calculated from the maximum ordinate of the sedimenting boundary. Analysis of boundary-spreading was carried out by using Fujita's (1956) equation as described by Baldwin (1957).

Approach-to-equilibrium experiments. These were carried out as described by Ehrenberg (1957) and by Schachman (1957); the most satisfactory rotor speed was 5227 rev./min. Molecular weights were calculated by the methods of Ehrenberg (1957), of Schachman (1957, 1959) or of Archibald (1947), in which $(dc/dr)/cr$ is plotted against r. Sedimentation coefficients were calculated by using Baldwin's (1953) transport equation for schlieren optical systems.

Equilibrium experiments. Between 0·10 and 0·15 ml. of enzyme solution was used, giving columns of between 0·218 and 0·311 cm. The rotor speed was 5227 rev./min. Equilibrium was attained 4–6 days after commencement of centrifuging.

The time to reach equilibrium was predicted by using the value of the sedimentation coefficient determined for the same experimental conditions in a separate sedimentation-velocity experiment, and Mason & Weaver's (1924) rule, modified as suggested by Van Holde & Baldwin (1958); the molecular weight of cytochrome b_2 was assumed to be 180 000. During the last 24 hr. before final photographs were taken, the temperature of the rotor did not change more than $\pm 0·15°$. Molecular weights were calculated by several different procedures (see the Results section).

Diffusion analysis

Diffusion experiments were carried out at approx. 1° in a modified Spinco model H diffusion–electrophoresis apparatus. The considerable absorption of light by cytochrome b_2, even at wavelengths above that of the α-band, prevented the use of the interference optical system, and schlieren optics were therefore used. The mercury arc lamp was replaced by a sodium lamp, and, for greater precision in observing the refractive-index gradient pattern, the schlieren knife-edge was replaced by a wire of bright 33-gauge nichrome. The accuracy of the optical systems was verified by measuring the diffusion coefficient, D, of a sucrose solution (0·75 g. of sucrose/100 ml. of water) at 1·44°. A value of $2·37 \times 10^{-6}$ cm.^{2}/sec. was obtained when corrected to 1°. Boundaries were sharpened as described by Gosting, Hanson, Kegeles & Morris (1949). Diffusion coefficients were calculated from enlargements of the diffusing boundary between cytochrome b_2 and lactate–pyrophosphate buffer by the height/area method.

Electrophoresis

This was carried out in an open Tiselius-type cell (vol. 2 ml.) with a model 38A electrophoresis apparatus (Perkin–Elmer Ltd.) equipped with a cold-water circulating system. Schlieren optics were used. A red filter which did not transmit wavelengths below 590 mμ was used with the tungsten light-source. Temperatures of 2·8–3·2° were obtained; the temperature did not vary more than 0·1° during any single experiment. Conductivities were measured at the temperature of the experiment. The boundaries were brought into view by slowly siphoning buffer from the buffer vessel on the ascending limb of the cell.

Viscosity. The viscosity of the buffer relative to water was measured at temperatures over the range 2–19°. By using an Ostwald viscometer (B.S. 186, 1957), with a flow time of 397 sec. for water at 20°, a value of 1·128 was obtained.

Density. The density of the buffer relative to water was determined with a pycnometer (vol. 10 ml.). A value of 1·030 was obtained over the range 2–4°.

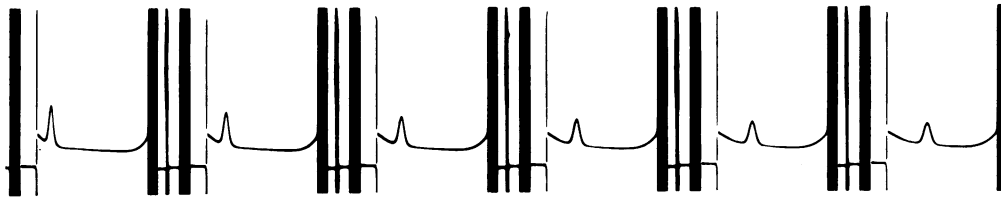

Fig. 1. Sedimentation pattern of cytochrome b_2. Sedimentation is from left to right. The photographs were taken at 8 min. intervals, 15 min. after reaching 59 780 rev./min. The protein concentration was 54 μM-cytochrome b_2 haem, the temperature 4·6° and the pH 6·8.

Table 1. *Sedimentation coefficient of cytochrome* b_2

The sedimentation coefficients were calculated from the apparent ($x_H^{app.}$) and corrected ($x_H^{corr.}$) maximum ordinates and from the second moment (μ_2) of the gradient curve obtained with a double-sector centre section in the analytical cell. Photographs were taken at 21, 37, 53, 69, 85, and 101 min. after reaching 44 770 rev./min. The temperature was 5·1°, the pH 6·8 and the protein concn. 31 μM-cytochrome b_2 haem.

Basis of calculation	$10^{13}\ S_{20,w}$ (sec.)	$\frac{100\,[S - S_{(\sqrt{\mu_2})}]}{S_{(\sqrt{\mu_2})}}$ (%)
$x_H^{app.}$	8·70	2·04
$x_H^{corr.}$	8·65	1·53
$\sqrt{\mu_2}$	8·52	0·0

Table 2. *Influence of temperature on the sedimentation coefficient of cytochrome* b_2

Separate portions of a sample of cytochrome b_2 (concn., 43 μM-cytochrome b_2 haem) at pH 8·0 were brought to approx. 4°, 10° and 20° and centrifuged at 59 780 rev./min.

Temp.	$10^{13}\ S_{20,w}$ (sec.)
4·19°	8·53
10·30	8·71
20·80	8·63
Mean	8·62 ± 0·09

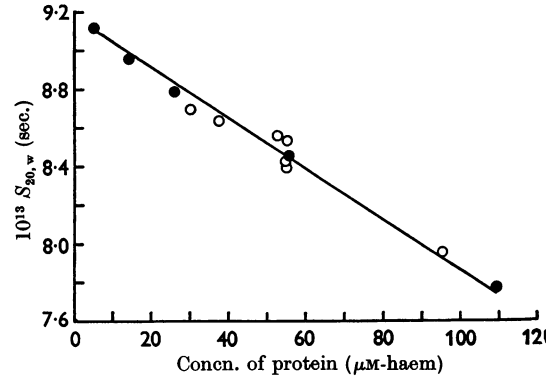

Fig. 2. Concentration-dependence of the sedimentation coefficient, $S_{20,w}$, of cytochrome b_2 (Type I) in lactate–pyrophosphate–EDTA buffer, pH 6·8. The line is fitted to the values shown thus ●, which were determined for dilutions of the one preparation; other values shown are for different preparations.

RESULTS

Sedimentation coefficient and analysis of boundary-spreading. Cytochrome b_2 sedimented with a single (apparently symmetrical) boundary, leaving a colourless supernatant above it (Fig. 1). The baseline was markedly curved, owing to a concentration gradient of buffer ions. Initially, the sedimentation coefficient of cytochrome b_2 was calculated from the apparent maximum ordinate of the gradient curve, the maximum ordinate (after subtraction of the buffer gradient), and the second moment of the gradient curve (Table 1). The values agreed within 2 % so that the apparent maximum ordinate was used throughout the remainder of these studies. The validity of the large temperature corrections to the sedimentation coefficient was verified by measurements on the same solution at 4°, 10° and 20° (Table 2).

Over the range 5–110 μM-haem, the sedimentation coefficient of cytochrome b_2 shows marked concentration-dependence (Fig. 2), which is linear and can be described by an equation of the form:

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

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 ($9{\cdot}17 \times 10^{-13}$ sec.) and k is constant, having the value $1{\cdot}40_5 \times 10^{-3}$ l./μmole of cytochrome b_2 haem.

Analysis of boundary-spreading, taking into account the effect of concentration-dependence of sedimentation (Baldwin, 1957), was carried out on four different preparations of cytochrome b_2 at concentrations between 40 and 110 μM-haem. The results of each experiment showed no upward curvature of the ratio of corrected area/height against time. Fig. 3 shows the apparent diffusion coefficients calculated in this way from the gradient curves at several times in one experiment. In this

and in other experiments, the deviation about the mean of the values of $D^{\text{app.}}_{20,w}$ determined at different times falls well within the limits of accuracy of the method, which Baldwin (1957) estimates as $\pm 5\%$ at best.

Molecular weight determined by approach-to-equilibrium sedimentation. A large number of experiments were carried out but there was considerable variation in the molecular weights obtained. The molecular weights obtained by different methods of calculation applied to results of a single experiment showed better agreement than did the molecular weights obtained by any one method of calculation applied to results of different experiments (Table 3). The results from these experiments were also used to calculate sedimentation coefficients by the transport method.

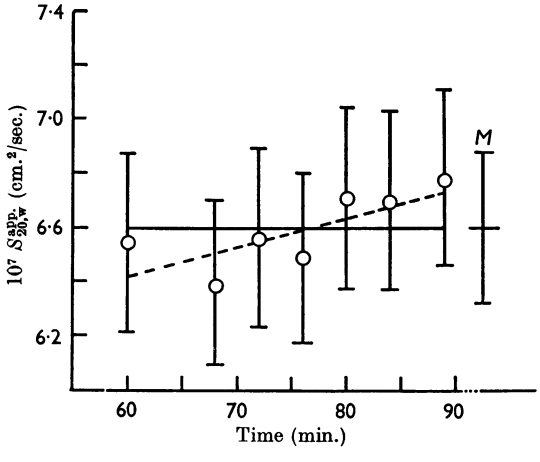

Fig. 3. Variation of the apparent diffusion coefficient, $D^{\text{app.}}_{20,w}$, of cytochrome b_2 with time. The concentration of protein was 109 μM-cytochrome b_2 haem, the temperature 2·5° and the pH 6·8. Photographs were taken at 36, 44 and 52 min. after reaching 59 780 rev./min., and thereafter at intervals of 4 min. to 88 min. The diffusion coefficients were estimated from boundary-spreading during sedimentation, as described by Baldwin (1957). The vertical lines show the estimated limits of accuracy of each value ($\pm 5\%$ at best; Baldwin, 1957). The line at *M* shows the 95% fiducial limits of the mean value ($6{\cdot}60 \times 10^{-7}$ cm.²/sec.). The slope of the broken line, which is fitted to the observed values, does not differ significantly from that of the horizontal solid line drawn through the mean value.

Table 3 shows results of two typical experiments. The suitability of the experimental procedures was examined by similar experiments with crystalline yeast alcohol dehydrogenase and with horse-heart cytochrome *c* (both from Sigma Chemical Co., U.S.A.). With Ehrenberg's (1957) method of calculation, values of 129 000 and of 14 600 ± 200 were obtained for the alcohol dehydrogenase and cytochrome *c* respectively.

Molecular weight determined by sedimentation equilibrium. Three experiments were carried out; the most satisfactory in terms of retention of enzymic activity during the experiment is reported in Table 4. By using the Lansing & Kramer (1935) equation, values of 205 000 and 170 000 for the molecular weight of cytochrome b_2 were calculated for the other two experiments, but in both of these the specific activities of the enzyme preparations had fallen markedly by the time the contents of the ultracentrifuge cell were assayed. However, in general the results of these two experiments were similar to those reported in Table 4.

Molecular weight determined from diffusion and sedimentation-velocity experiments. Static free diffusion was used to determine the diffusion coefficient. Initially, short periods (18–24 hr.) of dialysis of the cytochrome against buffer were used because it was considered that longer periods might result in loss of enzymic activity. In that grossly non-gaussian gradient curves and large zero times were obtained in these experiments, the results were unsatisfactory. More reproducible diffusion coefficients and small zero times were obtained when longer periods of dialysis (more than 72 hr.) were used; under the experimental conditions used, the enzymic activity was in fact largely retained throughout the period of dialysis and of diffusion (see Table 5).

Sedimentation-velocity experiments were carried out on the same samples of cytochrome b_2 before and after diffusion, and a single symmetrical sedimenting boundary was obtained in each experiment. Each of the samples used in the diffusion

Table 3. *Variation of molecular weight of cytochrome* b_2 *as determined by approach-to-equilibrium methods*

Values were calculated for the meniscus. Sedimentation coefficients shown in parentheses were determined for the same enzyme solution from a sedimentation-velocity experiment.

Expt. no.	Rotor speed (rev./min.)	Temp.	Time from full speed (min.)	Concn. of protein (μM-haem)	No. of photographs	Method of calculation	$10^{13}\ S_{20,w}$ (sec.)	Mol.wt.
1	5227	0·8°	32–192	109	5	Ehrenberg	10·45 (7·78)	343 700 ± 10 600
					5	Archibald	—	313 600 ± 3 600
2	5227	3·4	16–192	190	8	Ehrenberg	13·80 (7·70)	246 000 ± 4 600
					8	Archibald	—	247 800 ± 3 500

Table 4. *Molecular weight of cytochrome* b_2 *from equilibrium sedimentation*

The enzyme solution was dialysed against lactate–pyrophosphate buffer for 60 hr. at 2° before commencement of the experiment. The volume of enzyme solution was 0·15 ml., and the column height was 0·311 cm. Centrifuging was for 4½ days at 5227 rev./min. The protein concentration was 54 μM-cytochrome b_2 haem (4·32 mg./ml.); the specific activity was 7200 units before centrifuging and 5600 units after centrifuging. Sedimentation-velocity experiments were carried out on the same solution before (see Fig. 1) and after equilibrium sedimentation, and only a single sedimenting boundary was observed on each occasion. The molecular weight was calculated by three different methods: method A, graph of $(1/r)(dc/dr)$ against c; method B, graph of ln c against r^2; method C, Lansing & Kramer's (1935) equation, by using $[2(c_b - c_a)]/[c_0(b^2 - a^2)]$.

Bar angle for photographs	Molecular weight of cytochrome b_2: Method A* Top of cell	Method A* Bottom of cell	Method B† Top of cell	Method B† Bottom of cell	Method C
80°	213 000	365 000	127 700	195 000	173 000
70°	219 000	338 000	136 800	196 100	181 600
				Mean:	177 300

* Curves markedly concave upwards. † Curves moderately concave upwards.

Table 5. *Molecular weight of cytochrome* b_2 *from diffusion and sedimentation coefficients*

Expt. no.	Dialysis period (hr.)	Enzymic activity (units/mg.): Before dialysis	After dialysis	After diffusion	Concn. of protein: (μM-haem)	(mg./ml.)	$10^{13}\ S_{20,w}$* (sec.)	$10^7\ D_{20,w}$ (cm.²/sec.)	Mol.wt.†
1	30	8250	8480	7800	54·9	4·39	8·41	3·09‡	228 100
2	72	6170	6260	6400	55·0	4·40	8·39	3·86§	182 100
3	108	—	6900	6990	52·5	4·20	8·54	3·75	190 800
							Mean of Expts. 2 and 3		186 400

* From sedimentation after dialysis; a similar value was obtained after diffusion.
† A partial specific volume of 0·71 was calculated from the composition, as given by Morton *et al.* (1961). The mean value is based on Expts. 2 and 3 because of the apparent error in determination of the diffusion coefficient in Expt. 1.
‡ The value is probably in error owing to incomplete dialysis, since zero time in diffusion was unreasonably large.
§ From boundary-spreading during sedimentation, $D^{app.}_{20,w}$ for this sample was $5{\cdot}51 \times 10^{-7}$ cm.²/sec.

studies (Table 5) showed a single symmetrical sedimenting boundary, the spreading of which obeyed the Boltzmann assumption, and showed no evidence of polydispersity of the sedimenting material (cf. Fujita, 1956; Baldwin, 1957). A marked discrepancy exists between the diffusion coefficient calculated from the boundary-spreading and that measured by static free diffusion (see Table 5).

The molecular weight of cytochrome b_2 was calculated from the sedimentation and diffusion coefficients (from static free diffusion) determined on the same samples (Table 5). The anhydrous partial specific volume, $\bar{v}$, of cytochrome b_2 (0·71) was calculated from the amino acid and nucleotide composition (Appleby, Morton & Simmonds, 1960; Appleby & Morton, 1959*b*; Morton, Armstrong & Appleby, 1961), contributions from the haem and flavin groups being neglected (cf. Cohn & Edsall, 1943; Ehrenberg & Theorell, 1955). The error introduced by omission of these groups is probably small, since they constitute only about 1·2 % of the weight of the enzyme.

Electrophoresis of cytochrome b_2. On electrophoresis in lactate–pyrophosphate buffers over the pH range 5–8, cytochrome b_2 migrated as an anion with a single boundary (see Fig. 4). Some preparations contained a colourless minor component (less than 3 % of the area of the major component) which migrated rapidly toward the anode. The electrophoretic mobility of cytochrome b_2 at pH 6·8 in 0·3 M-sodium lactate–0·05 M-tetrasodium pyrophosphate–0·1 mM-EDTA is approx.

$$9{\cdot}5 \times 10^{-6}\ \text{cm.}^2/\text{sec./v}$$

and over the pH range 5–8 there is an approximately linear increase in mobility with increase of pH.

DISCUSSION

Cytochrome b_2 is a labile flavohaemoprotein (Bach *et al.* 1946; Appleby & Morton, 1954, 1959*b*; Morton, 1961*a*). In the work described here every effort was made to exclude from the enzyme both heavy metals and oxygen, both of which may cause substantial decline in activity accompanied by

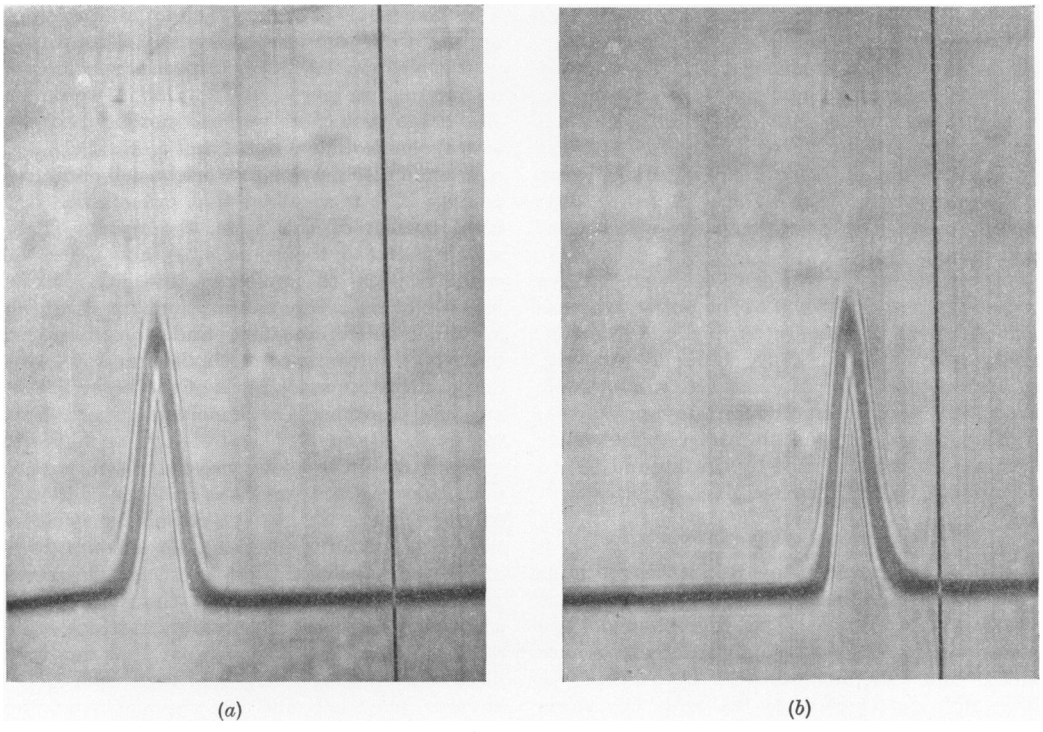

Fig. 4. Electrophoresis pattern of cytochrome b_2 in lactate–pyrophosphate buffer. The ionic strength was 0·63, the pH 6·9, the protein concn. 99·3 μM-cytochrome b_2 haem and the current 9·0 mA. (*a*) Descending boundary (cathodic); (*b*) ascending boundary (anodic), 6 hr. after commencement.

changes in physical properties (Appleby & Morton, 1959*b*; Armstrong, Coates & Morton, 1960, 1961). Nevertheless, on occasions there was considerable decline in activity during an experiment; measurements from such experiments were not used for determining the physical constants reported here, which refer to the enzymically active deoxyribonucleoprotein, Type I cytochrome b_2 (Morton, 1961*a*).

The deep-pink colour of the enzyme prohibited the use of interference optics in sedimentation, diffusion and electrophoresis experiments, and schlieren optics were therefore used. The measurement of schlieren photographs at high concentrations of the enzyme was also made difficult by the considerable absorption of the monochromatic light. Errors were reduced to a minimum by careful alignment of optical systems and by repetitive measurements of photographs.

The sedimentation behaviour of cytochrome b_2 is that of an apparently monodisperse solute in that a single symmetrical boundary was obtained in numerous sedimentation-velocity experiments. Within the limits of experimental error, analysis by Baldwin's (1957) procedure showed that, after correction for the concentration-dependence of the sedimentation coefficient, boundary-spreading during sedimentation was apparently solely due to diffusion. However, the values of the diffusion coefficients calculated by this procedure (cf. Fig. 3) are greater than those obtained from static free diffusion (Table 5); this is possibly due to the self-interaction of cytochrome b_2 (see below). R. L. Baldwin (personal communication) has indicated that the main use of Fujita's (1956) equation is to show that boundary-spreading during sedimentation occurs by diffusion only, i.e. as a criterion of homogeneity of sedimentation coefficient. A minor component (always less than 3 % of the total protein) was sometimes observed; it had a sedimentation coefficient corresponding to that of an oxygen-induced aggregated form of the enzyme (Armstrong *et al.* 1960, 1961).

Cytochrome b_2 (Type I) shows a greater dependence of sedimentation coefficient on concentration than is usually observed with globular proteins, the proportionate change of sedimentation coefficient with concentration being about 2·8 times that of bovine serum albumin. Because of the unsatisfactory state of present theories of the concentration-dependence of sedimentation coefficient, no adequate explanation of this behaviour can be offered. Because of the high ionic strength

(0·63) of the buffer system used, it is unlikely that it is due to the primary salt effect. However, intermolecular interaction associated with the deoxyribonucleic acid component may be responsible. Type II cytochrome b_2 (Morton, 1961*a*), which is free of the deoxyribonucleic acid component, shows a proportionate change of sedimentation coefficient with concentration (Armstrong *et al.* 1961; Armstrong, 1961) that is rather similar to that of bovine serum albumin.

The values of $S_{20,w}$ given here are corrected for both viscocity and buoyancy of the buffer, whereas the values given previously (Appleby & Morton, 1960; Armstrong *et al.* 1960, 1961; Armstrong, 1961; Morton *et al.* 1961; Morton, 1961*a*; Morton & Shepley, 1961) are uncorrected for buoyancy effect; these previous values, and the derived molecular weights, should be multiplied by the factor 1·087 to be comparable with the values given here.

Molecular weight of cytochrome b_2

The failure of approach-to-equilibrium methods to give consistent values for the molecular weight of cytochrome b_2 (Table 3) is unexplained. The molecular weight of yeast alcohol dehydrogenase (129 000) and of horse-heart cytochrome *c* (14 600 ± 200) determined by us with the same approach-to-equilibrium procedures are in agreement with published values for these proteins (Hayes & Velick, 1954; Tint & Reiss, 1950*a*, *b*) based on sedimentation and diffusion experiments. It is unlikely, therefore, that the variable results of Table 3 are experimental in origin, although incomplete dialysis may be one factor contributing to the variability. The discrepancy in the sedimentation coefficients as determined by sedimentation-velocity experiments and by calculation from results derived from approach-to-equilibrium experiments (Baldwin, 1953) on the same samples of cytochrome b_2 (Table 3) is sufficient reason for rejection of the approach-to-equilibrium experiments for the determination of the molecular weight of cytochrome b_2.

The three experiments which involved the use of sedimentation equilibrium to determine the molecular weight of cytochrome b_2 were in agreement but in two there was a considerable loss of enzymic activity, possibly due to the use of aluminium-filled epoxy resin centre-pieces in the analytical cells. The results shown in Table 4, in which there was satisfactory retention of enzymic activity, were obtained with fluorocarb (Kel-F) centre-pieces in the analytical cells. The mean value from Lansing & Kramer's (1935) equation (Table 4, method C) was 177 300, but values ranging from 127 000 to 365 000 were also obtained, depending on the method of calculation and the portion of the analytical cell examined (Table 4).

If the three procedures cited in Table 4 were applied to a polydisperse system of an ideal mixture of proteins of identical partial specific volume and refraction increment, method A would yield a *Z*-average molecular weight, method B a weight-average molecular weight, and method C a weight-average molecular weight for the cell contents as a whole. If it is assumed that cytochrome b_2 is an ideal system of this type, the results (Table 4) suggest that cytochrome b_2 is not homogeneous with respect to molecular weight. However, because of the many assumptions involved in such a simple interpretation, and the discrepancies between the results of methods A and B (Table 4), no quantitative assessment of the heterogeneity (if any) is possible. The conclusion from Table 4 (methods A and B) that cytochrome b_2 is heterogeneous with respect to molecular weight conflicts with that reached from the analysis of boundary-spreading (Fig. 3). In the calculations from sedimentation-equilibrium data, no account is taken of the variation of activity coefficient with concentration. As indicated above, the marked concentration-dependence of the sedimentation coefficient of cytochrome b_2 (Type I) probably is due to intermolecular interaction, and such non-ideal behaviour may account for the apparent heterogeneity indicated by Table 4 (methods A and B). However, there is good agreement between the molecular weights (177 300 and 186 400 respectively) as calculated by the Lansing & Kramer equation (Table 4, method C), and by combination (Table 5) of the diffusion coefficient (from static free diffusion) and the sedimentation coefficient (from sedimentation-velocity experiments).

The diffusion studies (Table 5) show the importance of adequate dialysis to ensure equilibrium of all components; with continuous movement of both the non-diffusible and diffusible material, the minimum period with cytochrome b_2 is 72 hr., and longer periods are desirable. The molecular weight of 228 100 obtained from the first diffusion experiment (Table 5) is rejected since the results indicated that true Donnan equilibrium was not attained. The stabilization of the enzyme for the period of about 10 days at 0–2° required for dialysis and diffusion follows from the work of Appleby & Morton (1954, 1959*b*) and of Armstrong *et al.* (1961) and was achieved by rigorous exclusion of oxygen and of heavy metals.

From analyses of several different preparations of crystalline cytochrome b_2 (Type I) (Appleby & Morton, 1954, 1959*b*; the present work; and R. K. Morton, unpublished work), the minimum molecular weight based on estimation of haem is 81 800 ± 900 (4), of flavin 83 300 ± 1700 (3) and of iron 67 900 (2). From the total composition (Morton *et al.* 1961; Montague & Morton, 1960) the

calculated molecular weight is 75 400, but some of the nitrogen is unaccounted for by the composition (Appleby *et al.* 1960; Morton *et al.* 1961). From the haem and flavin analyses, the best estimate is 82 300 ± 900 (7); the iron analyses are less reliable and are rejected. Based on haem analyses with non-crystalline preparations, other workers have reported minimum molecular weights of 230 000 (Boeri *et al.* 1955), 140 000 (Boeri & Rippa, 1961), 120 000 (Boeri & Cutolo, 1958) and 97 000 (Nygaard, 1959, 1961); haem analysis by Rippa (1961) on a crystalline preparation indicates a value of approx. 100 000.

The apparent conflict between conclusions based on results obtained with the velocity ultracentrifuge and the equilibrium ultracentrifuge would arise if cytochrome b_2 in solution is a self-associating system in which various polymeric forms are in rapid dynamic equilibrium. Insulin, α-chymotrypsin and chymotrypsinogen appear to show similar behaviour, and it may be a common feature of proteins. If the rates of interconversion of the various forms are sufficiently fast, they will not be resolved in the velocity ultracentrifuge but the heterogeneity will be revealed by the equilibrium ultracentrifuge. For the reasons already given, some of the estimates of molecular weight are rejected, and the mean (183 400, S.E.M. ± 3900) of the results from Table 4 (method C) and from Table 5 (Expts. 2 and 3) is taken as the best estimate of the molecular weight from the physicochemical studies. It is concluded that the predominant form at the concentrations studied has a molecular weight which is twice that of the minimum molecular weight (82 300) as estimated from the chemical composition. The discrepancy between the expected value of 164 600 and the estimated value of 183 400 may partly arise from some error in the value of $\bar{v}$, which was not determined experimentally. These physicochemical studies do not permit any conclusion about the predominant form of the enzyme at the concentrations used in kinetic studies of enzymic activity. However, a structure containing two haem groups and two flavin groups/molecule is consistent with the kinetic data of Armstrong (1961) and of Morton *et al.* (1961), which indicate intramolecular transfer between the flavin and haem groups of cytochrome b_2. It is also consistent with titration data of Boeri & Cutolo (1958, 1961) which show that, under certain conditions, reduction of the enzyme by lactate requires more than two reducing equivalents/haem group. Moreover, a dimeric structure accounts for the elevated extinction coefficients of ferrocytochrome b_2 as compared with other cytochromes, e.g. ferrocytochrome b_5, and with pyridine protohaemochrome (Morton, 1958, 1961*b*). A previous estimate of the molecular weight as 97 000, based on physicochemical measurements, and the consequent conclusion that there is one haem group/molecule (Morton, 1958), is incorrect as applied to cytochrome b_2 at concentrations of 5–110 μM-haem.

Table 6. *Calculation of net charge on cytochrome* b_2 *at* pH 6·8

Calculations were made from the nucleotide composition (Appleby & Morton, 1959*b*, 1960; Montague & Morton, 1960) and from the amino acid composition (Appleby *et al.* 1960). It was assumed that acidic and basic amino acid residues are fully ionized, and that histidine residues are 30% ionized; that *C*-terminal and *N*-terminal groups cancel each other; that the polynucleotide has one primary phosphate dissociation; and that the secondary phosphate dissociation is about 50% complete at pH 6·8. Calculations are expressed per mole of cytochrome b_2 haem.

Component	Total no. of residues	Charge at pH 6·8
Amino acid residues		
Free aspartyl and glutamyl*	—	−37·6
Lysyl	45·7	+45·7
Argininyl	21·0	+21·0
Histidinyl	6·2	+1·9
Polynucleotide residues		
Primary dissociation	1	−1
Secondary dissociation	15	−7·5
Riboflavin phosphate		
Primary dissociation	1	−1
Secondary dissociation	1	0·5
Total positive charge		+68·6
Total negative charge		−47·6
Net positive charge		+21

* The total number of aspartic and glutamic acid residues is 118·6; amide nitrogen amounts to 81 residues, and, by assuming that this amide is associated with aspartic and glutamic acid residues, free residues must be 37·6.

The frictional ratio of 1·18 was calculated by using Stoke's equation and the Svedberg & Pedersen (1940) theory from the molecular weight of 186 400 (Table 5) and the intrinsic sedimentation coefficient. This indicates that the molecule of cytochrome b_2 is probably spheroidal.

Electrophoresis of cytochrome b_2

The electrophoretic studies at high ionic strength and over the pH range 5–8 show only one charged component, as observed by Appleby & Morton (1960) at lower ionic strengths. J. S. Graham, R. K. Morton & K. Shepley (unpublished work) have also shown that cytochrome b_2 migrates as a single component on vertical starch-gel electrophoresis in lactate–pyrophosphate buffer at pH 6·8.

The mobility curve of Type I cytochrome b_2 over the pH range 5–8 is approximately 0·75 pH unit above that of Type II cytochrome b_2, indicating that Type I cytochrome b_2 has a larger negative

charge in this pH range owing to the presence of the deoxyribonucleic acid component in Type I cytochrome b_2. Extrapolation of the mobility–pH curve for Type I cytochrome b_2 in lactate–pyrophosphate buffer indicates zero mobility at about pH 4·1, but the isoelectric point has not been directly determined since flavin dissociates and enzymic activity is rapidly lost below pH 5 (see Appleby & Morton, 1959*b*, 1960).

There is a discrepancy between the observed negative charge on cytochrome b_2 at pH 6·8 and the expected net positive charge as calculated from the known ionizable groups (Table 6). This discrepancy may arise from intramolecular interactions which selectively mask some of the potentially charged groups, or from selective binding of one or more ions of the buffer system, or from a lower amide content than that determined by Appleby *et al.* (1960).

SUMMARY

1. The paper describes sedimentation, diffusion and electrophoresis studies on the crystalline, enzymically active deoxyribonucleoprotein, Type I cytochrome b_2 [L(+)-lactate–cytochrome *c* oxidoreductase of baker's yeast]. Enzymic activity of this labile flavohaemoprotein was substantially retained during the experiments by rigorous exclusion of oxygen and of heavy metals.

2. Cytochrome b_2 behaves as an apparently monodisperse protein as indicated by boundary analysis of sedimentation patterns according to Baldwin (1957) and by electrophoresis at I 0·63 and at pH 5–8.

3. Sedimentation-velocity experiments gave an intrinsic sedimentation coefficient, $S^0_{20,w}$, of $9{\cdot}17 \times 10^{-13}$ sec. There is marked concentration-dependence of the sedimentation coefficient, described by the equation $S^{c_0}_{20,w} = S^0_{20,w}\ (1 - kc_0)$, where c_0 is μM-cytochrome b_2 haem, k is $1{\cdot}40 \times 10^{-3}$ l./μmole of cytochrome b_2 haem.

4. Variable molecular weights for cytochrome b_2 were obtained by approach-to-equilibrium experiments, which also gave unsatisfactory sedimentation coefficients. The cause of this discrepancy is unexplained. Satisfactory values of 129 000 and of 14 600 ± 200 were obtained for the molecular weights of yeast alcohol dehydrogenase and of horse-heart cytochrome *c* by similar procedures.

5. From equilibrium-sedimentation data, a molecular weight of 177 300 was obtained by Lansing & Kramer's method, but other values were obtained by the equilibrium differential equation and the integrated form of the same equation. Possible reasons for this discrepancy are discussed; there appears to be a rapid dynamic equilibrium between various polymeric forms of the enzyme.

6. By static free diffusion, the diffusion coefficient, $D_{20,w}$, of cytochrome b_2 (Type I) at 0·4 % of protein is $3{\cdot}80 \times 10^{-7}$ cm.²/sec. The partial specific volume as calculated from the composition is 0·71. The molecular weight derived from these values and the sedimentation coefficient is 186 400.

7. The minimum molecular weight from analyses of flavin and of haem is 82 300 ± 900, and the apparent molecular weight from physical measurements is 183 400 ± 3900. At the concentrations studied (5–110 μM-haem) cytochrome b_2 thus appears to exist predominantly as a dimer, containing two flavin and two haem groups/molecule. The calculated frictional ratio is 1·18, indicating that the molecule is approximately spherical.

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Biochemical Studies of Toxic Agents

14. THE BIOSYNTHESIS OF ETHYLMERCAPTURIC ACID*

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The first evidence for the metabolic formation of an alkylmercapturic acid was obtained by Roberts & Warwick (1957, 1958), who showed by radiochromatographic methods that the administration of ^{14}C-labelled ethyl methanesulphonate,

$$CH_3 \cdot SO_2 \cdot O \cdot C_2H_5$$

to rats is followed by the excretion of ethylmercapturic acid, *N*-acetyl-*S*-ethyl-L-cysteine. By the use of a procedure for determining mercapturic acids Bray & James (1958) were led to conclude that the administration of 1- and 2-bromobutane and certain higher 1-bromoalkanes to animals is followed by the excretion of the corresponding alkylmercapturic acids, and Thomson, Maw & Young (1958) showed by chromatographic and radiochromatographic methods that ethylmercapturic acid is excreted by rats dosed with bromoethane. The present paper contains a more detailed account of the work on ethylmercapturic acid together with a description of the isolation of this compound from the urine of rats dosed with bromoethane or with *S*-ethyl-L-cysteine. While this work was in progress four other alkylmercapturic acids were isolated from the urine of animals dosed with 1-halogenoalkanes, namely *n*-propylmercapturic acid (Grenby & Young, 1959, 1960) and *n*-butyl-, *n*-pentyl- and *n*-hexylmercapturic acid (Bray & James, 1960; James, 1961).

* Part 13: Hopkins, Brooks & Young (1962).

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MATERIALS

All melting points are uncorrected. Elementary microanalyses were carried out by Weiler and Strauss, Oxford.

Preparation of S-*ethyl*-L-*cysteine.* Method 1. The procedure adopted closely resembled that described by Grenby & Young (1960) for the preparation of *S*-(*n*-propyl)-L-cysteine. Small leaflets of *S*-ethyl-L-cysteine were obtained in 48% yield from 12 g. of L-cystine. After being recrystallized twice from 85% (v/v) methanol in water the compound had m.p. 252–253° (decomp.), $[\alpha]_D^{19}$ −27° (*c* 1 in water) (Found: C, 40·5; H, 7·3; N, 9·2; S, 21·6. Calc. for $C_5H_{11}NO_2S$: C, 40·2; H, 7·4; N, 9·4; S, 21·5%).