CYTOCHROMES b_2 AND c OF BAKERS YEAST

Thesis submitted for the Degree

of

Doctor of Philosophy

by

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from

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PREFACE

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

J. McD. Armstrong

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Abbreviations, symbols etc.

Chemical

AS-YLD	YLD (q.v.). free from polynucleotide.
BAL	British anti-lewisite
cyt.	cytochrome, e.g. cyt.c - cytochrome c
DCPIP	2,6 dichlorophenol indophenol
DNA-ase	deoxyribonuclease
DPN	diphosphopyridine nucleotide
DPNH	reduced diphosphopyridine nucleotide
EDTA	ethylene diamine tetra-acetic acid
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
FeCN	ferricyanide
FMN	riboflavin monophosphate
Fp	flavoprotein
GSH	glutathione
IAM	iodoacetamide
NEM	N-ethyl maleimide
ox.	oxidised
PCMB	p-chloromercuric benzoic acid
PCMS	p-chloromercuriphenylsulphonic acid
red.	reduced
-SH	sulphydryl group
TPN	triphosphopyridine nucleotide
TPNH	reduced triphosphopyridine nucleotide
YLD	yeast lactate dehydrogenase

Spectrophotometric

α, β, γ, δ	absorption bands of cytochromes from red to blue end of spectrum
E	optical density; $\log \frac{Io}{I}$
$E_{x m\mu}$, E_{α}	optical density at a wavelength of $x m\mu$ or at the maximum of the α band. Unless specified a 1 cm depth of solution is indicated
$E^{1\%}$	optical density of a 1% solution
E^{1cm}	optical density of a solution contained in a cell of 1 cm. light path
E_o	optical density at time zero
E_t	optical density at time t
ε	molar extinction coefficient; optical density of a solution containing 1 mole of solute/litre, in a cell of 1 cm. light path.
$\varepsilon_{A'}$	molar extinction coefficient of the ion A'
ε_{HA}	molar extinction coefficient of the undissociated acid HA
$\varepsilon^{mM}_{x m\mu}$	millimolar extinction coefficient ($10^{-3}\varepsilon$) at a wavelength of $x\ m\mu$
I_o	light intensity with no absorbing substance in the light path
I	light intensity after passing through absorbing substances

Physical

C	concentration
D	diffusion coefficient
$D_{20,w}$	diffusion coefficient of solute at 20° in a solution having the viscosity and density of water
f	frictional coefficient of a particle of a given weight
f_o	frictional coefficient of a spherical particle of a given weight.
Ip	iso electric point
M	molecular weight
n	refractive index
S	sedimentation coefficient
$S_{20,w}$	sedimentation coefficient of a solute at 20° in a solution having the viscosity and density of water
$\bar{V}$	partial specific volume
x_m, x_p	distance to the meniscus (m) or plateau p
γ	activity coefficient
$\Gamma/2$	ionic strength $\frac{1}{2}\sum C_i z_i^2$
η	viscosity
μ_i	the i^{th} moment
ρ	density
τ	equivalent time of centrifugation ($=2w^2st$)
ω	angular velocity
z	valence of an ion

Kinetic

Apparent values denoted by prime

$[E]$	total enzyme concentration
k_i	rate constant of i^{th} reaction
Keq.	equilibrium constant
Kapp.	apparent equilibrium constant
K_A	Michaelis constant for A
K_m	Michaelis constant
p.	concentration of enzyme-substrate complex
$[s]_o, [s]_t$	substrate concentration at time o and time t
$t_{\frac{1}{2}}$	time required for p to fall from its maximum value (for any given set of conditions) to $p/_2$.
T.N.	turnover number $(= V/[E])$
v	velocity of reaction
v_{obs}	observed velocity
V	maximum velocity
V_A	maximum velocity at saturating concentrations of A
$V/[E]$ (lactate)	Turnover number in moles of lactate per mole of enzyme per second

other abbreviations

N.S.L.	National Standards Laboratory
$\sim$	approximately

SUMMARY

The general properties of the haemoprotein, cytochrome *c* as obtained from various sources are reviewed. Methods for preparation of yeast cytochrome *c* are discussed; it is concluded that the use of trichloroacetic acid during purification results in formation of artifact forms of the protein. Cytochrome *c* was prepared from bakers' yeast by resin chromatography. The product was shown to be very similar in spectral and other properties to cytochrome *c* from horse-heart, but differed from the latter in having a lower iso-electric pH and a higher molecular weight. Electrophoretic studies suggested some heterogeneity of the yeast cytochrome *c*.

Controversies concerning the nature and properties of cytochrome b_2 are discussed, especially in relation to the identity with L(+)-lactate dehydrogenase of aerobic yeast. The behaviour and properties of other cytochrome systems are discussed in relation to the possible functions of flavin-and haem-containing proteins in biological oxidations. It is concluded that the physiological role of cytochrome b_2 is as the L(+)-lactate-cytochrome *c* reductase of respiratory granules of aerobic yeast.

Crystalline cytochrome b_2 was isolated from bakers' yeast. The haem and iron content agreed well with previous estimates; only traces of non-haem iron were present. Cytochrome b_2 was found to contain 4-6 sulphydryl groups which reacted rapidly with

p-chloromercuriphenyl sulphonate. Further evidence is presented that the haem prosthetic group of cytochrome $\underline{b}_2$ is protohaem.

In relation to studies of the kinetic properties of cytochrome $\underline{b}_2$, detailed consideration was given to the mechanism of action of typical flavoproteins. Based on these considerations, it was concluded that ferricyanide is reduced by the enzyme at the flavin prosthetic group and cytochrome $\underline{c}$ at the haem prosthetic group of cytochrome $\underline{b}_2$. The kinetics of ferricyanide reduction by cytochrome $\underline{b}_2$ were found to be consistent with a rate equation of the form

$$v = \frac{V}{1 + \frac{K_{lactate}}{[lactate]} + \frac{K_{FeCN}}{[FeCN]} + \frac{K_{AB}}{[lactate]\,[FeCN]}}$$

while the kinetics of cytochrome $\underline{c}$ reduction by cytochrome $\underline{b}_2$ were consistent with the following rate equation

$$v = \frac{V}{1 + \frac{K_{lactate}}{[lactate]} + \frac{K_{cyt.\ \underline{c}}}{[cyt.\ \underline{c}]}}$$

A specific effect of chloride ion on the rate of ferricyanide reduction by cytochrome $\underline{b}_2$ was observed.

The enzyme oxidised L(+)-lactate but not D(-)-lactate.

The apparent Michaelis constant for L(+)-lactate and the inhibition constant for D(-)- lactate were calculated. A number of other α-hydroxy-monocarboxylic acids were oxidised by the enzyme. Very strong inhibition of lactate-ferricyanide reductase activity was found with both L(+)- and D(-)- malate; this behaviour appears anomalous in relation to other dicarboxylic acids.

Enzymic activity was inhibited by N-ethyl maleimide and by p-chloromercuriphenylsulphonate, but not by iodoacetamide.

Sedimentation and electrophoretic behaviour did not reveal more than one protein in preparations of cytochrome b_2. The enzyme was not dissociated into a number of components by treatment with urea. The effect of oxygen and of p-chloromercuriphenyl sulphonate in causing dissociation of flavin, and of oxygen in inducing formation of aggregated material, was investigated. It was concluded that the binding of the flavin prosthetic group to the protein involved a sulphydryl group.

A number of experiments using "approach-to-sedimentation-equilibrium" were carried out to determine the molecular weight of cytochrome b_2. Highly variable results for the molecular weight were obtained. No satisfactory explanation of this finding may be offered at present. By contrast, consistent behaviour was observed in sedimentation velocity experiments. Use of a diffusion coefficient obtained from analysis of boundary spreading by Fujitas' solution to the ultracentrifuge differential equation

gave a molecular weight of 123,000 for cytochrome b_2. This is not an integral of the minimum molecular weight (80,000).

The polynucleotide present in crystalline cytochrome b_2 appears to form a definite complex with the protein, and has a marked effect on the sedimentation properties of the cytochrome. The sedimentation behaviour of the free polynucleotide was studied. This material appears to aggregate in solution.

It is concluded that cytochrome b_2 is a flavo-haemoprotein with catalytic properties which confer upon it lactate dehydrogenase activity.

GENERAL INTRODUCTION

Certain haemoproteins are firmly associated with insoluble material of the cell. In many cases, such haemoproteins are resistant to extraction by buffer solutions except under conditions which cause substantial denaturation of the haemoproteins. However, the two haemoproteins known as cytochrome c and cytochrome b_2 present in yeast may be obtained in true solution by extraction of air-dried yeast with aqueous solutions. Other haemoproteins, such as cytochrome c_1, have been obtained in a soluble form from yeast but very special conditions of extraction are necessary. Hence cytochrome c and cytochrome b_2 may be referred to as 'soluble cytochromes of yeast', this description referring only to the relative ease of extraction into solution and not to localisation of these pigments within the cell *in vivo*, which is discussed elsewhere in this thesis. This thesis describes studies of the properties of these two soluble cytochromes from baker's yeast.

It is shown here that yeast cytochrome b_2 possesses lactate dehydrogenase activity. The term "yeast lactate dehydrogenase" is frequently used in this thesis as synonymous with "cytochrome b_2".

Cytochrome c of Baker's Yeast

I. INTRODUCTION

A. General Aspects of Cytochrome c

In 1925, Keilin rediscovered MacMunn's respiratory pigments, the histohaematins and myohaematins, in animal tissue, and also demonstrated their presence in plants and microorganisms. A typical four-banded absorption spectrum was observed in the region 520-605 mμ, and Keilin proposed the name cytochrome for the substances responsible for these bands; he recognised that cytochrome consisted of at least three components, which he named cytochromes a, b and c. A major consideration in establishing the multicomponent nature of cytochrome was the isolation of cytochrome c in a soluble form from baker's yeast. (Keilin, 1930). This provided the first unequivocal evidence for the role of cytochromes a, b and c, as components of a linked redox system for the transfer of electrons from substrate to oxygen.

Although a few other workers carried out investigations of the properties of yeast cytochrome c, Keilin's method of preparation was rather tedious and unpleasant, and when Theorell (1936) developed a simple method for the large scale extraction and purification of cytochrome c from heart muscle, studies of yeast cytochrome c were abandoned in favour of the much more simply prepared heart muscle cytochrome c. For this reason, most of our present knowledge concerning cytochrome c has been obtained for the pigment isolated from either horse or beef heart, although some comparative studies have been carried out with cytochrome c isolated from pig heart, chicken heart, salmon muscle (Tint and Reiss, 1950; Paleus, 1954; Atlas and

Farber, 1954) and from wheat germ (Goddard, 1944). Kamen and his co-workers (Kamen, 1955,1956) have studied purified c-type cytochromes obtained from a number of bacterial species; whilst the prosthetic group of the bacterial cytochromes was the same as that of heart muscle cytochrome c, in general they differed considerably from heart muscle cytochrome c in such ways as positions of spectral absorption bands, redox potential, basicity of the protein and activity in enzymic tests.

From about 1956 onwards, cytochrome c from a wide variety of sources has been obtained in a highly purified state, although few comparative studies have been made with these preparations. The sudden interest in cytochrome c can be attributed to the pioneer work of Paleus and Neilands (1950), Boardman and Partridge (1953) and Margoliash (1954) in developing ion exchange chromatographic methods for the purification of cytochrome c. In effect, these methods rely on the extremely basic nature of most cytochrome c's, and use weakly acidic ion exchangers for the chromatographic purification of these cytochromes. The ease of purification of cytochrome c by chromatography has resulted in the crystallisation of cytochrome c from a number of sources (Bodo, 1955; Hagihara, Morikawa, Sekuzu, Horio and Okunuki, 1956; Hagihara, Horio, Nozaki, Sekuzu, Yamashita and Okunuki, 1956; Hagihara, Tagawa, Nozaki, Morikawa, Yamashita and Okunuki, 1957; Hagihara, Tagawa, Morikawa, Shin and Okunuki, 1958; Hagihara, Tagawa, Sekuzu, Shin, Morikawa, Yoneda and Okunuki, 1958). The first preparations of heart muscle cytochrome c described by Theorell (1936) and by Keilin and Hartree (1937) contained 0.34% iron, corresponding to a minimum molecular

weight of 16,400. It was shown that these preparations were not homogeneous, and that by preparative free-solution electrophoresis a homogeneous product could be obtained, containing 0.43% iron (M=13,000) (Theorell and Åkeson, 1939, 1941). This purified cytochrome c was homogeneous by a number of criteria (Paul, 1951). However, the use of chromatography has resulted in preparations with even higher iron content, and some properties of these are summarised in Table 1, part B. Table 1, part C lists sedimentation, diffusion and electrophoretic data for cytochrome c obtained from a number of sources. Table 1, part A gives the general spectroscopic properties of "classical" cytochrome c. The available information warrants the classification of yeast cytochrome c as a classical cytochrome c. Such cytochromes have a characteristic absorption spectrum, a redox potential, E'_0, in the region of + 0.25 volt, high stability to heat, acidity and alkalinity, high activity with cytochrome oxidase and cytochrome c reductase, and are highly basic proteins.

B. Prosthetic Group of Cytochrome c

The spectrum of reduced cytochrome c is unlike that of any of the haemochromagens of protohaem, and is very similar to that of a mesohaemochromagen. This suggests that the prosthetic group of cytochrome c has no unsaturated side chains. Treatment of cytochrome c with HBr and acetic acid results in a haematoporphyrin which is indistinguishable from the haematoporphyrin obtained from protohaem (Hill and Keilin, 1930). This haematoporphyrin can be converted to mesoporphyrin dimethyl ester (Zeile and Reuter, 1933). Since acidified

Table 1A

General Spectroscopic Properties of Cytochrome c.

Reduced		Oxidised	
Wavelength of maximum mμ	Extinction coefficient x 10^{-3}	Wavelength of maximum mμ	Extinction coefficient x 10^{-3}
550	27.7	530	11
521	17	408	108
415	130	364	29
316	34		

$\Delta \varepsilon_{550}$ (reduced-oxidised) = 18.5

One of the isosbestic points, for reduced and oxidised forms occurs at $\sim$540 mμ.

Table 1B

Properties of Cytochrome c from Various Sources

Source	Iron (%)	$\frac{E_\alpha}{(E_{Pr})(ox)}$	$\varepsilon_\alpha \times 10^{-3}$ $cm.^{-1}M^{-1}$	$(M)_{Fe}$ *	Remarks
Horse heart					
(Margoliash (1954)	0.463	1.17	27.7	12,000	Chromatographically homogeneous
Leaf, Gillies and Pirrie (1958)	0.460	1.20	27.6	12,140	Chromatographically homogeneous
Tint and Reiss (1950)	0.456	-	27.8	12,250	Electrophoretically homogeneous
Munnikhoven (1958)	0.454	1.24	-	12,300	Chromatographically homogeneous
Beef heart					
Paleus and Neilands (1950)	0.466	1.17	26.9	11,980	
Tint and Reiss (1950)	0.453	-	26.4	12,330	Electrophoretically heterogeneous, corrected for impurity

* $(M)_{Fe}$ is the molecular weight calculated for 1 atom of iron per molecule of cytochrome c.

Table 1B contd.

Source	Iron (%)	$\frac{E_{\alpha}}{(E_{Pr'})(ox)}$	$\varepsilon_{\alpha} \times 10^{-3}$ cm^{-1} M^{-1}	(M) Fe	Remarks
Hagihara et al. (1956)	0.450	1.28	-	12,410	Chromatography for purification, crystalline
Paleus (1954)	0.449	1.17	27.9	12,440	Recalculated for observed iron content
Paleus and Theorell (1957)	0.435	-	29.2	12,840	Crystalline, electrophoretically heterogeneous (3 components)
Theorell and Åkeson (1939, 1941)	0.430	-	28.1	12,990	Electrophoretically homogeneous
Hagihara et al. (1956)	-	1.29	-	-	Crystalline, homogeneous by sedimentation and electrophoresis
Beef kidney					
Hagihara et al. (1958)	-	1.26-1.30	-	-	Chromatographically homogeneous, crystalline
Pig heart					
Tint and Reiss (1950)	0.430	-	28.0	12,990	Electrophoretically heterogeneous, corrected for impurity

Table 1B contd.

Source	Iron (%)	$\frac{E_a}{(E_{Pr})(ox)}$	$\varepsilon_a \times 10^{-3}$ $cm.^{-1} M^{-1}$	$(M)_{Fe}$	Remarks
Hagihara et al.	-	1.28	-	12,990	Crystalline
Chicken					
Paleus (1954)	0.434-0.436	1.13	26.9	12,810-12,870	
Tint and Reiss. (1950)	0.421	-	27.0	—	Electrophoretically heterogeneous, corrected for impurity
Pigeon					
Hagihara et al. (1958)	-	1.20	—	—	
Penguin					
Bodo (1956)	0.430-0.444	1.15	-	12,580-12,990	Crystalline

Table 1 B contd.

Source	Iron (%)	$\frac{E_a}{(E_{Pr})(ox)}$	$\varepsilon_a \times 10^{-3}$ cm^{-1} M^{-1}	(M) Fe	Remarks
Salmon					
Paleus (1954)	0.434-0.452	1.00	26.1-26.7	12,350-12,870	Recalculated on iron content
Bonito, Tunny					
Hagihara et al. (1957)	-	1.04	-	-	Crystalline
Wheat germ					
Hagihara et al. (1958)	-	0.95-1.00	-	-	Crystalline
Ustilago sphaerogera					
Neilands (1952)	0.28	1.10	-	19,940	Chromatographically homogeneous

Table 1B contd.

Source	Iron (%)	$\frac{E_{\alpha}}{(E_{Pr})(ox)}$	$\varepsilon_{\alpha} \times 10^{-3}$ $cm.^{-1}$ M^{-1}	$(M)_{Fe}$	Remarks
Baker's yeast Nunnikhoven (1958)	(0.424 (0.355	1.12 1.08	- -	13,170 15,730	Electrophoretically separated components, each homogeneous
Tuppy and Dus (1958)	0.37	1.21	-	15,090	Chromatographically homogeneous
Minakami (1955)	0.37	-	-	15,090	Chromatographically homogeneous microheterogeneous on electrophoresis
Li and Tsou (1956)	0.43	1.21	28.1	13,300	One of two components, electrophoretically homogeneous

Table 10

Physical Properties of Cytochrome c from Various Sources

Source	$\bar{v}$	$S_{20,w} \times 10^{13}$	$D_{20,w} \times 10^{7}$	$M \times 10^{-3}$	f/f_o	Ip	Iron (%)	pH for sedimentation and diffusion experiments
beef	0.707[7]	1.9[8] *	10.1[3]	15.69[4]	1.19[8]		(?0.34)	
	0.707	1.83[1] *	11.3[1]	13.47[1]	1.22[8]	10.65(0°)[7]	0.43[7]	
	(0.707)	2.5[4]	13.3[4]	15.63[4]	0.93[8]	10.6(0.5°)[5]	0.453[5]	7.7
	(0.707)	1.64 ± 0.01[6]	(11.3)[1]	12.07[6]	1.25[6]		0.42[10]	6.8
	0.728±0.005[12]	1.71[12]	11.4[12]	13.40[12]			0.46[14]	7.0
				13.43[12] **			0.42[13]	8.7
	0.707	1.83[15]	(11.3)[15]	13.47[15]			0.433[15]	
horse	(0.707)	2.1[4]	13.0[4]	13.43[4]	1.00[8]	10.8(0.5°)[5]	0.456[5]	7.7
	(0.707)	1.89 ± 0.15[6]	9.5[6]	16.54[6]	1.34[6]		0.41[10]	6.8

Table 1C contd.

Source	$\bar{V}$	$S_{20,w} \times 10^{13}$	$D_{20,w} \times 10^{7}$	$M \times 10^{-3}$	f/f_o	Ip	Iron (%)	pH for sedimentation and diffusion experiments
horse	(0.707)	1.61 ± 0.06[6]	(11.3)	11.85	1.41[6]		0.31[11]	6.8
	(0.707)	1.61 ± 0.02[6]	(11.3)	11.85	1.41[6]		0.36[11]	6.8
	(0.707)	1.55	(11.3)	11.41	1.43		0.36[11]	6.8
salmon	(0.707)	2.33 ± 0.15[6]	10.2[6]	19.00[6]	1.19[6]		0.41[10]	6.8
	(0.707)	2.19 ± 0.13[6]	(10.45)	17.43	1.22[6]		0.45[10]	6.8
	(0.707)	1.76 ± 0.10[6]	(10.45)	14.01	1.31[6]		0.48[10]	6.8
	0.750	1.50 ± 0.02[6]	10.7[6]	11.66[6]	1.25[6]		0.27[11]	6.8
	(0.707)	1.63 ± 0.10[6]	(10.45)	11.54	1.26		0.27[11]	6.8
pig	(0.707)	2.3[4]	12.3[4]	15.55[4]	1.01[8]	10.6(0.5°)[5]	0.430[4]	7.7
chicken	0.72[6]	1.63 ± 0.03[6]	(11.3)	12.00	1.23[6]	10.65(0.5°)[5]	0.37[6] 0.421[4]	6.8

Footnotes to Table 10

* May be incorrect because of error in calibration for rotor temperature in Uppsala oil driven ultracentrifuge (Shulman, 1953).

** By approach to equilibrium.

(1) Paul (1951) : (2) Theorell (1936 : (3) Polson (1939) : (4) Atlas ans Farber (1956) : (5) Tint and Reiss (1950) : (6) Ehrenberg and Paleus (1955) : (7) Theorell and Åkeson (1941) : (8) Pedersen (1952) : (9) Svedberg (1937) : (10) Paleus and Neilands (1950) : (11) Loftfield and Bonnichsen (1954) : (12) Ehrenberg (1957): (13) Hagihara et al (1956) : (14) Paleus and Theorell (1957) : (15) Ehrenberg and Theorell (1955).

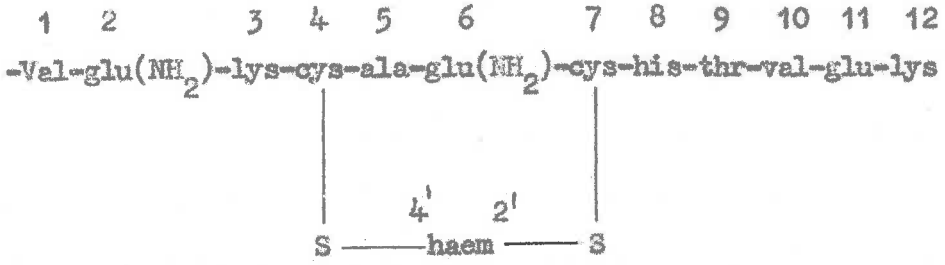
acetone will split off the haem from haemoglobin, catalase, peroxidase and other haemoproteins having protohaem as prosthetic group, but will not split off the haem from cytochrome c, this suggests a linkage between haem and protein in cytochrome c which is not present in protohaem-containing proteins. Hydrolysis of cytochrome c with HCl forms "porphyrin c", which releases L-cystine on treatment with HBr and acetic acid (Theorell, 1937, 1939). Theorell (1938) suggested that the prosthetic group of cytochrome c was protohaem, in which the vinyl side chains, at positions 2 and 4 on the haem, had reacted with cysteine residues of the protein to form thioether linkages between the protein and the haem. Paul (1950) was able to split the haem from cytochrome c with silver salts in weakly acidic solutions. The haem so obtained was free of sulphur and was an isomer of haematohaem. This treatment will split thioether bonds. As discussed later, the structure of the haemopeptide obtained by proteolytic digestion of cytochrome c confirms the existence of these thioether bonds.

The nature of ligands provided by the protein for coordination of the 5 and 6 valencies of the haem iron is still uncertain. While a considerable amount of evidence, both spectroscopic and titrimetric, implicates the imidazole nitrogens of histidine (Theorell and Åkeson, 1941; Paul, 1951, Palæus, 1954; Theorell, 1956), this is far from being completely established. It has been suggested (Falk, 1958) that one of the ligands for the 5 and 6 coordination valencies of the haem iron is a primary amino group, at least in oxidised cytochrome c . Stereochemical evidence discussed elsewhere in relation to the

proteolytic degradation product of cytochrome c suggests one of the ligands is a histidine residue of the protein.

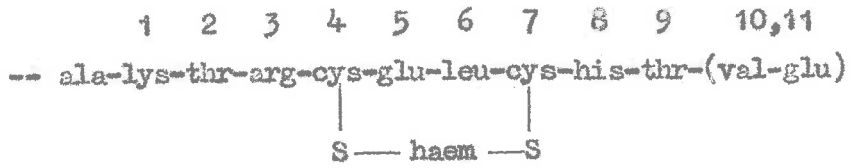
C. Structure of Cytochrome c

Tsou (1951) found that peptic digestion of horse heart cytochrome c yielded a "core" consisting of a polypeptide chain with the haem prosthetic group firmly attached. Tuppy and Bodo (1954) and Tuppy and Paleus (1955) investigated the composition and sequence of amino acids in the "core" obtained by tryptic and by peptic digestion of cytochrome c from various sources. The "core" was purified by chromatography and the haem was split off by treatment with silver sulphate-acetic acid (Paul, 1950), thus forming free sulphydryl groups in the polypeptide. These sulphydryl groups were oxidised to sulphonic acid groups and the polypeptide was subjected to the now-standard procedures for sequence analysis. As a result of these investigations, it was shown that the amino acid composition and sequence for the "core" from beef, chicken and pig cytochrome c was identical, and that the "core" had the structure

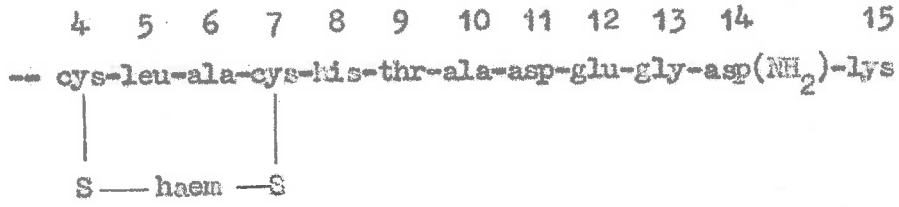


The "core" from salmon cytochrome c differed only in residue 5, which was serine in this case.

For yeast cytochrome c a somewhat different core was obtained (Tuppy and Dus, 1958)



and for Rhodospirillum rubrum cytochrome c (Paleus and Tuppy, 1959)



It may be seen that a sequence common to all these peptides is 7 8 9 cys-his-thr, and for all but the bacterial cytochrome, where such information is not available, that a basic amino acid occupies position 3, while the spacing between the two cysteine residues is two amino acid residues. These results show a clear cut species different, but it is noteworthy that the "cores" from the various animal cytochromes, representing very different species, should be so remarkably similar.

Ehrenberg and Theorell (1955) constructed models of the "core" from beef cytochrome c, using α, Υ and π helices for the amino acid residues. They found that a left-handed α helix permitted the two cysteine residues to project from the same side of the helix, at a separation suitable for forming thioether bonds with the vinyl side chains of protohaem. The axis of the α helix was very nearly parallel to the plane of the haem, and the imidazole group of residue 8 was able to form a strainless covalent bond with the haem iron. This was

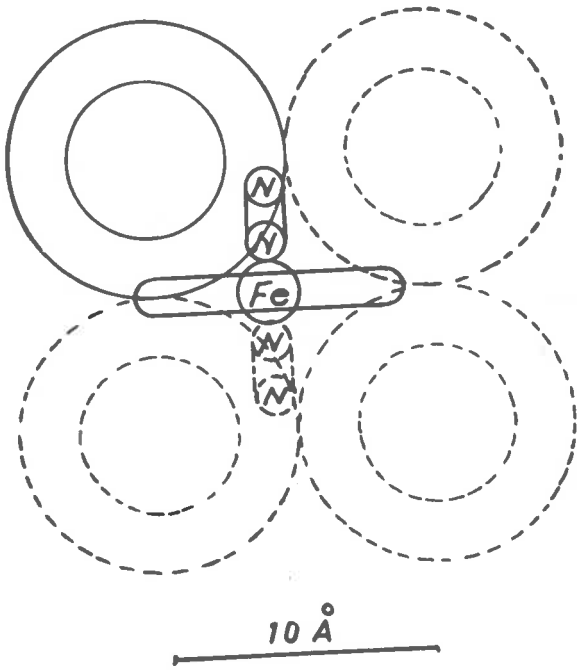
not the case for the γ and π helices. Arndt and Riley (1955) showed that the x-ray diffraction patterns of cytochrome c were those of a protein in the form of an α helix.

The partial specific volume for beef cytochrome c has been calculated from its amino acid composition. Assuming that the haem was built into the protein in such a way as to not increase the volume of the molecule, and a square packed arrangement of α-helices as in Figure 1, good agreement was obtained between the calculated and measured partial specific volumes (Ehrenberg and Theorell, 1955). More recent measurements of the partial specific volume of beef cytochrome c are higher than the earlier value (0.707 cc/g.), suggesting that the molecule may not need to be as closely packed as in the model (Ehrenberg, 1957).

In solution at near neutral pH values, the reduced "core" from beef heart has a slightly different spectrum from that of reduced cytochrome c. Sedimentation data indicated that the core existed as a pentamer or hexamer under these conditions. The polymer was dissociated into monomer on the addition of buffered histidine, while the spectrum in the visible region became indistinguishable from that of reduced cytochrome c. It seems therefore that the polymer results from the coordination of the remaining valency of the haem iron by a residue of another haemopeptide molecule, and that five or six of these form a closed ring. The coordinating residue is probably one of the lysine residues (Paleus, Ehrenberg and Tuppy, 1955).

Fig. 1. Diagrammatic structure of cytochrome c

A cross section in the region of the haem prosthetic group, showing the proposed location of the haem group in relation to four square-packed portions of an α-helix. The inner circles represent the polypeptide "backbone" of the α-helix, and the dashed outer circles the limit of the side groups. From Ehrenberg and Theorell (1955).


N
N
Fe
N
N
10 Å

Margoliash (1951) has carried out further sequence studies on cytochrome c, and has assigned positions to a large number of the residues. The analysis is as yet incomplete, but it seems likely that a number of lysine residues do not project into the water envelope unless the cytochrome c is denatured.

An interesting feature of cytochrome c is its stability. Since there are no disulphide bonds in beef heart cytochrome c (Paleus, 1955), the structural stability of the molecule must rely on other forms of interaction between neighbouring portions of the polypeptide chain to maintain its shape. Since dispersion forces would probably be insufficient, such interactions as hydrogen bonding, ionic interaction and micelle formation between lipophilic side chains must be considered to be likely. Cytochrome c contains about 12% of residues such as leucine, iso-leucine and valine, so that micelle formation could be substantial factor in stabilising the structure of cytochrome c. A large number of possibilities for hydrogen bonding exists. Increasing the ionic strength of solutions of cytochrome c markedly increases the rate of autoxidation of ferrocytochrome c (Margoliash, 1961). In view of the "crevice" theory concerning the inaccessibility of the haem of cytochrome c to oxygen, and from considerations of the model described by Ehrenberg and Theorell (1955) (see Fig. 1), the effect of ionic interaction in maintaining the structure of cytochrome c must be large.

D. Methods of Preparation of Yeast Cytochrome c

Keilin's (1930) procedure for the extraction of cytochrome c from yeast involved heating the yeast briefly at about 90°, extraction with sodium bisulphite to obtain ferrocytochrome c, oxidation by aeration at pH 3.5, and precipitation of the ferricytochrome c by saturation of the solution with sulphur dioxide, after addition of calcium chloride. The precipitated cytochrome c was then washed with acetone and dried.

Zeile and Reuter (1933) used the same extraction procedure, but took advantage of the fact the ferricytochrome c is absorbed on kaolin, from which it may be eluted with dilute alkali. Minakami (1955) obtained cytochrome c from baker's yeast by extraction of the heated yeast with sodium sulphite. The cytochrome was then concentrated and purified by chromatography, using Amberlite XE.64 (200-400 mesh Amberlite IRC-50, a resin having carboxylic acid functional groups). This preparation was homogeneous (or micro-heterogeneous) by paper electrophoresis. Nagihara et al. (1956) autolysed yeast in the presence of ethyl acetate, and extracted the cytochrome c with dilute ($\sim$0.45M) ammonium sulphate. After concentration of the cytochrome c by absorption on and elution from Duolite CS101 (a high capacity cation exchanger, probably with carboxylic acid functional groups) the cytochrome was purified by chromatography on Amberlite XE-64, concentrated, and crystallised, as ferrocytochrome c, from nearly saturated ammonium sulphate at pH9. The crystalline

cytochrome c was apparently homogeneous by sedimentation and by electrophoresis at pH 9.0.

Li and Tsou (1956) extracted baker's yeast (presumably dried) with M-sodium chloride, and then absorbed the cytochrome c on a zeolite. The eluted cytochrome c was then fractionated with ammonium sulphate and trichloroacetic acid. The resulting cytochrome contained two haemoprotein components which were readily separated by electrophoresis. One of these components was purified by ammonium sulphate fractionation, and was found to be electrophoretically homogeneous.

Nunnikhoven (1958) used Keilin's extraction procedure, but kept the cytochrome c reduced during SO_2 treatment, which precipitated other proteins but not cytochrome c. After twenty-fold concentration of the cytochrome by use of IRC-50 resin, fractionation with ammonium sulphate and trichloroacetic acid was carried out, followed by chromatography on IRC-50. The eluted cytochrome c contained two electrophoretically separable cytochrome components. These were separated on a large scale by prolonged paper electrophoresis, and were apparently homogeneous by free solution electrophoresis.

Tuppy and Dus (1958) extracted cytochrome c from baker's yeast with a solution about 4M in acetic acid, containing glycerol and dithionite, followed by chromatography on Amberlite XE-64. It may be seen that treatment with trichloroacetic acid is likely to cause modification of yeast cytochrome c. This has been confirmed by Nozaki et al, (1958), and is also true for cytochrome c from other

sources such as horse heart. In order to obtain cytochrome c with minimum modification, it is therefore desirable to avoid the use of trichloroacetic acid.

II MATERIALS AND METHODS

Yeast. The yeast used for the isolation of cytochrome c was specially grown by Barrett's Food Industries, Melbourne, and air-dried by them. It was a strain of aerobically-grown baker's yeast.

Resin. Amberlite IRC-50 was prepared as the ammonium salt, 100-150 mesh, by the method described by Margoliash (1954). To prepare the sodium form of the resin, as used in the later experiments, the resin was heated with an excess of 2N-NaOH at 80° for 10-15 minutes. The excess sodium hydroxide was removed by exhaustive washing with glass distilled water.

Spectrophotometry. The majority of the spectrophotometric measurements were carried out with an "Optica" CF4 grating spectrophotometer. This instrument has a dispersion of 16Å/mm. in the first order at the exit slit. The average band width was 0.8-1.5 Å. at the slit widths used, except in the ultraviolet, where it was necessary to operate at bandwidths up to 6Å., to avoid the instability associated with high photomultiplier voltage. The wavelength scale of the instrument was checked against the emission lines of mercury vapour and hydrogen lamps, and, after adjustment, agreed within 1 Å. with the positions of maximum intensities of the hydrogen and mercury lines. The absorbance scale was found to be satisfactory when checked with standard neutral filters supplied by

the National Standards Laboratory, Sydney.

Electrophoresis. This was carried out with a Perkin-Elmer 38A electrophoresis apparatus, which was fitted with the circulating system for refrigerated water supplied by the manufacturer. Philpott-Svensonn cylindrical lens Schlieren optics were employed, using a phase plate as the Schlieren diaphragm. The light source used was a high intensity, ribbon-type tungsten lamp, and the light was filtered with a red glass filter which cut off all light of wavelength below about 585 mμ. A 2ml. open cell assembly was used. The magnification factor for the camera lens was determined by measurement of the photographic image of a precision ruled scale ruled in 1 mm. divisions (Spinco Instruments). The scale was placed in the cell holder at a position corresponding to the centre of the cell, and was placed in the apparatus. The image of the scale was in focus. The magnification factor was 1.094. (1cm. in cell = 1.094 cm. on plate).

Boundaries were compensated using a siphon dipping into the buffer vessel on the ascending side of the cell. The siphon was adjusted to give 1 drop in 40 to 90 seconds. "Ilford" rapid process panchromatic cut film was used with 40 seconds exposure (20 seconds on either side of the desired time). The films were developed with Kodak D19 developer for high contrast.

Conductivity measurements were made with a "Philips" conductivity bridge, type GM 4249. This was modified by connection of a cathode ray oscilloscope to the voltage output of the bridge, as a null detector.

A variable capacitance unit was connected across the terminals R_x which permit use of external resistance and capacitance standards. The calibration of the bridge was checked against an N.P.L. standard resistance box, and was within 0.5% over all but the first 10% of the scale.

A "Philips" dip type conductivity cell (GM4221) was modified for use with small volumes (< 4 ml.). The holes in the jacket surrounding the electrode assembly were stoppered and the electrode assembly was then immersed in an ice-water bath contained in a "Thermos" flask, and shaken frequently. The solution resistance was measured until steady readings were obtained, and this was taken as being the solution resistance at 0°. The cell constant was not calculated directly, but standardised 0.1N KCl was used as a reference for all measurements.

A "Cambridge" null point pH meter was used for pH measurement. The electrode assembly used was a probe-type glass-saturated calomel pair designed for use with small volumes contained in test tubes. The pH was measured at room temperature.

The movement of the boundaries was measured from the photographs taken at various time intervals. These were measured with a "Beck" two dimensional measuring microscope at x 10 magnification. The photographs were aligned so that the direction of migration was parallel to the long axis of the microscope traverse. This was achieved by adjusting the position of the photograph until the reference edge of the Schlieren pattern remained centred between the

cross wires of the eyepiece as the microscope was traversed along its cross axis. All measurements were reproducible to within 20 microns. The"least squares"slope of the distance of the boundary from the reference edge against time was used for the calculation of mobility. The maximum error in the mobility values was estimated as ± 5%.

Sedimentation. Sedimentation velocity and approach to equilibrium experiments were carried out with a "Spinco" Model E analytical ultracentrifuge (E 239), fitted with Philpott-Svensonn Schlieren optics. A phase plate was used as the Schlieren diaphragm. The rotor temperature was measured using the RTIC unit supplied by the manufacturers. This unit consists essentially of a thermistor in contact with the rotor, and a bridge circuit for measuring the resistance of the thermistor, which is temperature dependent. The bridge was calibrated with the rotor at rest, using N.S.L. standardised calorimetry thermometers calibrated in 0.01 C°. Panchromatic plates (Kodak Spectroscopic plates1D-(2)) and a red filter were used for photography of the Schlieren patterns. The theory of sedimentation and experimental procedures are discussed elsewhere (p.172).

Estimation of cytochrome c. The samples were diluted with appropriate volumes of 0.1 M-sodium phosphate buffer, pH 7.0; the optical density was measured in the vicinity of 550 mμ. The cytochrome c in the sample was then reduced by the addition of solid

ascorbic acid (about 2-4 mg.) and the optical densities were again measured in the vicinity of 550 mμ, so that the position of the wavelength of maximum absorption could be located. An additional small quantity of ascorbic acid was then added to test for complete reduction. The cytochrome c content was calculated from the optical density of the fully reduced cytochrome c at the wavelength of the α band maximum, using $\varepsilon_{mM}^{\alpha\text{-band}} = 27.7$.

The percentage of reduced cytochrome c in the sample was calculated from the optical densities at the wavelength of the α band maximum, before and after addition of ascorbic acid. The following values of the extinction coefficients were used, ferrocytochrome c, $\varepsilon_{mM}^{\alpha} = 27.7$ and ferricytochrome c, $\varepsilon_{mM}^{550m\mu} = 9.2$ ($\Delta\varepsilon_{mM}^{550m\mu} = 18.5$). If E is the initial optical density for a sample in a cell of 1 cm. light path, and $E_{red.}$ the optical density at the maximum after reduction with ascorbic acid, then

$$\% \text{ ferrocytochrome c} = \frac{(E - \frac{E_{red.}}{27.7} \times 9.2) \times 100}{18.5 \times \frac{E_{red.}}{27.7}}$$

$$= \frac{(E - \frac{E_{red.}}{3.011}) \times 149.7}{E_{red.}}$$

The optical densities of the samples at 260 mμ, 278 mμ and 280 mμ were measured prior to the addition of ascorbic acid, and values for the ratios

$E_{red.}/(E_{278m\mu})_{ox.}$ and $E_{280\ m\mu}/\ E_{260\ m\mu}$ were calculated.

It may be seen that the optical densities of reduced and oxidised cytochrome c differ in the ultraviolet region (See Fig. 3). Since this will effect the above ratios, a set of figures giving the appropriate correction factors were calculated so that the ratios could be corrected to the values for ferricytochrome c. This correction is appreciable for the ratio $E_{red.}/(E_{278\ m\mu})_{ox.}$ but is extremely small for $E_{280\ m\mu}/\ E_{260\ m\mu}$. Fig. 2 shows the correction factors to be applied for yeast and horse heart cytochrome c.

Estimation of protein. The method described by Lowry, Rosenbrough, Farr and Randall (1951), which uses the Folin-Ciocalteau phenol reagent, was employed.

Estimation of iron. o-phenathroline was used to estimate iron, using a modification of Sandell's (1944) method, for a final volume of 10.0 ml. and 3-10 µg. iron. With a 4 cm. cuvette and at 507.5 mµ, optical densities of 0.08/µg. Fe were obtained. The reagent blank was usually in the region 3-4 µg.Fe.

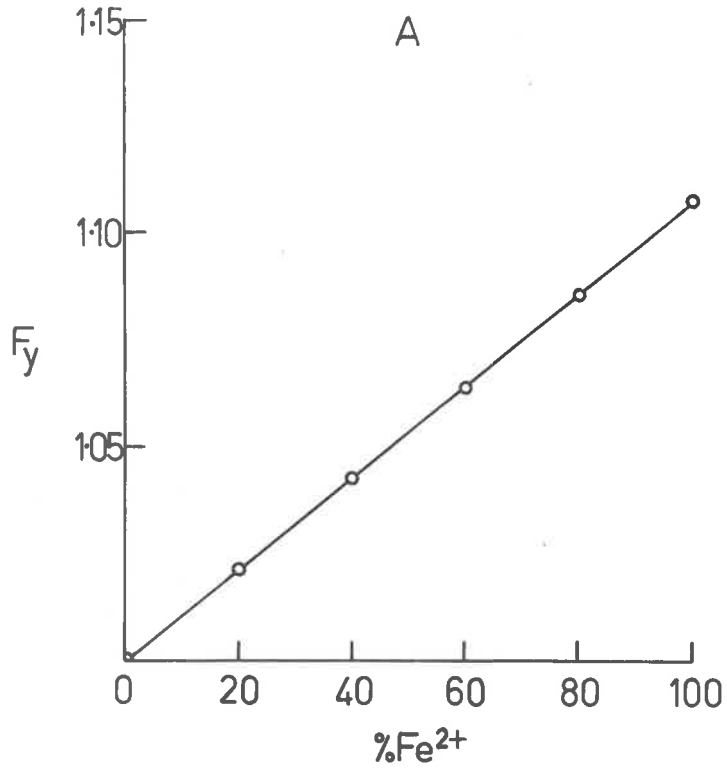
Samples containing iron in organic complexes were dried in vacuo over P_2O_5 to constant weight, using platinum micro crucibles (1-3 g. in weight). The platinum crucibles were placed in silica dishes and ashed in a muffle furnace at 600° for 3 to 4 hours. Ash weights were measured to correct for salt contamination.

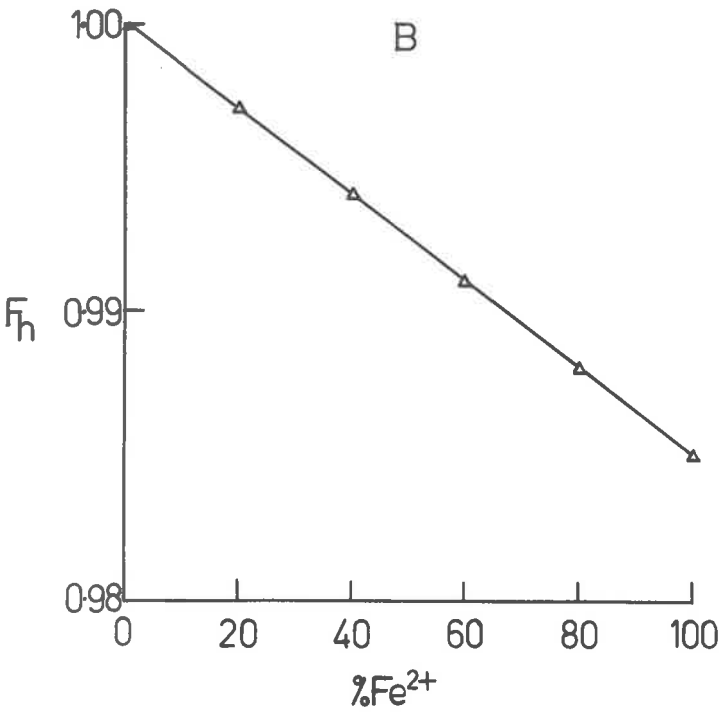
Fig. 2. Correction factors applied to $E^{red.}_{\alpha\text{-band}} / E_{276m\mu}$ for cytochrome c

The correction factor, F, was obtained from the optical density at 278 mμ for a mixture of oxidised and reduced cytochrome c, using the results shown in Figs 3 and 4. The factor, F, was multiplied by the observed value of $E^{red.}_{\alpha\text{-band}} / E_{278\ m\mu}$ to obtain the corrected value $(E^{red.}_{\alpha\text{-band}}/E_{278\ m\mu})_{corr.}$ used elsewhere in this thesis.

A. Ordinate, correction factor Fy, for yeast cytochrome; abscissa, % reduced cytochrome c.

B. Ordinate, correction factor Fh, for horse heart cytochrome c; abscissa, % reduced cytochrome c.


A
1·15
1·10
1·05
F_y
0
20
40
60
80
100
%Fe2+


B
1·00
0·99
0·98
F_h
0
20
40
60
80
100
%Fe2+

III. EXPERIMENTAL PROCEDURES AND RESULTS

A. Preparation of Cytochrome c from Baker's Yeast.

Since it was desired to compare the properties of mammalian and yeast cytochrome c, it was necessary to prepare the latter under the mildest conditions possible. This ruled out the use of trichloroacetic acid as a precipitant. Yeast cytochrome c is a by-product in the preparation of YLD and can be recovered from discarded fractions from the YLD preparation, or by further extraction of the yeast residue. It is present as ferrocytochrome c in these extracts. Some pilot experiments were carried out with residues rich in cytochrome c. These residues were the mother liquors from the initial crystallisation of YLD. Fractionation with ammonium sulphate at pH 7.0 was carried out, but cytochrome c did not precipitate even at saturation with ammonium sulphate. On standing for several days at 2°, some of the cytochrome c was precipitated. The purification achieved was approximately 2-fold. It was found that prolonged standing at -15°C in saturated ammonium sulphate resulted in loss of catalytic activity since the cytochrome c was not reduced by an active sample of YLD.

The extraction of cytochrome c from dried, finely ground yeast under various conditions of extraction was then investigated. Because of the strong absorption of the extracts at 550 mμ, it was not possible to estimate cytochrome c in the extracts reliably, but inspection with a spectroscope showed that extraction with 1M -NaCl was about as efficient as any other method for extraction of cytochrome c. On fractionation of such extracts with ammonium sulphate no precipitate

of cytochrome c was obtained at saturation and pH 5.6.

It was therefore decided to concentrate the cytochrome c by batch adsorption on IRC-50(NH_4^+) resin.

Five kg. of dried yeast was extracted with 15 l. of 1M-sodium chloride for an average period of 5 hr. The yeast residue was removed by centrifugation at 1,500 g. for 45 minutes, and re-extracted at 2° overnight. The supernatant was stored at 2°, and after centrifuging, the second extract was obtained. These were combined and had a volume of 16.1 l. after filtration through Hiflo Supercel pads.

Two One-litre portions were diluted with equal volumes of water, and were adjusted to pH 6.0 and pH 5.5 respectively. Five g. of IRC-50 resin was added to each solution, which was vigorously stirred for 30 minutes. The solution initially at pH 5.5 was maintained at that pH by the addition of HCl over the period of adsorption, while no adjustments were made to the solution initially at pH 6.0. The final pH of this solution was pH 6.45.

The resin was allowed to settle, the supernatant liquid decanted and the resin well washed with distilled water until the washings were clear. The supernatant solutions were examined with the spectroscope through a depth of liquid of about 10 cm. Adsorption of cytochrome c at pH 5.5 was virtually complete, while at pH 6.0 some cytochrome c was still detected in the supernatant.

One batch of the resin with adsorbed cytochrome c was washed with 25% saturated ammonium sulphate, pH 8.2, on a Buchner funnel. This was not efficient since a large volume of the solution was required

for elution of the cytochrome c. Subsequent washing of the resin with 0.5 N-ammonium hydroxide did not elute any further haem pigments.

The other batch of resin was made into a column and the cytochrome c was eluted with 33% saturated ammonium sulphate at pH 8. The cytochrome c moved down the column as a diffuse band and was collected in about 50 ml. of eluate. Batch adsorption of the remaining extract was carried out after dilution at pH 5.5 and the eluates were combined. Although the ratio $E_{\alpha\text{-band}}/E_{278\ m\mu}$ was 0.78, the total cytochrome c content (3.40 μmoles) and protein content (512 mg.) indicated that it was about 10% pure for a molecular weight of 15,000.

Another preparation was made from yeast but lactate was added to keep the cytochrome c reduced. After re-extraction the residue still showed a strong band in the region of 550 mμ, which was not extractable, and was probably due to cytochrome c_1.

DeCalso-F was used as adsorbent for the cytochrome c in the extract but large volumes of ~ 30% saturated $(NH_4)_2SO_4$, pH 8, were required for elution. The eluates from this and the preceding preparation were dialysed overnight against distilled water at 0 to 2° and adsorbed onto a column of IRC-50(NH_4^+), 3 x 23.5 cm. The adsorbed cytochrome c was eluted with ammonia/ammonium acetate buffer at pH 9.46. After 436 ml. of eluate had been collected, cytochrome c began to elute from the column. The estimated maximum purity obtained in the subsequent fractions was 10.8% and the maximum value of $E_{\alpha\text{-band}}/E_{278\ m\mu}$ was 0.186. A total of 1.49 μmoles was recovered, equivalent to 266 mg.

of protein and with $E_{\alpha\text{-band}} / E_{278\ m\mu} = 0.157$ (Table 2). It was decided that this was not a satisfactory chromatographic purification procedure for purification of yeast cytochrome c, and an attempt to increase the purity of the cytochrome was made, using solvent fractionation. The combined fractions from the previous experiment were dialysed overnight at 2° against 12 volumes of distilled water, adjusted to pH 7 with acetic acid and cooled in an alcohol bath. Cold acetone (-10°C) was added to a final concentration of 65% v.v.; no precipitate was formed, so magnesium acetate was added to a final concentration of ~0.002 M, but still no precipitate formed. In view of the fact that cytochrome c precipitates readily in YLD preparations* when butanol and acetone are present, n-butanol and acetone were added to a final concentration of 65% acetone, 5% butanol. No precipitate was observed, but on standing overnight at -11°, a brick-red precipitate was obtained. The precipitate was collected, suspended in 0.1 M-NaCl/0.002 M phosphate, pH 7.3, and centrifuged at 10,000 g. . A clear red supernatant was obtained which apparently contained cytochrome c, but there was a considerable amount of insoluble red material, and it was concluded that the fractionation procedure denatures cytochrome c. Therefore, no further solvent fractionations were carried out. Because yeast cytochrome c had been observed to be unstable in the presence of ammonium salts, it was decided to use sodium or potassium salts for chromatography of the cytochrome, following the general methods of Boardman and Partridge (1953).

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* It seems probable that cytochrome c is precipitated by acetone in YLD preparations as an ionic complex with nucleic acids, which are present in considerable amount in the precipitate.

Table 2

Chromatography of Yeast Cytochrome c at pH 9.46

The combined concentrates of cytochrome c were chromatographed on resin with ammonia-ammonium acetate buffer, pH 9.46. Protein was estimated according to Lowry et al. (1951). Purity was calculated for a molecular weight of 15,000.

Fraction No.	Volume (ml.)	Cytochrome c (μmoles)	Amount reduced (%)	Protein (mg./ml.)	$E_{\alpha\text{-band}}/E_{278\ m\mu}$	$E_{280\ m\mu}/E_{260\ m\mu}$	Purity %
1 - 4	435 ml.						
5	25	0.122	63	1.2	0.109	1.04	6.6
6	19.5	0.154	57	2.70	0.133	1.11	8.3
7	24	0.191	37	1.17	0.172	1.13	8.9
8	40	0.231	22	0.80	0.176	1.14	10.8
9	24.5	0.166	24	0.94	0.186	1.15	10.8
10	26	0.159	20.4	0.91	0.166	1.19	10.6
11	34	0.192	19	0.86	0.160	1.18	9.8
12	59	0.274	12.3	0.78	0.145	1.18	9.0
Bulked 5 - 12	252	1.489		1.05*	0.157*	-	8.4*

* Calculated

The yeast residues from the initial extraction for YLD preparations were re-extracted for 1-2 days at 2-3°. Various extracting solutions were used, such as 0.1 M sodium lactate pH 6.8, 0.5 M sodium chloride and 0.05 M sodium pyrophosphate, pH 9.0. The residues corresponded to 8 kg. of ground dried yeast. The diluted extracts were brought to pH 5.6-5.8 with HCl, the cytochrome c was adsorbed onto IRC-50($-NH_4^+$), and eluted from the well washed resin with 0.5 M KCl. After dialysis, a volume of about 2.2 l. of crude cytochrome c ($E_{\alpha\text{band}}/E_{278\ m\mu} = 0.29$), containing approximately 5.9 µmoles of cytochrome c was obtained. This was chromatographed on a column of IRC-50 (Na^+) essentially as described by Boardman and Partridge (1953). The first three drops of the eluting cytochrome c were not collected (Table 3A). It may be seen that a good recovery was achieved, and that the ratio, cytochrome c to protein, ($E_{\alpha\text{-band}}/E_{278\ m\mu}$) was considerably increased, indicating good purification. Fractions 2 to 9 were dialysed against distilled water at 2°, freeze-dried and dialysed again. The concentrated solution was chromatographed as before and the main fractions combined (Table 3 B). The combined cytochrome c was again chromatographed on IRC-50. It will be noted that although the ratio $E_{\alpha\text{-band}}/E_{278\ m\mu}$ improved on the second passage, it is somewhat decreased on the third passage, while the ratio $E_{280\ m\mu}/E_{260\ m\mu}$ increased on both the second and third passages. The somewhat random variation of these ratios suggests that although they are useful indicators of the purity of the cytochrome solution, these ratios are not particularly valuable in establishing absolute purity of the solution.

Table 3

Chromatography of Yeast Cytochrome g at pH 7.5

A. The crude concentrate, $E_{\alpha\text{-band}}/E_{280\ m\mu}$ = 0.29, approx. 5.9 µmoles, was adsorbed on a column, 2.5 x 21.5 cm., of IRC-50 ($-Na^+$). Elution was with 0.2M-sodium phosphate/0.14 M-NaCl, pH 7.5, (0.5 g. ions Na^+/l).

B. Fractions 2A to 9A, 5.36 µmoles, after dialysis and concentration by freeze drying. Developed with 0.2M-sodium phosphate/0.14M-NaCl, pH 7.5, (0.5 g. ions Na^+/l.), eluted with 0.2M-sodium phosphate/0.6M-NaCl, pH 7.5, (0.96 g. ions Na^+/l.).

C. Fractions 3B to 5B, 4.95 µmoles, after dialysis. Developed with 50 ml. 0.2M-sodium phosphate, pH 7.5, (0.36 g. ions Na^+/l.), followed by 30 ml. 0.2M-sodium phosphate/0.35M-NaCl, pH 7.5, (0.71 g. ions Na^+/l.) and eluted with 0.2M-sodium phosphate/0.75M-NaCl, pH 7.5, (1.11 g.ions Na^+/l.). Main fractions combined, dialysed and concentrated by freeze drying.

Fraction No.	Volume (ml.)	Cytochrome g (µmoles)	Amount reduced (%)	$E_{\alpha\text{-band}}/E_{278\ m\mu}$	$(E_{\alpha\text{-band}}/E_{278\ m\mu})$ corr.	$E_{280\ m\mu}/E_{260\ m\mu}$
A 1	15.0	0.36	33	0.46_5	0.48_1	1.02
A 2	13.5	0.80	33	0.90_0	0.93_2	1.01
A 3	16.0	1.23	31	0.92_0	0.95_6	0.99
A 4	11.0	0.85	31	0.90_0	0.93_0	0.93
A 5	12.5	0.84	42	0.89_4	0.93_4	0.92
A 6	10.0	0.68	22	0.93_5	0.95_6	0.84
A 7	8.5	0.33	38	0.97_0	1.00_9	1.00
A 8	10.0	0.28	22	0.96_5	0.98_7	1.04
A 9	20.0	0.35	11	1.03_7	1.04_9	1.27
A 10	31.5	Trace only	-	-	-	-

Table 3 contd.

Fraction No.	Volume (ml.)	Cytochrome c (μmoles)	Amount reduced (%)	$E_{\alpha\text{-band}} / E_{278\ m\mu}$	$(E_{\alpha\text{-band}} / E_{278\ m\mu})_{corr.}$	$E_{280\ m\mu} / E_{260\ m\mu}$
B3 - B5 (bulked)	24.0	4.95	23	1.05	1.07_6	1.11
$C_1 - C_4$ (batch I)	8.3*	2.65	30	0.98_4	1.01_5	1.14

* After concentration by freeze drying.

The eluate from the third passage, fractions C_1 to C_4, were combined, dialysed, freeze-dried and dissolved in water. This final solution constitutes batch I.

A sample of batch I was subjected to electrophoresis at pH 6.0 in sodium phosphate buffer, $\Gamma/2 = 0.2$, at 15 mA. and 2.5° for $4\frac{1}{2}$ hours. Unfortunately, at this time, the apparatus was fitted with interference optics, using the green mercury line, so that only gross movement of the boundary could be detected, since the light absorption by the cytochrome c was too severe to permit interference to occur. Over the $4\frac{1}{2}$ hour period the boundaries migrated nearly the full length of the cell centre section. The boundaries were then compensated back to about one third of the way up the centre section on the ascending limb and the cell was closed off. The cytochrome c in the ascending limb was removed separately from the cytochrome c in the rest of the cell. Analyses were carried out on the original solution and the two fractions from the electrophoresis (Table 4). Although the maximum iron content was obtained in the descending limb and bottom section of the electrophoresis cell, the optical purity of this fraction was lower, and it seems likely that an iron containing protein impurity may have concentrated in this fraction. However, calculation of purity in terms of dry weight of cytochrome c (from spectrophotometric data) divided by dry weight of combustible material indicates that this fraction is probably the most pure. It would seem that batch I was far from pure by chemical analysis.

Batch II was obtained from yeast residues from several YLD preparations, and was twice chromatographed on columns of IRC-50($-Na^+$),

Table 4

Analysis of Batch I Cytochrome c Before and After Electrophoresis.

	Original Sample	Ascending Limb	Descending and Bottom Limb
Cytochrome c	3.238×10^{-4} M	2.079×10^{-4} M	2.527×10^{-4} M
$(E_{\alpha\text{-band}} / E_{278\ m\mu})_{corr.}$	0.996	1.09	0.968
$E_{280\ m\mu} / E_{260\ m\mu}$	1.11	1.10	1.10
% ferrocytochrome c	9.5	0.3	6.0
Dry weight, mg./ml.	21.28	16.16	18.78
Ash weight, mg./ml.	12.03	9.52	11.98
Cytochrome c, mg./ml.	9.25	6.64	6.80
Iron, %	0.278 - 0.338	0.315	0.376 - 0.388
$\frac{\mu moles/ml. \times 15 \times 100}{mg./ml.\ cytochrome\ c}$	52.8%	47.0%	55.6

as for batch I. The final fractions ranged from values of $(E_{\alpha\text{-band}}/E_{278\ m\mu})_{corr.}$ of 0.86 to 0.97 and $E_{280\ m\mu}/E_{260\ m\mu}$ of 1.02 to 1.08. This was used for preliminary electrophoresis experiments, however, the currents used were too high and convection was observed. Batch III was prepared by recovery of the remaining cytochrome c from batches I and II, and from the crystal mother liquors from YLD preparations. This latter, after one passage through an IRC-50(Na^+) column, had values of $(E_{\alpha\text{-band}}/E_{278\ m\mu})_{corr.}$ and $E_{280\ m\mu}/E_{260\ m\mu}$ of 1.11. Batch III was used for the electrophoresis experiments described later (p. 29).

Batch IV was made by extraction of dried yeast. It was found that the yield was increased considerably (1.8 fold) by addition of the dried yeast to the extracting solution (0.5 M-NaCl or 0.1 M-sodium pyrophosphate) at a temperature of 65 to 70°. This resulted in a temperature of 58 to 65° after the addition of the yeast. After about 10 to 15 minutes, the yeast suspension was cooled by immersion of the stainless steel bucket containing the suspension in a large volume of water. About 30 to 45 minutes were required to decrease the temperature to about 30°. Four extracts were prepared in this way, corresponding to 13 kg. dried yeast, and the cytochrome c was concentrated from the extracts by batch absorption onto IRC-50(Na^+) and elution with strong salt solutions. The concentrate was dialysed and freeze dried. About 20% of the concentrated solution was retained for experiments on artifact production. The remaining cytochrome c was chromatographed on IRC-50(Na^+)(Table 5).

Table 5

Chromatography of Yeast Cytochrome c at pH 7.7

A. Crude concentrate. A column, 1.0 x 35.0 cm., of IRC-50-(Na^+) was equilibrated with 0.1M-NaH_2PO_4. Sodium phosphate buffer, pH 7.73, was used for development and elution. Cytochrome c was washed into the column with 30 ml. of buffer, 0.20 g. ions Na^+/l., spread with 20 ml. buffer, 0.38 g. ions Na^+/l., and eluted with buffer, 1.38 g. ions Na^+/l. .

B. Fractions 2 to 5, 29.32 µmoles, were dialysed against water and adsorbed onto column, 1.0 x 45 cm., of IRC-50-(-Na^+) which had been equilibrated with 0.1M-NaH_2PO_4. Cytochrome c was spread with 200 ml. 0.1M-Na H_2PO_4, followed by sodium phosphate buffer, pH 7.73, adjusted to the following strengths by dilution or addition of sodium chloride. (a) 0.19 (b) 0.38 (c) 0.69 (d) 1.38 g. ions Na^+/l. .

Fraction No.	Volume (ml.)	cytochrome c (µmoles)	Amount reduced %	$E_{\alpha\text{-band}}/E_{278\ m\mu}$	$(E_{\alpha\text{-band}}/E_{278\ m\mu})_{corr.}$	$E_{280\ m\mu}/E_{260\ m\mu}$
A						
1 (0.38 g. ions Na^+/l.)	26.0	4.36	75.6	0.88	-	1.16
2 (0.69 g. ions Na^+/l.)	20.0	12.98	98.0	0.23	-	1.00
3	11.6	12.31	97.2	0.58	-	0.94
4	8.5	4.03	97.3	0.50	-	0.92
5	9.0	Trace	-	-	-	-
6	10.5	Trace	-	-	-	-
Total		33.68				

Table 5 contd.

Fraction No.	Volume (ml.)	cytochrome c (μmoles)	Amount reduced %	$E_{\alpha\text{-band}}/E_{278\ m\mu}$	$(E_{\alpha\text{-band}}/E_{278\ m\mu})$ corr.	$E_{280\ m\mu}/E_{260\ m\mu}$
B						
1	20	3.75	77.0	0.935	1.01	1.17
2 (b)	20	3.09	41.9	1.075	1.12	1.17
3 (c)	10	1.31	54.4	1.00	1.06	1.17
4 (c)	10	9.53	78.3	1.128	1.22	1.17
5 (c)	10	3.64	74.9	1.063	1.15	1.10
6 (d)	10	1.32	70.2	1.012	1.09	1.17
	Total	22.64				

Fractions 2 to 6 stored. Batch IV.

For comparision, the chromatographic behaviour of a sample of horse heart cytochrome c (Sigma Chemical Co., U.S.A.) is given in Table 6 . A considerable amount of red-brown coloured material remained adsorbed on the resin at the top of the column, while a pale pink coloured fraction eluted as a separate band well ahead of the main, brick-red band.

It is felt that the ratio $E_{\alpha\text{-band}} / E_{278\ m\mu}$ is not a reliable indication of purity and is probably only a rough guide to the progress of purification, more particularly at high levels of purity. Paleus and Theorell (1957) comment on the inadequacy of this ratio for following the purification of cytochrome c .

Table 6

Chromatography of Sigma Horse Heart Cytochrome c at pH 7.5

Horse heart cytochrome c, 146 mg. ($E_{\alpha\text{-band}}/E_{278\ m\mu}$ = 0.82) was dissolved in 5.0 ml. of sodium phosphate buffer, pH 7.5, (0.1 g. ion Na^+/l.), adsorbed onto a column, 2.5 x 21 cm., of IRC-50 (Na^+) previously equilibrated with sodium phosphate buffer, pH 7.5, + NaCl (0.50 g. ions Na^+/l.). Elution was carried out with equilibration buffer, 1 ml./35 seconds.

Fraction No.	Volume (ml.)	Cytochrome c (μmoles)	Amount reduced (%)	$E_{\alpha\text{-band}}/E_{278\ m\mu}$	($E_{\alpha\text{-band}}/E_{278\ m\mu}$) corr.	$E_{280\ m\mu}/E_{260\ m\mu}$
1	2.0	0.27	19.9	1.07	1.07	1.10
2	5.1	1.91	14.4	1.13	1.13	1.11
3	4.9	2.10	11.3	1.14	1.14	1.11
4	5.0	1.25	5.6	1.22	1.22	1.11
5	5.2	0.45	22.5	1.17	1.16	1.09
6	5.0	0.11	29.9	1.19	1.18	1.33

B. Chemical Properties of Yeast Cytochrome c

1. Iron content.

Several unsuccessful attempts have been made to obtain reproducible iron analyses for horse heart and yeast cytochrome c; dry ashing in a furnace was used to obtain the iron in an inorganic form. Unfortunately, the temperature control of the furnace was not good and at stages during the ashing the temperature rose to in excess of 800°C. This resulted in the loss of iron from standard samples such as haemoglobin which were ashed at the same time. The iron loss was due either to volatilisation, probably as halide, or by cooking into the platinum ware used for the ashing. It is possible that the iron analysis obtained in the samples in Table 4 was unsatisfactory, although the temperature remained in the region of 600°.

2. Spectrum.

Spectra of reduced and oxidised cytochrome c are shown in Figs. 3, 4 . To obtain oxidised cytochrome c, the sample was treated with potassium ferricyanide in about 10-fold excess, adsorbed onto a small column of IRC-50(Na^+), washed with dilute buffer until the washings were colourless, and eluted with strong buffer. Reduced cytochrome c was obtained in the same manner, using an excess of neutralised ascorbic acid as reductant. Since dehydroascorbate has a high absorption at 305 mμ, its presence is easily detected. Attempts to keep the cytochrome c fully reduced by addition of lactate and YLD to

Fig. 3. Spectra of oxidised and reduced yeast cytochrome c

For conditions, see text (p.27); ——, oxidised cytochrome c; - - - -, reduced cytochrome c. In 0.1 M-sodium phosphate buffer, pH 7.73.

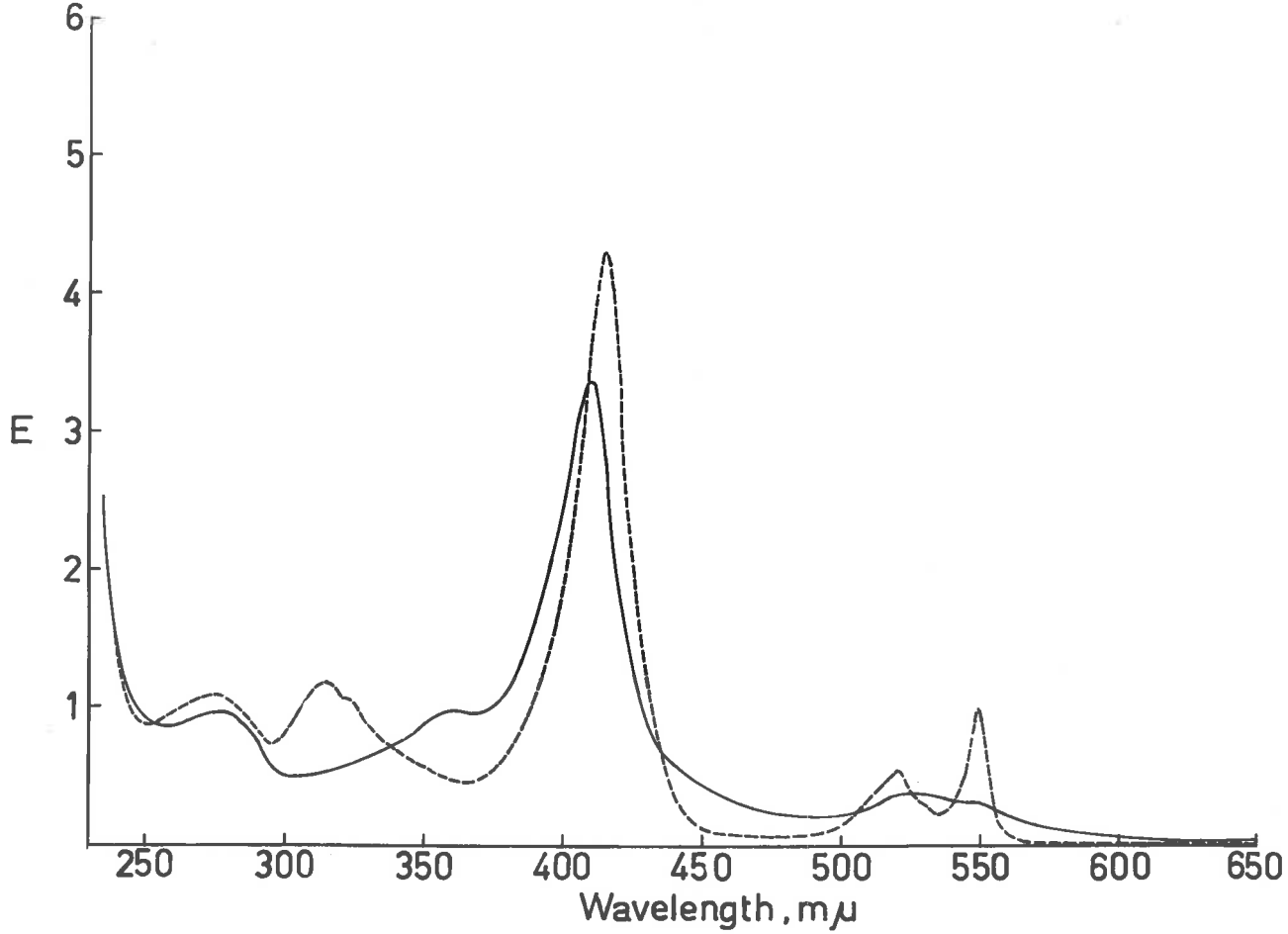
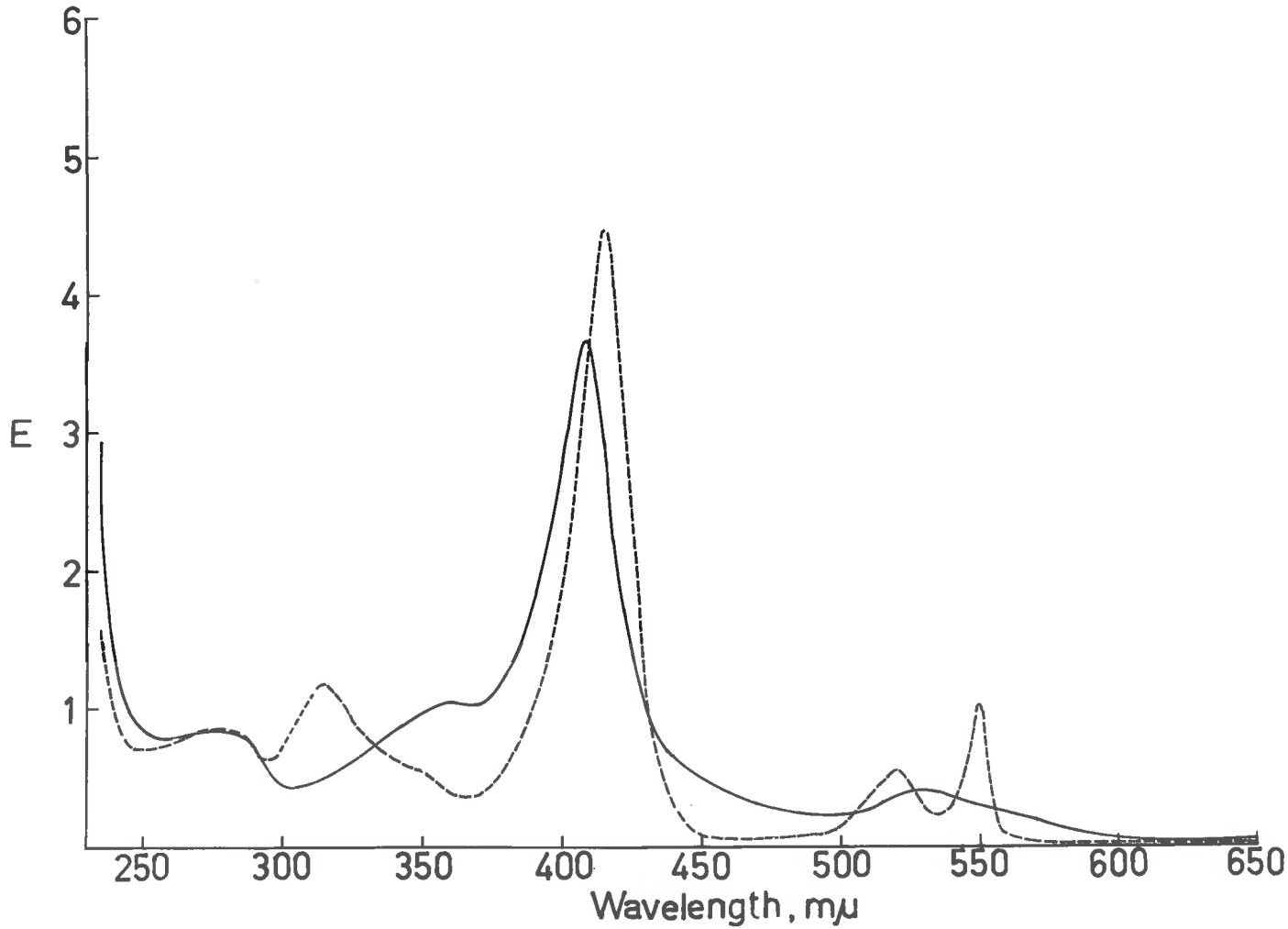

6
5
4
E 3
2
1
250
300
350
400
450
500
550
600
650
Wavelength , mμ

Fig. 4. Spectra of oxidised and reduced horse-heart cytochrome c

For conditions, see text (p.27); ——, oxidised cytochrome c; - - - -, reduced cytochrome c. In 0.1 M-sodium phosphate buffer, pH 7.20.


6
5
4
E 3
2
1
250
300
350
400
450
500
550
600
650
Wavelength, mμ

both blank and cytochrome c were no more successful than those obtained by use of ascorbate and chromatography. The best reduction achieved by either of these methods was 97%.

The positions of the absorption maxima for oxidised and reduced cytochrome c from yeast and from horse heart are given in Table 7. It may be seen that the maxima of the α, γ and δ bands for yeast ferrocytochrome c occur at slightly lower wavelengths than for horseheart ferrocytochrome c. The spectrum of horse heart cytochrome c differs from that obtained by Margoliash (quoted in Keilin and Slater, 1953) in the u.v. region. Margoliash found that the extinction of ferrocytochrome c was considerably higher than that of ferricytochrome c in this region. As may be seen, whilst this is true for yeast cytochrome c, it is not the case for the sample of horse heart cytochrome c used in this instance.

3. Stability.

When yeast cytochrome c was mixed with silver sulphate and acetic acid (Paul, 1950), a rapid change in colour was observed. The same colour change took place with horse heart cytochrome c, but very much more slowly. This colour change is presumably related to the splitting of the prosthetic group from the protein, since it is associated with the disappearance of the cytochrome c spectrum and the appearance of a three-banded spectrum in the visible region, which has a characteristic absorption band in the red at about 615 mμ. This would suggest that yeast cytochrome c has a rather less stable molecular

Table 7

Absorption Maxima for Cytochrome c

Results are given for phosphate buffer at pH values between pH 7.0 and pH 7.8. For the reduced form, corrections have been made for the amount of oxidised cytochrome c present, and where possible (i.e. α to Soret band) an excess of ascorbate was added to obtain complete reduction. Figures in brackets show the relative extinctions of the bands at the maxima.

Yeast		Horse Heart	
Oxidised	Reduced	Oxidised	Reduced
278 mμ (0.965)	276 mμ (1.080)	278 mμ (0.838)	274 mμ (0.847)
361 mμ (0.968)	314 mμ (1.173)	360 mμ (1.051)	315 mμ (1.178)
409.5 mμ (3.360)	414.7 mμ (4.295)	408.5 mμ (3.660)	415.4 mμ (4.470)
526 mμ (0.378)	520.5 mμ (0.544)	529.5 mμ (0.404)	520.2 mμ (0.551)
	549.3 mμ (1.000)		549.8 mμ (1.000)

$E_{\alpha\text{-band}} / E_{278m\mu_{(ox.)}} = 1.03_6$ $\qquad$ $E_{\alpha\text{-band}} / E_{278m\mu_{(ox)}} = 1.19_3$

configuration than horse heart cytochrome c, and that the haem thioether linkages (Hill and Keilin, 1930; Theorell, 1938, 1939) are much more accessible in the yeast cytochrome.

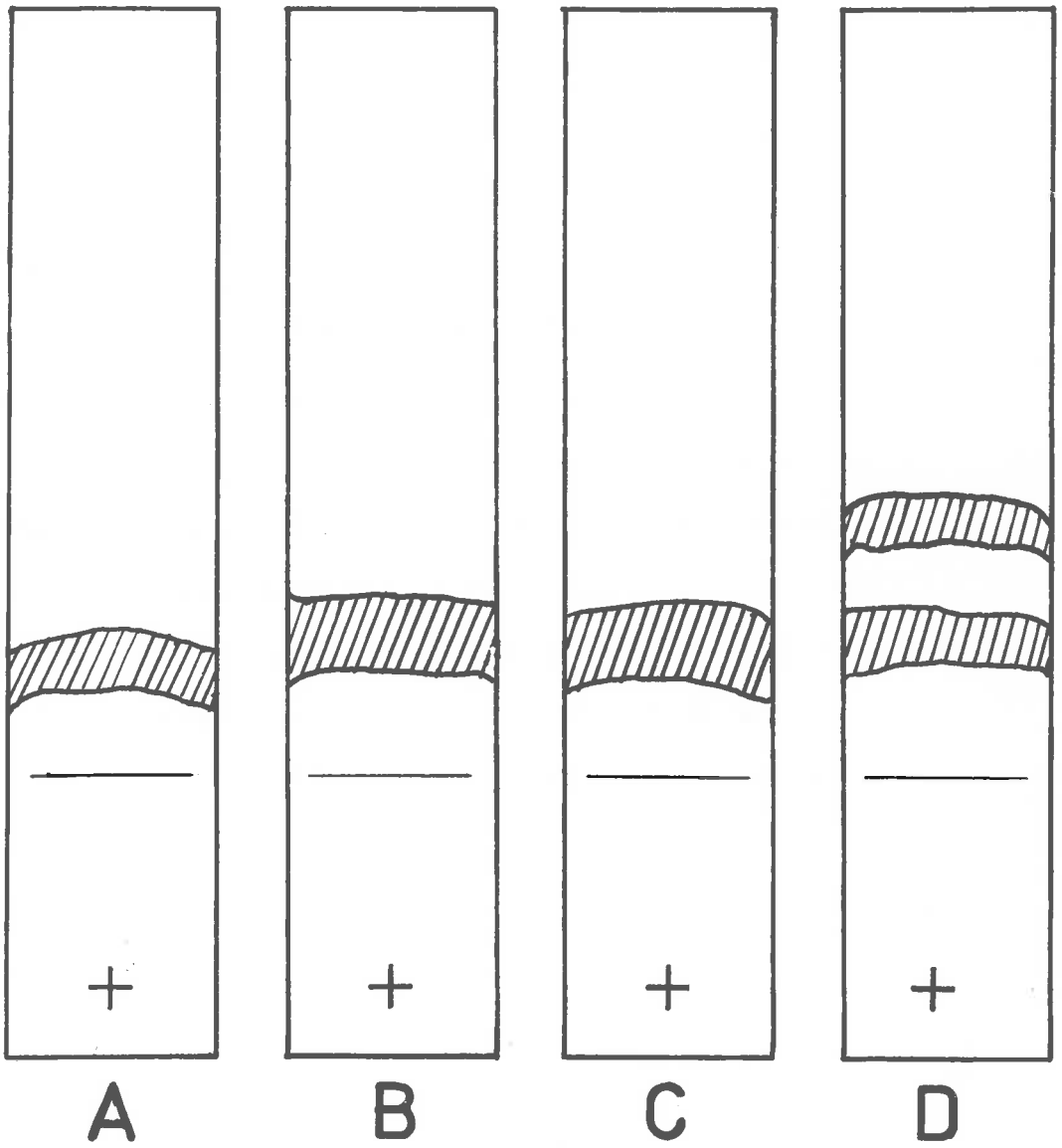
In view of the two cytochrome c components obtained from yeast by Li and Tsou (1956) and by Nunnikhoven (1958), where trichloroacetic acid was used as a precipitant for the cytochrome c in both cases, some experiments were carried out with a partially purified sample from batch IV to see whether such components could be obtained. Paper electrophoresis in veronal buffer at pH 8.5, $\mu/_2 = 0.1$, was used to test for the formation of new components. Heating in boiling water bath for several minutes, and treatment with ammonium sulphate to 90% saturation had no effect, but on one occasion a new electrophoretic component was observed after precipitation of cytochrome c from 90% saturated ammonium sulphate by addition of trichloroacetic acid (Fig. 5). This new component moved more rapidly than the untreated cytochrome c. Although this result could not be reproduced, it would seem to confirm the fact that yeast cytochrome c is much more readily affected by trichloroacetic acid than heart muscle cytochrome c, although artifact forms can be obtained from the latter by treatment with trichloroacetic acid. (Margoliash, 1954; Mizushima, Nozaki, Horio and Okunuki, 1958). The latter workers have found that yeast cytochrome c is very readily modified by a number of treatments.

C. Electrophoresis of Cytochrome c

Theorell and Åkeson (1941) carried out electrophoresis of beef heart cytochrome c in buffers of ionic strength, 0.1, and observed in

Fig. 5. Formation of artifact from yeast cytochrome c by treatment with trichloroacetic acid

Samples of yeast cytochrome c were treated as follows. (A) no treatment, (B) heated 100° for 2 mins., (C) ammonium sulphate added to 90% saturation, (D) precipitate obtained after addition of ammonium sulphate to 90% saturation and trichloroacetic acid to a final concentration of 0.8%. Electrophoresis was carried out in veronal buffer, pH 8.0, $\Gamma/2 = 0.05$ on Whatman 3 MM paper. The strips were stained with bromphenol blue.


+
+
+
+
A
B
C
D

some cases that the initial single boundary degenerated into a number of spiked boundaries. Tint and Reiss (1950) observed this phenomemom with 0.1 ionic strength buffers, and found that it could be overcome by increasing the ionic strength to 0.15-0.2. They attributed the boundary break-up to convection. For this reason, buffers of ionic strength 0.2 were used with yeast cytochrome c. About 10 to 20 mg. of ascorbic acid was added to the buffers ($\sim$ 500 ml.) to reduce the cytochrome c.

Preliminary experiments were carried out at a current of 15 mA., which according to the manufacturers, was within the range of heat dissipation which would not cause convection. However, at this current, spiked convection boundaries appeared within one to two hours. Calculation showed that the energy dissipation in the cell at 15 mA was 0.28 watts/cc., which according to Alberty (1951), is too great a heat dissipation in the cell for convection-free electrophoresis, even at low temperatures. It was calculated that a current of 11 mA. resulted in an energy dissipation of 0.15 watts/cc., the maximum permissable value, and accordingly all subsequent experiments were carried out at currents smaller than this (5 to 8 mA.).

The position of the boundary was measured at the maximum ordinate rather than the centroidal ordinate. Although the use of the maximum ordinate for measuring the boundary position introduces an error in the mobility determinations, the computation involved in determining the centroidal ordinate of the gradient curve is tedious and time consuming, so that for moderately symmetrical boundaries the use of the maximum ordinate is permissable if some sacrifice in accuracy is acceptable. For

several typical well spread boundaries which were obtained for cytochrome c, the error introduced by using the position of the maximum ordinate instead of the position of the centroidal ordinate was less than 2%. The electrophoretic behaviour of yeast cytochrome c at various pH values is given in Table 8. For comparison, this data is plotted, together with the data for beef heart cytochrome c obtained by Theorell and Åkeson (1941) and by Tint and Reiss (1950) (Fig. 6). A maximum error of 5% was assumed for the mobility values of the beef cytochrome. A smooth curve, fitted by visual inspection, was drawn for both cytochromes, since there seemed no justification for any other procedure. It will be noted that the mobility of beef heart cytochrome c at acid pH values is higher at ionic strength 0.1 than at ionic strength 0.2, but that the differences are much smaller at neutral and alkaline pH values, where the mobility is smaller. This is to be expected as increase in ionic strength decreases the mobility of protein molecules.

Tint and Reiss (1950) claimed that a plateau region exists in the pH-mobility curve of all the animal cytochrome c's which they examined, occurring in the pH region of the histidine ionisation. However, they have insufficient data to demonstrate this convincingly and probably place overmuch reliance on the accuracy of the mobility values obtained. Titration curves for most of the cytochromes which they examined indicate that from 3 to 4 equivalents are titrated in this region (Paleus, 1954), so that it seems highly unlikely that the net charge of the cytochromes would be unaffected by such changes, which occur above the range where carboxyl ionisations might be expected. It is also necessary to

Table 8

Electrophoretic Mobilities of Cytochrome c from yeast in Various buffers.

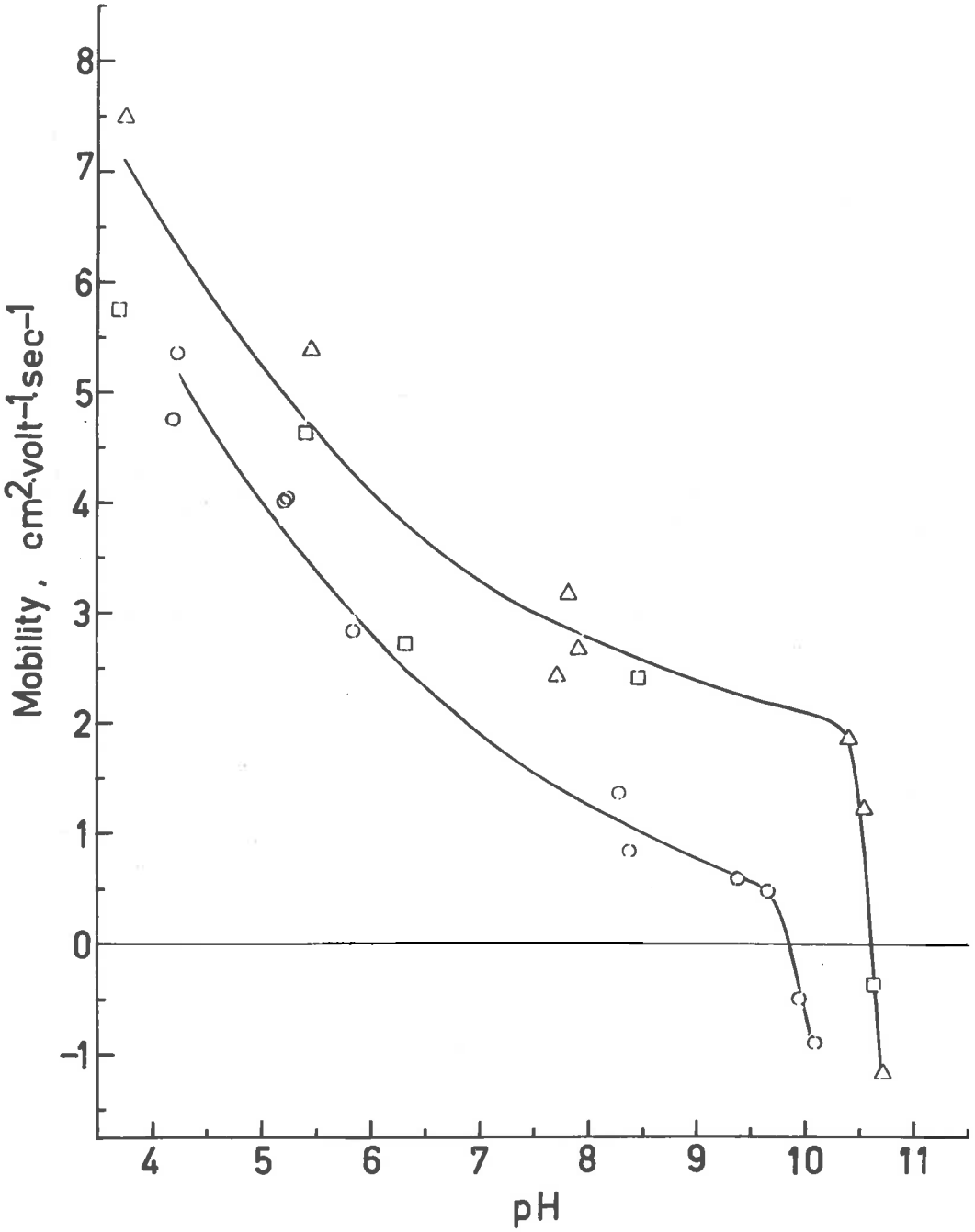
pH	4.18	4.21	5.19	5.20	5.83	8.32
mobility ($cm.^2\ v.^{-1}\ sec.^{-1}$)	+4.76	+5.37	+4.02	+4.04	+2.84	+1.37
Buffer	acetate$^-$ NaCl	acetate$^-$ NaCl	acetate$^-$ NaCl	acetate$^-$ NaCl	cacodylate$^-$ NaCl	veronal$^-$ NaCl
$\Gamma/_2$	0.2	0.2	0.2	0.2	0.2	0.2
Current (mA)	5	5	5	6	6	7
Duration (hr.)	8.0	8.5	8.0	7.5	5.0	10.5
Temperature	2.8°	2.8°	2.6°	2.8°	3.2°	2.6°

pH	8.37	9.39	9.68	9.93	10.10
mobility ($cm.^2\ v.^{-1}\ sec.^{-1}$)	+0.84	+0.57	+0.48	-0.50	-0.79
Buffer	veronal$^-$ NaCl	glycine$^-$ NaCl	glycine$^-$ NaCl	glycine$^-$ NaCl	glycine$^-$ NaCl
$\Gamma/_2$	0.2	0.2	0.3	0.2	0.2
Current (mA.)	7.0	7.0	8.0	6.0	8.0
Duration (hr.)	12.0	7.3	6.5	3.0	6.3
Temperature	3.2°	2.8°	3.2°	2.9°	3.2°

Fig. 6. pH-mobility curves for beef-heart and yeast cytochrome c

Upper curve for beef cytochrome c; results of Theorell and Åkeson, (1941) Δ, $\Gamma/2 = 0.1$, and Tint and Reiss (1950), □, $\Gamma/2 = 0.2$.

Lower curve for yeast cytochrome c, O, $\Gamma/2 = 0.2$


Mobility, cm^2-$volt^{-1}$-sec^{-1}
8
7
6
5
4
3
2
1
0
-1
4
5
6
7
8
9
10
11
pH

consider the likelihood of specific ion effects, since protein interaction with certain ions is a well established phenomenon (Alberty, 1951), and the nature of the buffer salts used can have a marked effect on protein mobility.

The pH at which the cytochrome c has zero mobility (i.e. the iso electric point) differs considerably between the yeast and beef heart cytochromes. It was found that the position of this intercept was relatively unaffected by the manner in which the line passing through the points on either side of zero mobility was drawn. The iso-electric point for yeast cytochrome c in glycine buffers at an ionic strength of 0.2 at 0° was estimated to occur at pH 9.85 ± 0.05. This is considerably lower than that for beef heart cytochrome c at 0° (pH 10.65), and this is consistent with the lower lysine and arginine content and higher aspartic acid and glutamic acid content of the yeast cytochrome (Nunnikhoven, 1958; Leaf, Gillies and Pirrie, 1958). Although the iso-electric point of proteins is affected by ionic strength (Alberty, 1951), the effect of ionic strength between 0.1 and 0.2 would probably be small. This seems to be borne out by the data for beef heart cytochrome c; therefore it is probable that a valid comparison can be made between the yeast and beef heart cytochromes with respect to iso-electric pH.

At most pH values, only a single migrating boundary was observed, however at pH 8.37, 9.39 and 9.68, a small boundary, migrating to the anode, was observed to separate from the main component, but only in the ascending limb. It is possible that this represents a minor component,

however, it disappeared within one hour and no such boundary was observed in the descending limb. Only occasionally were δ boundaries observed, and ε boundaries were never seen.

The boundaries obtained were never completely symmetrical about the maximum ordinate, and it is uncertain as to whether this was due to heterogeneity or whether it arose during boundary formation and compensation. (Fig. 7 A-F).

The technique of reversible boundary spreading at the iso-electric point has been used to detect heterogeneity by electrophoresis. The rationale of this method is as follows. At the iso-electric pH, spreading of the boundary for a homogenous ampholyte will be due to diffusion alone, and will therefore be unaffected by the polarity of the cell. If, however, the system is heterogeneous with respect to iso-electric pH, then spreading of the boundary at the average iso-electric pH will be due both to diffusion and electrophoretic migration, and will be affected by the polarity of the cell. If the polarity of the cell is reversed after some spreading of the boundary has occurred, the boundary will sharpen for a heterogeneous system because of the reversal of the direction of migration of the components not at their iso-electric pH. It seems questionable as to whether this is a completely valid test for electrophoretic heterogeneity, since migration of buffer ions in the electric field results in small pH changes in the solution, so that maintaining the system at its iso-electric pH may be impossible.

In the experiment carried out with yeast cytochrome c at pH 9.93,

Fig. 7A. Electrophoresis of yeast cytochrome c at pH 5.19

→ Ascending		← Descending
1	15 mins	2
3	75 mins	4
5	134 mins	6
7	434 mins	8

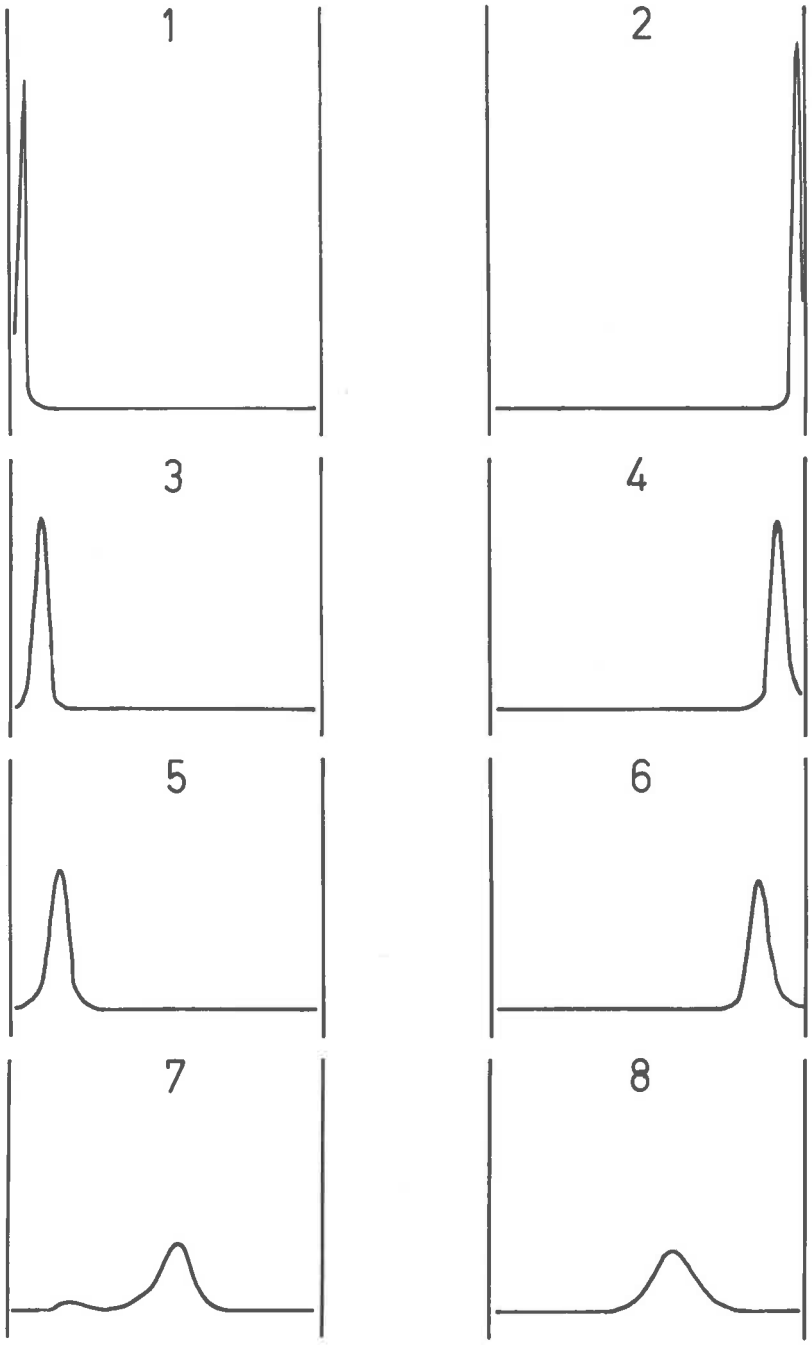

1
2
3
4
5
6
7
8

Fig. 7B. Electrophoresis of yeast cytochrome c at pH 5.83

→ Ascending		← Descending
1	30 mins	2
3	120 mins	4
5	210 mins	6
7	330 mins	8

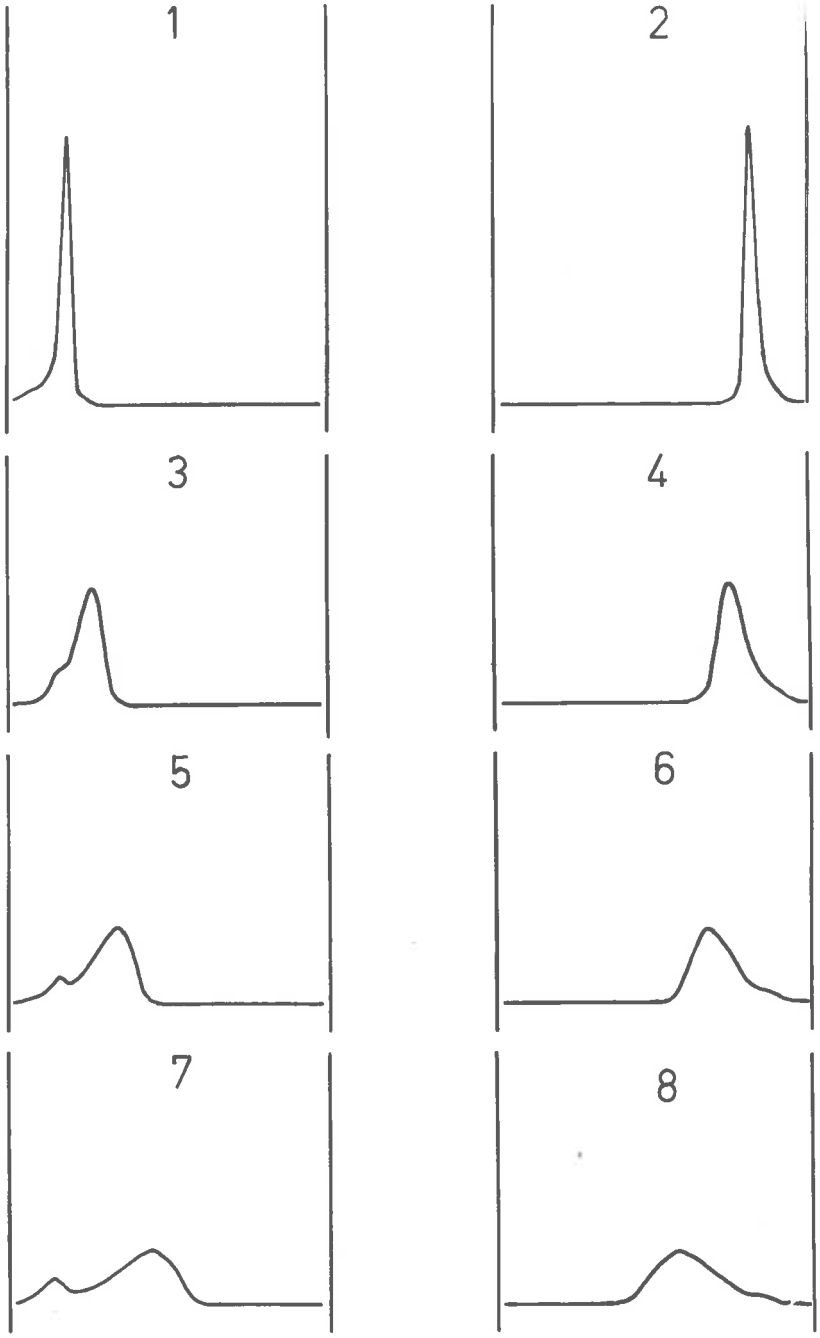

1
2
3
4
5
6
7
8

Fig. 7C. Electrophoresis of yeast cytochrome c at pH 8.32

→ Ascending		← Descending
1	0 mins	2
3	90 mins	4
5	210 mins	6
7	390 mins	8

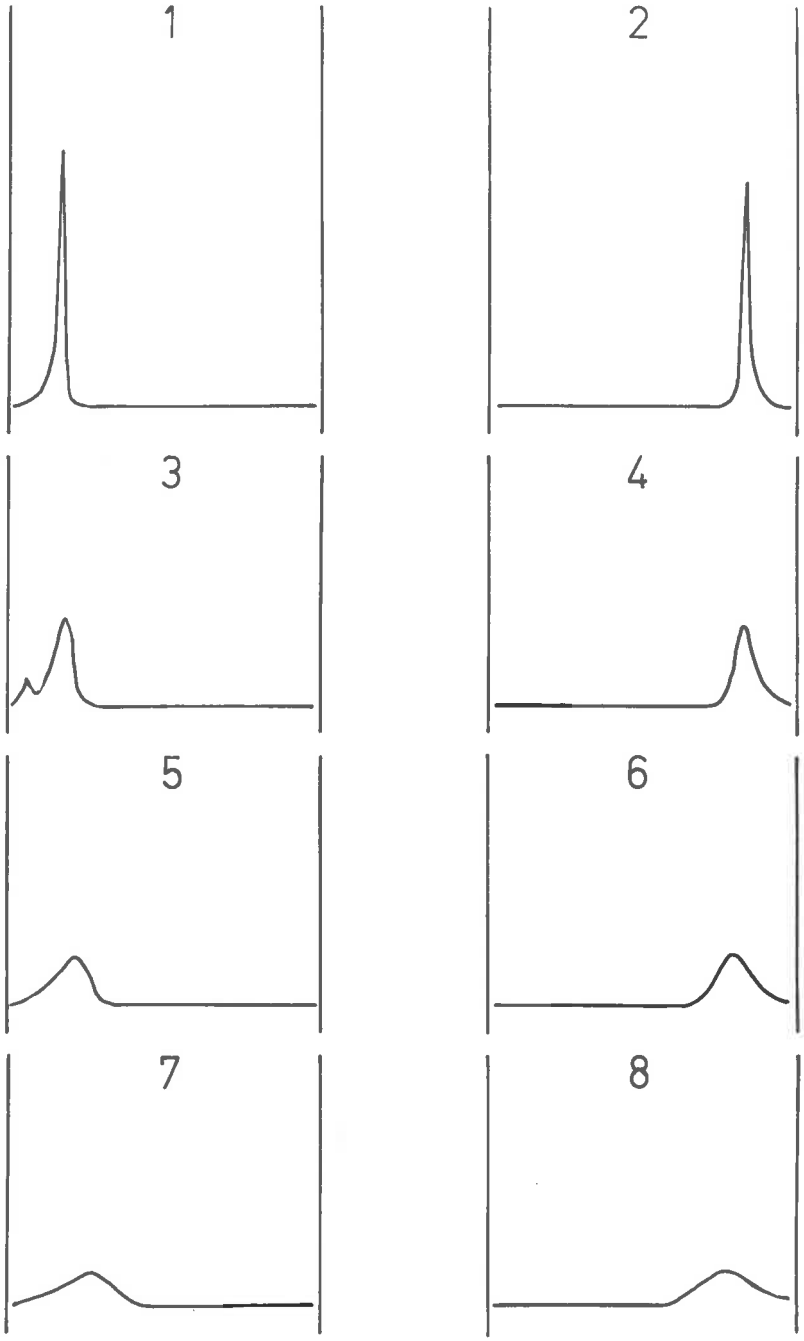

1
2
3
4
5
6
7
8

Fig. 7D. Electrophoresis of yeast cytochrome c at pH 9.39

→ Ascending		← Descending
1	0 mins	2
3	75 mins	4
5	200 mins	6
7	375 mins	8

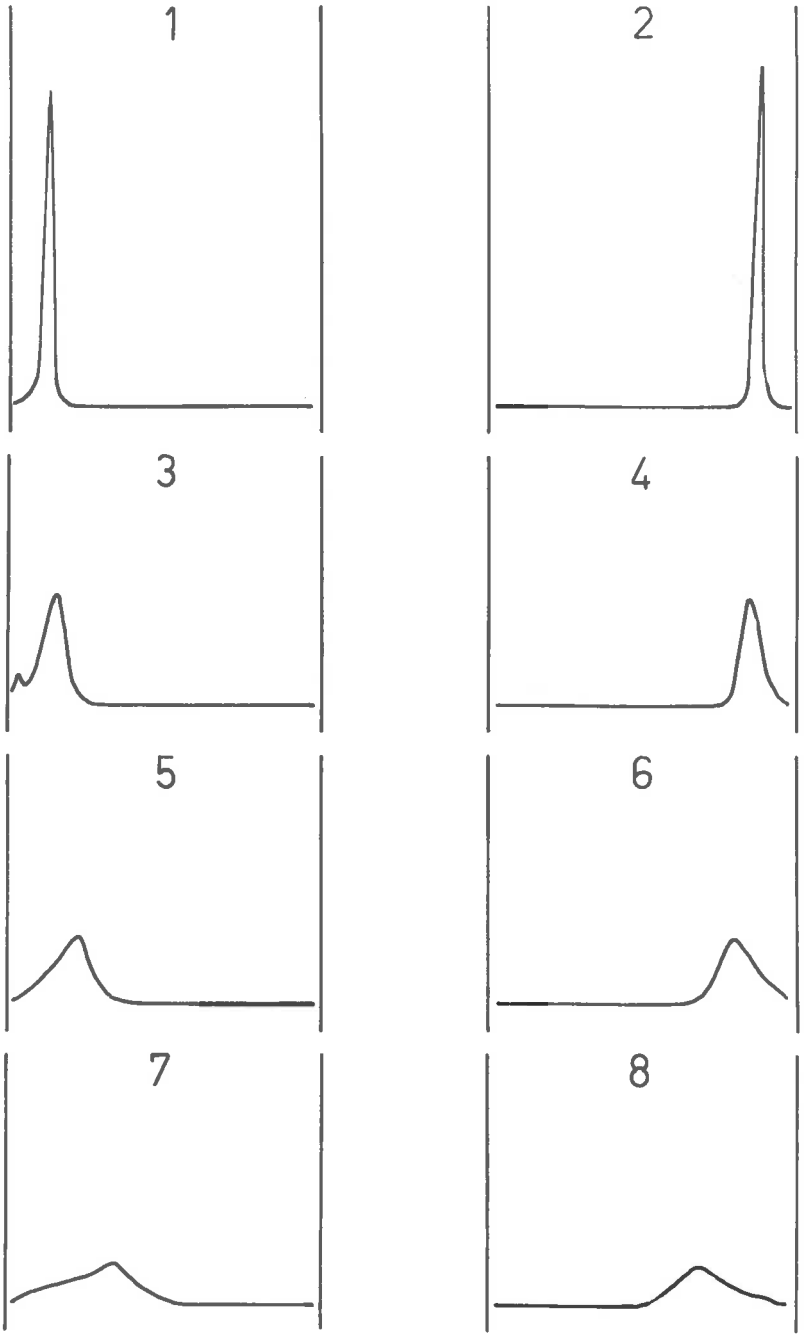

1
2
3
4
5
6
7
8

Fig. 7E. Electrophoresis of yeast cytochrome c at pH 9.68

→ Ascending		← Descending
1	0 mins	2
3	65 mins	4
5	190 mins	6
7	250 mins	8

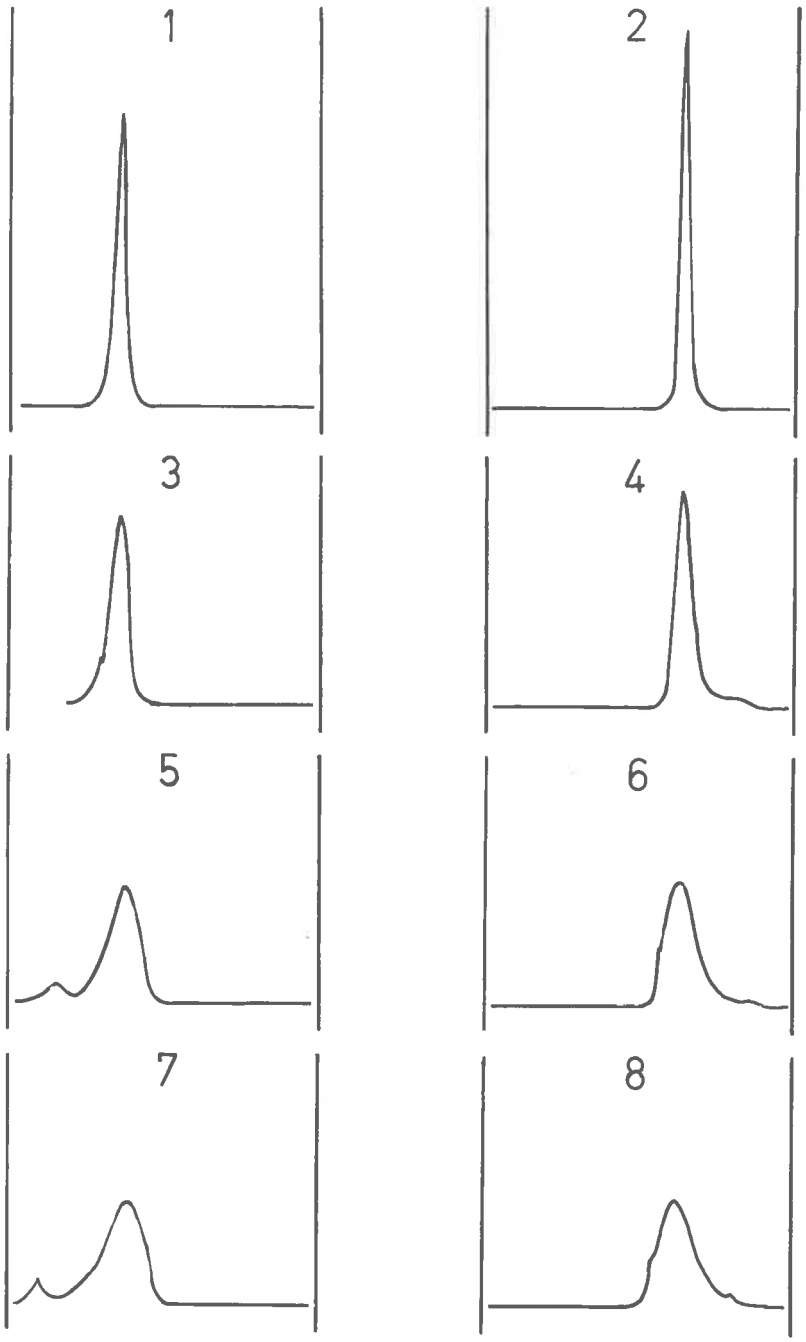
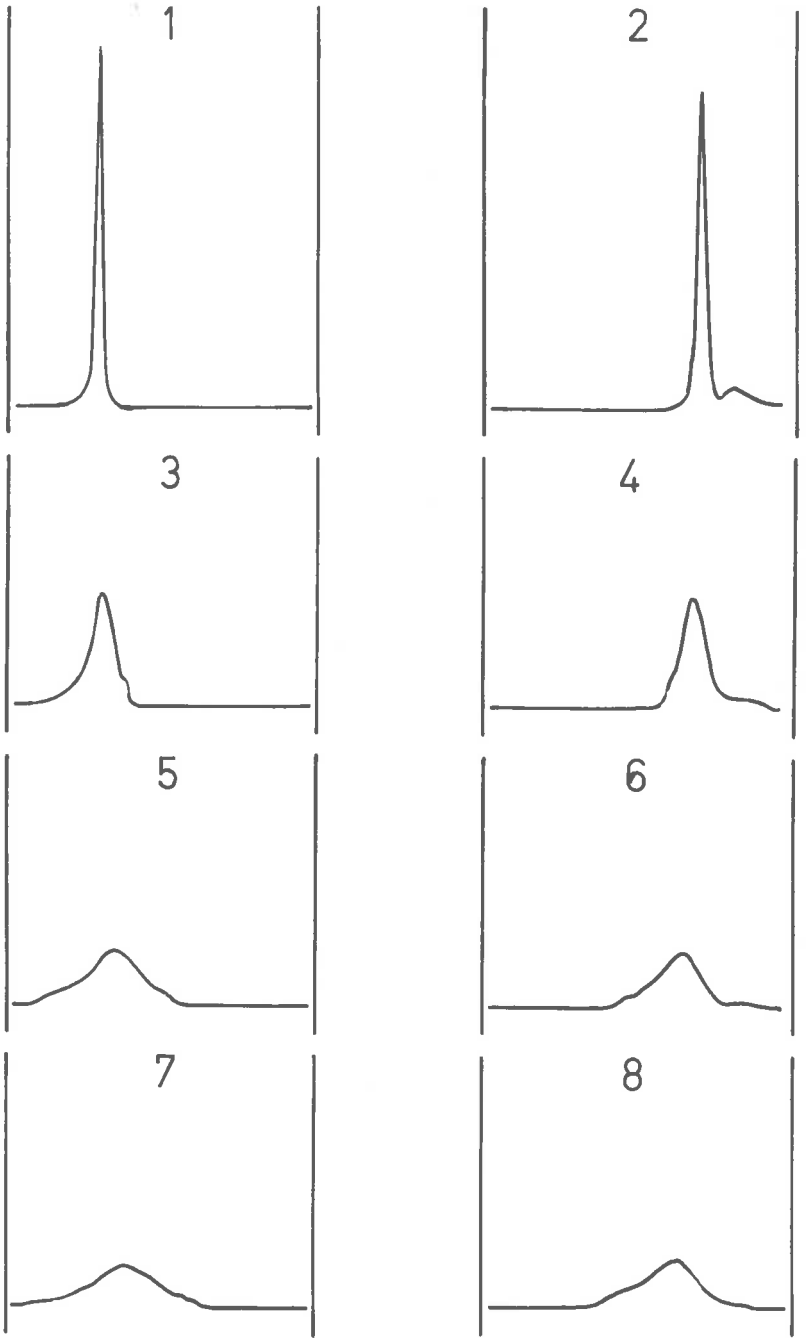

1
2
3
4
5
6
7
8

Fig. 7F. Electrophoresis of yeast cytochrome c at pH 10.10

→ Ascending		← Descending
1	0 mins	2
3	80 mins	4
5	200 mins	6
7	260 mins	8


1
2
3
4
5
6
7
8

the cell polarity was reversed after 180 minutes. Movement of the maximum ordinate of both boundaries from 180 minutes to about 280 minutes was negligible, which suggests that the solution pH was sufficiently altered, at least in the region of the boundaries, to approach the iso-electric pH. Over this time period, sharpening of the boundaries was observed. The trailing side of both boundaries (after reversal), sharpened considerably and a small spike was observed to move towards the leading side of the boundary. (Fig. 8). Although this experiment was not specifically designed for testing heterogeneity by reversible boundary spreading, the results obtained suggest that, at the time of reversal of cell polarity, the pH, in the region of the boundaries at least, was very nearly the iso-electric value. The observed sharpening of the boundaries, and the small migrating component therefore indicate electrophoretic heterogeneity of yeast cytochrome c, provided that reversible boundary spreading is a valid test. It would not be altogether surprising that some other charged component should be present in cytochrome c purified by chromatography alone, since the purification is based on charge differences at one pH, so that it may be concluded that these preparations of yeast cytochrome c contained a contaminant of similar charge which was not separated from the main component by chromatography.

D. Sedimentation of Cytochrome c

Because of the small sedimentation coefficients reported for cytochrome c (see Table I, C), it was decided to carry out sedimentation velocity experiments using a synthetic boundary cell. This cell creates

Fig. 8. Electrophoresis of yeast cytochrome c at pH 9.93

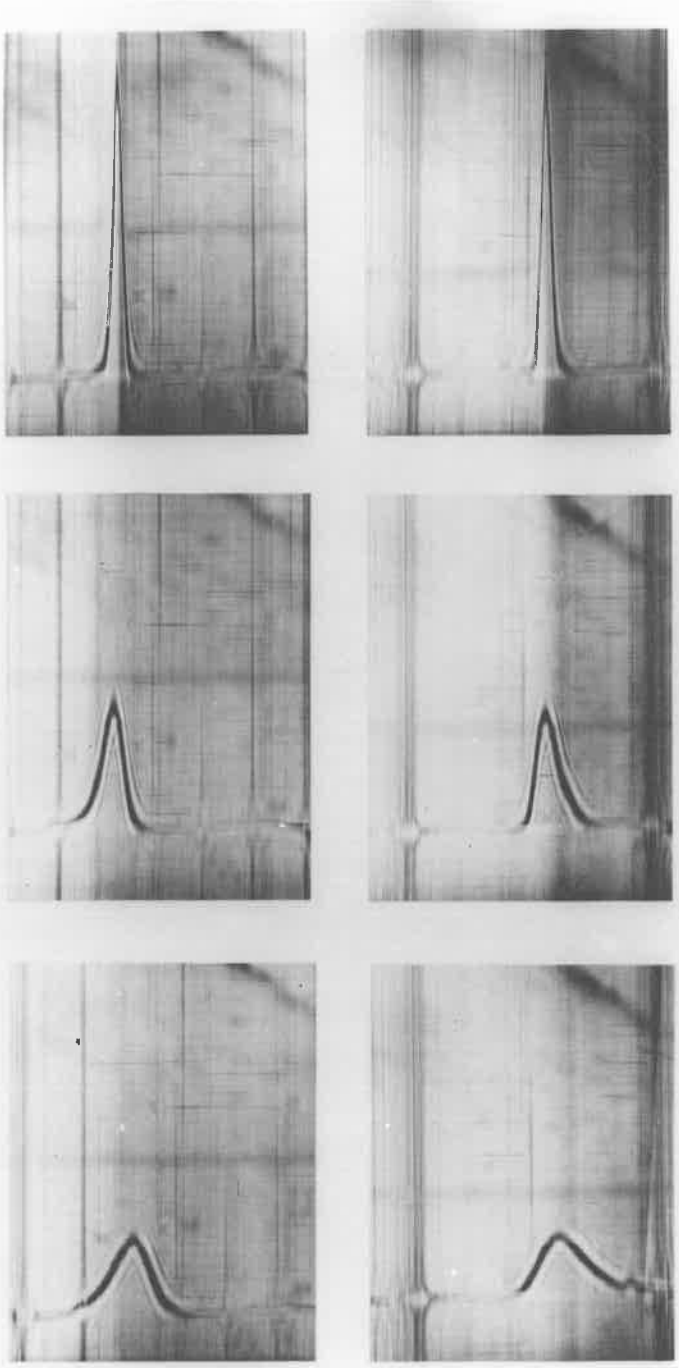
A.

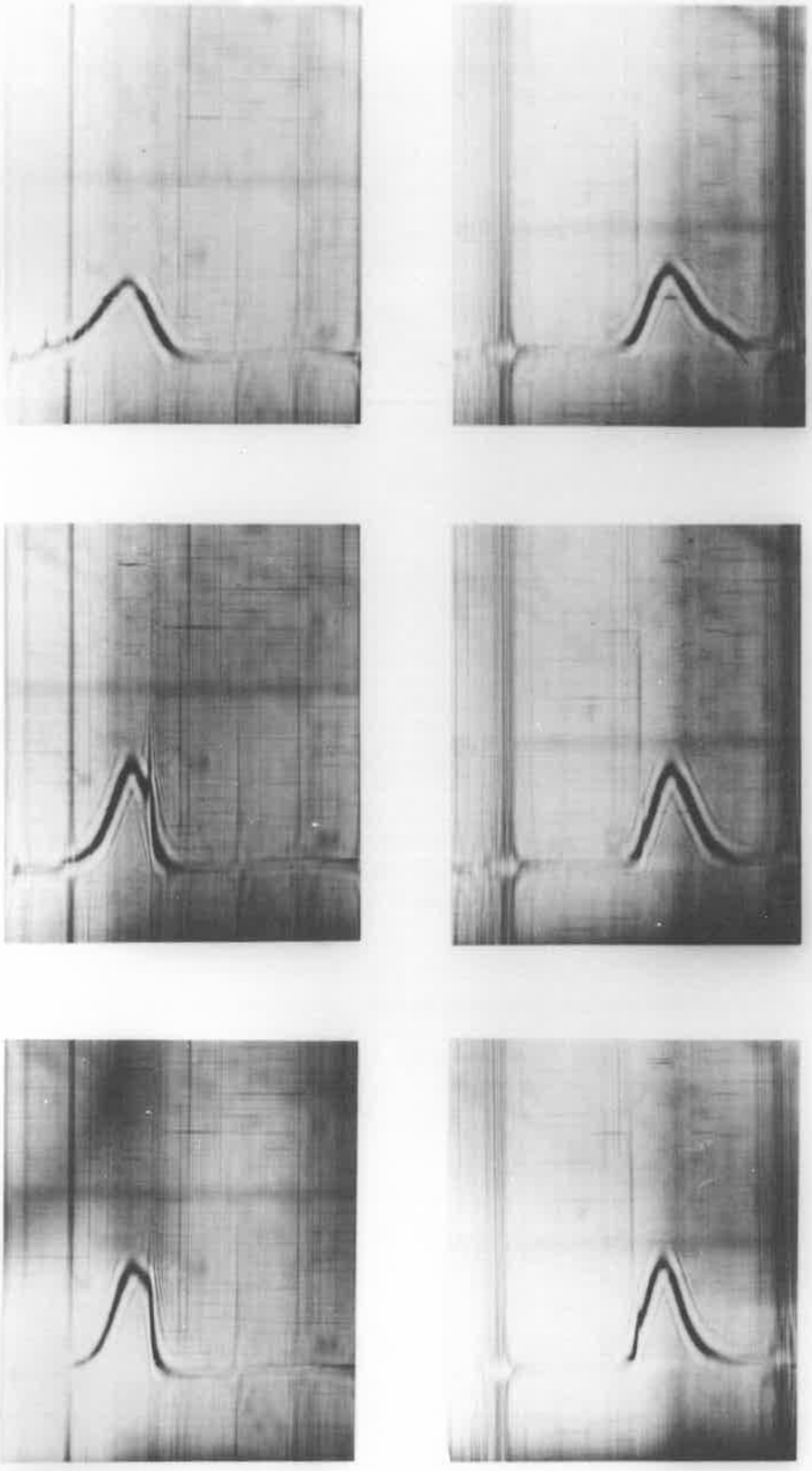
Cathodic ⟶	Anodic ⟵
"Ascending"	"Descending"
0 mins	0 mins
120 mins	120 mins
180 mins	180 mins

B.

cell polarity reversed at 180 mins.

Anodic ⟵	Cathodic ⟶
"Ascending"	"Descending"
240 mins	240 mins
300 mins	300 mins
335 mins	335 mins





an artificial boundary between solution and solvent, relying on a centrifugally operated valve to layer solvent over the solution. The artificial boundary is stabilised by the density difference between solvent and solution, which is magnified greatly by the centrifugal field, thus permitting the layering to occur with the minimum of disturbance. This enables the rate of sedimentation of small molecules to be measured easily by moving boundary methods. Sedimentation velocity experiments were carried out in buffers of ionic strength 0.2, at pH 5.3, pH 6.0 and pH 9.8, at temperatures close to 20°. The plots of log x vs. time (where x is the radial distance of the maximum ordinate of the boundary) were straight lines. The sedimentation coefficients varied considerably (Table 9). As may be seen in Fig. 9 only a single sedimenting boundary was obtained.

Molecular weight determinations by the approach to equilibrium method (see later, p. 181) were carried out for both yeast and horse heart cytochrome c. The general experimental procedure followed was that described by Schachman (1955) and the molecular weights were calculated either by trapezoidal integration of the schlieren patterns to obtain concentrations (see later p. 184), or by mechanical integration using a planimeter (see later p. 186 and Ehrenberg, 1957). For one experiment the extrapolation procedure described by Archibald (1947) was also used (see later p. 185). Molecular weights were evaluated for the meniscus only, except for one case, because of the extremely steep refractive index gradients which were obtained at the bottom of the cell. The results of these experiments are given in Table 10. It will be noted the values of M vary with time for yeast cytochrome c, and in the first

Table 9.

Sedimentation Coefficient of Yeast Cytochrome c

Viscosity corrections have been made on data obtained from International Critical Tables.

Batch	Temperature	pH	Buffer	Rotor speed (rev./min.)	$S_{20,w}$	Method of determination
II	21.9°	6.0	phosphate/ NaCl, $\Gamma/_2$ = 0.2	59,780	2.29	moving boundary
III	16.9°	9.8	glycine/ NaCl, $\Gamma/_2$ = 0.2	13,153	1.89	moving boundary
III	17.1°	9.8	glycine/ NaCl, $\Gamma/_2$ = 0.2	19,927	2.30	moving boundary
	16.7°	9.8	glycine/ NaCl, $\Gamma/_2$ = 0.2	19,927	1.88	transport, from approach to equilibrium data.
III	18.4°	5.3	acetate/ NaCl, $\Gamma/_2$ = 0.2	19,927	2.30	moving boundary
	18.4°	5.3	acetate/ NaCl, $\Gamma/_2$ = 0.2	19,927	1.62	transport, from approach to equilibrium data.
III	16.5°	9.8	glycine/ NaCl, $\Gamma/_2$ = 0.2	59,780	1.64	moving boundary

Table 10

Molecular Weight of Ferrocytochrome c by Approach to Equilibrium Centrifugation

Source	Rotor speed (rev./min.)	pH	Buffer	Temperature	Time (min.)	Molecular weight
						(Ehrenberg's Method)
Yeast (Batch III)	19,926	9.8	glycine/ NaCl, $\Gamma/_2$ = 0.2	16.7°	30	15,450 (28,500*)
					60	16,090
					90	15,460
					120	14,550
						Mean 15,390 ± 630
						(Archibald's Method)
					30	15,500
					60	15,500
					90	15,800
					120	14,900
					150	13,600
						Mean 15,060 ± 880

* Bottom of cell.

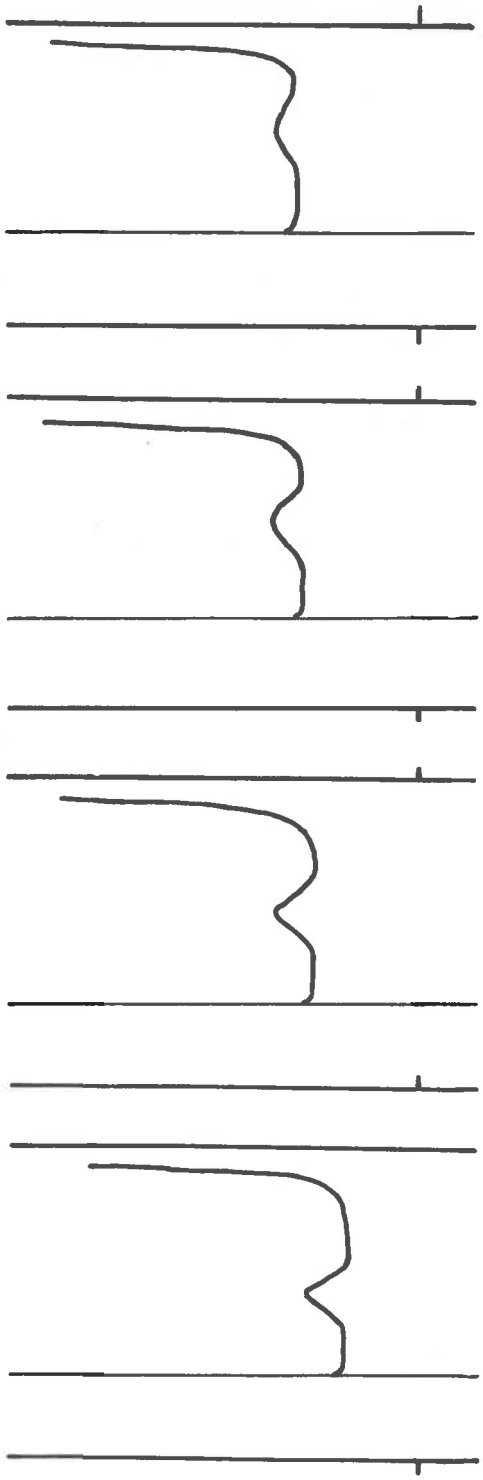
Table 10 contd.

Source	Rotor speed (rev./min.)	pH	Buffer	Temperature	Time (min.)	Molecular weight (Ehrenberg's Method)
Yeast (Batch III)	19,926	5.3	Acetate$^-$ / NaCl, $\mu/_2$ = 0.2	18.4°	20	16,810
					60	16,280
					80	15,720
					120	16,900
						Mean 16,430 ± 540
Yeast			Mean			15,910 ± 780 (Ehrenberg's Method)
Horse heart (Sigma Chemical Company) (see Table 6)	19,926	9.8	glycine / NaCl, $\mu/_2$ = 0.2	16.7°	20	14,860
					40	14,430
					60	14,450
					80	14,600
						Mean 14,590 ± 200

Difference between the mean molecular weight of yeast cytochrome c (pH 5.3 and pH 9.8) and of horse heart cytochrome c (t = 2.426, 10 degrees of freedom) is significant at 5% level.

Fig. 9. Sedimentation pattern of yeast cytochrome c

Sedimentation was carried out in glycine buffer, pH 9.8 $\Gamma/2 = 0.2$, at 59,780 rev./min. A synthetic boundary cell was used, and the solution of cytochrome c was layered over with glycine buffer against which it had been dialysed. Sedimantation is from left to right and photographs were taken at 30 min. intervals. Tracings of the photographs are shown because the marked light absorption of the cytochrome c prevented satisfactory photographic reproduction of the plate.



experiment (pH 9.8), the general tendency is for the value of M to decrease, while M calculated from the cell bottom is considerably greater than the corresponding value at the meniscus. Since no tendency for M to decrease with time was observed in the second experiment, at pH 5.3, the apparent heterogeneity indicated by the first experiment may possibly be due to an aggregation of the cytochrome c at the higher pH. The mean value of the molecular weight from these experiments is significantly different at the 5% level from the value obtained for the horse heart cytochrome c. The molecular weight of the latter as found in this study will be seen to be greater than that determined by other methods (see Table I, B, C). This discrepancy may well be due to an uncertainty as to the position of the meniscus in the ultracentrifuge cell, since an error of 0.1 mm. in locating the meniscus can result in errors of 5% or more in molecular weights determined by approach to equilibrium methods (Cheng, 1957; Trautman, 1958) particularly for experiments of short duration. Trautman (1958) has discussed methods for reducing the uncertainty of the meniscus position. The major source of error arises from positioning of the light source off the optical axis of the schlieren optical system. Since critical light source positioning was not carried out in the optical alignment of the ultracentrifuge, it is possible that a systematic error might be introduced when measuring the schlieren patterns obtained during centrifuging.

Another possible source of error is the use of data for the partial specific volume ($\bar{V}$) of beef heart cytochrome c. If $\bar{V}$ for yeast and horse heart cytochrome c differed to any extent from $\bar{V}$ for beef heart

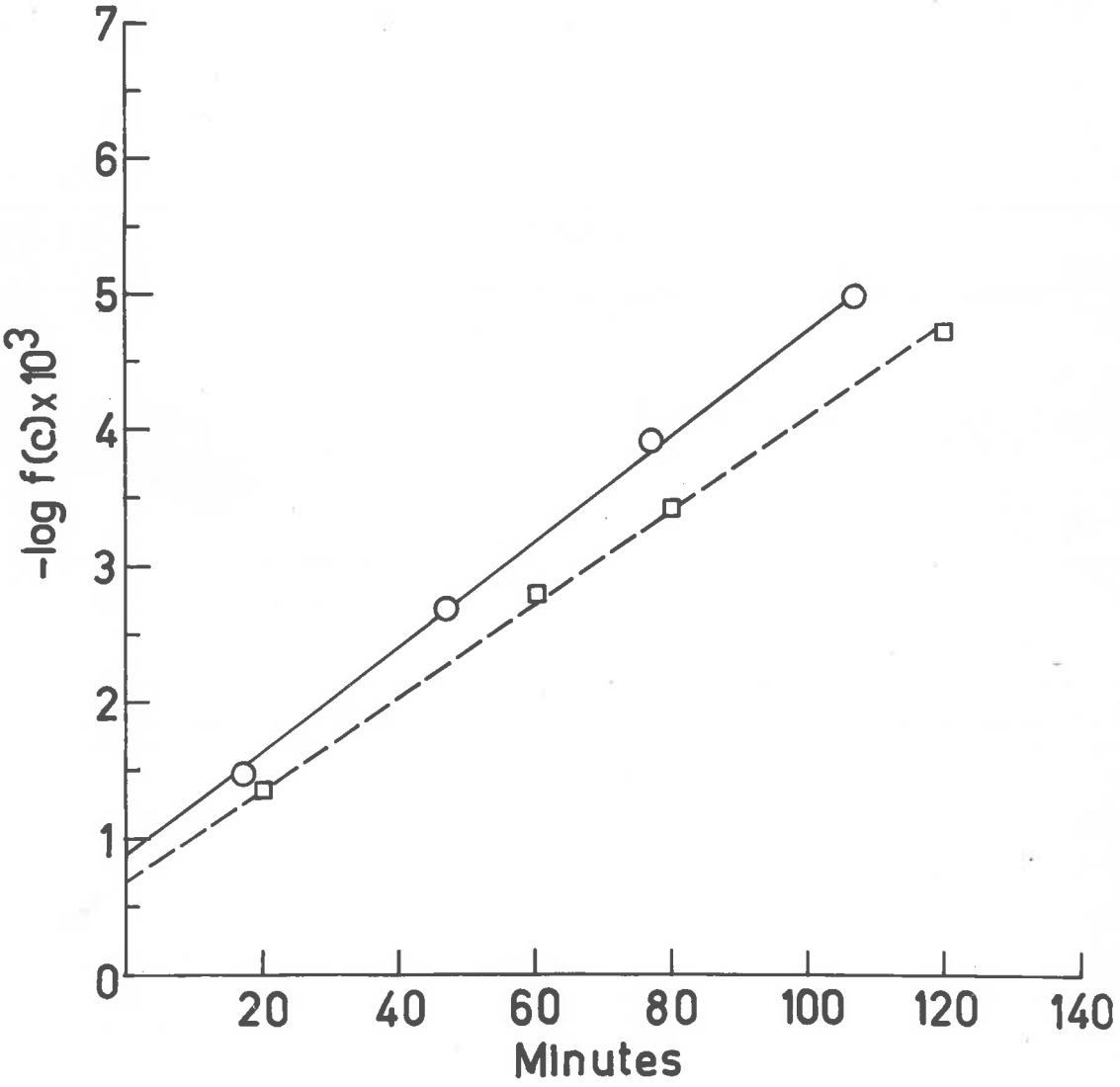
Fig. 10. Test of transport method as applied to the sedimentation of yeast cytochrome c

The function $-\log \left\{ \frac{1 + x_m^2 \int_{x_m}^{x_p} \frac{dn}{dx} dx - \int_{x_m}^{x_p} x^2 \frac{dn}{dx} dx}{x_m^2 c_o} \right\}$,

(-log f(c)), was calculated from the results obtained in approach to equilibrium experiments (p. 35).

O——O. glycine buffer pH 9.8, $\Gamma/2$ = 0.2. 19,927 rev/min.

□ - - □ acetate buffer, pH 5.3, $\Gamma/2$ = 0.2 , 19,927 rev/min.


7
6
5
4
3
2
1
0
-log f(c)x 10^3
20
40
60
80
100
120
140
Minutes

cytochrome c ($\bar{V}$ = 0.728, Ehrenberg, 1957) then a considerable error would be introduced in the calculated value of the molecular weight by using $\bar{V}$ for beef cytochrome c.

Although it might be expected that $\bar{V}$ for cytochrome c from various sources would not differ greatly, this is probably not justified, since $\bar{V}$ is markedly dependent on the tertiary structure of protein molecules, because it is related to the tightness with which the tertiary structure is packed.

IV. DISCUSSION

A. Preparative Procedures and Purification

Although chromatography on IRC-50 resin has proved invaluable in the purification of cytochrome c it is questionable as to whether chromatography alone is satisfactory in obtaining pure cytochrome c. This is suggested by the results presented in Table IV, and more particularly by Nunnikhoven's (1958) findings that yeast cytochrome c contained persistent contaminants of a polysaccharide nature, probably containing amino sugars. Such contaminants would not be expected to absorb much light in the region 260-280 mμ, so that they would not be detected from the value of the ratio $E_{\alpha\text{-band}} / E_{278\ m\mu}$, although they would decrease the purity when estimated on a dry weight basis. The presence of such contaminats, which are very probably involved in the cell wall of the yeast cell, may be rather affected by the conditions of extraction. Batch IV of cytochrome c probably contained very little of such impurities, since the value of $E^{1\%,1cm.}_{\alpha\text{-band}}$ was 20.49, corresponding to a molecular weight of 13,650 (assuming $(\epsilon_\alpha)_{mM.} = 27.7$ for yeast cytochrome c). This provides evidence that the particular extraction conditions used in the preparation of batch IV did not extract the contaminants. Since batches I, II and III were obtained from yeast extracted with n-butanol, which might well be expected to disrupt the cell wall structure, and since batch I was far from pure ($E^{1\%,1cm.}_{\alpha\text{-band}} = 9.70$), it seems likely that such contamination can be avoided by suitable extraction procedures. Nunnikhoven's (1958) extraction procedure may not extract cell wall

components. Although the ratio $E_{\alpha\text{-band}}/E_{(278\ m\mu)\ ox.}$ should be constant for purified cytochrome c from any particular source, this ratio is probably an unreliable guide to the purity of cytochrome c at high levels of purity, since the light absorption of aromatic residues of the protein in this region is affected by the tertiary structure. Hence cytochrome c which has been only slightly modified during purification, probably differs somewhat from native cytochrome c in the ratio $E_{\alpha\text{-band}}/E_{(278\ m\mu)\ ox.}$ Yeast cytochrome c seems less stable than animal cytochrome c (see later, p.41) so that any small changes in the tertiary structure of the protein could possibly influence the value of $E_{\alpha\text{-band}}/E_{(278\ m\mu)\ ox.}$ without affecting the charge or other properties of the molecule. Paleus, et. al. (1957) have expressed some criticism of the use of this ratio as the sole guide to purity of cytochrome c.

Whether or not the rather random variation in $(E_{\alpha\text{-band}}/E_{278\ m\mu})_{corr.}$ which was found between various fractions in both yeast and horse heart cytochrome c is due to heterogeneity, or to minor modifications of the protein tertiary structure is uncertain.

B. Minimum Molecular Weight

Although no satisfactory iron analysis was obtained for yeast cytochrome c in this work, some comments are necessary in relation to the molecular weight of yeast cytochrome c. Munnikhoven (1958) has made corrections for the presence of contaminants in calculating the iron content of yeast cytochrome c, but there must be some reservations

in accepting his figures. The two components which he obtained almost certainly represent modified forms of cytochrome c, arising because of treatment with trichloroacetic acid which may hydrolyse a number of amino acid residues from the protein. Since his corrections are made on the basis of (protein + haematin) dry weight and actual dry weight, it seems difficult to reconcile these corrections with the very good agreement which he obtained from between the total nitrogen (dry weight) and nitrogen content estimated from the amino acid composition and porphyrin nitrogen. Nunnikhoven's (1953) values for the iron content of yeast cytochrome c (0.474% Fe (M=11,820) and 0.443% Fe (M=12,660) are therefore probably incorrect. The iron content of yeast cytochrome c obtained by Minikami (1955) and by Tuppy and Dus (1958) corresponds to a molecular weight of 15,090. Although the purity of these preparations was not well established, the probability of modification of the protein was certainly small for Minikami's preparation, so that two values of 0.37% Fe obtained with yeast cytochrome c prepared in widely different ways probably indicates that the molecular weight is about 15000.

C. Spectroscopic Properties

The difference in the positions of the light absorption maxima for yeast and horse heart ferro-cytochrome c is unlikely to be due to incomplete reduction, since calculation shows that the equilibrium constant is very much in favour of reduction of cytochrome c ($\Delta E_o' = 0.210$ volt.) and an excess of ascorbic acid was used. Since the prosthetic group is the same for both cytochromes, (Hill and Keilin,

1930; Theorell, 1938), such differences must arise from differences in the protein. The differences may arise from differences in the ligands between the haem iron and the protein; from the effects of neighbouring residues on the ligands; from interactions between the protein and the prosthetic group. It will be seen that the intensities of the absorption bands are also slightly different. Nunnikhoven (1958) found that the α-band of yeast cytochrome c occurred at 549.5 mμ, as compared with 550.0 mμ for horse heart cytochrome c. The slight difference between his figures and those found in the present study are possibly of instrumental origin; also, Nunnikhoven used somewhat greater band widths which would result in some loss in accuracy in finding the absorption maximum of this sharp band. Estabrook (1956) has examined the low temperature ($\overline{ca}$ -180°) spectra of heart muscle and yeast ferrocytochrome c. The α-band for heart muscle cytochrome c split into three bands, whereas only two bands were observed for yeast cytochrome c. The α_1 band of yeast cytochrome c was at 546.6 mμ, compared with 548.6 mμ for heart muscle cytochrome c. Similarly, the β band for heart muscle cytochrome c had some seven component bands, as against five for yeast cytochrome c.

D. Stability

During the course of these experiments, the general impression obtained was that yeast cytochrome c was not particularly stable. Thus, prolonged exposure to strong ammonium sulphate in the cold resulted in loss of catalytic activity, although reduction by dithionite was still

obtained. Margoliash (1961) found that high ionic strength increased the rate of autoxidation of cytochrome c. Hence the loss of catalytic activity by yeast cytochrome c may be simply a result of the effect of ionic strength in decreasing ionic interaction between amino acid residues of the protein, followed by loss of tertiary structure. However, such an effect was not observed for cytochrome c in strong sodium chloride solutions. This may possibly be a specific ion effect. The extremely rapid splitting of the prosthetic group by silver salts in acid solution also seems to indicate a rather unstable protein tertiary structure, as does the effect of trichloroacetic acid in producing electrophoretically separable cytochrome c components from yeast (see also Li and Tsou, 1955; Munnikhoven, 1958). The ease with which these components may be separated (Li and Tsou, Munnikhoven) suggests that the duration and conditions of exposure to trichloroacetic acid may determine the extent of modification of the protein. The comparative study carried out by Okunuki and his coworkers (Nozaki, Yamanaka, Horio and Okunuki, 1957; Nozaki, Mizushima, Horio and Okunuki, 1958; Yamanaka, Mizushima, Nozaki, Horio and Okunuki, 1959) shows clearly that yeast cytochrome c is much more readily modified than heart muscle cytochrome c.

E. Electrophoresis Studies and Heterogeneity

The results of the electrophoretic experiments suggest that batch III yeast cytochrome c was heterogeneous; this is probably most readily indicated by the presence of a minor, negatively charged

component at pH 8 and above. It is unlikely that this represents modified cytochrome c in view of the high negative mobility of this component. It may therefore be concluded that the procedure used for preparation of yeast cytochrome c in this work prevents the formation of the modified cytochrome c components as observed by Nunnikhoven (1958) and by Li and Tsou (1955). The preparations obtained by these workers had net positive charge at approx. pH 6 and at pH 8.3. The question then arises as to whether the heterogeneity is due to components like the amino sugar polysaccharides observed by Nunnikhoven. It seems likely that such compounds might give rise to the negatively charged component which was observed above pH 8 since the pK' of amino sugars occur at about pH 6 to pH 7; a negatively charged, non-protein, ninhydrin-positive component was obtained by Nunnikhoven (1958). Several similar components, having high positive mobilities (much greater than the cytochrome) were also observed by Nunnikhoven. The small sharp spike in the boundaries at pH 9.93 (Fig. 8) has a net negative charge and therefore probably the same component as that observed in other experiemnts (see Fig. 7). Since this component was not resolved in the descending limb of the cell it is not possible to say whether it was coloured or not; however, the high negative mob ility of this component at pH 8.3 makes it very unlikely that this was a modified cytochrome c.

The maximum ordinate of both boundaries was stationary in the experiment in which cell polarity was reversed, from about 30 minutes before to 90 minutes after the polarity reversal. Hence the sharpening observed over this period is probably due to heterogeneity of the

system with respect to iso-electric pH. Some doubts have been expressed as to the validity of reversible boundary spreading at the average iso-electric pH as a meaningful test for heterogeneity (e.g. Longsworth, 1959). The net charge of an electrolyte such as a protein is an average value, and hence a certain proportion of the molecules have positive charge (or negative charge) at the average iso-electric pH, even though any individual weak electrolyte molecule is undergoing random fluctuations in charge. Thus it is to be expected that a pure protein will exhibit some reversible boundary spreading.

F. Sedimentation Studies

The sedimentation experiments are not particularly informative as to the heterogeneity of the particular yeast cytochrome c samples examined. Although it is apparent that there is no gross heterogeneity in sedimentation coefficient, resulting in the formation of two or more boundaries, a detailed boundary analysis was not carried out, and therefore heterogeneity in S cannot be ruled out.

There is some uncertainty as to which range of values of the sedimentation coefficient is most likely. On one hand it might be expected that cytochrome c which had lost some of its tertiary structure would have a larger axial ratio (or frictional coefficient) and that the sedimentation coefficient would therefore be smaller. On the other hand, for rapidly spreading boundaries, the maximal ordinate of the moving boundary is difficult to determine precisely, whereas the sedimentation coefficient as determined by transport methods does not suffer from such a subjective error. It is clear that the sedimentation

coefficient determined by the transport method is smaller than that determined in the same experiment, by the moving boundary methods, and examination of Table IC leads to the conclusion that the most common value in the more reliable experiments by other workers is 1.6 - 1.9 S for cytochrome c of animal origin. For the reasons given above, it is felt that the sedimentation coefficient of yeast cytochrome c also lies in this range.

The molecular weight of yeast cytochrome c as determined by approach to equilibrium methods is significantly different from that of horse heart cytochrome c, at the 5% level of confidence. The discrepancy in the observed value of the latter and that obtained by other workers is most probably due to a systematic error introduced by the misalignment of the light source of the optical system. At pH 9.8, the molecular weight of yeast cytochrome c tended to decrease with time when evaluated for the solution meniscus, while there was a considerable discrepancy between the molecular weights determined at the meniscus and at the cell bottom. Since the refractive index gradient was steep at the cell bottom, and since there was an infinite gradient between the solution and the dense immisiscible artificial cell bottom (Dow Corning Silicone No. 555) there is some uncertainty as to the accuracy of the one value of M obtained for the cell bottom. However, it seems likely that this value would probably be an underestimate, because the refractive index gradient was steep and curving upwards. Therefore, it is possible that yeast cytochrome c was heterogeneous in M at pH 9.8. Since there was no trend in M with time at pH 5.3, the

cytochrome was probably homogeneous in M at this pH, although there is insufficient data available to claim this with any certainty. If, however, this is the case, then yeast cytochrome c undergoes a pH dependent aggregation.

Cytochrome b_2 of baker's yeast

I. INTRODUCTION

A. Yeast Lactate Dehydrogenase

1. Historical

The lactate dehydrogenase of yeast (YLD) is known to differ considerably from the lactate dehydrogenase of muscle. The obvious major differences are (a) the lack of a dialysable coenzyme (b) the oxidation of lactate to pyruvate at neutral pH in the absence of a pyruvate-trapping agent and (c) the ability to reduce cytochrome c. These are all properties of the yeast enzyme which the dehydrogenase from muscle does not possess (Green and Brosteaux, 1936).

Since YLD was the only known cytochrome-reducing dehydrogenase which was readily obtained in a soluble form in aqueous extracts of cellular material, Bach, Dixon and Zerfas (1946) set about the purification of the enzyme from autolysates of dried Delft baker's yeast. At an early stage in the purification, they observed that the addition of lactate to the fraction containing YLD resulted in the rapid appearance of an absorption band at 556 mμ. This band also appeared on addition of dithionite. As the purification proceeded, the enzymic activity was observed to run parallel with this pigment. Spectroscopic examination of the dithionite- or lactate-reduced pigment revealed that it had a typical haemochromagen spectrum, and it was named cytochrome b_2 (Bach, Dixon and Keilin, 1942). Yeast lactate dehydrogenase prepared by a rather different procedure from another strain of baker's yeast also showed this relationship between cytochrome b_2

and YLD. As a result of these studies, it was proposed that cytochrome $\underline{b}_2$ was part of YLD, possibly the primary (substrate activating) dehydrogenase. Small but variable amounts of flavin were associated with these preparations, which were extremely unstable at high levels of purity, and the ability of the enzyme to catalyse the reduction of cytochrome c was in doubt.

In 1954, Appleby and Morton (1954) crystallised cytochrome $\underline{b}_2$, using a wild strain, highly pigmented baker's yeast as their starting material. Solutions of the crystalline protein were electrophoretically homogeneous at pH 7.0, and repeated recrystallisation of cytochrome $\underline{b}_2$ did not alter the ratio of cytochrome $\underline{b}_2$ to the extremely active lactate dehydrogenase activity. Furthermore, the ratios of the lactate reductase activities for ferricyanide, cytochrome c and methylene blue were the same for the crystalline enzyme as for the crude extracts. Cytochrome $\underline{b}_2$ contained equimolar amounts of protohaem and riboflavin monophosphate (FMN); Mg^{++} and haem bound iron were the only metals present. The bands of reduced cytochrome $\underline{b}_2$ appeared rapidly, in enzymically active preparations of the crystalline enzyme, on addition of lactate; in preparations where most of the activity had disappeared, cytochrome $\underline{b}_2$ was reduced slowly by lactate, while completely inactive preparations were unable to reduce cytochrome $\underline{b}_2$ in the presence of lactate. In all cases, cytochrome $\underline{b}_2$ was rapidly reduced by dithionite. Oxidised cytochrome $\underline{b}_2$ showed a typical parahaematin spectrum. Crystalline cytochrome $\underline{b}_2$ was about 15 times more active than the best preparations of Bach, Dixon and Zerfas (1946). Appleby and Morton postulated that

crystalline cytochrome b_2 was identical with YLD, and that YLD was a single protein having two prosthetic groups, the primary dehydrogenase being associated with the flavin moiety of the enzyme, while the haem moiety functioned as a built-in intermediate electron acceptor.

In the following year, Boeri, Cutolo, Luzzati and Tosi (1955) reported purification of YLD by a method rather similar to that described by Bach, Dixon and Zerfas (1946). This preparation was about one third pure (taking crystalline cytochrome b_2 as completely pure); the enzymic behaviour was very similar to that of crystalline cytochrome b_2, and the preparations contained equimolar amounts of protoheem and FMN. The YLD activity was shown to be stabilised by addition of ethylenediamine tetraacetate (EDTA), and the enzyme was specific for L(+)-lactate. Boeri and Tosi (1956) found that their preparation contained 8 atoms of non-haem iron per haem, and observed that increasing ionic strength decreased lactate-cytochrome c reductase activity. This they attributed to a salt effect on the binding of FMN to the protein. Boeri's group also postulated that cytochrome b_2 and YLD were identical, and represented one protein with two prosthetic groups; however, because of the presence of iron they concluded that YLD was a metallo-flavo-haemo-protein, the non-haem iron being involved in electron transport (see p.85). Since crystalline cytochrome b_2 contained only haem iron, this conclusion cannot be valid.

This raises a question of nomenclature. Since cytochromes are in general first identified on spectroscopic grounds, when a cytochrome is isolated as a homogeneous protein, any enzymic activity

associated with the purified cytochrome is a property of the cytochrome. It is therefore proposed that the term cytochrome b_2 should be restricted to preparations of that cytochrome which possess YLD activity, and that the portion of cytochrome b_2, responsible for the parahaematin spectrum be referred to as the haem portion or moiety. Thus YLD is a flavohaemoprotein and not a flavocytochrome, and cytochrome b_2 and YLD are synonymous.

2. Controversies concerned with the nature and mechanism of action of yeast lactate dehydrogenase

There are several aspects concerned with YLD in which the viewpoints of various investigators have differed. These fall roughly into four groups: i, the substrate specificity of YLD; ii, YLD as a flavohaemoprotein; iii, the mechanism of electron transfer in YLD, and iv, the physiological role of YLD.

i. Substrate specificity. Bernheim (1928) found that aqueous extracts of acetone-dried yeast catalysed the reduction of methylene blue by DL-lactate and DL-α-hydroxybutyrate. A number of other short chain aliphatic hydroxy and amino acids, glyceric acid, β-hydroxybutyric acid, acetaldehyde and glucose were not reactive. Both Mahler and Huennekens (1953) and Boeri et al. (1955) found that YLD was specific for L(+)-lactate, and Boeri et al. stated that they were unable to find a substrate for YLD other than L(+)-lactate. Dickens and Williamson (1956) using a YLD preparation supplied by Boeri, found that L(+)-glyceric acid was a substrate for YLD, while Yamashita, Horio and Okunuki (1958) and Yamanaka, Horio and Okunuki (1958) have found that

TPNH and malate are substrates for a partly purified YLD preparation.

Appleby and Morton (1959) found that crystalline cytochrome b_2 catalysed oxidation of the following compounds in addition to lactate; glycollate, α-hydroxy-n-butyrate, α-hydroxy-caproate, but not β-hydroxy-butyrate or α-hydroxy-iso-butyrate.

ii. Yeast lactate dehydrogenase as a flavohaemoprotein.

Yamashita et al. (1957) obtained a crystalline haemoprotein from baker's yeast, which was homogeneous on electrophoresis and by chromatography. The spectrum of this haemoprotein in both the oxidised and reduced state is very similar to that of crystalline cytochrome b_2 but no lactate dehydrogenase is associated with this preparation. It is, however, reduced by lactate and YLD. These workers considered that this protein was cytochrome b_2 and that YLD was cytochrome b_2 plus some other components.

Further studies (Yamashita et al., 1958; Yamanaka et al., 1958) showed that the haem ("cytochrome b_2") of partially purified YLD was reduced by lactate, L-malate and TPNH, and it was suggested that "cytochrome b_2" occupied an important position in the terminal oxidase system of yeast. It was later found that enzymically inert "cytochrome b_2" was reduced by lactate and YLD at about 1/50 th the rate of the haem in YLD (Yamashita et al., 1958). The differential inactivation of the lactate-phenazine reductase and lactate-methylene blue reductase activities of partially purified YLD has led these workers to conclude that "cytochrome b_2" is intimately associated with the primary dehydrogenase in YLD and that "cytochrome b_2" is a degradation product of YLD (Horio, Yamashita and Okunuki, 1959). It now seems acceptable to all the

workers in the field of cytochrome b_2 that the "double-headed" enzyme as proposed by Appleby and Morton (1954) is indeed correct, although there is still some question as to whether YLD is composed of some complex of a flavoprotein and haem which could be split under suitable conditions.

iii. Mechanism of electron transfer in yeast lactate dehydrogenase

The rapid reduction of the YLD haem by lactate led Appleby and Morton (1954) and Boeri et al. (1955) to conclude that the haem functioned in electron transport, by analogy with the respiratory chain in mitochondria (see p. 58), and similar conclusions were reached by Okunuki and his co-workers, although they included TPNH and malate as substrates.

Chance and Boeri (1961) examined a preparation of YLD in a spectrophotometric flow apparatus and obtained results which suggest that this might not be the case, and that reduction of cytochrome c and ferricyanide by YLD and lactate was too rapid to permit the inclusion of the YLD haem on the direct pathway of electron transfer from lactate since the latter was reduced at a slower rate than the acceptors. This conclusion is open to the criticism that the discrepancy in the rates of reduction of flavin, ferricyanide and cytochrome c on the one hand, and YLD haem on the other could be explained by the presence of enzymically inactive YLD, which would be reduced more slowly than enzymically active YLD (see p.51), so that the total haem reduction rate would be slower than the rates of reduction of flavin, ferricyanide or cytochrome c. In view of the instability of YLD, the presence of inactive material in such a preparation would not be unlikely, more

particularly as the preparation used had been transported from Italy to the United States and was several weeks old at the time the measurements were performed (Boeri, personal communication to R. K. Morton).

Unfortunately, crystalline cytochrome $\underline{b}_2$ has not been examined by these methods; since enzymically inert cytochrome $\underline{b}_2$ is not readily crystallised by the method of Appleby and Morton (1959), it would be possible to perform critical experiments in the flow apparatus with freshly recrystallised cytochrome $\underline{b}_2$.

Nygaard (1958, 1959a, b, 1960) has obtained a number of lactate cytochrome c reductases from yeast, by column chromatography of partially purified extracts from yeast. While the major component of the L(+)-lactate-cytochrome c reductase is very similar in its properties to crystalline cytochrome $\underline{b}_2$, a minor component with L(+)-lactate-cytochrome c reductase activity has been obtained, in which the enzyme haem is not reduced by lactate; this enzyme is regarded as being a modified form of YLD. Unfortunately, this has been quoted by other workers (Ziegler and Doeg, 1959; Ziegler and Doeg, 1959a) as being true for YLD.

Nygaard (1960) has also found that aerobic yeast contains a D(-)-lactate-cytochrome c reductase, in contrast to the results of Boeri and coworkers (1955). He suggests that the variety of lactate-cytochrome c reductases obtained by him arise in the following way. Anaerobic baker's yeast possesses a D(-)-lactate dehydrogenase, which will not reduce cytochrome c. (Labeyrie, Slonimski and Naslin, 1959). It is proposed that the various D(-)-and L(+)-lactate

cytochrome c reductases observed in aerobic yeast represent the products of stepwise modification of the anaerobic D(-)-lactate dehydrogenase to L(+)-lactate cytochrome c reductase (cytochrome b_2) in the changeover from anaerobic to aerobic metabolism (or vice versa). This hypothesis does not seem very probable for a number of reasons, as follows; (a) L(+)-lactate dehydrogenase, whether crystalline or not, is not very stable, and can undergo changes in the relative rates of reduction of various acceptors very easily (Appleby and Morton, 1959a; Boeri et al., 1955; Horio et al., 1959). (b) It seems unlikely that the active centre of a highly stereospecific enzyme could be brought about by modification of the active site of an already existing enzyme. (c) The process of enzymic adaptation seems always to involve the de novo synthesis of the enzyme from the amino acid pool of the organism, rather than from existing proteins, except by complete hydrolysis of proteins to amino acids (Spiegelman, 1953).

iv. The physiological role of yeast lactate dehydrogenase. Considerable controversy exists as to the physiological role of YLD, both with respect to substrate and acceptor. Since baker's yeast carries out alcoholic fermentation rather than glycolysis, and since a pyridine nucleotide linked lactate dehydrogenase has not been detected in yeast, it has been widely assumed that yeast does not form lactic acid from sugars. However, it is known that yeast will produce small amounts of lactic acid during alcoholic fermentation (Hohl and Joslyn, 1941) and the early literature concerning alcoholic fermentation is full of hypotheses on the involvement of lactic acid as an intermediate in

alcohol formation. The origin of the lactic acid is uncertain, but some observations by Neuberg and Kobel (1927) seem relevant. When methyl glyoxal (pyruvaldehyde) is supplied to fresh or dried yeast, D(-)-lactic acid accumulates. If, however, methyl glyoxyl is added to yeast press juice, DL-lactic acid accumulates. Although the widely studied glyoxylase forms D(-)-lactic acid (Racker, 1954), this seems evidence for a glyoxylase which forms L(+)-lactic acid, since the accumulation of D(-)-lactic acid by yeast cells suggest that any L(+)-lactate formed is oxidised, probably by YLD. The presence of lactate isomerase would result in complete disappearance of lactic acid. Methyl glyoxal can arise from threonine or from glycine and acetyl-CoA, via α-amino-β-keto-butyric acid and amino acetone (Elliot, 1959), as well as non-enzymically from triose phosphates. Thus the search for other possible substrates for YLD is probably unnecessary, since it would be advantageous to the yeast to oxidise the lactic acid which it forms.

The nature of the physiological acceptor for YLD has been complicated by the conclusion that YLD is an enzyme of the "cell sap", due to the fact that it is obtained in the soluble fraction of extracts from dried yeast. This poses questions as to the accessibility of cytochrome c and cytochrome oxidase to YLD, since the latter, at least, is known to be present in yeast mitochondria. Similar arguments have been raised with respect to the microsomal pyridine nucleotide-cytochrome c reductases of animals and plants, both of which are analogous to YLD in that they involve b type cytochromes (see pp. 67-71). Some experiments carried out by Nossal, Keech and Morton (1956) suggested that YLD was associated with yeast mitochondria, but these experiments were

inconclusive due to the ease with which YLD could be removed. from the mitochondrial fraction of yeast cell homogenates. However, Linnane (personal communication) has been able to show that YLD is a mitochondrial enzyme. Therefore, YLD has access to the respiratory chain and cytochrome c is probably the physiological acceptor for the enzyme.

In view of the controversies discussed above, it was felt that clarification of the following matter was necessary.

i. The substrate specificity of YLD.

ii. The mechanism of acceptor reduction by YLD.

iii. The stoichiometry of the reaction with respect to the reduction of enzyme, i.e. the number of active sites per enzyme molecule.

iv. As to whether YLD is a complex of a flavoprotein and a haemoprotein, or a single protein with two prosthetic groups.

The experimental section of this thesis is concerned with work carried out towards clarification of these issues.

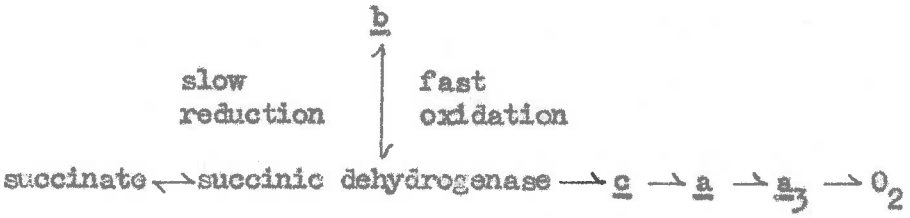
B. Flavoprotein-haem Systems

1. Succinate dehydrogenase system

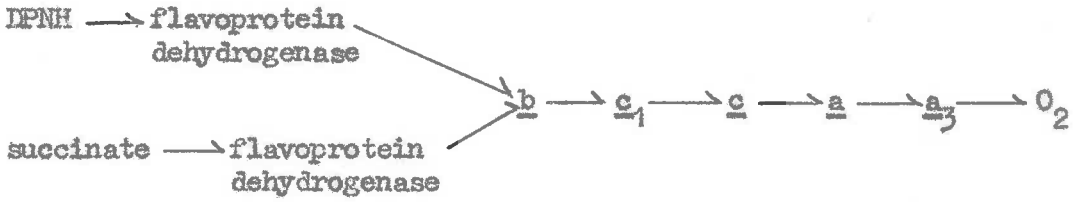
i. The role of cytochrome b in electron transport.

Preparations from heart muscle (Keilin-Hartree preparations) were demonstrated to reduce cytochrome b on addition of succinate (Keilin and Hartree, 1939); this reduction occurred rapidly and concomitant with the reduction of cytochromes a and c on exhaustion of oxygen (Slater, 1949), but was not observed when DPNH was used instead of succinate (Slater, 1950). From this, Slater concluded that cytochrome b was on the pathway of electron transfer from succinate, but not from DPNH, to oxygen.

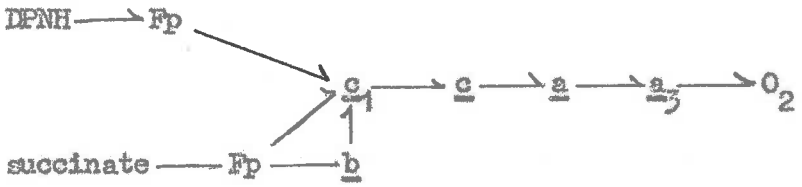
When Keilin-Hartree preparations were examined by Chance (1952, 1955) he found that the reduction of cytochrome b occurred rather later, and at a much slower rate, than the reduction of cytochromes a_3, a and c. From this he concluded that the sequence of electron transfer in such preparations should be written (where a, b, c etc. stand for cytochromes)



Later studies by Chance (1955) with mitochondria still capable of oxidative phosphorylation, and with yeast cells, indicated that in such systems the sequence of electron transfer should be written



and for the Keilin-Hartree preparation: *



By this time it had been shown that succinic dehydrogenase was a flavoprotein (Morton, 1950; Singer and Kearney, 1954), and that the dehydrogenase was not cytochrome b, as had been earlier suggested (Cooper, Ball and Anfinsen, 1947; Slater, 1949).

Slater (1958) considers it difficult to picture a system in which the displacement of cytochrome b from its normal position in the phosphorylating respiratory chain causes no loss in the rate of succinate oxidation by oxygen. Some criticism of Chance's (1952) experiments is made, and calculations made from Chance's data suggest that in the absence of cyanide, there is no reason for not placing cytochrome b on the respiratory chain in Keilin-Hartree preparations. Some experiments carried out by Slater and Colpa-Boonstra (1961) show very little difference in the rates and extent of reduction of cytochromes a_3 and b

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* Abbreviations used in these schemes are as follows: Fp, flavoprotein dehydrogenase; b, c etc. are the corresponding cytochromes.

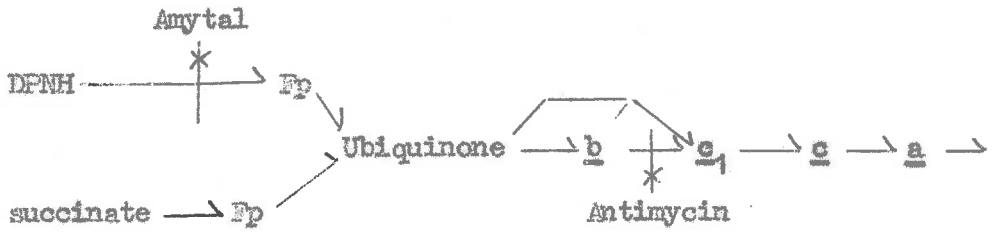
in Keilin-Hartree preparations, either with succinate or high concentrations of DPNH. Low DPNH concentrations, however, cause a very slow and incomplete reduction of cytochrome b, as was earlier observed (Slater, 1950). It was found that malonate caused a discrepancy in the relative rates of reduction of cytochromes a_3 and b, of the type observed by Chance (1952). Chance (1961) suggested that Slater and Colpa-Boonstra's results might result from the time response of the spectrophotometric system used, but Slater (1961) indicated that considerable care had been exercised to exclude this possibility.

The behaviour of cytochrome b in sub-mitochondrial particles or soluble preparations (Chance, 1959; Ziegler and Doeg, 1959) suggest that cytochrome b is not on the main pathway of electron transfer in these non-phosphorylating mitochondrial fragments. A lipide-soluble quinone which can be extracted from mitochondria and related particles has been recently isolated. This compound, known as ubiquinone (R. A. Morton, 1958) or coenzyme Q (Crane, 1957) has been shown to undergo cyclic oxidation and reduction in mitochondria (Hatefi, 1959) and to stimulate electron transfer in particles which have been extracted with lipid solvents (Crane, 1959). This has been shown to be the case in phosphorylating and non-phosphorylating preparations, and it is therefore considered to be a constituent of the respiratory chain by both Crane and coworkers and by R. A. Morton and coworkers.

The antimycin insensitivity of ubiquinone reduction places it on the substrate side of the antimycin-induced block of the respiratory

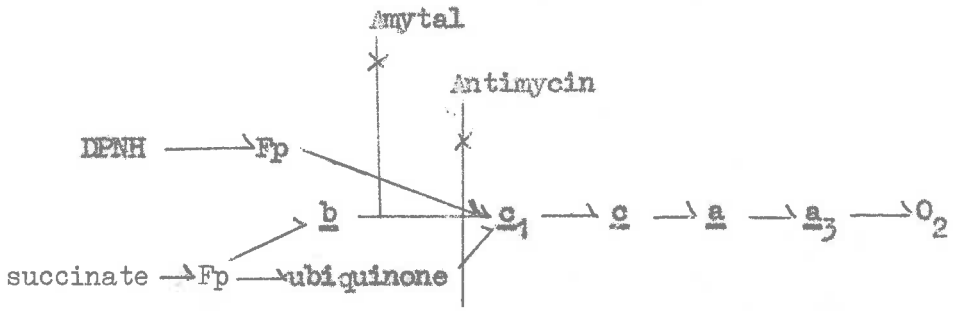
chain (Redfearn, 1959). Experiments with sub-mitochondrial particles and soluble preparations lead to the conclusion that antimycin completely inhibits the oxidation of reduced ubiquinone by cytochrome $\underline{c}_1$ (Green, Ziegler and Doeg, 1959).

Since ubiquinone in phosphorylating preparations can be completely reduced by DPNH or succinate, it seems that the respiratory chain should be written



$$\underline{a}_3 \longrightarrow O_2$$

(see also Chance, 1959), and for the non-phosphorylating chain



Slater's factor (Slater, 1949) is not included in this scheme, as there is some doubt as to whether it represents another as yet unidentified component of the respiratory chain, or whether it can be explained in terms of the existing respiratory chain. Various

workers (Hopkins, Morgan and Lutvak-Mann, 1938; Straub, 1942) showed that it was possible to bring about inhibition of succin-oxidase activity, without any marked affects on succinate dehydrogenase and cytochrome oxidase activity, by treatment of heart muscle preparations with bile salts.

Slater (1949, 1950) showed that incubation of Keilin-Hartree preparations with 2,3 dimercaptopropanol (BAL) in the presence of air inactivated the succinoxidase system without appreciably affecting succinate dehydrogenase or cytochrome c reductase. Furthermore, the addition of succinate to BAL-treated preparations caused the appearance of the absorption bands of ferrocytochrome b, while cytochromes c and a remained oxidised. Since added cytochrome c had no effect on the BAL-induced inhibition of succinoxidase activity, it was concluded that an additional factor was involved between cytochromes b and c. Similar results were observed with DPNH oxidase, except that cytochrome b was only slowly and incompletely reduced by DPNH. Thus the site of action of BAL is located between cytochrome c and the flavoprotein dehydrogenases for succinate and DPNH. Depending on whether or not cytochrome b is on the electron transfer chain in Keilin-Hartree preparations, the site of BAL action can possibly be considered as lying bewteen cytochromes b and c_1 and is probably not cytochrome c_1 (Slater, 1958).

In conclusion, it may be said that cytochrome b is involved in electron transfer between flavoprotein enzymes and cytochrome c_1 in the intact respiratory chain of phosphorylating mitochondria. Conclusions derived from the behaviour of cytochrome b in non-

phosphorylating preparations are of little significance in the understanding of the intact mitochondrial respiratory chain. The position of cytochrome b in cellular oxidation processes is therefore not as anomalous as is indicated from *in vitro* studies with functionally damaged sub-cellular particles.

ii. Succinate dehydrogenase. Since the discovery that minced muscle preparations would catalyse the reduction of methylene blue by succinate (Thunberg, 1916), there has been considerable interest in the enzyme system which catalysed this reaction. It was found that the enzyme was associated with insoluble particulate matter in tissue homogenates and that the oxidation of succinate could be coupled also to the reduction of cytochrome c (Ogston and Green, 1935), cytochrome b or oxygen (Keilin and Hartree, 1940). On the basis of these observations, succinate dehydrogenase was classified as a cytochrome-reducing dehydrogenase and considerable speculation as to the nature of the primary dehydrogenase ensued. As will be seen, the major difficulty to be overcome for successful solubilisation and purification of succinate dehydrogenase was the selection of a suitable electron acceptor for assaying succinate dehydrogenase.

The first solubilisation of the enzyme was achieved by Hogeboom (1946) from acetone powders of mitochondria. A partially purified soluble preparation of the dehydrogenase was obtained by Morton (1950) by butanol treatment of a Keilin-Hartree preparation from heart muscle, the extract subsequently being fractionated with

ammonium sulphate (Morton, 1955a, b). A soluble, apparently homogeneous, amber-coloured protein was obtained from acetone powders of beef heart mitochondria; the purification achieved was approximately 100-fold from the initial extract. (Singer and Kearney, 1954, 1956). A preparation from yeast respiratory particles was obtained, which was about 40-50% pure (Singer, Massey and Kearney, 1957). Both proteins had succinate-acceptor reductase activity with phenazine compounds and with ferricyanide, while various dyes such as methylene blue, indophenol, brilliant cresyl blue and cytochrome c were reduced too slowly to allow for their direct coupling with the enzyme in particulate preparations. The spectrum suggested that the enzyme was possibly a flavoprotein, but the nature of the prosthetic group remained in doubt for some time, since conventional methods for releasing flavins from flavoproteins were not successful with succinate dehydrogenase. It was found that a FAD-like compound was released on tryptic digestion of the dehydrogenase, and it has been concluded that the prosthetic group is FAD which is associated, by covalent bonds, with amino acid residues of the protein (Singer and Kearney, 1957; Wang, Tsou and Wang, 1956). The enzymic activity of the protein was rather labile. The enzyme also contained iron, which could be released on treatment with acid; there were 4 gram atoms of Fe per mole of flavin. Light scattering and flavin analysis indicated a molecular weight of about 200,000.

Since the enzymic activity was inhibited by iron-chelating agents, and since the chelated iron was still associated with the protein, it was concluded that the iron was involved with the electron

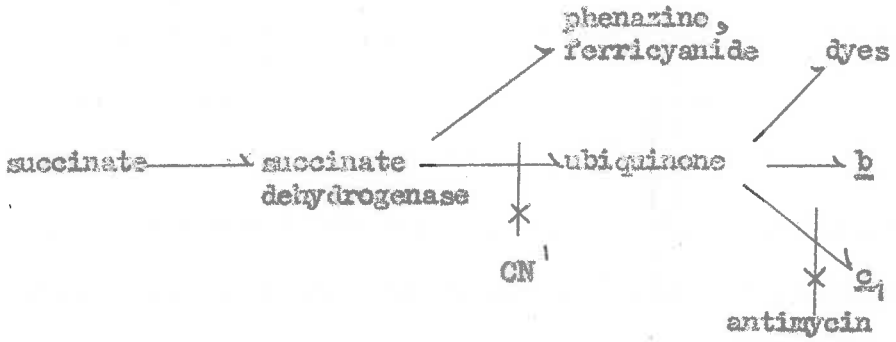
transport process for reduction of acceptors. However, Massey (1958) showed that the inhibition obtained with iron chelating agents occurred because denaturation of the enzyme was caused by these agents under the conditions in which they were tested. If suitable precautions were taken, then two of the four atoms could be co-ordinated without denaturation of the enzyme or loss of enzymic activity; the remaining two iron atoms were only accessible to the chelating agents after denaturation. In view of these results, and the general lack of evidence for the involvement of transition metals in electron transport from flavoproteins, and the unspecific ability of iron compounds to act as acceptors for reduced flavoproteins, Massey concluded that the iron was required for the maintenance of a specific structure of the enzyme, rather than as an electron transport mediator.

Tsou (1951) demonstrated quite clearly that the succinate dehydrogenase activity of Keilin-Hartree preparations (using either methylene blue or cytochrome c as an acceptor) was inhibited by cyanide. The activity of the soluble enzyme towards phenazine methosulphate and ferricyanide is unaffected by cyanide. Keilin and King (1958, 1960) showed that the methylene blue and cytochrome reductase activity and the succinoxidase activity of alkali-treated heart muscle particles, which have lost these activities, could be restored by addition of a soluble succinate dehydrogenase (which, however, was only about 60% pure). Furthermore, the added dehydrogenase was found to be incorporated into the deficient particles in a definite amount, and could not be removed by washing. The reconstituted particles appeared to be very similar

to normal Heilin-Hartree preparations, the succinate dehydrogenase activity was cyanide sensitive, and the dehydrogenase had regained the stability of the enzyme normally observed in particulate preparations. It was concluded that the soluble enzyme could not represent a form of succinate dehydrogenase which had been modified, at least to any significant extent.

The question therefore arises as to why some "solubilised" preparations of succinate dehydrogenase are able to reduce dyes rapidly (Ball, 1956; Stotz, 1956), while more purified forms of the enzyme will reduce only phenazine and ferricyanide at significant rates. Some observations of Green, Ziegler and Doeg (1959) and Ziegler and Doeg (1959a, b) are relevant to this point. Green and his coworkers have been concerned with the separation of the components of the respiratory chain into smaller and smaller subunits. Such preparations as possess succinate dehydrogenase activity are very potent succinate-ubiquinone reductases, and also possess the ability to reduce dyes such as indophenol, until the fragmentation has progressed to a point where a soluble flavoprotein, which is possibly identical with purified succinate dehydrogenase (Ziegler and Doeg, 1959b) is obtained. This flavoprotein will catalyse the reduction of phenazine and ferricyanide, but not ubiquinone, by succinate; it is obtained by solvent treatment of a soluble flavin-containing protein. It has been observed that soluble succinate dehydrogenase cannot bring about the reduction of ubiquinone. Since ubiquinone is hydrophobic, these results suggest that it may not be able to approach the soluble purified dehydrogenase sufficiently

closely for electron transfer to occur, whereas this may be facilitated in less purified preparations where the possibility of a lipoprotein structure still exists, as seems indicated for mitochondria and submitochondrial particles (Green, 1959). It is therefore suggested that the following sequence of electron transfer takes place, at least in non-phosphorylating systems (Slater, personal communication).



This succinate dehydrogenase, which for many years has been the "type" enzyme for cytochrome-reducing dehydrogenases, can no longer be included in this group.

2. Reduced diphosphopyridine nucleotide - cytochrome c reductases

Homogenates of various animal and plant tissues catalyse the reduction of cytochrome c by DPNH. The reductase activity is partially inhibited by antimycin A in unfractionated homogenates (Potter and Reif, 1952). Complete inhibition by antimycin can be obtained with some mitochondrial preparations and Keilin-Hartree preparations (Chance and Williams, 1956; Potter and Reif, 1952). The antimycin-insensitive activity is associated with the microsomal fraction of tissue homogenates (Potter and Reif, 1952; Martin and Morton, 1956).

although in certain circumstances mitochondria can exhibit antimycin-insensitive DPNH-cytochrome c reductase activity (Potter and Reif, 1952; Martin and Morton, 1956). The microsomal system will be discussed first since there is some agreement on the general behaviour of this system. Moreover, it may represent a somewhat more physiological system than those solubilised from mitochondria.

i. Microsomal reduced diphosphopyridine nucleotide reductase system. C.Strittmatter and Ball (1952) observed, in liver microsomes, a haemoprotein having an α-band at 556 mμ. This they named cytochrome m, and demonstrated that it was reduced by DPNH and oxidised by oxygen. Chance and Williams (1954) carried out a kinetic study of this cytochrome, which they denoted as cytochrome b_5. This name was also used independantly by Baillie and Morton (1955) to describe a cytochrome component, first detected in microsomes from cow's milk, and subsequently in cow mammary gland and from intestinal mucosa. The term cytochrome b_5 has now become a generic name for cytochromes of animal origin having a spectrum similar to that of pyridine protohaemochromagen.

P. Strittmatter and Velick (1956), using mild conditions, extracted cytochrome b_5 from rabbit liver microsomes and obtained it as a low molecular weight protein ($M \simeq 17,000$) which was homogeneous by sedimentation and was slowly resolved into two haemoproteins on electrophoresis. However, they were of the opinion that one of the electrophoretic species represented a slightly modified form of the other. The spectrum closely resembled that of cytochrome b_2, although the intensities at the absorption maxima were somewhat lower.

The redox potential was determined by titration with a number of dyes, and was found to be + 0.02 volt. All the iron could be accounted for as haem. The reduction of cytochrome b_5 by DPNH or TPNH required the presence of microsomes, and a partially purified DPNH-cytochrome reductase was obtained on fractionation of an extract of lipase-treated microsomes. It was found that this enzyme would not catalyse the reduction of cytochrome c unless cytochrome b_5 was added (Strittmatter and Velick, 1956a).

A homogeneous flavoprotein carrying out the reactions of the crude microsomal extract was isolated, having FAD as prosthetic group, and containing 2 moles of Mg^{++}/mole of flavin. This was the only metal present in the enzyme in more than trace amounts, and it appears that it may be required for the reduction of cytochrome b_5 by the enzyme, since this reaction, but not the reduction of ferricyanide, was inhibited by reagents which might be expected to chelate Mg^{++}. The reduction of cytochrome c by DPNH in the presence of the flavoprotein dehydrogenase and cytochrome b_5 was not sensitive to antimycin or to dinitrophenol, but the reductase activity of the flavoprotein was completely inhibited by organic mercurials such as PCMB and PCMS. This inhibition could be prevented by the prior addition of DPNH and DPNH analogues, but not by the oxidised form of these substrates (Strittmatter and Velick, 1957). Titration of the flavoprotein showed that it contained three moles of -SH/mole of flavin in the native enzyme. Only two of these reacted rapidly with PCMB or NEM; PCMB was found to react preferentially with the sulphydryl group involved in the binding of the substrate (DPNH),

whereas NEM only reacted with this thiol group after the other group had reacted with the reagent. Only one of the -SH groups, the PCMB-sensitive one, was involved in the reductase activities of the enzyme (Strittmatter, 1959). Strittmatter (1959a) obtained spectroscopic evidence for the existence of a complex between the flavoprotein and DPNH. This will be discussed further in the section dealing with mechanism of action of flavoprotein enzymes (p. 138).

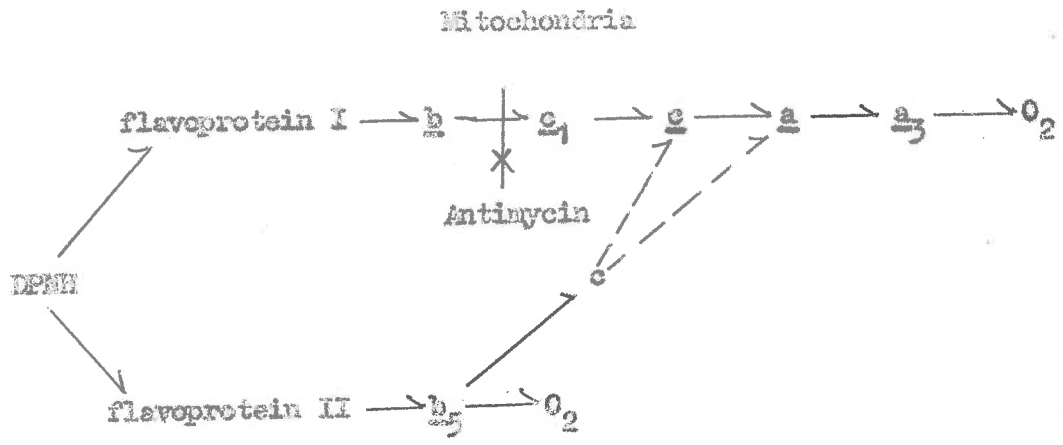
Recently, a cytochrome has been isolated from pig liver, having very similar properties to cytochrome b_5 of Strittmatter and Velick, but differing in several ways, particularly in redox potential, acid stability, and reducibility by cysteine (Raw, Molinari, do Almaral and Mahler, 1958). In view of the results of Henderson and Rawlinson (1961) on the change in redox potential of cytochrome c with progressive denaturation, the difference in redox potential may not be of significance. Mahler and his coworkers (Mahler, Raw, Molinari and do Amaral, 1958) have also isolated a DPNH cytochrome reductase from pig liver. The properties of this flavoprotein are somewhat different from the reductase obtained by Strittmatter and Velick (1957). In particular, the origin of Mahler's enzyme is in doubt, but it is quite clear that the extraction procedure used by Mahler fails to extract, or inactivates, the reductase from rabbit liver microsomes, which Strittmatter and Velick partially purified. Moreover, the extraction released considerable quantities of reductase from mitochondria. It seems most likely that Mahler's flavoprotein is derived from mitochondria, since it shows the same sensitivity to snake venom as does mitochondrial DPNH oxidase. The extraction from liver mitochondria of a cytochrome having a spectrum like cytochrome b_5

(Schmukler and Polis, 1958) did seem to lend credence to the possibility that cytochrome b_5 is not exclusively located in the microsome fraction. However, this haemoprotein has been shown to be identical with mitochrome, which is a degradation product released by mitochondria (Hülsemann, Elliot and Slater, 1960). Therefore, the likelihood that Mahler's enzyme can react with cytochrome b_5 *in vivo* seems extremely small, and one must consider the possibility that his reconstructed system is an artifact, which fortuitously has the ability to reduce cytochrome b_5.

A haemoprotein with an α-band at 556 mμ has been observed in insect issues (Sanborn and Williams, 1950); it is particularly concentrated in the midgut of the silkworm (Cecropia) together with cytochrome oxidase, and only traces of cytochromes b and c are present. In washed homogenates of Cecropia midgut (Pepperheimer and Williams, 1954), the reduced cytochromes appeared slowly under anaerobic conditions. The addition of DPNH to the anaerobic tissue resulted in the rapid appearance of the reduced absorption bands of all the cytochromes; KCN did not prevent the oxidation of reduced cytochrome b_5 by oxygen. Succinate rapidly reduced cytochromes b and c and cytochrome oxidase; cytochrome b_5 was reduced slowly at a rate which was probably not much different from the rate of its appearance in the absence of succinate or DPNH.

The rate of oxygen uptake with DPNH as substrate in antimycin-treated homogenate was inhibited by KCN. This inhibition was considerably greater when cytochrome c was added. Since the cytochrome b system was inhibited by antimycin, this oxygen uptake was due to the

oxidation of DPNH through cytochrome b_5, although the cyanide inhibition indicates that cytochrome oxidase was involved. Kinetic studies of the homogenate (Chance and Pappenheimer, 1954) showed that the aerobic oxidation of cytochrome b_5 was inhibited $\sim$ 80% by 1 mM. KCN; higher cyanide concentrations had no further effect. The inhibition showed the same dependence on cyanide concentrations as was obtained for heart muscle preparations and it may therefore be concluded that a considerable part of the oxygen uptake was brought about by cytochrome oxidase. In view of the oxidation of reduced cytochrome b_5 by oxygen, the following scheme of electron transport seems reasonable for this system



The problem of the *in vivo* function of cytochrome b_5 remains unsolved. Its slow rate of reaction with oxygen suggests that it is unlikely to function as a terminal oxidase (C. Strittmatter, 1961), more particularly in view of the α-glycerophosphate system for oxidation of extramitochondrial DPNH by mitochondria (Zabe, Delbruck

and Bucher, 1959; Boxer and Shonk, 1960). Similarly, its ability to transfer electrons to soluble cytochrome c need not imply that coupling to the mitochondrial respiratory chain is possible, especially since the accessibility of extramitochondrial cytochrome c to the respiratory chain does not seem likely (Chance and Williams, 1955; Slater, 1958).

Since the endoplasmic reticulum, from which the microsomes are derived, is recognised as the site of a number of synthetic reactions which require a reductant, it has been suggested that cytochrome b_5 may fulfill the function of the reductant, particularly in view of its low redox potential (C. Strittmatter, 1961).

ii. Mitochondrial reductase systems

(a) Nomenclature. In 1939, Straub isolated a fluorescent, FAD-containing protein from pig heart. This enzyme, which he named diaphorase, catalysed the rapid reduction of various dyes by DPNH; and a slow reduction of cytochrome c (Lockhart and Potter, 1941; Straub, 1942). The derivation of the word diaphorase implies that it was considered that this flavoprotein was an intermediate carrier in the oxidation of DPNH, although more recent work shows that this is without justification. Subsequent to the discovery of diaphorase, any enzymic reduction of a dye, such as methylene blue, by DPNH was attributed to diaphorase, and for some time it was considered that the mitochondrial DPNH-cytochrome c reductases and diaphorase were identical. However, it has been convincingly demonstrated that Straub's diaphorase represents a modified form of lipoyl dehydrogenase, which has lost its ability to reduce oxidised lipoic acid. Lipoyl dehydrogenase is a component of the α-ketoglutarate dehydrogenase complex (Searls and Sanadi, 1959;

Massey, 1958, 1960a, b, c). It seems that there is some virtue in restricting the term diaphorase to Straub's enzyme (Massey, 1960a), since the physiological function of this enzyme differs considerably from other enzymes to which the name diaphorase has been applied.

An earlier indication of this is found in the study of plant cellular constituents by Martin and Morton (1955), where the distribution of the DPNH-cytochrome c reductase and diaphorase-like activities in the various fractions were not parallel. Accordingly, where enzymic dye reduction by DPNH occurs, this will be referred to as dye reductase activity, in the absence of convincing evidence that the enzyme responsible is diaphorase.

(b) Nature of the system. Although soluble flavoproteins catalysing the reduction of cytochrome c by DPNH had been isolated by conventional classical procedures (Haas, Horecker and Hogness, 1940; Horecker, 1950), the mitochondrial DPNH-cytochrome c reductases were not amenable to these procedures, and therefore the involvement of flavoprotein dehydrogenases in DPNH oxidation was uncertain. In 1952, the Wisconsin group (Edelhoch, Kaiyaishi and Tegley, 1952; Mahler, Sarkar, Vernon and Alberty, 1952) obtained a soluble, homogeneous flavoprotein from submitochondrial particles of beef heart which possessed DPNH-cytochrome c reductase and diaphorase-like activity. The prosthetic group was very similar to, but not identical with FAD, and the protein contained 4 atoms of non-haem iron for each molecule of flavin. It was postulated that the iron was involved in the reduction of cytochrome c by the flavoprotein and DPNH, but not in dye reduction. This was based on the finding that various chelating agents, and in

particular, pyrophosphate and citrate, behaved as competitive inhibitors for cytochrome c reduction, but not for dye reduction (Mahler and Elowe, 1954). Recent work suggests that the extraction procedure employed modifies the normal FAD prosthetic group to the FAD-like compound observed by Mahler et al. (Green, Ziegler and Doeg, 1959b). Indeed it is suggested on the basis of this later study that the enzyme isolated by Mahler is a modified form of diaphorase, whereas Mahler concluded earlier that diaphorase was formed from DPNH-cytochrome c reductase by loss of iron. It is difficult to reconcile this viewpoint with Massey's results (1960a) which show quite clearly that Mahler's enzyme and diaphorase may be differentially extracted under mild conditions. The lipoflavoprotein isolated by Green, Ziegler and Doeg (1959b) is claimed to be related very closely to diaphorase, but this does not seem to be supported by the spectrum of the lipoflavoprotein, which is atypical for a flavoprotein, and not at all like that of Straub's diaphorase (Savage, 1957). The enzymic role of this protein must remain in doubt, although its specificity towards acceptors is rather like that of diaphorase. It does not seem likely that it represents a modified DPNH-cytochrome c reductase.

The metallo-flavoprotein concept will be discussed elsewhere (p. 85), but it is worth noting that microsomal DPNH-cytochrome c reductase (Strittmatter and Velick, 1956a, 1957), which contains only Mg^{++} in addition to flavin, is inhibited by many of the same reagents as Mahler's enzyme, in particular, by pyrophosphate and citrate. It may, therefore, be concluded that the iron in DPNH-cytochrome c reductase

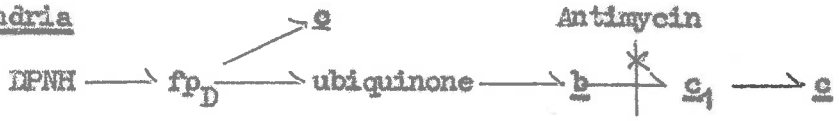
is probably not involved directly in electron transport.

It is necessary to consider whether the direct reduction of cytochrome c by the DPNH-flavoprotein dehydrogenase has any physiological significance. As discussed earlier, it seems that in mitochondria and submitochondrial particles, cytochrome c is separated from the primary mitochondrial dehydrogenases by several components and that electron transfer through this system is inhibited by antimycin A. The available evidence implicates ubiquinone as the acceptor for the primary dehydrogenases, and also suggests that antimycin A inhibition is caused by an effect on cytochrome c_1, which prevents it from being reduced by either cytochrome b or ubiquinone. As mentioned earlier, mitochondria possess, in some cases, an antimycin A-insensitive DPNH-cytochrome c reductase activity. It seems likely that this activity is due to the same enzyme as soluble DPNH-cytochrome c reductase from mitochondria which is not sensitive to antimycin A (Mahler and Elowe, 1954; De Bernard, 1957). Since both DPNH-cytochrome c reductase and mitochondrial DPNH oxidase exhibit the same sensitivity towards snake venom (Mahler et al., 1958), it is probable that the flavoprotein dehydrogenase is also the same in these cases.

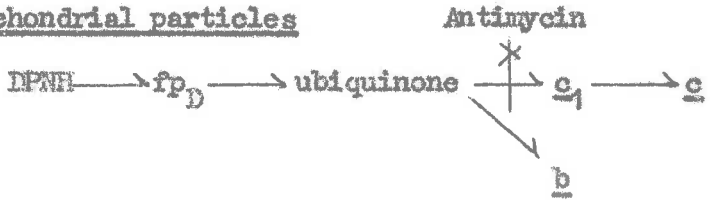
None of the DPNH-cytochrome c reductases solubilised by mitochondria are susceptible to inhibition by amytal, which Chance has shown to act between DPNH and the flavoprotein dehydrogenase in mitochondria (Chance, 1956). If, therefore, the soluble reductases are derived from the respiratory chain dehydrogenase, it must be assumed that the effect of amytal on the respiratory chain is physical rather than chemical. It seems reasonable to conclude, therefore, that

mitochondrial DPNH-cytochrome c reductase is very unspecific towards acceptors, and that the specificity may become modified during purification of the soluble enzyme. It has been shown that soluble cytochrome c_1 will not act as an acceptor for DPNH-cytochrome c reductase nor for succinate cytochrome c reductase (Green, Jarnefelt and Tisdale, 1959). The following reactions are all apparently possible.

Mitochondria

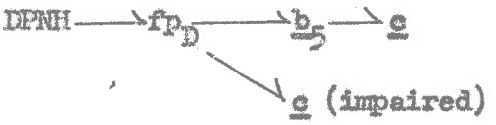


Submitochondrial particles



Soluble enzyme from beef heart particles

Soluble enzyme from pig heart



Particulate preparations from Azotobacter vinelandii, containing cytochromes b_1, c_4, c_5 and a_2 possess DPNH-cytochrome c_4 reductase activity; the reduction of mammalian cytochrome c is very much slower than the reduction of the bacterial cytochrome (Bruemmer, Crane, Glenn and Wilson, 1957). The pathway of electron transport for this system is not known.

In summary, the direct reduction of cytochrome c by DPNH-flavoprotein dehydrogenases does not occur *in vivo*, and is mediated by one or more intermediate electron carriers with redox potentials intermediate between flavoprotein and cytochrome c. The *in vitro* reduction of cytochrome c by soluble preparations of the flavoprotein dehydrogenases is probably due to a modification of the dehydrogenase, although it is possible that this may be simply due to the dehydrogenase becoming accessible to cytochrome c by virtue of the loss of the ordered structure of respiratory carriers present in particluate material. For the same reasons as given for microsomal DPNH-cytochrome c reductase, the direct reduction of cytochrome c by the mitochondrial DPNH dehydrogenase is of little or no physiological significance in electron transport.

3. Reduced triphosphopyridine nucleotide-cytochrome c reductase

i. From yeast. TPNH-cytochrome c reductase was purified from autolysates of dried top yeast and was shown to have FMN as its prosthetic group (Haas, Horecker and Hogness, 1940). Slightly impure preparations contained a haem pigment, having an absorption at 413 mμ when the enzyme was in the oxidised state. On addition of TPNH, the band at 413 mμ disappeared and was replaced by bands at 423 mμ and 557 mμ. (Haas, Horecker and Hogness, 1942). The haem pigment was not essential for cytochrome c reduction, since further purification removed it and increased the specific activity of the reductase. Since cytochrome b_2 had been reported as being associated with yeast lactate dehydrogenase earlier in the same year (Bach, Dixon and Keilin, 1942), Haas *et al.*

(1942) concluded that the haem pigment observed in impure TPNH-cytochrome c reductase was cytochrome b_2, and that it was reducible by TPNH. Bach, Dixon and Zerfas (1946) considered that there was insufficient evidence for such an identification. They pointed out that the difference between the two systems (activating lactate and TPNH respectively) made it improbable that the pigment was cytochrome b_2, and furthermore, that if the pigment of Haas et al. was cytochrome b_2, it seemed unlikely that reduction of the pigment was due to TPNH, since minute traces of lactate would bring about the reduction of cytochrome b_2 in their yeast lactate dehydrogenase preparations.

Yeast lactate dehydrogenase, prepared as described by Bach et al. (loc. cit.) possesses TPNH- and malate-cytochrome c reductase activity (Yamashita et al., 1957; Yamanaka, Horio and Okunuki, 1958; Yamashita et al., 1958). Furthermore, a haem pigment from yeast, described as cytochrome b_2* (Yamashita et al., 1957) was reduced by TPNH or by malate in the presence of lactate dehydrogenase (Yamashita et al., 1958). It should be noted, however, that the rate of reduction of the so-called 'cytochrome b_2' by lactate, TPNH or malate was about $^1/_{50}$ th of the rate of reduction of cytochrome c.

Nygaard (personal communication) has found that the malate cytochrome c reductase activity of partially purified ($\sim$ 10% pure) lactate dehydrogenase disappears on further purification and other workers (Boeri, 1961; Morton, Armstrong and Appleby, 1961) have found that malate and TPNH do not act as substrates for lactate dehydrogenase.

* discussed elsewhere (p. 51).

In view of these results it is likely that the pigment observed by Haas *et al.* was cytochrome b_2 or denatured cytochrome b_2, and that the reduction of the pigment was due to TPNH. However, whatever the state of the cytochrome b_2, it is not known whether the reduction of the haem pigment was brought about directly from the TPNH activating flavoprotein or whether the flavin prosthetic group of lactate dehydrogenase was required.

ii. *From liver.* A FAD-containing TPNH-cytochrome *c* reductase has been isolated from liver, and has been shown to be present in the mitochondria (Horecker, 1950).

A TPNH-cytochrome *c* reductase has been demonstrated to be present in microsomes (Bailie and Morton, 1955; Martin and Morton, 1955; Velick and Strittmatter, 1956). This enzyme is probably analogous in behaviour to microsomal DPNH-cytochrome *c* reductase.

The physiological significance of the mitochondrial enzyme has not been established. It is still uncertain as to whether both the DPN- and TPN- requiring *iso*citrate dehydrogenases are involved in the oxidation of *iso*citrate by mitochondria (Purvis, 1958; Ernster, 1959; Ernster and Gladsky, 1960). However, Purvis (1958) has established quite clearly in guinea pig liver mitochondria and in rat sarcosomes that the oxidation of *iso*citrate requires the TPN-specific dehydrogenase, and that at least some of the TPNH so formed is oxidised by transhydrogenase and DPNH-oxidase. The role of transhydrogenase in TPNH oxidation by mitochondria is not yet settled, and the possibility is conceded that both TPNH-oxidase and transhydrogenase-DPNH oxidase are involved in the oxidation of TPNH by mitochondria (Ernster, 1959, 1960; Slater, 1959).

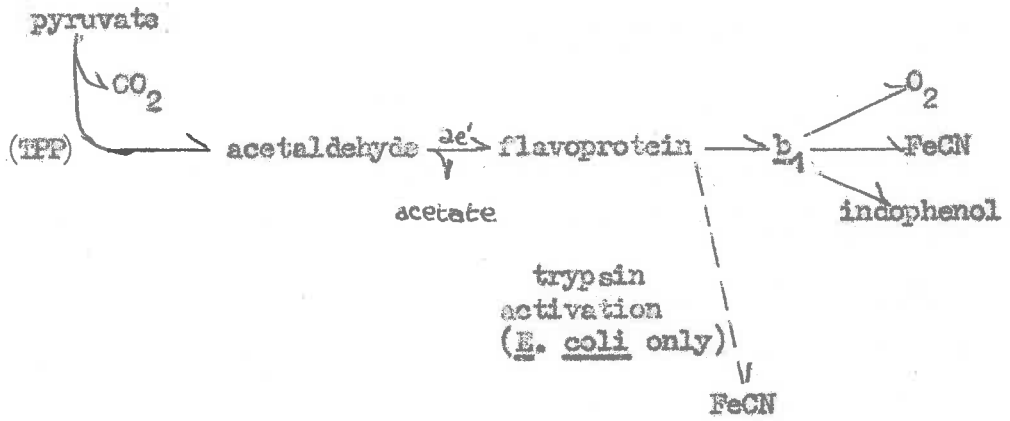
The relationship of TPNH-cytochrome c reductase to TPNH "diaphorases" is not known, and in fact they may possibly be the same enzyme (Mahler, 1955) except in the case of TPNH diaphorase from chloroplasts (Avron and Jagendorf, 1956).

4. Bacterial pyruvate oxidase system.

In addition to the normal CoA-dependent, DPN-linked pyruvate oxidase, Escherichia coli possesses a system which oxidises pyruvate to acetate and carbon dioxide, the only cofactor required being thiamine pyrophosphate (Razzel and Gunsalus, 1954). This system is also present in Proteus vulgaris (Moyed and O'Kane, 1956), and ferricyanide and indophenol will substitute for oxygen as electron acceptors. The system can be separated into soluble and particulate fractions, both of which are required for reaction with oxygen as acceptor (Moyed and O'Kane, 1956; Hager, 1957). Indeed, both fractions are required for reaction with any of the acceptors in preparations from E. coli (Hager, 1957). Moyed and O'Kane (1956) have shown that the particulate fraction from P. vulgaris contains cytochromes a_1, a_2 and b_1, which are reduced in anaerobic conditions by pyruvate and the soluble fraction. The soluble fraction from E. coli has been purified, and is a non-autoxidisable flavoprotein, with 1 mole of FAD/200,000 g. of protein. This enzyme will not react with oxygen or ferricyanide in the absence of the particulate fraction. However, treatment with trypsin modifies the flavoprotein so that it will act directly with ferricyanide (Hager, 1951, 1959). The active fraction of the E. coli particles is released by digestion with trypsin, and has been partially purified. It contains a pigment with a spectrum

somewhat similar to that of cytochrome b_1, although the position of the α-band as reported is lower than that attributed to cytochrome b_1 (Morton, 1958). This cytochrome is reduced by pyruvate in the presence of the soluble flavoprotein, and by dithionite. It is apparently autoxidisable. (Williams and Hager, 1960).

It is suggested, therefore, that the reaction mechanism is the decarboxylation of pyruvate to acetaldehyde, which is then oxidised to acetate.



It would seem that the soluble flavoprotein is a complex of enzymes, involving a carboxylase and a flavoprotein dehydrogenase. This is very similar to the α-ketoglutarate dehydrogenase of pig heart, which has been separated into two proteins, one containing thiamin pyrophosphate and lipoic acid, the other a flavoprotein, lipoyl dehydrogenase (Massey, 1960). Before suitable treatment α-ketoglutarate dehydrogenase appears homogeneous in the ultracentrifuge, as does the soluble flavoprotein of the bacterial pyruvate oxidase (Hager, 1959).

An interesting feature of the flavoprotein from E. coli is

its failure to reduce ferricyanide unless modified by trypsin. This treatment results in a decrease in sedimentation coefficient of the enzyme.

C. Other Lactate Dehydrogenases with Flavin Prosthetic Groups.

1. Lactate oxidative decarboxylase of Mycobacteria.

Mycobacteria can oxidise lactate; cell free extracts carry out the reaction

$$\text{lactate} + O_2 \longrightarrow \text{acetate} + CO_2 + \text{water}$$

Anaerobically, with methylene blue as acceptor, these extracts will oxidise lactate to pyruvate, although added pyruvate is not converted to acetate in aerobic conditions. (Edson, 1947, 1951). It was postulated that acetate was formed by the non-enzymic oxidation of pyruvate by hydrogen peroxide, produced by the reaction of oxygen with a reduced flavoprotein lactate oxidase. However, the aerobic reaction with lactate is not inhibited by catalase (Sutton, 1954) and only traces of peroxide and pyruvate are formed under these conditions (Cousins, 1956). The enzyme is specific for L-(+)-lactate (Cousins, 1956), and has been crystallised as a homogeneous flavoprotein, containing 2 moles of FMN per mole of enzyme, and having a molecular weight of approximately 260,000. The oxidation of lactate is competitively inhibited by pyruvate and DL-glycidate (2,3 epoxypropionate). (Sutton, 1955, 1956). It was shown that oxygen from the gaseous phase was incorporated into the acetate formed, but not into the CO_2 (Sutton and Hayaishi, 1956).

$$CH_3\text{-}CHOH\text{-}COO^- + O_2{}^{18} \longrightarrow CH_3\text{-}COO^{18-} + CO_2 + H_2O^{18}$$

These results are consistent with the hypothesis that a ternary complex of enzyme, lactate and oxygen is formed, and that acetate arises directly from this complex, without dissociation of pyruvate and hydrogen peroxide from the enzyme.

2. Possible flavoprotein lactate dehydrogenases

Casida and Knight (1954) partly purified a lactate dehydrogenase from Penicillum chrysogenum. This enzyme was presumably a flavoprotein, since there was no requirement for DPN or TPN as a coenzyme and lactate oxidation was stimulated by FMN and FAD and inhibited by high concentrations of quinine. The oxidation of lactate was also stimulated by cytochrome c, and it was suggested that the enzyme was a cytochrome-linked lactate dehydrogenase.

Haugaard, (1959) obtained a particulate preparation from Escherichia coli, which oxidised D- and L- lactate to pyruvate. The particles contained cytochromes. Acetone powders of cell-free extracts would oxidise only D-lactate; the addition of methylene blue was necessary to obtain oxidation. This suggested that separate dehydrogenases for D-and for L-lactate are present in E. coli. These enzymes may possibly be flavoproteins. Previously, Szulmajster, Grunberg-Manago and Delavier-Klutcho (1953) had reported the preparation of a soluble lactate dehydrogenase from particles obtained from E. coli. There was no direct relationship between flavin content and enzymic activity. However, since the enzyme was not very highly purified, this finding does not negate the likelihood that the lactate dehydrogenase of E. coli is a flavoprotein.

Moyed and O'Kane (1954) reported substantial purification of an L-lactate dehydrogenase from Proteus vulgaris. The enzyme reduced 2:6-dichlorophenol indophenol and did not require added DPN or TPN; The authors were unable to show the presence of any flavin or haem pigment.

Snoswell (1959) has obtained a highly purified flavoprotein from Lactobacillus arabinosus. This protein has L(+)-lactate-indophenol reductase activity. He later was able to separate a D(-) lactate dehydrogenase which he claimed had no organic prosthetic group (Snoswell, 1959a). More recent studies have shown that the D-lactate dehydrogenase was grossly impure, and that a flavoprotein dehydrogenase for D-lactate could be separated by chromatography of the crude preparation (Snoswell, personal communication). Thus it would appear that both the L- and D- lactate dehydrogenases of this organism are flavoproteins.

The L-amino acid oxidase of mammalian tissues catalyses the rapid oxidation of a number of L-α-hydroxy acids, including lactate (Blanchard, Green, Nocito and Ratner, 1944, 1945; Winitz, Bloch-Frankenthal, Izumaya, Birnbaum, Baker and Greenstein, 1956). This enzyme has FMN as the prosthetic group. Baker demonstrated the presence of at least two enzymes in particles from pig kidney, which catalyse the aerobic oxidation of a number of D- and L-α hydroxy acids. This oxidation is stimulated by FMN.

Tubbs and Greville (1959) have obtained, in soluble form, a specific D-lactate dehydrogenase from an acetone powder of rabbit kidney mitochondria; the enzyme is probably a flavoprotein; it does not require added DPN or TPN for activity.

Glycollate oxidase from spinach leaves also catalyses the oxidation of L-lactate. The enzyme has FMN as prosthetic group (Zelitch and Ochoa, 1953). L-α-hydroxy acid oxidase of green leaves catalyses the aerobic oxidation of a number of L-α-hydroxy acids including lactate. The formation of hydrogen peroxide suggests that the enzyme is a flavoprotein (Tolbert, Clagett and Burns, 1949; Clagett, Tolbert and Burns, 1949; Kenton and Mann, 1952). This enzyme is probably distinct from the glycollate oxidase described by Zelitch and Ochoa (1953). It therefore appears that only the L-amino acid oxidase of mammalian tissues, the glycollate oxidase of spinach leaf, the lactate oxidative decarboxylase of Mycobacteria and cytochrome b_2 of baker's yeast have been clearly demonstrated to contain flavin prosthetic groups, which are involved in lactate oxidation.

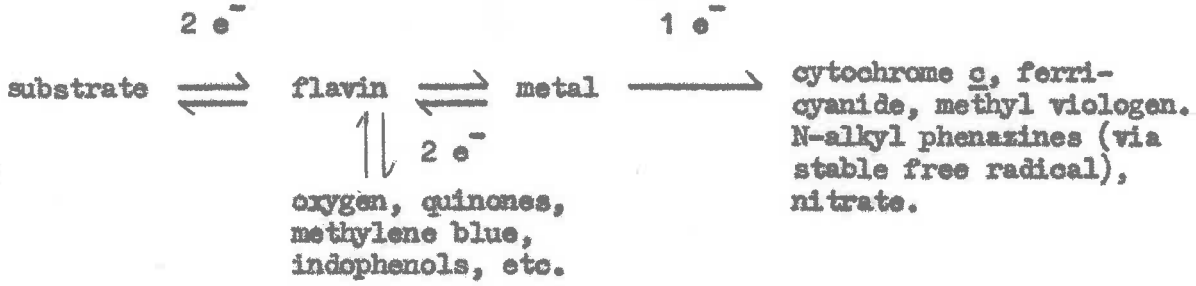
D. Metallo-Flavoprotein Hypothesis.

The requirement for metal ions for numerous enzyme catalysed reactions is well established and in general has been interpreted in terms of substrate (or cofactor) binding and activation. In 1954, a new concept for the role of metal ions in reactions catalysed by flavoproteins was suggested (Mahler and Green, 1954). This theory proposed that metal ions functioned as intermediary electron carriers between the flavin prosthetic group and one-electron acceptors, and the term metallo-flavoprotein was coined. The theory gained wide acceptance and within a short period, a large number of purified flavoproteins were shown to contain firmly bound metal ions; a number of flavoproteins, at various levels of purity, were found to be inhibited by metal chelating reagents. On the

basis of these and other findings, an elaborate theory for the mechanism of action of flavoprotein enzymes was constructed (Mahler and Glenn, 1956; Mahler, 1956). This section is concerned with an examination of this theory.

Although the first flavoprotein for which a metal ion requirement was established was xanthine oxidase (Westerfield and Richert, 1949; 1953; DeRenzo, Kaleita, Heytler, Hutchings and Williams, 1953), the theory discussed here arises from the demonstration of iron in DPNH-cytochrome c reductase (Mahler and Klowe, 1953, 1954) and copper in butyryl CoA dehydrogenase, a green flavoprotein (Mahler, 1953, 1954).

It was found that cytochrome c reduction, but not dye reduction, by DPNH-cytochrome c reductase was inhibited by phosphate, citrate, pyrophosphate and reagents such as 8-hydroxy-quinoline (Mahler and Klowe, 1954). The green colour of butyryl CoA dehydrogenase, which was attributed to copper on spectroscopic evidence, was bleached on reduction of the enzyme, and cytochrome c reduction was inhibited by dialysis against cyanide. Since both xanthine oxidase and nitrate reductase (Nicholas, Nason and McElroy, 1953, 1954a) require molybdenum for their *in vivo* synthesis, and the metal is present in the purified enzymes, this lent some support to the idea that metals were involved in flavoprotein catalysis. Furthermore, it had been shown that riboflavin could form stable coordination complexes with a number of metal ions (Albert, 1953). The mechanism proposed to account for these findings was



Thus, for two electron acceptors, the transfer of two electrons from reduced flavin to acceptor was assumed to be simultaneous, in that the flavin-acceptor complex existed long enough for the stepwise transfer of two electrons as postulated by Michaelis (1951). For one electron acceptors, the kinetics do not permit a ternary collision between reduced flavoprotein and two one-electron acceptor molecules, nor the addition of the acceptor molecules in sequence (Mahler, Vernon and Sarkar, 1952; Frieden, 1957). Thus, a semiquinone (free radical) form of the flavoprotein is required (see p.137), and it was postulated that the metal acted by stabilising the semiquinone by increasing the resonance of the system, or by assisting in the formation of a diradical from the reduced flavin by uncoupling of an electron pair. (The diradical mechanism as presented by Mahler is not satisfactory for the explanation of the kinetics of DPNH-cytochrome c reductase, but this is a matter of detail).

In view of the fact that a relatively stable free radical form of riboflavin was known to exist (see p.136) and that the model (non-enzymic) system

$$\begin{matrix}\text{DPNH} \\ \text{or} \\ \text{TPNH}\end{matrix} + 2\ \text{cyt}\ c^{+++} \rightleftharpoons \begin{matrix}\text{DPN} \\ \text{or} \\ \text{TPN}\end{matrix} + 2\ \text{cyt}\ c^{++}$$

took place in the absence of metal ions, (Singer and Kearney, 1950), these hypotheses seem rather unnecessary, and the data on which they rest is now examined.

The green colour of butyryl-CoA dehydrogenase is not due to copper, and the green enzyme is irreversibly converted to a yellow form on reduction by substrate and reoxidation by an acceptor. The presence of copper for enzymic activity is unnecessary, since preparations with much higher specific activities, and containing only traces of copper have been obtained (Beinert and Steyn-Parve, 1959). The observed reactivation, by copper, of the cyanide-inactivated enzyme has been attributed to a non-enzymic reaction between reduced flavin and acceptor mediated by copper (Singer and Massey, 1957).

By far the greatest weight of the metallo-flavoprotein hypothesis rests on results obtained with DPNH-cytochrome c reductase. The complete loss of activity of the enzyme towards cytochrome c as acceptor (but only partially towards dyes) on removal of the iron, and the reactivation by added Fe^{+++} (at high concentrations) seemed to be good evidence for the hypothesis, as did the finding that the iron was more reduced after addition of DPNH to the enzyme, and that cytochrome c reductase activity, but not indophenol reductase activity, was inhibited by metal complexing reagents (Mahler and Elowe, 1954). However, some caution is necessary in interpreting these results. It is known that

inorganic iron and protein chelates of iron can catalyse the reduction of cytochrome c by reduced flavins (Singer and Massey, 1957). The valency state of the iron is affected by various conditions. Thus, under the conditions used by Mahler, o-phenathroline reduces ferric iron to ferrous iron (Singer and Massey, 1957); DPNH will reduce ferric iron non-enzymically (Weber, Lenhoff and Kaplan, 1954); thirdly, as has been observed for succinate dehydrogenase, reduction of ferric iron is also brought about by sulphydryl groups which may be released by denaturation of the protein, in the extraction of the iron. (Singer and Massey, 1957; Massey, 1958), and finally, ferrous iron is oxidised by cytochrome c (Weber, Lenhoff and Kaplan, 1954). The inhibition of cytochrome c reductase by 8-hydroxyquinoline and o-phenathroline is not necessarily an indication that the iron participates in electron transport, as similar inhibitions were observed with succinate dehydrogenase, and were found to be due to denaturation of the enzyme. The inhibition of enzymic activity by citrate, phosphate, pyrophosphate and ethylenediamine tetraacetate has also been observed in microsomal DPN-cytochrome c reductase (Strittmatter and Velick, 1957), which contains no iron, only magnesium. It seems possible that this effect might be explained by competition of the enzyme with these anions for the positively charged cytochrome c.

The situation with regard to molybdenum-containing flavoproteins is not yet clear. Thus it has been unequivocally demonstrated that Mo is required for the synthesis of xanthine oxidase and nitrate reductase, and is present in the purified enzymes. A reversible inactivation of nitrate reductase on removal of Mo has been observed, and the levels of the metal

required for reactivation are small (Nicholas and Nason, 1954, 1954a). In xanthine oxidase, reduction of the Mo by substrate and by acceptor have been observed by electron spin resonance (Bray, Malstrom and Vängård, 1959), and it is believed that this is of significance in the enzymic reaction.

The evidence for the participation of iron in the reduction of phenazine and of ferricyanide by reduced succinate dehydrogenase is discussed elsewhere (p. 64), and Massey and Palmer (1959) have recently shown that iron is not present in D-amino acid oxidase, as had been earlier reported (Kubo et al., 1958). It has been known for some time that crystalline cytochrome b_2 contains no non-haem iron (Appleby and Morton, 1954), although the preparations of cytochrome b_2 studied by Boeri and his coworkers (Boeri and Tosi, 1956; Boeri and Rippa, 1961) have been found to contain variable quantities of non-haem-iron. The data for most other enzymes classified as metallo-flavoproteins is insufficient for any conclusions to be drawn as to the role of the metal in catalysis. In view of the ability of proteins to bind metal ions, considerable care should be exercised before the presence of a metal in an enzyme is taken as evidence that the metal is involved in the catalytic function of the enzyme, rather than being present as a firmly bound contaminant. Similarly, inhibition by metal chelating agents is not necessarily due to a direct effect of these reagents on the accessibility of the metal, but may be due to denaturation of the protein, or some other effect of the reagent which is not concerned with its capacity as a metal chelator (Singer and Massey, 1957).

The necessity for invoking such a concept as the metallo-flaveprotein hypothesis turns largely upon the stability of the semi-quinone form of the flavin prothetic group which must exist during the oxidation of the enzyme by one electron acceptors. The stability of the semiquinone form of riboflavin seems adequate evidence that a relatively stable free radical can be formed (Michaelis, Schubert and Smythe, 1936), so that Mahler's suggestions for metals acting by/stabilising a free radical form of the flavin prothetic group seem unnecessary.

It cannot be doubted that certain flaveproteins contain firmly bound metal ions which are essential for their enzymic activity. However there are numerous other ways in which this requirement can be explained without postulating, on rather dubious evidence, that the metal is an intermediate electron carrier.

II. CHEMICAL PROPERTIES OF CYTOCHROME b_2

A. Materials and Methods

1. Preparation of Yeast Lactate Dehydrogenase

During the course of the work described here, many batches of YLD prepared from air dried bakers yeast, essentially as described by Appleby and Morton (1959). The method of preparation is set out in Fig. 11. A number of minor modifications were made to obtain the best yields of enzyme from the particular yeasts used. Initially the yeast was obtained as air-dried material from Barretts Foods Ltd., Melbourne. Later, compressed yeast, obtained from Effront Yeast Company, Melbourne, was broken into small lumps, air-dried and then used.

Modifications were made in

(a) the fractionation with acetone to obtain the first precipitate

(b) the concentrations of acetone and of lactate in the extraction of the first precipitate

(c) the temperature of the final fractionation.

These variations are indicated in Fig. 11, which sets out the general procedure for the preparation of YLD.

The time at which first crystals appeared during dialysis ranged from about 18 to 70 hours. It was found that crystallisation could often be induced in well dialysed solutions by removing the dialysis sac from the dialysate and allowing it to warm slightly. Typical first crystals are shown in Fig. 12. The laminated appearance of the crystals which is just visible in these crystals, was clearly visible to the eye, and is

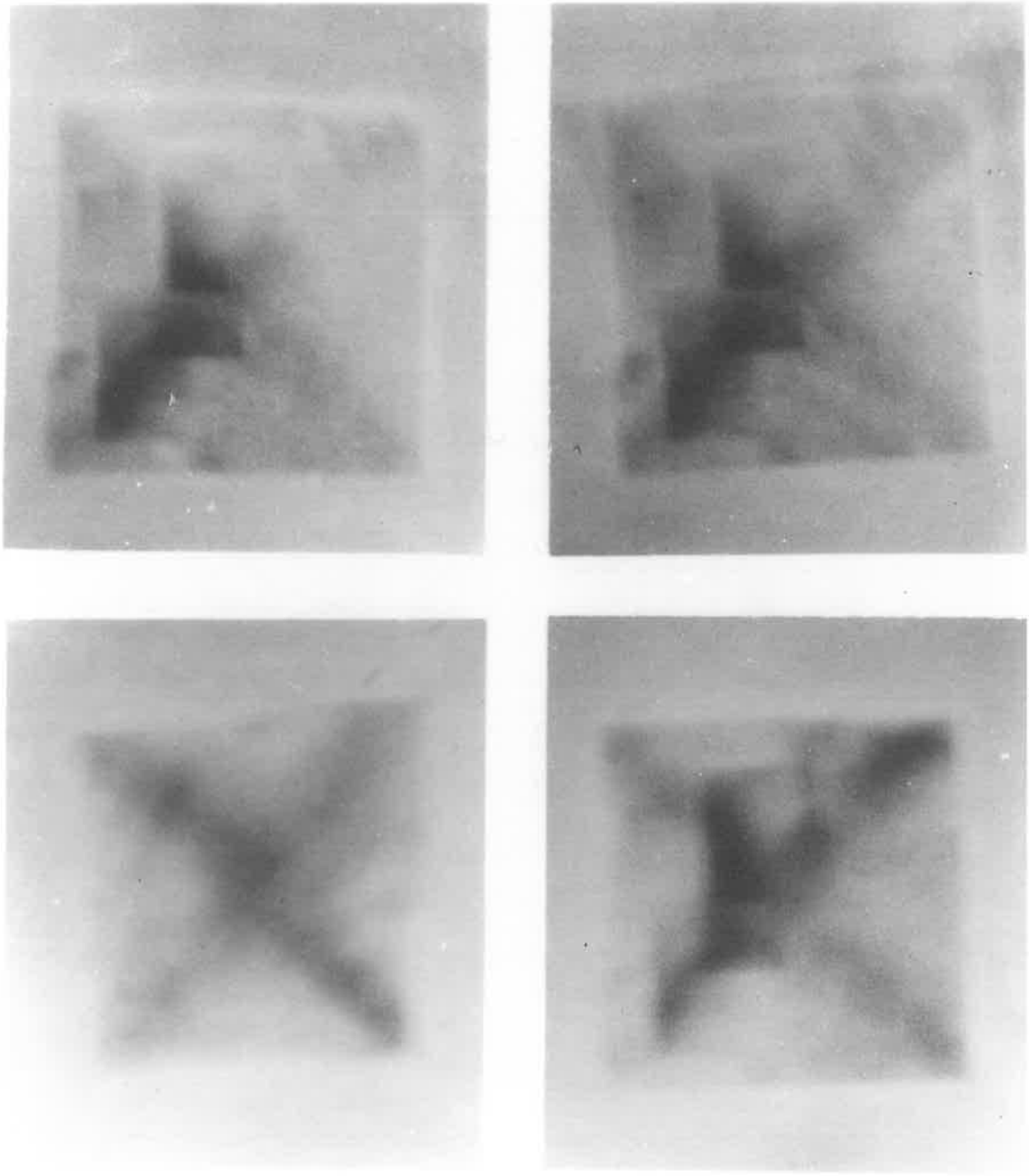
Figure 11.

Air dried yeast, ground in ball mill for 18-24 hrs. at 2°, extracted with 3.5 l./kg. of n-butanol, for 1 hr., centrifuged 10 mins. at 1200 g. Yeast residue extracted with 0.1M-Na lactate/10^{-4}M-EDTA, pH5.1, 4-6 l./kg., saturated with n-butanol, for 2-3 hrs., centrifuged 1,200 - 2,00 g./35-60 mins.

- fatty supernatant layer discarded
- precipitate discarded
- Clear supernatant (may be stored overnight at -15° and then thawed) cooled to -2 to -3°, in cold alcohol bath (-10 to -15°), cold acetone slowly added with mechanical stirring to 16-20% v/v final concentration, centrifuged 1,200 g./-5°/10 mins.
  - precipitate discarded
  - Supernatant, cooled to -5 to -6° in alcohol bath, acetone added to 30-32% v/v final concentration, final temperature -8 to -10°. Allowed to stand 45 minutes at 2°, poured off bulk of clear supernatant, suspended precipitate, centrifuged 1,200 g./-10°/10 mins.
    - supernatant discarded
    - Red brown gummy precipitate gently suspended in 22 to 25% acetone/0.1 to 0.2M-lactate, pH6.8/10^{-4}M-EDTA at -2° to -5°; this required up to 90 mins.; centrifuged 3000 g./-2°/20 mins.
      - precipitate discarded if cytochrome b_2 not spectroscopically detected
      - Supernatant, at -2 to -5°, fractionated with small volumes of acetone, centrifuging to remove pinkish brown precipitate, until XLD began to precipitate. Acetone then added to 40-45% v/v final concentration, until bands of cytochrome b_2 no longer detected in supernatant; centrifuged 3000 g./-10°/20 mins.
        - supernatant discarded
        - Precipitate mixed with small volume, 15-49 ml., of 0.04M-sodium pyrophosphate/0.1M-lactate/10^{-4}M-EDTA, pH6.8, centrifuged 10,000 g./2°/15 mins.
          - brown gummy precipitate discarded
          - Viscous supernatant placed in dialysis bags, sealed with bubble of N_2, placed in 5l. of 0.05M-lactate/10^{-4}M-EDTA, pH6.8, (outgassed by boiling, cooled to 2° under N_2); this was changed every 18-24 hrs. until crystals were formed. Crystals harvested 10,000 g./2°/15 mins., washed with 0.05M-lactate/10^{-4}M-EDTA, pH6.8, dissolved in 0.5M-lactate/10^{-4}M-EDTA, pH6.8, stored at -15° under N_2.

Fig. 12. Crystals of yeast lactate dehydrogenase

Crystals of YLD, obtained in the initial crystallisation, harvested by centrifuging at 10,000 g, in crystal mother liquor. Phase contrast illumination, total magnification, x 2160.



possibly evidence for the growth of the crystals by spiral dislocation. The diagonal cross is presumably related to the bipyramid structure of YLD crystals (Appleby, 1957).

Recrystallisation was rapid, generally commencing within 2 to 4 hours after dialysis was begun. Short periods of dialysis, up to 14 hours, gave the most satisfactory purification by recrystallisation; amorphous white polynucleotide material precipitated if dialysis was allowed to proceed for longer times. In general, after two recrystallisations, no further purification was obtained, and the ratio $E_{265 m\mu}/E_{423 m\mu}$ became constant (approx. 0.9). It was observed that recrystallisation removed enzymically inactive YLD, which remained in the mother liquor.

2. Preparation of yeast lactate dehydrogenase free from polydeoxyribonucleotide

Appleby and Morton (1960) found that when solutions of crystalline YLD were dialysed under nitrogen against 65 to 70% saturated ammonium sulphate in 0.1M-lactate, the pink-coloured precipitate so obtained could no longer be crystallised by the usual method for crystallisation of YLD. Similarly, YLD treated with DNA-ase would not crystallise. It was found that this change in the properties of YLD was caused by the loss of the polynucleotide component of the crystalline enzyme, either by dissociation with ammonium sulphate, or by enzymic digestion of the polynucleotide with DNA-ase. The loss of polynucleotide from the protein was reflected in a decrease in the ratio $E_{265 m\mu}/E_{\gamma\text{-band}}$.

It was decided to investigate some of the properties of nucleotide-free cytochrome b_2, which is henceforth designated as AS-YLD. It was felt

that the prolonged dialysis against ammonium sulphate which was necessary to split off the polynucleotide could possibly result in modifications to the structure of the protein precipitate, and therefore attempts were made to prepare AS-YLD by direct addition of solid ammonium sulphate to solutions of recrystallised YLD. It was found that cytochrome $\underline{b}_2$ tended to precipitate with the nucleotide still attached under these conditions, even with the additions spaced over about 1 hour from 50% to 70% saturated ammonium sulphate. The precipitate was rather oily and tended to flow easily, even after centrifugation at 10,000 g for 10 minutes. On one occasion the nucleotide was split off during the precipitation and washing of the precipitate, but in another experiment none of the polynucleotide was removed. It was therefore decided to revert to the precipitation of AS-YLD by dialysis for 14 to 24 hours against ammonium sulphate; the precipitation takes place more gradually with this method. The pink precipitate obtained by this method was packed well on centrifuging, and resembled the more usual ammonium sulphate precipitates obtained with other proteins. It was found that the ratio ($E_{265 m\mu}/E_{\gamma\text{-band}}$) obtained at pH8.0 was the same as at pH6.8. This procedure has been used routinely for the preparation of AS-YLD, and the polynucleotide from YLD (Montague and Morton, 1960). Table 11 gives some of the spectral properties of AS-YLD as compared with YLD. The spectrum of preparation 1 is given in Fig. 13. This is compared with a sample of the same batch of YLD before ammonium sulphate treatment.

The enzymic activity of YLD was unaffected by removal of the polynucleotide, as was previously observed by Appleby and Morton (1960). The ratio of ferricyanide reduction to cytochrome $\underline{c}$ reduction was constant, if corrections were made for the variation in cytochrome $\underline{c}$ concentration in the various assays.

Table 11

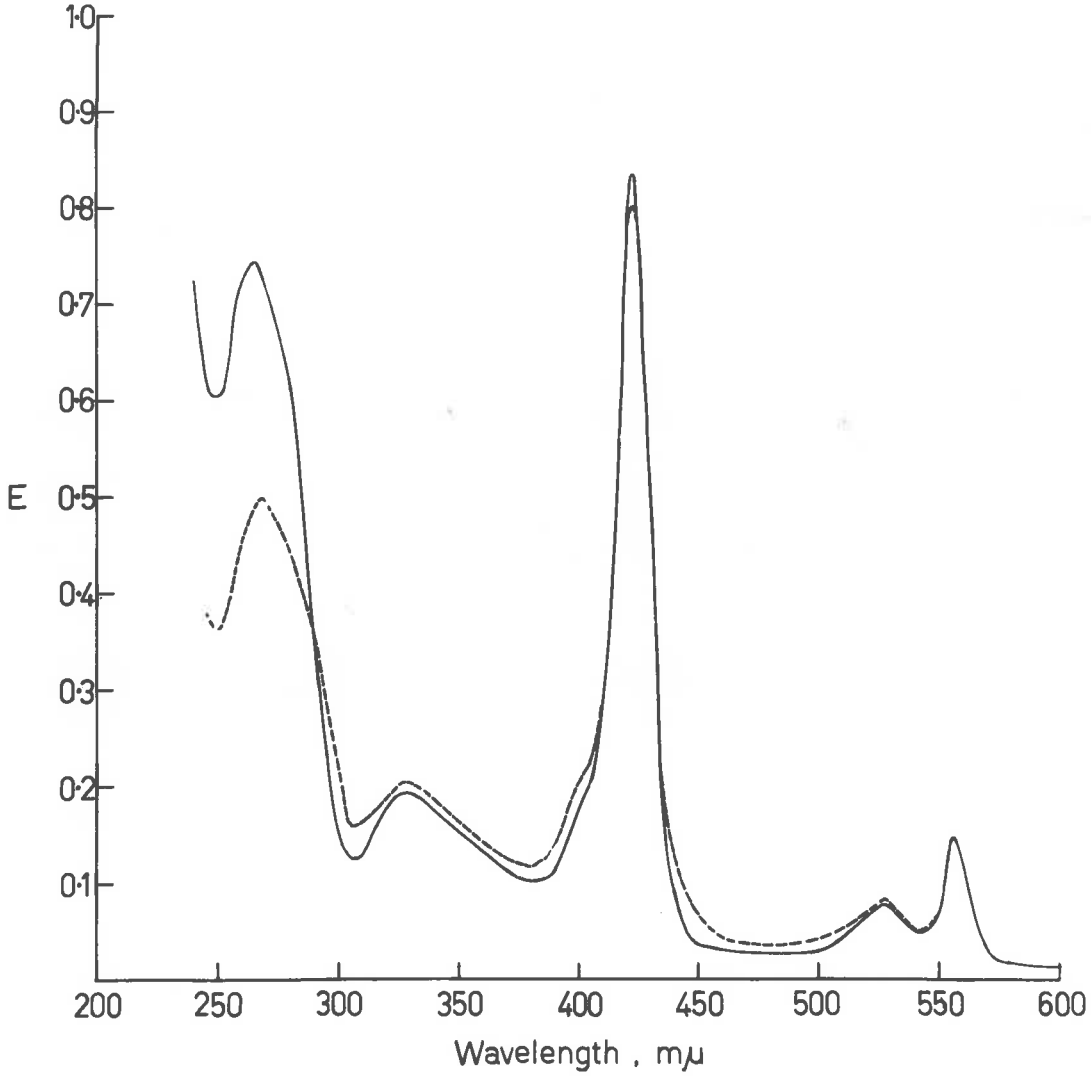
Spectral Properties of crystalline and Nucleotide-free YLD

Precipitation of the protein was either brought about by the addition of solid ammonium sulphate of solutions of recrystallised YLD (direct) or by dialysis of YLD solutions against 70% saturated ammonium sulphate in 0.1M-sodium lactate / 10^{-4} M-EDTA, pH 6.8, under nitrogen.

Preparation	Method of Precipitation	Crystalline YLD		YLD after ammonium sulphate precipitation	
		$\lambda_{max.}$ (mμ)	$\frac{E_{max.}\ U.V.}{EYred.}$	$\lambda_{max.}$ (mμ)	$\frac{E\ max.\ U.V.}{EYred.}$
1	direct	265	0.894	268-9	0.620
2	direct	265	0.870	265-6	0.893
3	dialysis pH 6.8	265-6	0.935	268	0.654
	dialysis pH 8.0	265-6	0.935	267	0.665
4	dialysis pH 6.8	-	-	267-8	0.611

Fig. 13. Spectrum of reduced YLD and reduced AS-YLD.

Solutions of freshly prepared YLD (———) and AS-YLD (- - - -) in 0.3M-sodium lactate, 0.05M-pyrophosphate buffer, 10^{-4}M-EDTA, pH6.8, in 1 cm. cells, using a manually operated Optica spectrophotomet No precautions in excluding oxygen were taken during spectrophotometric measurements, however the solutions were initially free of oxygen.


1·0
0·9
0·8
0·7
0·6
E 0·5
0·4
0·3
0·2
0·1
200
250
300
350
400
450
500
550
600
Wavelength , mμ

3. Analytical Methods

i. Iron. This was estimated as described elsewhere in this thesis, using a water-washed crystal pellet of YLD. It is possible that a small quantity of sodium lactate remained in the crystal pellet, however, Appleby (1957) found that sodium lactate corresponded to 0.7% of the dry weight of unwashed crystal pellets (i.e. crystal suspension in 0.05M-sodium lactate, pH6.8), and therefore water-washed pellets might be expected to contain less sodium lactate. Therefore corrections to the dry weight of the pellet are unnecessary.

ii. Protohaem. This was estimated as the pyridine haemochromagen (Appleby and Morton, 1959) although initially the protohaem was estimated by the method described by Strittmatter and Velick (1956).

iii. Sulphydryl Groups. Boyer (1954) studied the reaction of sulphydryl groups with mercuriphenyl derivatives, and found that formation of the derivative was accompanied by a change in the absorption spectrum. He suggested that p-chloromarcuribenzoate be used as a reagent for the determination of -SH groups. For other studies reported in this thesis p-chloromercuriphenylsulphonate was used, so that the spectral changes accompanying the reaction of sulphydryl groups with PCMS were measured. The spectrum of PCMS-glutathione has its maximum difference in optical density from PCMS alone at 235 mμ. However, the product of the reaction of EDTA with PCMS has some absorption in this region, but very little absorption at 240 mμ. Although EDTA was omitted from YLD samples in which -SH was to be measured and was probably $< 10^{-6}$M, measurements of optical density change were made at 240 mμ. The difference extinction coefficient

for PCMS-GSH at 240 mμ is 7.72×10^3 $cm^{-1}.M^{-1}$. This was determined using a solution of GSH, standardised by a spectrophotometric method against N-ethyl maleimide, for which the difference extinction coefficient is known (Gregory, 1955).

Because of the strong absorption of YLD at 240 mμ it was necessary to use a blank of YLD, so that optical density changes could be readily measured.

4. Spectrum of yeast lactate dehydrogenase haem prosthetic group.

Acidified methyl ethyl ketone has been used by Teale (1959) to split off the prosthetic group from haemoglobin. The major virtue claimed for this method as compared with acidified acetone is the lower ketone concentration necessary to precipitate the apoprotein when methyl ethyl ketone is used. However, where YLD is concerned, the usefulness of this method lies in the fact that two phases, organic solvent and aqueous layer, are obtained. Since acidified acetone will split off the flavin prosthetic group as well as the haem group from YLD, this procedure results in a solution containing both flavin and haem, the flavin interfering markedly with the haem spectrum in the region 360 - 460 mμ. Since riboflavin and its derivatives are much more soluble in aqueous than in organic solvents, methyl ethyl ketone provides a method whereby flavin and haem prosthetic groups may be separated by a simple operation.

Prior to the publication of Teale's procedure, a method involving chloroform extraction of solutions of acidified acetone containing flavin and haem was investigated. In this procedure, an aqueous solution of haemoglobin and FMN was mixed with 20 volumes of acidified acetone, and

the protein precipitate removed by centrifuging. The supernatant acetone solution was mixed with equal volumes of chloroform and aqueous sodium chloride and shaken vigorously. The FMN was extracted quantitatively into the aqueous phase and the haem remained in the chloroform-acetone phase. The spectrum of the haem derivative was virtually identical with that of haem in acidified acetone, although the intensity of absorption was slightly lower. The spectrum was independent of the acetone concentration in the organic phase over the range investigated, and satisfactory recoveries were obtained. The major defect of this method was the difficulty in drying the chloroform-acetone phase after the extraction, since the organic phase was frequently turbid. For this reason, this procedure was abandoned in favour of the simple, one-step procedure using methyl ethyl ketone, although both procedures give satisfactory results. It was found that ether extraction of acid acetone solutions of haem gave variable results, depending largely on the amount of acetone remaining in the organic phase.

5. Yeast for yeast lactate dehydrogenase preparations.

At the commencement of this study, a considerable quantity of dried yeast was available, remaining from the earlier studies of Appleby and Morton. This yeast was a different strain from that initially used by these workers, and had a rather lower content of cytochrome c and cytochrome oxidase, and yielded extracts containing about one-third of the amount of YLD obtained with the first-used yeast (Appleby, 1957). It seems probable that storage of the yeast resulted in a decrease in the extractable YLD,

since the activities obtained in this study indicated that the YLD content had fallen to approximately half the initial level. For some unknown reason, only about one-third of the preparations with this yeast resulted in crystalline YLD, in very small yield, between 1 and 2 mg. of YLD per kg. of dried yeast. Several other yeasts made available by Barretts Foods Ltd. were tested, but were little or no better than the stored yeast.

It was decided to test the compressed yeast manufactured by the Effront Yeast Company, since Appleby (1957) had found that it was very nearly as satisfactory as the strain of Barretts yeast which he used initially. This yeast yielded about 3 times the amount of extractable YLD obtained with Barretts yeast, and was accordingly used for most of the studies reported here. The yield of first crystals of YLD ranged between 8 and 15 mg./kg. of dried yeast. The type of yeast which is likely to yield reasonable quantities of YLD is usually rather dark, and dries to a definitely brown material, as compared with the lighter-coloured yeasts, which when dried usually have a yellowish colour and yield relatively small amounts of crystalline YLD. Spectroscopically, the dark yeasts are characterised by strong absorption bands, especially of cytochrome c and cytochrome oxidase. Bach, Dixon and Zerfas (1946) found that Delft yeast, which is a dark coloured yeast, was a much better starting material than the pale coloured English yeasts.

B. Results.

1. Iron content.

In view of Mahler's (1956) comments, and Boeri's finding that his preparations of YLD contained non-haem iron (Boeri et al., 1956),

Table 12.

Iron Content of Crystalline YLD.

Twice recrystallised cytochrome b_2; the crystal pellet was washed with oxygen-free water, drained, and dried over P_2O_5 *in vacuo*. Haemoglobin from ox blood, re-crystallised once, was dialysed free of salts, freeze-dried, and dried over P_2O_5 *in vacuo*. $E^{4\ cm.}_{507.5m\mu}/\mu g.$ iron as ferrous o-phenanthroline complex, 0.0714.

	Cytochrome b_2	Haemoglobin.
Crystal pellet wet weight (mg.)	1590	-
Dry weight of sample (mg.)	7.325	3.020
Iron (µg.)	5.5	10.5
Iron (%)	0.075%	0.331%
Minimum molecular weight	74,370	16,060 (x 4 = 64,240)

crystalline YLD was analysed for its iron content to confirm the finding by Appleby and Morton (1959a) that only traces of non-haem iron were present. The cytochrome was recrystallised twice, the final crop of crystals harvested by centrifuging, washed with oxygen-free 0.05M-sodium lactate /10^{-5}M-EDTA, pH6.8, harvested and drained, and the crystal pellet rapidly washed with 10 ml. of cold (2°) oxygen-free distilled water. The suspension was centrifuged, the crystal pellet drained, weighed, and dried to constant weight over P_2O_5 *in vacuo*. A sample of the dried YLD was ashed at 600° in a muffle furnace, and a sample of haemoglobin was also analysed at the same time. The results of this analysis are given in Table 12.

2. Haem Content.

Initially, the haem content was estimated by splitting the haem prosthetic group from the protein with acidified acetone. This method was chosen in preference to the pyridine haemochronagen method because of the difficulties in obtaining satisfactory, reproducible results with the latter procedure (Appleby, 1957). A sample of the dried YLD used for iron analysis (see above) was suspended in 0.1 M-NaCl, and finally solubilised by addition of a small amount of sodium hydroxide. After the addition of a quantity of 10 N-HCl calculated to give a final concentration of 0.09 N-HCl, 20 volumes of acetone were added, the precipitate removed by centrifuging and the spectrum of the solutions measured. As discussed earlier (p. 96), the interference of flavin prevented satisfactory estimation of the haem content. Therefore, the acetone was evaporated with a

Table 13.

Haem Content of Crystalline YLD b_2.

For conditions, see text; a value of $\epsilon_{mM}^{\alpha\text{-band}} = 32.5$ for pyridine haemochromagen was used.

	Acid Acetone Treatment	Direct Formation of Haemochrome
sample weight (mg.)	1.375	1.110
haem as pyridine haemochrome (mM.)	0.00578	0.00458
haem (%)	0.822	0.806
Minimum molecular weight	79,400	80,900

stream of nitrogen, and the dried residue was converted to the pyridine haemochromagen. A slight turbidity which appeared after reduction with dithionite was removed by centrifuging. Another sample of dried YLD was converted to the pyridine haemochromagen directly. The results of these analyses are given in Table 13. Although these analyses are not entirely satisfactory, the agreement between the haem and iron content is reasonably good, and both are consistent with the earlier analyses obtained for crystalline YLD (Appleby and Morton, 1959). The minimum molecular weight based on these results is lower than that calculated by Appleby (1957) on iron content, and is very similar to the minimum molecular weight calculated by him for haem and FMN content. A minimum molecular weight of approx. 80,000 is also predicted on the amino acid composition, although the minimum molecular weight here, based on amino acid composition only, is 25,000 (Appleby, Morton and Simmonds, 1959). It is clear, however, that only traces of non-haem iron are present in crystalline YLD. Since it is apparent (Morton, Armstrong and Appleby, 1961), despite earlier comments to the contrary (Boeri and Tosi, 1956) that both crystalline YLD and YLD prepared by Boeri and his coworkers have essentially the same enzymic properties, it must be concluded that the non-haem iron in Boeri's preparations of YLD is a non-functional contaminant. This provides further evidence against the already insecurely-based metallo-flaveprotein hypothesis of Green and Mahler (1954) which is discussed elsewhere (p.85).

3. Sulphydryl Content.

To a solution of YLD in lactate-pyrophosphate buffer at pH 7.0 was added a neutralised solution of PCMS to a final concentration of 7.5×10^{-4}M,

and optical density changes at 240 mμ were measured against a blank solution containing the same final concentration of YLD. It was calculated that the YLD blank solution had an optical density of approximately 3 at 240 mμ. As a consequence of the high light absorption of the blank, the stray light reaching the detector from the monochromator constituted 7.6% of the total light, although under normal conditions the stray light was less than 0.2% at 240 mμ. Corrections have been made for the stray light in the following way. Optical densities were converted to transmittances and $\log \frac{I_o}{I}$ calculated after subtraction of 0.076 from I_o and I (as transmittances). The optical density increment due to PCMS was measured, and the sulphydryl content was calculated from the corrected value of the optical density difference between (YLD + PCMS) and (YLD) + (PCMS). The results of this experiment are given in Table 14 .

Since YLD contains the equivalent of 18 ½-cystine residues per haem (Appleby, Morton, and Simmonds, 1959), it is to be expected that an even number of -SH groups would react with PCMS if it is assumed that all -SH groups react with PCMS. It is possible, however, that there may be -SH groups in YLD which react very slowly with PCMS, so that an odd number of -SH groups may be found when the determination is carried out with PCMS. Since there was a 2.5 fold excess of PCMS present, it is unlikely that there were any rapidly reacting -SH groups which had not reacted with PCMS.

Although a number of assumptions were made in this determination, YLD probably contains 4-SH groups per haem, although this figure must be

Table 14.

Sulphydryl Content of YLD

To 0.6 ml. of YLD in lactate-pyrophosphate buffer, pH 7.0, was added 0.025 ml. of 6×10^{-2}M-PCMS. Final concentrations, YLD, 6.47×10^{-5}M; PCMS, 7.5×10^{-4}M. The optical density changes at 240 mμ were followed in 2 mm. cuvettes, reading against a blank containing 6.47×10^{-5}M-YLD in lactate-pyrophosphate buffer, pH 7.0. The optical density increment at 240 mμ due to 7.5×10^{-4}M-PCMS was measured in lactate-pyrophosphate buffer. Corrections for stray light reaching the detector have been made (see text).

Time (mins.)	$(E^{2mm}_{240\ m\mu})$ corr.	$\Delta(E^{2mm}_{240\ m\mu})$ corr.	-SH mM	-SH per haem
35	0.960	0.452	0.287	4.46
50	0.969	0.461	0.293	4.52
57	0.969	0.461	0.293	4.52

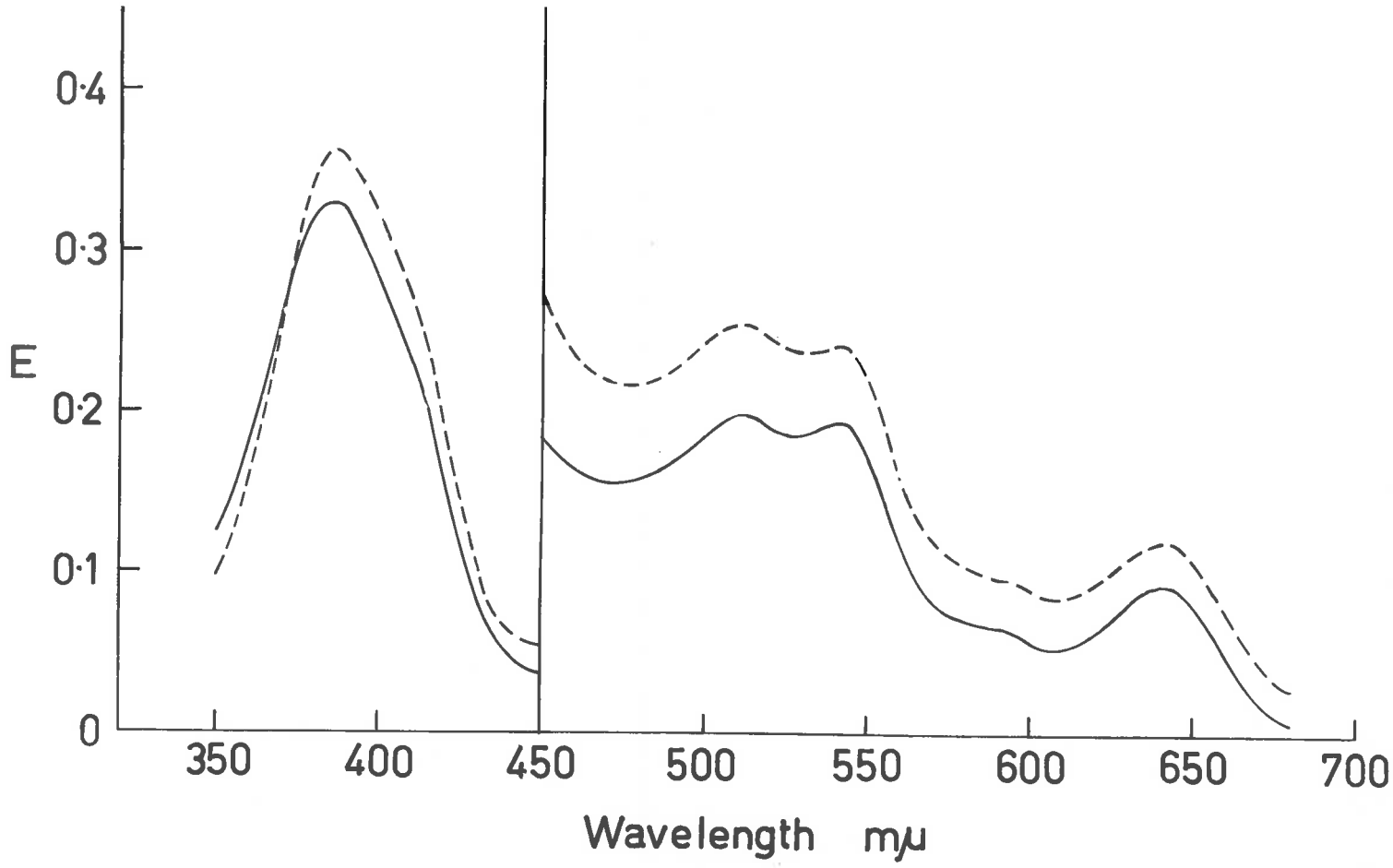
accepted with some reservations. An approximate estimate of the -SH content of YLD was made indirectly and with a number of assumptions from inhibition studies with YLD (p.170), and it was calculated that there were possibly 6-SH groups per haem in YLD. These two figures agree reasonably well. Other studies of the inhibition of YLD by sulphydryl reagents (Appleby and Morton, 1954, 1959a; Beeri et al, 1955; this thesis p.170) suggest that only one of these groups is essential for enzymic activity, and this essential group is masked since inhibition occurs with PCMB, PCMS and NEM, only slightly with iodosobenzoate and not at all with monoiodoacetate or iodoacetamide.

4. Haem Prosthetic Group.

The haem prosthetic group of YLD and of cytochrome b_2 was tentatively identified as protohaem on the similarity of the spectrum of the pyridine haemochromagens of YLD and protohaem. (Bach, Dixon and Zerfas, 1946; Appleby and Morton, 1959). Further proof of identity of the haem of YLD with protohaem is provided by comparison of the spectra of the methyl ethyl ketone extracts of YLD and haemoglobin (Fig. 14). A slight difference occurs in the region 350 to 450 mμ, but the maxima and minima are identical. The difference in the blue-violet region may probably be attributed to the presence, in the methyl ethyl ketone, of a small amount of flavin extracted from YLD. It is therefore concluded that the prosthetic group of YLD is protohaem, since it is split off from the protein by acidified dimethyl ketone and methyl ethyl ketone, and because of the spectrum of the prosthetic group which is virtually identical with the spectrum of protohaem in methyl ethyl ketone. The

Fig. 14. Spectrum of extracts of haemoglobin and YLD in methyl ethyl ketone

The extracts were prepared as described in the text (p. 96), From 350 mμ to 450 mμ, 2 mm. cuvettes and from 450 mμ to 680 mμ 1 cm. cuvettes were used. Spectra were measured in an Optica double beam recording spectrophotometer. ---- extract from haemoglobin; ——— extract from YLD.


0·4
0·3
E
0·2
0·1
0
350
400
450
500
550
600
650
700
Wavelength mμ

spectrum of YLD is nearly identical with that of the haemochromagens of protohaem with heterocyclic bases such as pyridine, except that the intensity of the absorption bands is greater. Therefore, the ligands supplied by the protein of YLD to the 5 and 6 coordination positions of the haem must be extremely basic, and the possibility that the ligands are primary amino groups seems unlikely.

III. KINETIC STUDIES ON YEAST LACTATE DEHYDROGENASE.

A. Introduction.

1. Enzyme Reaction Kinetics and the Michaelis-Menten Equation

Observations of the effect of specific enzymes on the rate of hydrolysis of disaccharides showed that the initial velocity of hydrolysis was (a) a linear function of the enzyme concentration under all conditions and (b) a hyperbolic function of the substrate concentration at constant enzyme concentrations (Brown, 1902; Henri, 1902; Michaelis and Menten, 1913). The reaction rate appeared to be first order at low substrate concentrations, and zero order at high substrate concentration. It was postulated that the conversion of the substrate (S) to the product or products (P) by the enzyme (E) took place through an intermediate compound ES, and a rate equation for the overall reaction

$$S \xrightarrow{\text{enzyme}} P \qquad (1)$$

was derived on this basis for the mechanism

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (2)$$

It was pointed out by Briggs and Haldane (1925) that the assumption, $k_2 \gg k_3$, used in deriving this equation, was not necessarily valid, and that a more rigorous derivation could be obtained by setting $\frac{d[ES]}{dt} = 0$. This is a steady state approximation and is valid because, in general, $\frac{d[ES]}{dt}$ changes to a much smaller extent than $\frac{d[S]}{dt}$ or $\frac{d[P]}{dt}$, either of which may be used as a measure of the reaction velocity.

The rate equation so derived for the initial velocity (v) is

$$v = \frac{V}{1 + \frac{K_m}{(S)}} \qquad (3)$$

where $V = k_3[E]$, the maximum attainable velocity, and $K_m = \frac{k_2 + k_3}{k_1}$.

It may be readily shown that $K_m = [S]$ when $v = \frac{V}{2}$, and this is indeed the only satisfactory definition of K_m, which is not, as assumed in the earlier derivation, the equilibrium constant for the dissociation of the complex, ES, to E and S.

Integration of equation (3) between zero time and time t after initiation of the reaction leads to

$$V = \frac{1}{t} K_m \ln \frac{[S]_o}{[S]_t} + \frac{1}{t} ([S]_o - [S]_t) \qquad (4)$$

The first and second terms of the right hand side of (4) are those obtained, on integration of the differential equations, for a first and a zero order reaction, respectively. This serves to explain the effect of substrate concentration on the reaction velocity since the first term will predominate when $[S]_o \ll K_m$ while the converse is true when $[S]_o \gg K_m$.

Equation (3) has been applied to a large number of enzymic reactions, and the fact that experimental data for these reactions fit the equation is not necessarily because they follow mechanism (2). The apparent ubiquity of this mechanism stems from the fact that the criteria are readily fulfilled by other mechanisms as well.

The mechanism is inadequate if more than one substrate is involved in the reaction, since no term for a second reactant appears in equation (3).

The equation is most useful, however, as a general basis for discussion of the relationship between kinetic data and mechanism; although the only satisfactory approach to such problems for any particular reaction is the derivation of the rate equations for all mechanisms which seem reasonable in the light of present knowledge of the reaction and analogous reactions.

The basic postulates of the Michaelis-Menten equation can thus be extended to allow for departure from mechanism (2) while still retaining the concept of the enzyme substrate intermediate which can decompose to free enzyme and to substrate or product.

The rate equations (for the overall reaction) of a number of mechanisms based on the Michaelis-Menten mechanism are given by Alberty(1956) and similar information is available elsewhere (e.g. Dixon and Webb, 1958; Reiner, 1959). A common feature of most of these mechanisms is that they fulfil the requirements of equation (3) if only one of the experimental variables is altered at a particular time. The observed parameters, V' and K'_m, for any set of conditions are usually related to the real rate parameters in the following way.

$$V'_A = \frac{V}{1 + \frac{K_B}{[B]} + \ldots} \qquad (5a)$$

$$K'_{m_A} = \frac{K_m}{1 + \frac{K_B}{[B]} + ; \ldots} \qquad (5b)$$

where V'_A and K'_{m_A} are the observed values of these parameters when the concentration of B (and other variables) is held constant, and only [A]

is varied. Some of these mechanisms do not result in a truly hyperbolic relationship between substrate concentration and reaction velocity. For example, the rate equation which fits the data for peroxidase most adequately is derived for the mechanism (this thesis p.119).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES\ (I) \quad ; \quad ES\ (I) + AH\ (\text{or } \tfrac{1}{2}BH_2) \xrightarrow{k_5} ES\ II + A\ (\text{or } \tfrac{1}{2}B)$$

$$ES\ (II) + AH\ (\text{or } \tfrac{1}{2}BH_2) \xrightarrow{k_3} E + P + A\ (\text{or } \tfrac{1}{2}B) \qquad (6)$$

and is

$$v = \frac{k_3 [E]\ [AH]}{1 + \frac{k_3}{k_5} + \frac{k_3(k_2 + k_5 [AH])}{k_1 k_5 [S]}} \qquad (7a)$$

whence

$$K'_{m_S} = \frac{\frac{k_3 (k_2 + k_5 [AH])}{k_1 k_5}}{1 + \frac{k_3}{k_5}} \qquad (7b)$$

and

$$V'_S = \frac{k_3 [E]\ [AH]}{1 + \frac{k_3}{k_5}} \qquad (8)$$

It will be noted that both V ($= k_3 [E]\ [AH]$) and K_m ($= \frac{k_3(k_2 + k_5 [AH])}{k_1 k_5}$) are functions of $[AH]$ and approach infinity as $[AH]$ approaches infinity. Thus, the saturation effect of substrate concentration on the initial

reaction velocity is observed only if the donor concentration AH_2 is held constant. This reaction will be discussed more fully elsewhere in this thesis.

2. Analysis of Mechanism from Rate and Equilibrium Studies.

The analysis of enzymic reaction mechanisms in terms of the "Michaelis-Menten" theory or extensions thereof can be approached in several ways. A consideration of mechanism (2) shows that there will be three phases after the initiation of the reaction. In the first phase both enzyme and substrate will disappear rapidly as the concentration of the enzyme-substrate complex increases (i.e. $\frac{d[ES]}{dt} > 0$). This phase usually has a duration of a fraction of a second and has been referred to as the pre-steady state. The second phase is that in which the concentration of the intermediate, ES , has reached its maximum value for the particular conditions, and $\frac{d[ES]}{dt} = 0$. This is the true steady state and is in theory an instantaneous phenomenon, although if [S] is high enough, it may have a practical lifetime of considerable duration. The third phase is entered when the concentration of ES begins to fall (i.e. $-\frac{d[ES]}{dt} > 0$), and occurs because [S] has fallen below the level required for $[ES]_{max}$. This condition is still referred to as the steady state since $\frac{-d[ES]}{dt}$ is much smaller than $\frac{-d[S]}{dt}$ or $\frac{d[P]}{dt}$ until the reaction is virtually completed.

It is therefore possible to study the reaction in a number of ways. The most general approach has been to follow the overall reaction in terms of $\frac{-d[S]}{dt}$ or $\frac{d[P]}{dt}$ over a range of conditions after the third phase of the reaction has been entered. It is necessary that the reaction

rate be measured at an early stage in the course of the reaction because the rate equation derived for a mechanism in terms of the overall reaction is based on the assumptions that $\frac{d[ES]}{dt} = 0$, that the concentration of substrate is the initial concentration and that the concentration of the product is zero. In practice it is usual to extrapolate the concentration measurements of substrate or product to zero time and to use the velocity so obtained as the initial velocity of the reaction.

If the reaction proceeds at measurable velocities in both the forward and reverse reactions, the Michaelis-Menten rate parameters may be obtained for both the forward and reverse reactions. At equilibrium we have

$$-\frac{d[S]}{dt} = \frac{d[P]}{dt} = \frac{V_f[S]/K_s - V_r[P]/K_p}{1 + [S]/K_s + [P]/K_p} \qquad (4)$$

for rate equations of type (3). The subscripts f, r, s and p refer to the rate parameters for the forward and reverse reactions respectively. The apparent equilibrium constant ($K_{app.}$) for the overall reaction is thus

$$K_{app.} = \frac{[P]_{eq.}}{[S]_{eq.}} = \frac{V_f K_p}{V_r K_s} \qquad (5)$$

Similar relationships are obtained for other mechanisms, the form of the equation depending on the nature of the mechanisms. It is possible in this way, for instance, to distinguish between a mecha nism involving two binary enzyme-substrate complexes and one in which a single ternary complex is formed, although the criteria for the steady state rate equations of both mechanisms are the same (Alberty, 1956).

Where an enzymic reaction involves two or more substrates, it may be possible to measure either the rate of formation or disappearance of (or the equilibrium constant for) the complex of one of the substrates with the enzyme. Such data may then be used as a check on the steady state rate parameters, and as a means of obtaining individual rate constants for steps in the overall reaction.

The kinetics of all three phases of a reaction are known as transient state kinetics. In general the study of transient state kinetics requires a method for the estimation of both reactants and the intermediates, and apparatus which is suitable for measuring the concentration changes occurring at very short periods after the initiation of the reaction. A technique which has proved very successful is known as the rapid flow method. The reactants are forced into a mixing chamber and their concentrations (or those of intermediates) are measured at some point along the outflow tube of the mixing chamber. The time after mixing is determined from the position of the observation point and the flow velocity of the mixed reactants. Apparatus of this type has been developed by Chance (1940, 1947, 1951) from the apparatus designed by Roughton and Millikan (1936) and has been found to be suitable for the study of extremely rapid reactions. (For a first order reaction, rate constants up to 200 $sec.^{-1}$, second order reactions, rate constants up to 2×10^7 $M.^{-1}sec.^{-1}$). The same apparatus may also be used to follow the reaction after flow has stopped, so that information concerning the true and approximate steady states, as well as for the pre-steady state may be obtained in one experiment. The individual rate constants for the

distinguishable steps in the overall reaction are readily obtained in this way and may be substituted into the rate equations for the steady rate, for comparison with the rate parameters of the overall reaction, V and K_m. The application of these approaches to various enzymic reactions is outlined below.

i. Fumarase (Alberty, 1956). The overall reaction catalysed is

$$\underset{(F)}{\text{fumarate}} + \text{water} \rightleftharpoons \underset{(M)}{\text{malate}}$$

The enzyme is inhibited by high concentrations of either fumarate or malate, but at slightly lower concentrations of fumarate, activation is obtained; for low concentrations of either compound the experimental data are in satisfactory agreement with equation (3), and also with equation (5)

i.e.

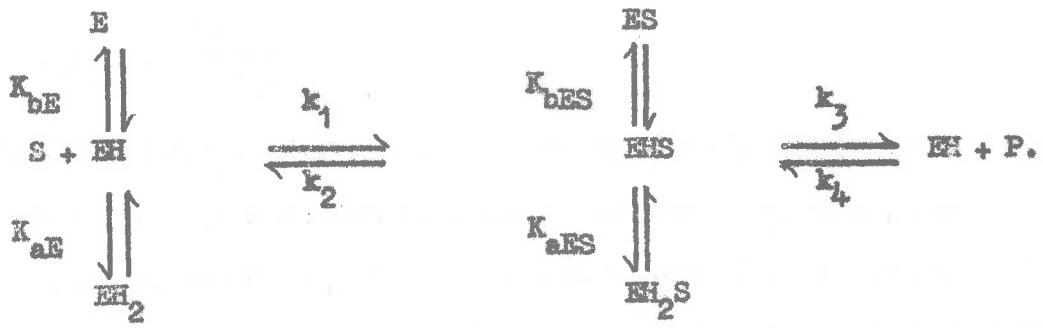
$$v_f = \frac{V_F}{1 + \frac{K_F}{[F]}} \qquad v_r = \frac{V_M}{1 + \frac{K_M}{[M]}}$$

$$K_{app.} = \frac{V_F K_M}{V_M K_F}$$

There is also a marked effect of the ionic environment on the reaction velocity; the variation of V and K and the pH optimum, for forward and reverse reactions, with the concentration of a particular ionic species is interpreted as consistent with the presence of two binding sites for anions on the enzyme, one the enzymic site, the other site where binding affects the properties of the enzyme. Because of such effects, it was necessary to select a suitable buffer for study of the effect of pH on the reaction.

The effect of pH on enzymic reactions has been interpreted in a number of ways. The bell shaped activity/pH plots are explained in the changing degree of ionisation of the enzyme. In order to account for a maximum in such plots, there must be at least two ionisable groups involved in the active centre of the enzyme, the enzyme-substrate complex only forming or breaking down to yield product where one of the groups is ionised and the other not. This then results in a curve of activity against pH which is symmetrical about the maximum value. The effect of substrate ionisation is readily allowed for and appears only in the Michaelis constant Km, since V implies saturation of the enzyme with the substrate with which it combines.

The ionisation of the enzyme is usually written in the following way, with the proviso that the ionisations refer to the active site of the enzyme alone, and not to the whole enzyme, the numerical values for protons being only of relative significance.



The pH dependence of V is expressed as follows, the V'_x terms being pH independent.

$$V_s = \frac{V'_s}{1 + \frac{[H^+]}{K_{aES}} + \frac{K_{bES}}{[H^+]}} \qquad \text{where } V'_s = k_3[E]_o$$

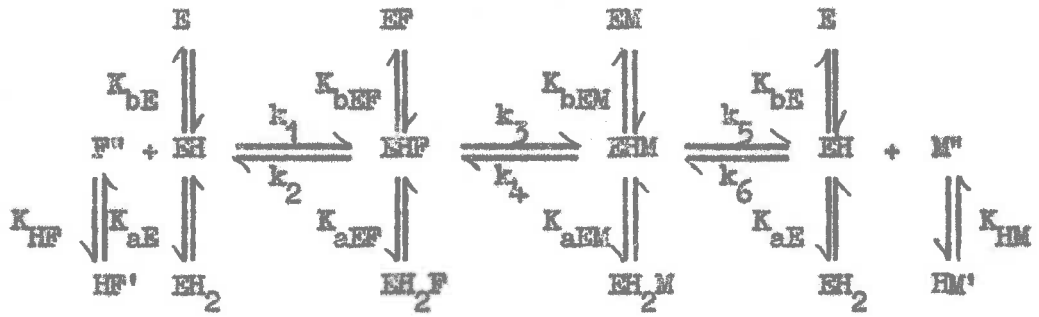
$$V_p = \frac{V'_p}{1 + \frac{[H^+]}{K_{aES}} + \frac{K_{bES}}{[H^+]}} \qquad \text{where } V'_p = k_2[E]_o$$

Since the shape of the plot V vs. pH depends on the variable, $[H^+]$ and the constants K_{aES} and K_{bES}, the same variation in V with pH should occur for both the forward and the reverse reactions.

This is not the case with fumarase, and therefore the Michaelis-Menten mechanism is inadequate. The following mechanism yields a rate equation which is indistinguishable from (3) on available criteria at any one pH, although the individual rate constants, k_i, involved in V and K_m are different.

$$F + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EF \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EM \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + M.$$

On introduction of the various ionic species of the reactants and intermediates we obtain.



It is obvious that the reactions governed by k_1 in the forward direction, and k_6 in the reverse reaction, cannot be rate limiting towards V because of the saturation effect of the infinite substrate concentration. This leaves the two cases, (a) k_5 or k_2, (b) k_3 or k_4 as rate limiting conditions. Case (a) will result in V_F and V_M having the same pH dependence, because the ionisations of EHF and EHM will enter into both terms. This leaves k_3 or k_4 as the rate limiting process, and it may be seen that, if the ionisation constants of the complexes EHF and EHM are different, the pH dependence of V_F and V_M will be different. For this case

$$V_F = \frac{k_3 k_5 [E]}{(k_3 + k_4 + k_5)} \Big/ 1 + \frac{[H^+]}{K_{aEF}} + \frac{K_{bEF}}{[H^+]}$$

$$V_M = \frac{k_2 k_4 [E]}{(k_2 + k_3 + k_4)} \Big/ 1 + \frac{[H^+]}{K_{aEM}} + \frac{K_{bEM}}{[H^+]}$$

From a knowledge of the two pH values at which the reaction (in any one direction) is occurring at half the rate at the optimum pH, it is possible to obtain values for K_{aES} and K_{bES}. Therefore it is possible to calculate theoretical plots of V vs. pH for the assumed mechanism, and Alberty has shown that the observed data for fumarase fit the calculated curves extremely well. The Michaelis constants have the same denominators as their respective V, and the numerator consists of the pH independent Michaelis constant, and the terms for ionisation of the free enzyme and the substrate. If the Haldane equilibrium condition is obeyed, then

$$K_{app.} = \frac{[M]_{Tot.}}{[F]_{Tot.}} = \frac{V_F \cdot K_M}{V_M \cdot K_F} = \frac{Keq. (1 + [H^+]/K_{HM})}{(1 + [H^+]/K_{HF})}$$

where Keq is the equilibrium constant in terms of the double charged anion. This has been verified for fumarase.

If $\frac{V_M}{K_M}\left(1 + \frac{[H^+]}{K_{HM}}\right)$ and $\frac{V_F}{K_F}\left(1 + \frac{[H^+]}{K_{HF}}\right)$ are plotted against pH, then the same bell shaped curve should be obtained, due to the two ionisations of the free enzyme. It is also possible to compute values for the ionisation constants of the enzyme. From these, a curve for the plot $\frac{K_F}{V_F}\frac{(1 + [H^+])}{K_{HF}}$ vs. pH may be constructed. The observed values and the calculated values are in good agreement and conform to the expected bell shaped curve.

Thus it may be seen that the kinetics of the fumarase reaction are adequately described, in terms of the available criteria, by the last given mechanism, which may therefore be accepted as being a possible mechanism.

It has not been possible to evaluate any of the individual rate constants separately from these steady state measurements, because the value for any single rate constant is not known.

ii. Peroxidase. This enzyme catalyses the reaction

$$R\text{-}OOH + AH_2 \longrightarrow ROH + H_2O + A.$$

where R = H, CH_3, C_2H_5, ----------, and AH_2 is a compound capable of donating electrons (e.g. phenols, amines, leuco-dyes, ene-diols). The addition of hydrogen peroxide to peroxidase was found to cause the colour of the peroxidase solution to change from brown to red, and this colour change could be rapidly reversed by the addition of a donor compound; (Keilin and Mann, 1937). This was attributed to the formation of a peroxidase-peroxide complex and its subsequent reaction with a donor. Theorell (1941) showed that a green compound preceded the formation of the red complex and suggested that this was the reactive complex, while the red compound was inactive.

The development of sensitive, rapid flow photometers by Chance (1940, 1947, 1951) made possible the study of the kinetics of these reactions. The early experiments (Chance, 1940a, 1943) were carried out with a filter photometer, and it was not possible to distinguish the reactions of the red and green complexes, but the subsequent use of a grating monochromator made it possible to obtain the spectra of the complexes and to follow the kinetics of formation and disappearence of each of the complexes (Chance, 1949).

The mechanism initially proposed and tested by Chance (1943) was

$$\underset{([E]-p)}{E} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(p)}{ES}$$

$$ES + AH_2 \xrightarrow{k_3} E + "SH_2" + A$$

$$v = \frac{k_4 [E]}{1 + \frac{k_2 + k_4}{k_1 [S]}} \qquad 11\ (a)$$

where $k_4 = k_3 [AH_2]$ and $[E]$ is the total enzyme concentration.

The differential rate equations are

$$dp / dt = k_1 [S] ([E] - p) - (k_2 + k_3 [AH_2])p \qquad 11\ (b)$$

$$- d[S] / dt = k_1 [S] ([E] - p) - k_2 p \qquad 11\ (c)$$

$$- d[AH_2] / dt = k_3 [AH_2]\ p \qquad 11\ (d)$$

Solution of 11 (b) for the condition $dp / dt = 0$ (and $p = p_{max.}$, the maximum attainable level of p for any given $[S]$) results in

$$\frac{p_{max.}}{[E]} = \frac{[S]}{[S] + Km} \quad \text{where } Km = \frac{k_2 + k_4}{k_1}$$

Although it is difficult to determine when the inequality $[S] \gg \frac{k_2 + k_4}{k_1}$ is reached, due to inactivation of the enzyme by high peroxide concentrations, under this condition $p_{max.} = [E]$ and k_4 may be obtained directly from 11 (d).

$$k_4 = \frac{1}{[E]} \left(- \frac{d[AH_2]}{dt} \right) = \frac{1}{[E]} \left(- \frac{d[S]}{dt} \right). \qquad 11\ (e)$$

$$\text{i.e.} \quad k_3 = \frac{1}{[E]\,[AH_2]} \left(- \frac{d[AH_2]}{dt} \right)$$

When $[S] \ll \frac{k_2 + k_4}{k_1}$, and $k_2 \ll k_4$, $p_{max.} \ll [E]$ and

$$k_1 = \frac{1}{[E]\,[S]} \cdot \left(- \frac{d[S]}{dt} \right) = \frac{1}{[E]\,[S]} \cdot \left(- \frac{d[AH_2]}{dt} \right) \qquad 11\ (f)$$

When the approximation $k_2 \ll k_4$ is made then 11 (a) reduces to

$$v = \frac{[E]}{\frac{1}{k_4} + \frac{1}{k_1[S]}} = \frac{[E]}{\frac{1}{k_3[AH_2]} + \frac{1}{k_1 S}} \qquad 11\ (g)$$

Thus, when $k_3[AH_2] \gg k_1[S]$, the reaction becomes independent of the donor concentration, and a <u>pseudo</u> "saturation" effect for donor will be observed if $[AH_2]$ is varied over a sufficiently wide concentration range while $[S]$ is held constant. Similarly, saturation with substrate will be observed when $k_1[S] \gg k_3[AH_2]$ and $[S]$ is varied while $[AH_2]$ is held constant, provided inactivation by substrate does not occur. Since peroxide at high concentrations does inactivate peroxidase, the direct

evaluation of k_3 (or k_4) by equation 11 (e) is not particularly reliable, and therefore direct determination of this constant from the kinetic behaviour of the complex, ES, is more suitable.

Adding equations 11 (b) and 11 (c)

$$\frac{d\,p}{d\,t} + \frac{d[S]}{d\,t} = -k_3\,[AH_2]\,p \qquad 11\ (h)$$

Integrating 11 (h) between $t = o$ and $t = t$

$$[S] = [S]_o - p - k_3\,[AH_2]\int_o^t p\,dt + c. \qquad 11\ (i)$$

For the limits $t = o$ and $t = \infty$

$$k_3 = \frac{[S]_o}{[AH_2]_o \int_o^{\infty} p\,dt} \qquad 11\ (j)$$

where $\int_o^{\infty} p\,dt$ may be obtained from the area under the curve of p vs. t for the formation and complete disappearance of the complex ES.

Use of a differential analyser showed that 11 (j) may be rewritten as

$$k_3 = \frac{[S]_o}{[AH_2]_o} \cdot \frac{1}{p_{max.} \cdot t_{\frac{1}{2}}} \qquad 11\ (k)$$

where $t_{\frac{1}{2}}$ is the time required for p to fall to $\frac{p_{max}}{2}$ (Chance, 1943).

It is obvious that in the absence of acceptor, the equilibrium constant for the formation of ES can be measured, and is $\frac{k_1}{k_2} = \frac{p}{[S](E-p)}$. Thus a value for k_2 may be calculated, if the rate of formation of ES is measured.

The values of k_1 and k_2 so obtained were $1.2 \pm 0.4 \times 10^7\,M^{-1}.sec^{-1}$ and $0.2\ sec^{-1}$, while k_4 was found to vary linearly with the acceptor concentration. Good agreement between the values of k_1 and k_4 obtained directly

from the transient state kinetics and those obtained by manipulation of the steady state rate equation was observed.

Later studies by Chance (1949), carried out with grating spectrophotometer, showed that the mechanism should be amended to

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Complex I} \xrightarrow{k_5} \text{Complex II}$$

Complex II + AH_2 $\xrightarrow{k_3}$ E + ROH + A + H_2O. where E = Per.OH, Complex I = Per. COR (I) and Complex II = Per.COR (II).

The spectra of complexes I, II and a third, inactive form were measured directly in the flow apparatus. The value of the second order rate constant k_1, for hydrogen peroxide was $0.9 + 0.2 \times 10^7 M^{-1}$. $sec.^{-1}$, while, for the slower reaching methyl and ethyl hydrogen peroxides, values of 1.5×10^6 M^{-1}. sec^{-1}. and 3.6×10^6 M^{-1}. sec^{-1}. respectively, were obtained.

The conversion of complex I to complex II is somewhat complicated by the effect of donor on the reaction. It was observed that the value of k_5 (in the absence of added donor) was high at low peroxide concentrations, and decreased to about one-twentieth of the value at slightly higher peroxide concentrations. This effect could be overcome by pretreating the peroxidase with a small successive additions of peroxide, and was attributed to the presence of an endogenous donor.

The first order constant, k_2, when measured with treated peroxidase is very small (< 0.02 $sec.^{-1}$), indicating that complex I is extremely stable in the absence of donor.

The value for k_5 (with untreated peroxidase containing donor) was approximately 4 $sec.^{-1}$, which is far too small to account for the turnover rate of peroxidase in the overall reaction. It was observed in these

experiments, however, that although k_5 was virtually independent of the peroxide concentration, it was dependent on the concentration of added donor so that it was obvious that the transformation

$$\text{complex I} \xrightarrow{k_5} \text{complex II}$$

was more complicated than indicated. By completely removing the endogeneous donor, it could be shown that the transition of complex I to complex II required one equivalent of a reducing agent, so that the reaction is more correctly written

$$\text{complex I} + \tfrac{1}{2}AH_2 \xrightarrow{k_7} \text{complex II} + \tfrac{1}{2}A$$

The value of k_7 was very large (2×10^7 M^{-1}. $sec.^{-1}$ for nitrous acid) and was consistent with the high turnover rate of peroxidase in the overall reaction (Chance, 1952, 1952a).

The value of k_3 depended on the donor used and varied between 10^1 to 10^8 M^{-1}. $sec.^{-1}$. The reactivity with donors of complex II formed with alkyl hydrogen peroxides is the same as for complex II hydrogen peroxide, but the rate of formation of complex I is slower for the alkyl peroxides than for hydrogen peroxide.

The values of k_1 and k_3 obtained from the overall reaction compare very well with those measured directly; they are independent of the hydrogen ion activity between pH 4 and 10 (Chance, 1949a).

The good agreement of the kinetic data with the earlier proposed mechanism arose firstly because of the inability of the apparatus to distinguish between complexes I and II, secondly because of the presence of endogeneous donor, and thirdly, because the reaction of complex I with a donor proceeds much more rapidly than the reaction of complex II with donor.

The steady state rate equation for the probable mechanism of peroxidase action is, as stated earlier,

$$V = \frac{k_3 [E] [AH_2]}{1 + \frac{k_3}{k_7} + \frac{k_3 (k_2 + k_7 [AH_2])}{k_1 k_7 [S]}} \qquad (12a)$$

$$= \frac{k_7 [E] [AH_2]}{1 + \frac{k_7}{k_3} + \frac{k_2 + k_7 [AH_2]}{k_1 [S]}} \qquad (12b)$$

for $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES\ (I)$; $ES\ (I) + \frac{1}{2}AH_2 \xrightarrow{k_7} ES\ (II) + \frac{1}{2}A$; $ES\ (II) + \frac{1}{2}AH_2 \xrightarrow{k_3} E + P + A$.

Equation 12 (a) is obtained by solving the differential equations in terms of complex II while 12 (b) is obtained if solved in terms of complex I. Equation (12b) may be obtained from (12a) by multiplying numerator and denominator by $\frac{k_7}{k_3}$.

Alternatively equation 12(a) may be written in the form

$$\frac{P_{max.}}{[E]} = \frac{1}{1 + \frac{k_3}{k_7} + \frac{k_3 (k_2 + k_7 [AH_2])}{k_1 k_7 [S]}}$$

Since $k_3 \ll k_7 \quad (\simeq 0.02 k_7)$

$$\frac{P_{max.}}{[E]} = \frac{1}{1 + \frac{1}{50} + \frac{k_3 k_7 [AH_2]}{k_1 k_7 [S]}} \simeq \frac{1}{1 + \frac{k_3 [AH_2]}{k_1 [S]}}$$

Since the first mechanism tested also results in this approximation (see p.117) it is easy to see why such good agreement between the direct and steady state values of k_1 and k_4 was obtained on the basis of this mechanism.

iii. Liver Alcohol Dehydrogenase (ADH).

(a) Equilibrium studies. This enzyme was obtained as a crystalline homogeneous protein from horse liver (Bonnichsen, 1950). At low concentrations of the enzyme (10^{-6}M or less) the equilibrium constant for the overall reaction

$$R - CH_2OH + DPN^+ \rightleftharpoons R\text{-}CHO + DPNH + H^+.$$

$$Keq. = \frac{[R\text{-}CHO] \cdot [DPNH] \cdot [H^+]}{[R\text{-}CH_2OH] \cdot [DPN]}$$

is independent of both pH and enzyme concentration, and is nearly identical with that measured with yeast alcohol dehydrogenase (i.e. 10^{-11}M). As the enzyme concentration is increased, however, the equilibrium constant increases to a maximum value and also becomes pH dependent. It was known that in the presence of ADH, the absorption maximum for DPNH shifted from 340 mμ to 325 mμ, the spectra being isosbestic at 328 mμ (Theorell, 1951). This change was attributed to the formation of a complex between ADH and DPNH, 2 moles of DPNH being bound per mole of ADH at pH 7.0, while at pH 10.0, 1 mole of DPNH was bound. Since it could be demonstrated that DPN competed with DPNH for the same site on ADH, it was suggested that an ADH.DPN complex also existed. By assuming that the two binding sites for DPNH were independent, enzymically identical and had the same intrinsic dissociation constant, it was possible to measure the velocity constant for the formation of the ADH.DPNH complex and the dissociation constant for the complex. This was achieved in the following way. Since the absorption spectra of free and bound DPNH are isosbestic at 328 mμ, and the

absorption of free DPNH is equal at 328 and 354 mμ, there will be no difference signal obtained from the light detector of a photometer by alternately flickering light at wavelengths of 328 and 354 mμ through a solution of free DPNH. However, a difference signal will be obtained when ADH.DPNH complex is present, since the absorption of the complex is not the same at these two wavelengths. The absorption at 328 mμ is therefore a measure of the total DPNH, while the difference signal 328-354 mμ is proportional to the concentration of bound DPNH. This method may be used to measure the equilibrium constant for the DPNH complex, by titration of ADH with DPNH. The substitution of a flow apparatus for the cuvette makes the study of the reaction kinetics possible. The value obtained for the dissociation constant of the ADH . DPNH complex was 1×10^{-7} M at pH 7.0 and 3×10^{-6} at pH 10.0, while the velocity constant at pH 7.0 for the formation of the complex was 4×10^{6} M^{-1} sec^{-1}. (Theorell and Chance, 1951).

Alberty (1953) has considered the effect of binding of reactant or product by an enzyme present in large concentrations. For the reaction

$$A + B \overset{E}{\rightleftharpoons} C + D$$

the apparent equilibrium constant is defined in terms of the total quantities of the reactant and products,

$$K_{app.} = \frac{[C]_T \quad [D]_T}{[A]_T \quad [B]_T}$$

For vanishingly small concentrations of the enzyme, where negligible amounts of product or reactant are bound by the enzyme, the true equilibrium constant is measured; i.e.

$$K_{eq.} = \frac{[C]\,[D]}{[A]\,[B]}$$

The relationship between these two constants is

$$K_{app.} = K_{eq.}\,(f_A\, f_B / f_C\, f_D).$$

where f_A, ---------- represent the fraction of the indicated reactant or product <u>not</u> bound by the enzyme. It may be seen that the binding of product alone by the enzyme will increase $K_{app.}$ when measurable quantities of product are bound, while binding of reactant alone by the enzyme will decrease $K_{app.}$

If, for example, only C is bound then

$$f_C = \frac{K_{eq.}}{K_{app.}} = 1 - \frac{[EC]}{[C]_T}$$

For the case $EC \rightleftharpoons E + C$ and $D_{EC} = \frac{[E]\,[C]}{[EC]}$

if $[E]_T \gg [C]_T$ then

$$[EC] = \frac{[C]_T}{1 + D_{EC}/[E]_T}$$

or if $[E]_T \ll [C]_T$

$$[EC] = \frac{[E]_T}{1 + D_{EC}/[E]_T}$$

The ratio $K_{app.}/K_{eq.}$ at high enzyme concentrations may be obtained and it may be seen that

$$\left(\frac{K_{app.}}{K_{eq.}}\right)_{E \longrightarrow \infty} = \frac{[E]_T}{D_{EC}}$$

Thus, at high enzyme concentrations, the apparent equilibrium constant for the overall reaction will be directly proportional to the enzyme concentration.

Since, for ADH, a maximum value of $K_{app.}$ was obtained, it is necessary to see if binding of both reactant and product will produce this effect. For binding of B and C by the enzyme

$$\frac{K_{app.}}{K_{eq.}} = \frac{f_B}{f_C} = \frac{1 - (1 + D_{EB}/[E]_T)^{-1}}{1 - (1 + D_{EC}/[E]_T)^{-1}}$$

for which $(\frac{K_{app.}}{K_{eq.}})_{[E]_T \longrightarrow \infty} = \frac{D_{EB}}{D_{EC}}$. That is, at high enzyme concentrations, the apparent equilibrium constant becomes independent of both the enzyme and reactant concentrations. By use of this equation, for the case that DPN and DPNH compete for the same site on ADH, Alberty (1953) was able to show good agreement between his values, calculated on the basis of the dissociation constants of the DPN- and DPNH- ADH complexes, and those observed by Theorell and Bonnichsen (1951).

(b) Rate Studies. From a consideration of the equilibrium data obtained by Theorell and Bonnichsen (1951), Theorell and Chance (1951) proposed and tested the following mechanism for ADH action, using the special spectrophotometric method described earlier.

$$\underset{([E] - p_1 - p_2)}{\text{ADH}} + \text{DPNH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(p_1)}{\text{ADH.DPNH}}$$

$$\underset{(p_1)}{\text{ADH.DPN}} + \underset{(\text{ald})}{\text{R-CHO}} + H^+ \underset{k_6}{\overset{k_4}{\rightleftharpoons}} \underset{(p_2)}{\text{ADH.DPN}} + \underset{(\text{alc})}{\text{R-CH}_2\text{OH}}$$

$$\underset{(p_2)}{\text{ADH.DPN}} \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \underset{([E] - p_1 - p_2)}{\text{ADH}} + \text{DPN}$$

The differential rate equations for the reduction of aldehyde by DPNH are

$$dp_1 / dt = k_1 [DPNH] ([E] - p_1 - p_2) - (k_2 + k_4 [ald]) p_1$$

$$dp_2 / dt = k_4 [ald] p_1 - k_3 p_2 + k_5 [DPN] ([E] - p_1 - p_2)$$

$$- d[ald] / dt = k_4 [ald] p_1$$

By solving for the steady state condition $dp_1 / dt = dp_2 / dt = 0$,

$$p_1 = \frac{[E] - p_2}{1 + \frac{K_{m_1}}{[DPNH]}} \quad \text{where} \quad K_{m_1} = \frac{k_2 + k_4 [ald]}{k_1}$$

$$p_2 = \frac{[E] - p_1 \left\{ 1 - \frac{k_4 [ald]}{k_5 [DPN]} \right\}}{1 + \frac{K_{m_2}}{[DPN]}} \quad \text{where} \quad K_{m_2} = k_3 / k_5$$

It will be noted that while K_{m_1} has the usual form of a Michaelis constant, K_{m_2} is the dissociation constant of the complex, ADH-DPN . By combination of these equations

$$\frac{p_1}{[E]} = \frac{1}{\left(1 + \frac{[DPN]}{K_{m_2}}\right)\left(1 + \frac{K_{m_1}}{[DPNH]}\right) - \frac{[DPN]}{K_{m_2}} + \frac{k_4 [ald]}{k_3}}$$

Now the turnover number (TN) is

$$\frac{1}{[E]} \cdot \frac{d[ald]}{dt} = k_4 [ald] \cdot \frac{p_1}{[E]}$$

$$\therefore \quad TN = \frac{k_4[ald]}{\left(1 + \frac{[DPN]}{K_{m_2}}\right)\left(1 + \frac{K_{m_1}}{[DPNH]}\right) - \frac{[DPN]}{K_{m_2}} + \frac{k_4[ald]}{k_3}}$$

$$= \frac{1}{\frac{1}{k_4[ald]}\left(1 + \frac{[DPN]}{K_{m_2}}\right)\left(1 + \frac{K_{m_1}}{[DPNH]}\right) - \frac{[DPN]}{K_{m_2}} + \frac{1}{k_3}}$$

If the initial reaction velocity (of DPNH oxidation) is measured, the concentration of DPN is negligible in comparison to K_{m_2}, and then

$$TN = \frac{1}{\frac{1}{k_4[ald]}\left(1 + \frac{K_{m_1}}{DPNH}\right) + \frac{1}{k_3}} = \frac{1}{\frac{1}{k_1[DPNH]} + \frac{1}{k_4[ald]}\left(1 + \frac{k_2}{k_1[DPNH]}\right) + \frac{1}{k_3}}$$

If the aldehyde concentration is very high then

$$TN = \frac{1}{\frac{1}{k_1[DPNH]} + \frac{1}{k_3}}$$

Thus, at very large DPNH and aldehyde concentrations

$$TN = k_3$$

and $[DPNH]$ at $\frac{TN}{2}$ and infinite aldehyde concentration is $\frac{k_3}{k_1}$. For these conditions, then, the velocity of the reaction depends on the dissociation velocity of the ADH.DPN complex, and not on the dissociation of the ADH.DPNH complex.

By similar manipulations for very large $[DPNH]$ and small $[ald]$ (which also implies $k_3 \gg k_4[ald]$)

$$TN = k_4[ald] \text{ and } [DPNH]_{\frac{TN}{2}} = \frac{k_2 + k_4[ald]}{k_1}$$

Only where $k_4[ald] \ll k_2$ is it possible to measure the dissociation constant of the ADH.DPNH complex, otherwise what is measured is the Michaelis constant, and this is only obtained when $[ald] \ll [DPNH]$ and $k_4[ald] \ll k_3$. Similar approximations may be applied to the effect of aldehyde concentration and the significance of the apparent K_m for aldehyde. The results of these approximations are given in Table 14A. (Theorell & Chance, 1951).

The values of k_1, k_2 and k_4 so computed from steady-state rate data are in good agreement with values for these constants obtained directly by the rapid flow method, particularly for the steady-state rate data obtained with low concentrations of enzyme and reactant, using a fluorimetric method for following DPNH oxidation (Theorell, Nygaard and Bonnichsen, 1955).

Alberty (1953) has derived the overall rate equations, for a number of reaction mechanisms, for the overall reaction.

$$A + B \underset{}{\overset{E}{\rightleftharpoons}} C + D.$$

This involved the introduction of a new rate parameter K_{AB} (or K_{CD} for the reverse reaction). The general form of the rate equation is

$$-\left(\frac{d[A]}{dt}\right)_o = v_{init.} = \frac{V_f}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A][B]}}$$

$$-\left(\frac{d[C]}{dt}\right)_o = v_{init.} = \frac{V_r}{1 + \frac{K_C}{[C]} + \frac{K_D}{[D]} + \frac{K_{CD}}{[C][D]}}$$

K_{AB} may be obtained in the following way. If $[B]$ is held constant at the concentration B_o, which is of the order of magnitude of K_B, and $[A]$ is varied then

Table 14A

Variation of Turnover Number of ADH with Aldehyde and DPNH Concentration

(Theorell and Chance, 1951)

DPNH (x_1)	∞	$\frac{k_3}{k_1}$	∞	$\frac{k_2 + k_4 a_1}{k_1}$	∞	∞
aldehyde (a_1)	∞	∞	small	small	variable	k_4
$\frac{1}{E} \cdot \frac{da_1}{dt}$ (IN)	k_3	$\frac{k_3}{2}$	$k_4 a_1$	$\frac{k_4 a_1}{2}$	$\frac{1}{\frac{1}{k_4 a_1} + \frac{1}{k_3}}$	$\frac{k_3}{2}$

Thus it is possible to obtain values for k_1, k_2, k_3 and k_4.

$$-\left(\frac{d[A]}{dt}\right)_o = \frac{V'_f}{1 + \frac{K'_A}{[A]}} \quad \text{where } V'_F = \frac{V_f}{1 + \frac{K_B}{[B]_o}}$$

$$K'_A = \frac{K_A [B]_o + K_{AB}}{K_B + [B]_o}$$

whence

$$K_{AB} = K'_A \cdot K_B + [B]_o (K'_A - K_A)$$

K_A and K_B are obtained in the usual way, varying the concentration of one of the reactants while the second is held at a constant concentration, high enough to prevent it becoming rate limiting. K_C, K_D and K_{CD} may be obtained in a similar manner for the reverse reaction. If the same total enzyme concentration is used in obtaining these parameters for the forward and reverse reactions, it may be shown that the equilibrium constant $K_{eq.}$ is

$$K_{eq.} = \frac{V_f K_{CD}}{V_r K_{AB}}$$

For the mechanism proposed for ADH (and using the notation of rate constants given by Theorell and Chance, 1951)

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA$$

$$EA + B \underset{k_6}{\overset{k_4}{\rightleftharpoons}} EC + D$$

$$EC \underset{k_5}{\overset{k_3}{\rightleftharpoons}} E + C$$

$$V_f = k_3[E]_o \,, \quad K_A = \frac{k_3}{k_1} \,, \quad K_B = \frac{k_4}{k_5} \text{ and } K_{AB} = \frac{k_2 k_3}{k_1 k_4}$$

$$V_r = k_2[E]_o \,, \quad K_C = \frac{k_2}{k_5} \,, \quad K_D = \frac{k_2}{k_6} \text{ and } K_{CD} = \frac{k_2 k_3}{k_5 k_6}$$

$$\text{Whence } K_{eq.} = \frac{k_1 k_3 k_4}{k_2 k_5 k_6}$$

Since the same criterion holds for a number of mechanisms obeying the general rate equation, this is not a satisfactory test for the proposed ADH mechanism. However another relationship which is true for the proposed ADH mechanism, but not for other mechanisms described by Alberty, is

$$K_{eq.} = \frac{(V_f) \overset{3}{\cdot} K_C \cdot K_D}{(V_r) \overset{3}{\cdot} K_A \cdot K_B}$$

$$K_{eq.} = \frac{(k_3) \overset{3}{\cdot} (E_o) \overset{3}{\cdot} \frac{k_2}{k_5} \cdot \frac{k_2}{k_6}}{(k_2) \overset{3}{\cdot} (E_o) \overset{3}{\cdot} \frac{k_3}{k_1} \cdot \frac{k_3}{k_4}} = \frac{k_1 k_3 k_4}{k_2 k_5 k_6}$$

The earlier values of the rate constants $k_1 - k_6$ obtained by Chance, Theorell and Bonnichsen did not permit the elimination of all the mechanisms described by Alberty, but as Theorell, Nygaard and Bonnichsen (1955) later point out, the fact that the earlier data fit an alternative mechanism better at pH 7 and pH 8 arises because of the sensitivity of $K_{eq.}$ when $(V_f)^3$ and $(V_r)^3$ are used. Experiments carried out by these workers give results fitting the proposed mechanism over the range pH 5.3 - 10. No evidence of a ternary complex was obtained from these experiments.

It is interesting to note that experiments with yeast alcohol dehydrogenase fulfil the criteria for a ternary complex very well, and that the mechanism proposed for liver ADH was not suitable to describe the kinetic properties of the yeast enzyme. Values of the dissociation constants obtained from the kinetic data agree well with those obtained by Hayes and Velick (1954) measured directly by ultracentrifuge separation experiments.

3. Interpretation of kinetic information.

It may be seen how several approaches have been applied, with considerable measure of success, to the determination of the probable mechanism of enzyme action. Use of steady state data for the overall reaction does not provide sufficient information for the complete elucidation of mechanism since the approximations involved in the evaluation of specific rate constants required comparison of the results with directly determined values for these constants as an estimate of the accuracy of the approximations. The investigation of the kinetics of fumarase shows how far such studies can be extended if a great number of criteria are applied to experimentally obtainable data.

Although the liver alcohol dehydrogenase system has proved amenable to interpretation of the mechanism from the data obtained by the kinetics of the overall reaction, the confirmation of this mechanism rests largely on the direct experimental determination of several of the specific rate constants. The same is true for peroxidase, although here it has been possible to measure all the observable specific rate constants directly.

The good agreement between the overall kinetics and the observed constants for the earlier incomplete mechanism show the danger of the approximations which were used in this case, and indeed, one is left wondering whether some other intermediate step in the reaction sequence for peroxidase may not have been included, although it is difficult to see where such a step might occur, since kinetically detectable ternary complexes were not observed. It is certainly true that transformations of the type

$$(ES_1) \rightleftharpoons (ES_2) \ldots\ldots\ldots\ldots (ES_n)$$

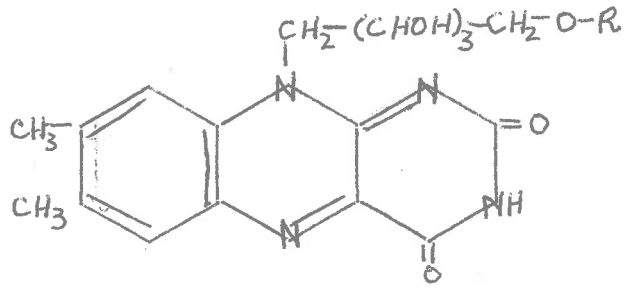
which may be considered to be analogous to the tautomeric transformations of an aromatic nucleus, are not allowed for, but unless these complexes exist for a reasonable period, there is as yet no way in which any but the rate limiting transformation state may be detected.

B. Mechanism of Action of Flavoprotein Enzymes.

1. Introduction.

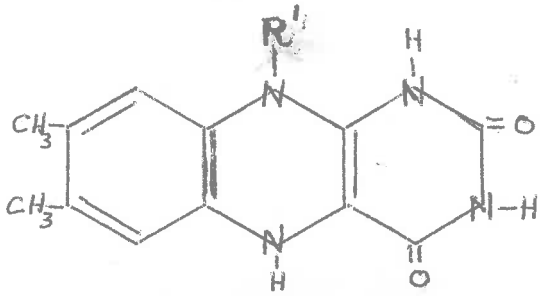
Flavoproteins are oxidising enzymes which contain tightly bound nucleotide derivatives of riboflavin. The bound flavin is referred to as a prosthetic group and undergoes reduction by the substrate for the enzyme and oxidation by some acceptor. The oxidised form is characterised by a two-banded spectrum in the violet-blue region of the spectrum; this portion of the spectrum is greatly diminished on reduction. The prosthetic group is not considered as dissociating from the protein during the reduction-oxidation cycle of the enzyme in its catalytic reaction. This, in fact, serves to distinguish a prosthetic group from a coenzyme, where such dissociation is necessary for the successful operation of the enzyme, if

the coenzyme is at any time associated with the enzyme. The structure of the flavin prosthetic groups in their oxidised form is



where R is H (FMN), or 5' adenylic acid (FAD).

The reduced form is conventionally written as



2. Classification of Flavoproteins.

Three general groups of flavoproteins are recognised on their enzymic behaviour. The basis for the classification depends on the acceptors with which the enzymes will react rapidly. A generalised scheme for these groups is as follows (Singer and Massey, 1957).

$RH_2 + O_2 \longrightarrow R + H_2O_2$ rapid for oxidases; some dehydrogenases react sluggishly.

$RH_2 + A \longrightarrow R + H_2A$ — A is a compound such as methylene blue, indophenol;

$RH_2 + 2A^{n+} \longrightarrow R + 2A^{(n-1)+} + 2H^+$ — A^{n+} such as ferricyanide, cytochrome c.

dehydrogenases, reductases and oxidases

$RH_2 + H_2O_2 \longrightarrow R + 2H_2O$ — peroxidases.

The justification for this classification is that it provides easy recognition of the predominating acceptor reaction. In terms of the overall reaction, however, there is nothing to distinguish one group from the other except the nature of the acceptor. There may be reasons for further classification of flavoproteins on the basis of the kinetically significant intermediates in the enzymic reaction, although the overall reactions suggest that there is no difference between the various groups from a mechanistic viewpoint.

3. Binding of Prosthetic Group to Protein.

It has been postulated that the linkage between the prosthetic group and the protein involves an ionic bond between the phosphate of the flavin nucleotide and a positively charged group on the protein (Theorell 1935, 1937). This would explain the effect of salts and pH on the displacement of flavins from flavoproteins. Theorell and Nygaard (1954, 1955) have shown that a primary amino group in old yellow enzyme is necessary for the binding of FMN to the apoprotein. The adenylic acid moiety of FAD

provides another possible site for binding, and it has been shown that such bonding occurs in D-amino acid oxidase, because competition between FAD and 5'-adenylic acid, adenosine-5'-sulphonate or phenyl carboxylates has been observed (Burton, 1950; Egami and Yagi 1956). It has been shown that riboflavin monosulphonate competes with the flavin moiety of FAD for another site on D-amino acid oxidase (Egami and Yagi 1956). In a number of flavoproteins, the spectrum of the bound flavin differs from that of the free flavin, the position and relative intensities of the bands in the violet-blue region of the spectrum being affected. Except for diaphorase (Straub, 1939; Massey, 1960) all flavoproteins so far isolated are either non-fluorescent, or have much less fluorescence than the free flavin. Titration of the 3-imino group of riboflavin with alkali results in the loss of fluorescence. The *sog* protein of old yellow enzyme will bind riboflavin but not 3-methyl riboflavin. It has been concluded that FMN is bound to old yellow enzyme through the 3-imino group of the flavin (Theorell, 1937; Kuhn and Rudy, 1936; Kuhn and Boulanger, 1936) and this has been often assumed as being true for flavoproteins in general, based largely on the incorrect belief that 3-methyl riboflavin is non-fluorescent (Harbury and Foley, 1958). Theorell and Nygaard (1954, 1955) have shown that a tyrosyl residue of old yellow enzyme is probably involved in the binding of FMN and riboflavin to old yellow enzyme, and suggest that hydrogen bonding occurs between the 3-imino group of the flavin and the tyrosyl hydroxyl group. Some recent studies of molecular interaction of *iso* alloxazine derivatives (Harbury and Foley, 1958; Harbury, La Neue, Loach and Amick, 1959) indicate that there is an alternative interpretation

of flavin binding by proteins. It is suggested that the binding may involve the formation of complexes between protein and flavin by molecular charge transfer (Mulliken, 1952; 1952a; Orgel, 1954), since hydrogen bonding was not a feature of the interactions of flavins in the systems studied, and the interactions were compatible with the charge transfer interpretation. This interpretation can still only be regarded as possible, since no unequivocal evidence in its favour has yet been obtained. The type of complex envisaged involves interaction between the π electron systems of the iso-alloxazine nucleus and aromatic amino acid residues of the protein. Interactions which are apparently of this type have been observed between flavins and tryptophan (Isenberg and Szent-Györgyi, 1958; Harbury et al., 1959), tyrosine, and phenylalanine (Harbury et al., 1959), the latter being contrary to the findings of Isenberg and Szent-Györgyi (1958). No interaction between flavin and imidazole has been observed, although calculations based on molecular orbital methods have indicated that such interaction was very likely (Pullman and Pullman, 1958). The available evidence suggests that molecular charge transfer is very much favoured where the flavin is dissolved in a solvent (or solution) which is capable of hydrogen bonding.

4. Mechanism of Flavoprotein Catalysis.

Michaelis, Schubert and Smythe (1936) and Kuhn and Wagner-Jauregg (1934) showed that the reduction and oxidation of flavins in aqueous solution proceeded by discrete transfers of one electron at a time, passing through a free radical form of the flavin which Michaelis denoted as a semiquinone. Below pH 1, this semiquinone was red, and above pH 1 a

greenish brown. Haas (1937) showed that a red form of old yellow enzyme could be obtained with TPN and dithionite, and attributed this to a semiquinone form of the flavoprotein.

Beinert (1956) has shown that the reduction of free flavins is accompanied by the transient appearance of an absorption band at 565 mμ, which he attributes to the semiquinone form of the flavin. An absorption band in this region, again attributed to a semiquinone, has been observed during the reduction by substrate of acyl CoA dehydrogenases, L amino acid oxidase and possibly old yellow enzyme (Beinert, 1956a), although the red form of old yellow enzyme reported by Haas was not obtained. Massey (1960) has obtained evidence for a semiquinone intermediate for D-amino acid oxidase, xanthine oxidase and lipoyl dehydrogenase. The stability of the substrate produced semiquinones is very high, although the semiquinone produced by reduction of acyl CoA dehydrogenases by dithionite is not very stable, and it is suggested that the semiquinone for this group of enzymes is probably a diradical of the form E-F$^{\bullet}$ —— S$^{\bullet}$ or E  S (Beinert and Page, 1957). Dolin has observed a band in the same region in the difference spectrum of DPNH peroxidase from S. faecalis, when the enzyme is reduced with DPNH, but not when reduced by dithionite. The band appeared an addition of DPN, TPN or NMN to the dithionite-reduced enzyme (Dolin, 1956; 1960). For both the acyl-CoA dehydrogenases and DPNH peroxidase, the flavin absorption was much less decreased by reduction with substrate as compared to reduction by dithionite. In view of the stability of the substrate produced semiquinones and the spectral differences between substrate-reduced and dithionite-reduced enzyme, it seems reasonable to suggest that the stabilisation of the substrate-produced semiquinones is

due to the formation of a diradical. This could either be a complex between the flavin and the substrate, where the electrons donated by the substrate are separated in the complex sufficiently to prevent their interaction, or by interaction between the semiquinoid flavin and another free radical on the enzyme (Massey, 1960a).

i. Spectroscopic and other evidence for flavoprotein-substrate complexes involving flavin. Strittmatter (1959) could not demonstrate an absorption band in the region 500 to 600 mμ for mixtures of DPNH and microsomal DPNH-cytochrome reductase; the difference spectrum (DPNH-reduced minus oxidised) was similar to that of reduced minus oxidised flavin, except for the presence of a band at 315 mμ. This band was not found with dithionite-reduced enzyme and was attributed to a complex between the enzyme and DPNH. It seems therefore, that in this case the protein has increased the electron withdrawing capacity of the flavin sufficiently so that most of the DPNH electron pair involved are donated to the flavin portion of the complex. Ehrenberg and Ludwig (1958) have studied the formation and properties of the red form of old yellow enzyme. It is produced by the addition of a large excess of TPNH to old yellow enzyme, and contains bound TPN. The spectrum is identical to that of oxidised old yellow enzyme in the violet-blue region except that it is shifted some 20 mμ to the red. There is no absorption band in the region of 340 mμ which could be attributed to TPNH. An electron spin resonance signal characteristic of an organic free radical was observed in preparations of this red complex, and was exceptionally stable in the absence of oxygen, but disappeared rapidly in the presence of oxygen. The red colour

was unaffected by oxygen, and furthermore, the calculated concentration of the free radical was far too small to account for the amount of the red complex obtained. The red complex was concluded to be a complex between TPN and the flavin prosthetic group of old yellow enzyme, being formed during the oxidation of a TPNH-enzyme complex.

It may be seen that there is a fair amount of evidence which indicates that a complex is formed between a flavoprotein and its substrate, so that what is written as EFH_2 is more probably EF ⟶ S. Whether this appears as a semiquinone or as the fully reduced flavoprotein depends to some extent on the substrate/enzyme ratio, on the presence of other oxidising groups in the enzyme and on the relative donor characteristics of the substrate and the acceptor capacity of the flavoprotein.

The nature of these complexes is not known, but as a tentative unifying hypothesis it is suggested that they are charge transfer complexes, involving the π electron system of the flavin. The nature of the substrate electrons involved would depend on the substrate, but it is suggested that they are p electrons. Thus for substrates such as DPNH, xanthine, orotic acid etc, a complex between two π systems is suggested, while for substrates such as hydroxy- or amino acids, a complex involving the flavin π system with a pair of p electrons in the group to be oxidised (e.g. N or O lone pair electrons) would be formed. The case of succinate and the acyl-CoA thioesters is somewhat anomalous, but since these substrates undergo α-β unsaturation on oxidation, it is suggested that an sp electron on the α carbon has some p character conferred by the strong electron withdrawal of the carboxyl or thioester group. Thus, to the extent that a

hydrogen ion can be ionised from the α carbon, there is an electron with p character on that carbon, the p character being conferred by the p character of the electron withdrawing group.

The nature of charge transfer complexes implies a certain amount of flexibility of the relative positions occupied by the substrate and the flavoprotein, but there will of course be favoured regions of the flavin prosthetic group where there is a high probability of localisation of the substrate. Such tendency to localisation will be increased by the orienting effect of groups on the protein which confer substrate specificity. In π - π complexes, the two components are usually pictured as occupying parallel planes, in order to permit maximum interaction of the two π systems. Such an arrangement, rather than an edge to edge juxtaposition, is predicated by the vertical distribution of π systems with respect to the plane of the molecule. This stacked arrangement would permit the flavin prosthetic group to lie fairly flat on the protein surface, and would permit the protein to confer the substrate specificity without imposing rather too severe requirements of substrate molecule orientation for a successful collision to occur, as might be the case if some specific group of the flavin had to be approached. A similar arrangement is suggested for the postulated p-π complexes with amino and hydroxy acids.

The semiquinone forms of the enzymes which are produced by substrate can either represent a particular electron distribution of such a complex, or a product derived from the decomposition of such a complex, stabilised by interaction with another free radical. This latter hypothesis is necessary to account for the kinetic behaviour of D-amino acid oxidase and lipoyl dehydrogenase (Massey, 1960).

ii. Kinetic evidence for flaveprotein-substrate complex involving flavin. Slater and Bonner (1952) described a method whereby the individual rate constants for the Briggs-Haldane solution of the Michaelis-Menten mechanism (see p.105) could be evaluated. This was possible where the apparent Michaelis constant could be made large (in the particular instance, for succinate dehydrogenase by using competitive inhibitors) and the maximum velocity varied by varying the concentration or nature of the acceptor. Slater (1955) extended this method to the case where the maximum velocity, V, could be varied, and suggested that flaveproteins were ideal for this since V could be varied by varying either the concentration or nature of the acceptor. The basis of the method is

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\,S \xrightarrow[+A]{k_3} E + P + H_2A$$

$$v = \frac{V}{1 + \frac{K_m}{[S]}} \quad \text{where } V = k_3[E] \text{ and } K_m = \frac{k_2 + k_3}{k_1} = \frac{k_2}{k_1} + \frac{V}{k_1[E]}$$

and k_3 is dependent on the nature and concentration of the acceptor. Thus if K_m vs. V is plotted for various values of these parameters obtained with the same enzyme concentration and different acceptors, the intercept on the K_m axis is k_1/k_2 at $V = 0$, and the slope is $\frac{1}{k_1[E]}$. The earlier method is very similar but here $V/v - 1$ is plotted against V. It is implicit in this derivation that k_3 is a pseudo first order rate constant dependent on the nature and concentration of the acceptor.

It has been pointed out (Dixon, 1955; Laidler, 1955; Bernhard, 1955) that Slater's method is only strictly valid for the simple Briggs-Haldane rate equation, and that for the method to be applicable, it is

necessary to show that this mechanism satisfactorily explains the data. It can be easily shown that modification of the mechanism results in changes in the individual rate constants which constitute K_m and V, so that it may not be possible to obtain individual rate constants by this method. There is, however, one important consequence which arises out of the discussion of this treatment (Dixon, 1955; Slater, 1955b). If the product of the enzymic oxidation is released before the reaction with the hydrogen acceptor takes place, then the plot of K_m vs. V (or V/v - 1 vs.V) will always pass through the origin, no matter what the relative magnitudes of the individual rate constants. As a corollary, if the product is not liberated until reaction with the acceptor occurs (i.e. ES is oxidised by A), then the plot will have a positive intercept on the ordinate (K_m or V/v -1), although the relative magnitudes of the individual rate constants may tend to obscure this, so that it appears to pass through the origin. Thus a positive intercept on the ordinate indicates that the enzyme substrate (ES) complex rather than reduced flavin (EH_2) is oxidised by the acceptor, whereas interception at the origin does not permit any conclusion to be drawn. Plots of K_m vs. V for glucose dehydrogenase of Aspergillus oryzae (Ogura, 1952) and of V/v -1 vs. V for succinate dehydrogenase (Slater and Bonner, 1952) give positive intercepts on the ordinate, while the plots of K_m vs. V for DPNH-cytochrome c reductase and notatin pass through the origin (Slater, 1955a). Thus it is uncertain in the two latter cases as to whether the product is dissociated from the enzyme prior to or following the reaction with the acceptor.

There are therefore two basic mechanisms which need to be considered in relation to flavoprotein catalysis. These mechanisms cover the two cases with respect to sequence of release of product. The basic forms of the mechanisms and the corresponding general steady state rate equations are given here for two-electron acceptors. The same forms of rate equations are also obtained for one-electron acceptors. More explicit forms of the rate equations are given elsewhere (see p.227). The mechanisms are derived for the assumption that substrate reacts first with the enzyme, but in most cases there is little justification for this except that it seems more probable in view of the reduction of flavoproteins by substrate, and that it is easier to derive rate equations for specific rather than random order of addition of reactants to the enzyme. For the case of one-electron acceptors, a favoured order of reaction of substrate and acceptor with the enzyme is necessary if terms involving the square of the acceptor concentration are to be avoided.

Group 1. Product dissociated following reaction with acceptor.

I.A. Single Binary Complex of Enzyme and Substrate.

This is really a special form of 1 B or 1 C where only one kinetically detectable complex of the enzyme exists.

$$E + S \rightleftharpoons ES; \quad ES + A \longrightarrow E + P + H_2A.$$

K_s and V are simple linear functions of [A], and approach infinity as [A] approaches infinity. Although an apparent Michaelis constant and maximum velocity may be measured for A no such terms are apparent in the rate equation except that K_S and V are linear functions of [A]. Similarly, the apparent steady state parameters of A approach infinity as [S] approaches

infinity. A positive intercept on the ordinate will be obtained when K_S vs. V_S or K_A vs. V_A are plotted, provided that the relative magnitudes of the individual rate constants do not obscure this. Lineweaver-Burk plots of 1/v vs. 1/[S] at several fixed concentrations of A will not be parallel since K_S/V_S is a function of [A]. The same is true of K_A/V_A.

1.B. Two Binary Complexes with the enzyme.

For the particular case considered, it is immaterial whether the second binary complex involves a complex of product or reduced acceptor with the enzyme.

$$E + S \rightleftharpoons E S \; + \; ES + A \rightleftharpoons \begin{matrix} EP + H_2A \\ (\text{or } EH_2A+P) \end{matrix} \; ; \; \begin{matrix} EP \longrightarrow E + P \\ (EH_2A \longrightarrow E + H_2A) \end{matrix}$$

$$-\frac{d\,S}{d\,t} = v = \frac{V}{1 + \frac{K_S}{[S]} + \frac{K_A}{[A]} + \frac{K_{AS}}{[A]\,[S]}}$$

where V, K_S, K_A and K_{AS} do not contain terms in [S] or [A].

The apparent Michaelis constants and maximum velocities for S and A (denoted by prime) are functions of the concentration of the other reactant and have finite values K_S, K_A and V, at infinite concentration of the other reactant. Both V'_S and V'_A are hyperbolic functions of [A] and [S] respectively, while K'_S and K'_A are complex functions of the concentration of the other reactant, containing terms which are either hyperbolic or inverse functions of the concentration of the other reactant. Lineweaver-Burk plots of 1/v vs. 1/[S] at several fixed concentrations of A will not be parallel, since K'_s/V'_s is a function of [A]. This will also occur for similar plots of 1/v vs. 1/[A] since K'_A/V'_A is a function of [S]. Plots of K'_A vs. V'_A and K'_S vs. V'_S

will have a positive intercept on the ordinate, although this may be too small to be detected.

1.C. Single Ternary Complex between Enzyme, Substrate and Acceptor.

$$E + S \rightleftharpoons ES \; ; \quad ES + A \rightleftharpoons ESA \longrightarrow E + P + H_2A$$

$$v = \frac{V}{1 + \frac{K_S}{[S]} + \frac{K_A}{[A]} + \frac{K_{AS}}{[A][S]}}$$

The general form of the rate equation for this mechanism is the same as for mechanism 1.B.; and there is no way of distinguishing between the two mechanisms on the basis of steady state rate data alone. A choice between the two mechanisms can be made from transient state kinetics and also from equilibrium data, provided the equilibrium permits satisfactory measurements of the forward and reverse reactions to be made. (see p. 109).

2. The product is dissociated prior to reaction with the acceptor

$$E + S \rightleftharpoons ES \longrightarrow E'' + P \; ; \; E'' + A \longrightarrow E + H_2A.$$

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_S}{[S]}}$$

V, K_A and K_S contain no terms in [S] or [A] . The apparent parameters V'_S, V'_A, K'_S and K'_A are hyperbolic functions of the concentration of the other reactant, and approach V, K_S and K_A respectively at infinite concentrations of the other reactant. Both K'_S/V'_S and K'_A/V'_A are constant and independent of the concentration of the other reactants, so that Lineweaver-Burk plots of 1/v vs. 1/[S] (or 1/[A]) at several fixed concentrations of A (or S) are parallel. When plotted by Slater's method, the line passes through the origin.

Graphical methods for distinguishing between these mechanisms are given in Fig. 15.

iii. Kinetic behaviour of flaveprotein enzymes. As mentioned earlier, glucose dehydrogenase and succinate dehydrogenase satisfy the criterion of Slater's method for enzymes where ES is oxidised by the acceptor, whereas it is uncertain by this criterion as to whether this is the case for DPNH-cytochrome c reductase and notatin. The available data for the first two enzymes does not permit any conclusion to be made as to whether a ternary complex between enzyme, substrate and acceptor is a kinetically detectable intermediate, although the insensitivity of the apparent maximal velocity and Michaelis constants to acceptor concentration for glucose dehydrogenase suggest either mechanism 1.B. or 1.C. Ogura's (1952) statement that a ternary complex is involved, and that it is formed from a binary complex of enzyme and acceptor cannot be supported on the experimental data presented, and his interpretation arises from the particular form in which the rate equation he derived is written and which he failed to see is really the steady state equation for mechanisms 1.B. and 1.C. All that is required is that $K_A/[A]$ and $K_{AS}/[A][S]$ should be very small so that $K_S/[S]$ is the only significant term in the denominator of the rate equation.

Ingraham and Markower (1954) have examined Laser's (1952) data for the variation of the rate parameters of notatin with glucose and oxygen concentration, and postulate a mechanism involving a ternary complex of enzyme, glucose and oxygen in order to explain the non-linear variation of the apparent Michaelis constants and maximal velocities with glucose and oxygen concentration. They had not considered a mechanism of type 1.B.

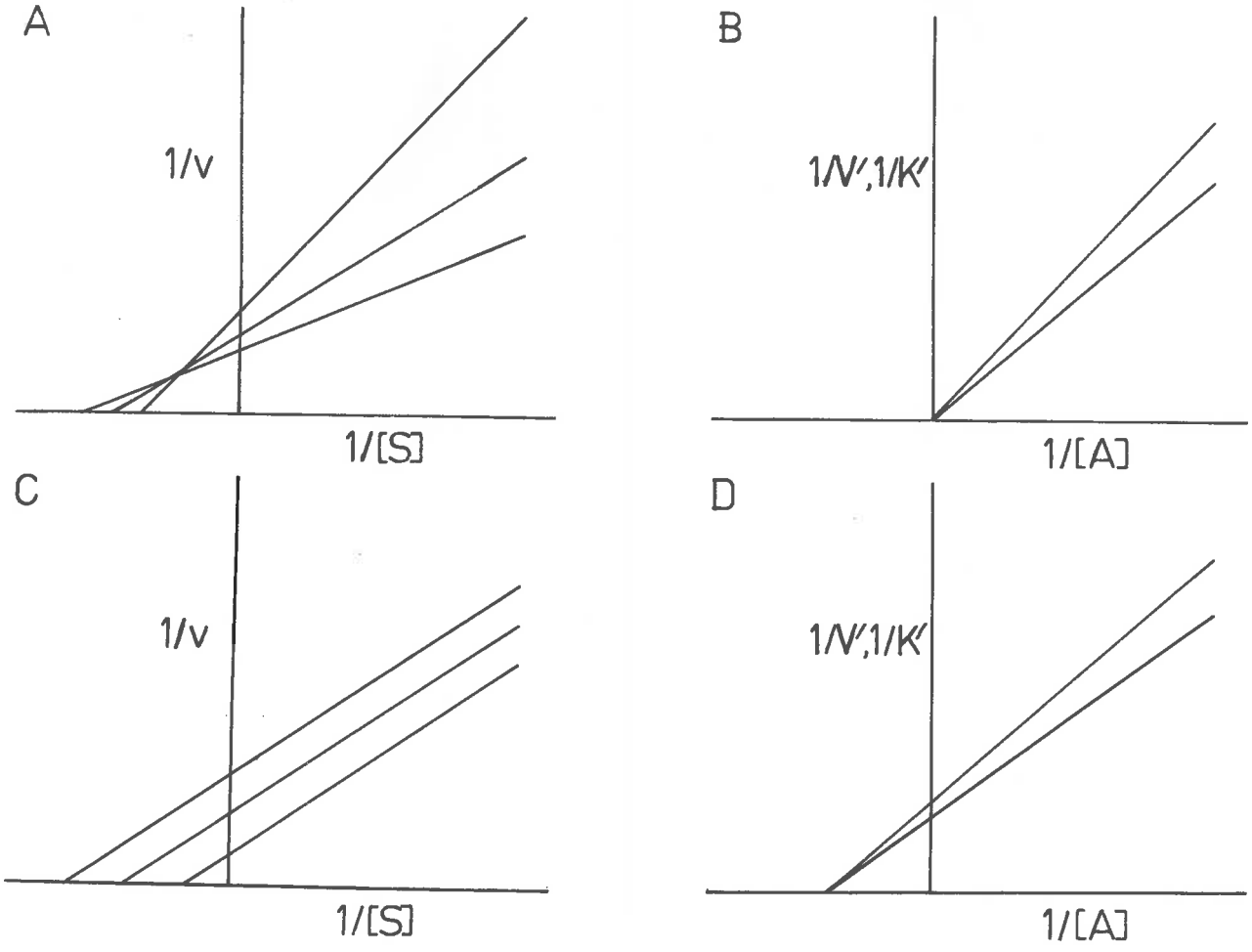
Fig. 15. Graphical methods for distinguishing mechanisms of action of flavoprotein enzymes.

A. Lineweaver-Burk plot for reaction of acceptor with enzyme substrate complex, at three different acceptor concentrations: Upper line, lowest acceptor concentration; bottom line, highest acceptor concentration.

B. Double reciprocal plot of the apparent Michaelis-Menten parameters V', K' in A, against acceptor concentration, for mechanisms of Group IA.

C. Lineweaver-Burk plot for reaction of acceptor with reduced enzyme; otherwise as in A.

D. Double reciprocal plot of the apparent Michaelis-Menten parameters in C, against acceptor concentration for mechanism II


A
1/v
1/[S]
B
1/V′,1/K′
1/[A]
C
1/v
1/[S]
D
1/V′,1/K′
1/[A]

however, and this would serve to explain the results just as well. While mechanism 2 would account for the behaviour of the apparent maximal velocities, it does not conform to the observed behaviour of the Michaelis constants.

Frieden (1957) has carried out extensive kinetic studies with DPNH-cytochrome c reductase. The experimental data at any one pH fit the empirical rate equation for two substrate mechanisms.

$$v = \frac{V}{\left(1 + \frac{K_{DPNH}}{[DPNH]}\right)\left(1 + \frac{K_{cyt.c}}{[cyt.c]}\right)}$$

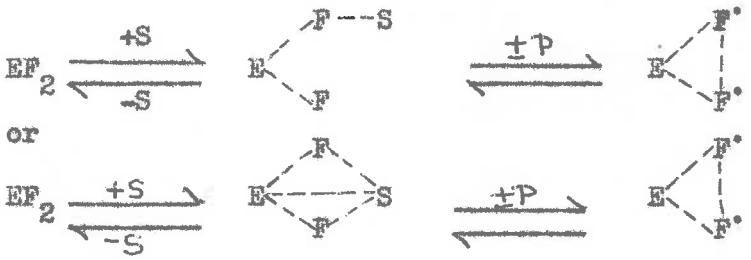
This eliminates mechanisms 1.A. and 2. In order that the velocity be proportional to the first power, rather than the square of cytochrome c concentration, it is necessary to postulate a free radical form of the enzyme substrate complex. This will result in a rate equation corresponding to that for mechanism 1.C. and, with certain restrictions, mechanism 1.B. The restrictions for 1.B. required that three binary complexes of the enzyme are kinetically significant, corresponding to the complexes of enzyme with the substrate, the substrate monoradical and the product or reduced acceptor respectively. It is therefore required that the product is not dissociated from the enzyme until the second acceptor molecule has oxidised the intermediate free radical if a mechanism of type 1.B. is to be considered. No such limitations are necessary for mechanisms of type 1.C. provided that the rate of oxidation of the semiquinone intermediate is not rate limiting.

Frieden observed that v showed a marked positive deviation from Michaelis Menten kinetics in plots of 1/v vs. 1/[cyt.c] at concentrations 2 to 3 times the Michaelis constant for cytochrome c. In order to explain this

effect it is necessary to postulate an effect of cytochrome c concentration on the values of K_{AS} and/or K_S, acting in such a way as to decrease these constants at relatively high concentrations of cytochrome c.

Gutfreund and Sturtevant (1959) have studied the kinetics of the oxidation of xanthine by xanthine oxidase and oxygen, using a stopped flow technique. They concluded that a mechanism where the product was dissociated from the enzyme substrate complex prior to the reaction with oxygen would fit the experimental data well. Since Massey (1960d) has found that a transient semiquinone state is obtained on reduction of xanthine oxidase with excess xanthine, and in view of Morell's (1952) findings that flavin reduction was biphasic, as is also the case for D-amino acid oxidase, it is probable that a semiquinoid flavin form of the enzyme is required for reaction with the acceptor.

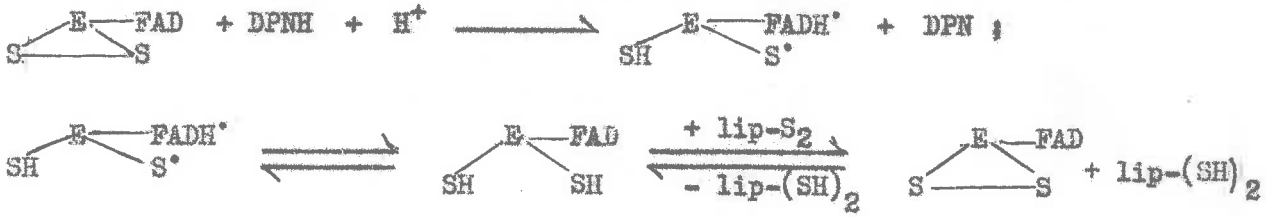
Massey (1960d) has found that the oxidative deamination of amino acids by D-amino acid oxidase follows kinetics which require a rate equation of the form for mechanism 2. Furthermore, he has observed that the fully reduced enzyme is produced too slowly for the form "$EFADH_2$" to have kinetic significance, while a semiquinoid form of the enzyme is produced at a rate consistent with it being a reaction intermediate. Since the enzyme contains 2 molecules of FAD per molecule of enzyme, he postulates the semiquinoid form of the enzyme is $E\begin{matrix}FAD^{\cdot} \\ FAD^{\cdot}\end{matrix}$, and suggests that the two flavin groups are close to one another. The kinetic behaviour requires that the product be released prior to the formation of the semiquinone, and it is therefore questionable as to whether the mechanism of formation of the semiquinone is



Similar conclusions may also apply to xanthine oxidase. Massey (1960d) points out that a considerable number of oxidases contain 2 moles of flavin per mole of enzyme (e.g. old yellow enzyme, glycollate oxidase, D- and L- amino acid oxidases and notatin) and suggests that the following mechanism may be applicable to all of them.

However, the kinetic behaviour of notatin does not correspond to a mechanism of this type (Laser, 1952; Ingraham and Markower, 1954).

Kinetic behaviour conforming to mechanism 2 has been observed for lipoyl dehydrogenase (DPNH + lip-S_2 ⟶ DPN^+ + lip-$(SH)_2$) and for succinate dehydrogenase in the anaerobic reduction of phenazine. (Massey, 1960d). Lipoyl dehydrogenase undergoes reduction of a disulphide bridge to a dithiol which is then re-oxidised during the catalytic process, and the following mechanism has been suggested by Massey (1960d).



The results obtained with soluble succinate dehydrogenase are somewhat anomalous in view of those obtained for other dehydrogenases such as glucose dehydrogenase and DPNH-cytochrome c reductase, and for particulate succinate dehydrogenase. However, it is to be remembered that succinate dehydrogenase is somewhat different in its acceptor requirements to other flavoprotein dehydrogenases, and that it may proceed by a mechanism akin to that for lipoyl dehydrogenase, although a semiquinone intermediate has not been identified as yet. (Massey, 1960d).

Results suitable for such an analysis are somewhat lacking for other flavoprotein enzymes, so that any conclusions drawn as to general mechanisms of action for flavoproteins may need to be modified in the light of further data.

It may be seen, therefore, that there are two general reaction mechanisms for flavoprotein enzymes, and that they differ as to the point at which the product is released from the enzyme-substrate complex. This occurs before the reaction with the acceptor takes place for D-amino acid oxidase, lipoyl dehydrogenase, soluble succinate-phenazine reductase and possibly for xanthine oxidase. The acceptor reacts with the enzyme-substrate complex for glucose oxidase, glucose dehydrogenase and DPNH-cytochrome c reductase, and possibly succinate dehydrogenase in heart muscle particles. There is insufficient information available for generalisations to be made about the possible mechanisms of action of flavoprotein enzymes, except that it seems likely where the "Michaelis" intermediate is the semiquinoid flavin state, that the product has been dissociated from the enzyme prior to or during the formation of this semiquinone. Thus it may be possible to classify flavoproteins on the basis of the conditions for

semiquinone formation. Where this occurs in the absence of acceptor (e.g. D-amino acid oxidase, lipoyl dehydrogenase), it would seem probable that the product is dissociated at this stage so that kinetic behaviour conforming to mechanism 2 is obtained. This implies the formation of a diradical (see p.138).

C. Experimental Procedures, Results and Discussion.

1. Kinetics of Lactate Oxidation by Yeast Lactate Dehydrogenase.

i. Lactate-ferricyanide reductase activity.

(a) General studies. Appleby and Morton (1959a) and Boeri et al. (1955, 1956) found that the lactate-ferricyanide reductase activity of YLD followed zero order kinetics, while the reduction of cytochrome c followed first order kinetics. During experiments on the substrate specificity of YLD, which were carried out with the recording spectrophotometer and with ferricyanide as acceptor, it was found that the optical density of the solution at 420 mμ did not decrease linearly with time. Since it was possible that this was an instrumental error, the rate of reduction of ferricyanide by lactate and YLD was measured with a manually operated spectrophotometer for which the optical density scale was known to be accurate. Particular care was taken in measuring optical densities and the elapsed time was obtained by another person to obviate the difficulties in carrying out two operations at once. Here again, non-linear dependence of the rate of ferricyanide reduction with time was obtained (Fig. 16), and it was found that log E_o/E_t, where E_o is the initial optical density, and

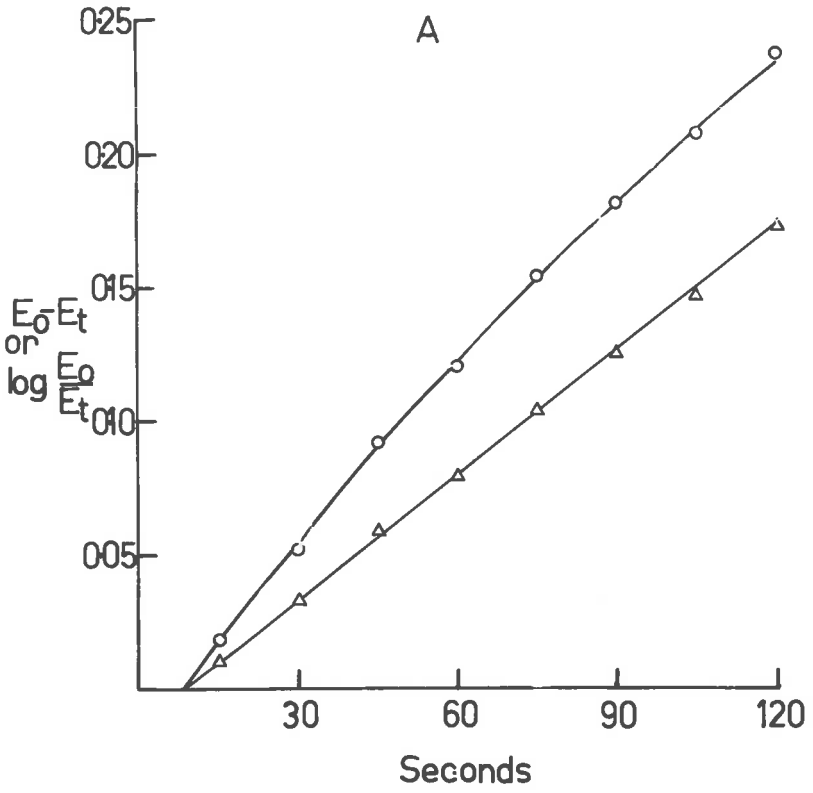
Fig. 16. Reduction of ferricyanide by ascorbate and by lactate and YLD.

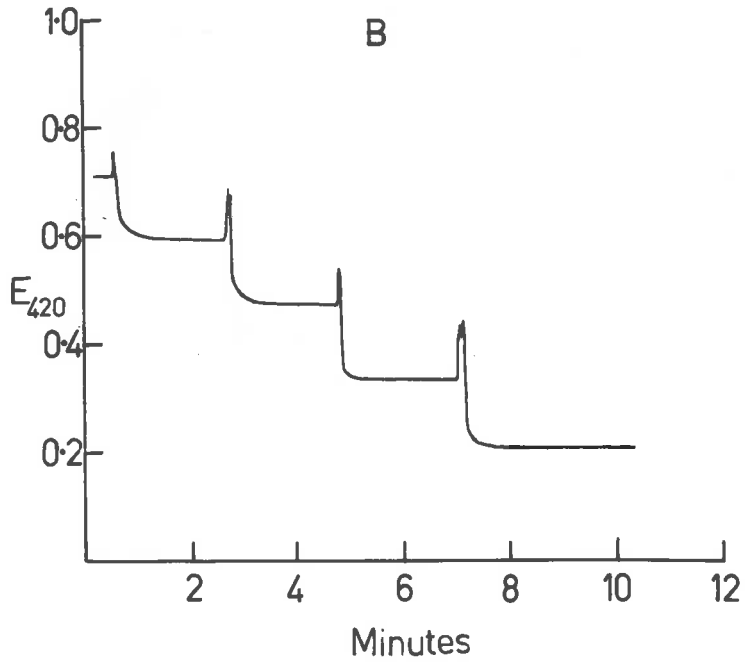
All measurements were made in an Optica double beam recording spectrophotometer at 420 mμ.

A. Change in optical density; 0.8 mM-ferricyanide, 0.033 M-pyrophosphate buffer, pH 8.0, 0.1 M-DL-sodium lactate, 10^{-5}M-EDTA; YLD added at zero time; 1 cm. cells.

O——O $E_o - E_t$, $\Delta - \Delta \log \frac{E_o}{E_t}$ ($\equiv$ $\log \frac{a}{a-x}$).

B. Successive 10 μl volumes of sodium ascorbate were added to 3 ml. of 0.8 mM-ferricyanide in 0.033 M-pyrophosphate buffer, pH 8.0, in a 1 cm. cell. The increases in optical density occur during the addition and mixing of the ascorbate solution with the cell contents, due to the insertion of a stirrer in the light path.


A
0·25
0·20
0·15
0·10
0·05
$E_o - E_t$
or
$\log \frac{E_o}{E_t}$
30
60
90
120
Seconds


B
1·0
0·8
0·6
0·4
0·2
E_{420}
2
4
6
8
10
12
Minutes

E_t the optical density at time t, was a linear function of time. A further check on the recording instrument was obtained by addition of successive equal volumes of sodium ascorbate to a solution of ferricyanide in pyrophosphate buffer at pH 8.0 (Fig. 16). A uniform optical density change was obtained for each addition. Corrections for volume change were negligible.

It is therefore concluded that the non-linear decrease of optical density with time is real and not instrumental in origin. This behaviour has been observed with many different samples of YLD.

First order kinetics of ferricyanide reduction have been obtained over the range 0.10 to 3.14 mM ferricyanide. At 3.40 mM ferricyanide, linear plots of $(E_o - E_t)$ vs. time and log E_o/E_t vs. time were obtained. The plots of log E_o/E_t vs. time were linear for 25% or greater extent of the reaction. The pseudo first order rate constant (k') for lactate-ferricyanide reductase activity was determined over a range of ferricyanide concentrations. Fig. 17 shows the non-linear variation of k' with concentration, and indicates that k' is not a true first order rate constant. It is possible that this effect may be explained by an inactivation of YLD by ferricyanide.

The apparent Michaelis-Menten parameters were estimated from Lineweaver-Burk plots of $1/v_{obs.}$ vs. $1/[FeCN]$ and $1/k'[FeCN]$ vs. $1/[FeCN]$ (Fig. 17). Table 15 compares the values of $v_{obs.}$, the initial velocity obtained as the tangent to the recorder tracing, and $k'[FeCN]$. The apparent Michaelis-Menten parameters are also given. The higher values for the initial velocity given by $k'[FeCN]$ suggest that the fitting of the tangent for obtaining $v_{obs.}$ was not satisfactory.

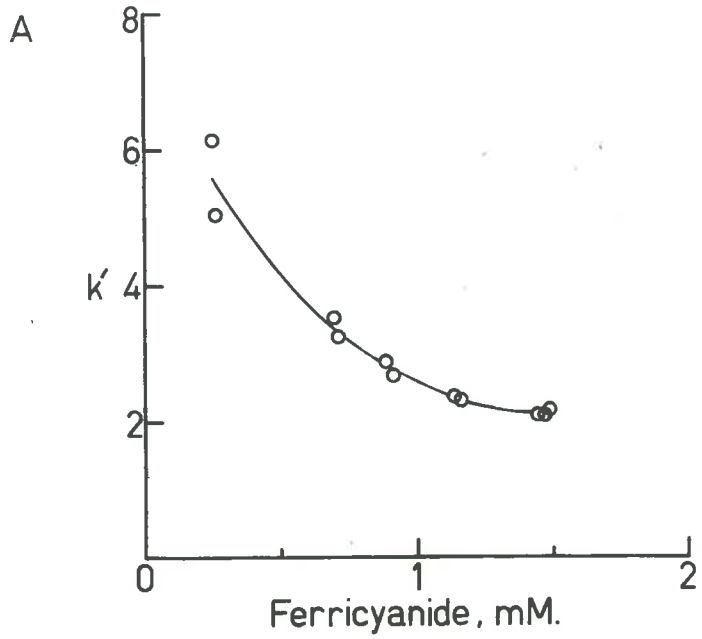
Fig. 17. Kinetic plots for ferricyanide reduction by YLD

The rate of ferricyanide reduction by YLD was measured in 1 cm. cells at 420 mμ with a recording spectrophotometer at varied ferricyanide concentrations in 0.1 M-DL-sodium lactate, 0.033 M-pyrophosphate buffer, pH 8.0, 10^{-5}M-EDTA at 26° (see Table 15).

A. The variation with ferricyanide concentration of the apparent pseudo first-order rate constant (k') for ferricyanide reduction by YLD and lactate.
Ordinate, k', $sec.^{-1} \times 10^{3}$, abscissa, mmoles of ferricyanide/litre.

B. Lineweaver-Burk plot of initial velocity of ferricyanide reduction as a function of ferricyanide concentration.
Ordinate, min./ $E^{1\ cm.}_{420\ m\mu}$, abscissa, litre/ mmole of ferricyanide.

O———O, $1/v_{obs.}$, obtained from visually fitted tangent to the recorder tracing; Δ———Δ, $1/k'[S]_o$, obtained from data in A.


A
8
6
k' 4
2
0
1
2
Ferricyanide, mM.

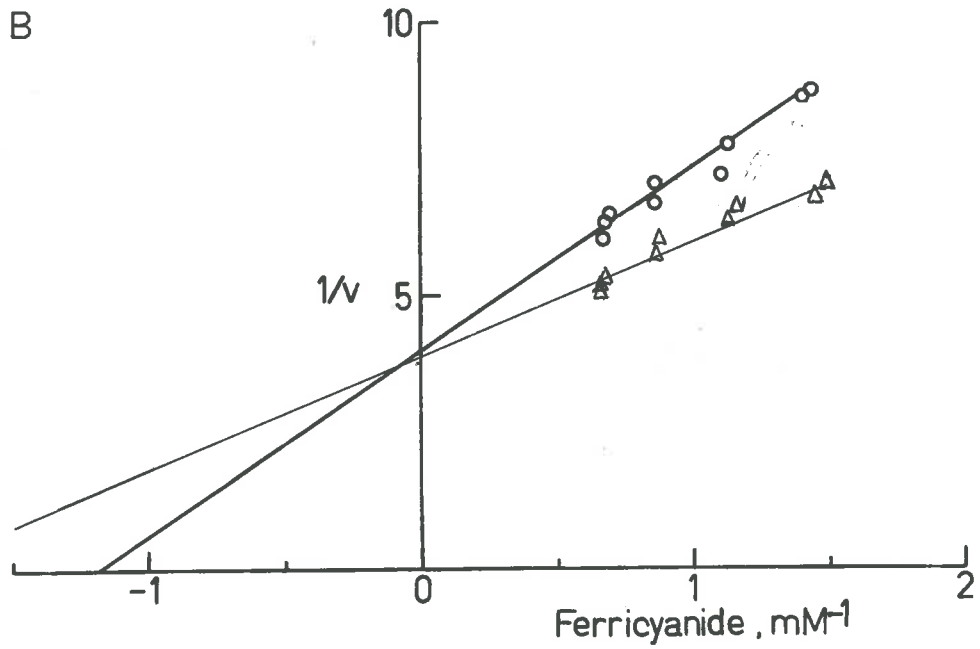

B
10
1/v
5
-1
0
1
2
Ferricyanide, mM-1

Table 15.

Apparent Michaelis-Menten Parameters for Ferricyanide with YLD.

The reduction of ferricyanide by lactate in the presence of YLD was followed in a recording spectrophotometer at 420 mμ, at ferricyanide concentrations of 0.36 mM, 0.70 mM, 0.89 mM, 1.15 mM and 1.47 mM. Concentrations of other reactants were DL-lithium lactate, 0.1 M, sodium pyrophosphate / HCl buffer, pH 8.0, 0.033 M, EDTA, 10^{-5} M. The reaction was started by addition of YLD to the other reactants. Temperature 26°. Velocities are given as $E^{1\ cm.}_{420\ m\mu}$ / min.

Ferricyanide (mM)	0.36	0.70	0.89	1.15	1.47
$v_{obs.}$	0.100	0.114	0.121	0.141	0.155
	0.104	0.116	0.130	0.150	0.158
					0.166
k' [FeCN]	0.114	0.145	0.149	0.161	0.191
	0.120	0.154	0.163	0.172	0.193
					0.199

Apparent Michaelis-Menten Parameters

	K'_{FeCN} M x 10^4	V'_{FeCN} M/min x10^4
$v_{obs.}$	8.3	2.4
k' [FeCN]	5.3	2.5

(b) Mechanism of action of yeast lactate dehydrogenase with ferricyanide as acceptor. The mechanism of action of the lactate-ferricyanide reductase activity of YLD was investigated in a series of experiments in which the initial velocity of ferricyanide reduction was measured at various concentrations of lactate and ferricyanide. DL-lithium lactate was used as substrate and it was assumed that only the L-isomer acted as a substrate, and that the D-isomer was not inhibitory, (however, see p.168).

The initial velocities were estimated from the tangent to the recorder tracing of $E_{420\ m\mu}$ vs. time. Lineweaver-Burk plots for both lactate and ferricyanide as the varied reactant are shown in Fig. 18. For the latter, only the points at the two lower ferricyanide concentrations are used, since it is quite clear from the results that the higher ferricyanide concentration was inhibitory. The values of 1/v used in 1/v vs. 1/[FeCN] are obtained from the least squares plots of 1/v vs. 1/[lactate]. There is no evidence for inhibition by high lactate concentrations in these experiments, but preliminary experiments carried out over the range 10 mM to 500 mM DL-lactate showed that YLD was inhibited by high concentrations of lactate.

The values of the apparent Michaelis-Menten parameters are given in Table 16. Fig. 19 shows the double reciprocal plots of the apparent parameters vs. the con centration of the second reactant. It will be noted that the plot of $1/K'_{FeCN}$ vs. 1/[lactate] shows marked curvature. For the mechanisms discussed earlier, V' is, in general, a hyperbolic function of the second reactant (except for the peroxidase-type mechanism,

Fig. 18. Lineweaver-Burk plots for lactate-ferricyanide reductase activity of YLD

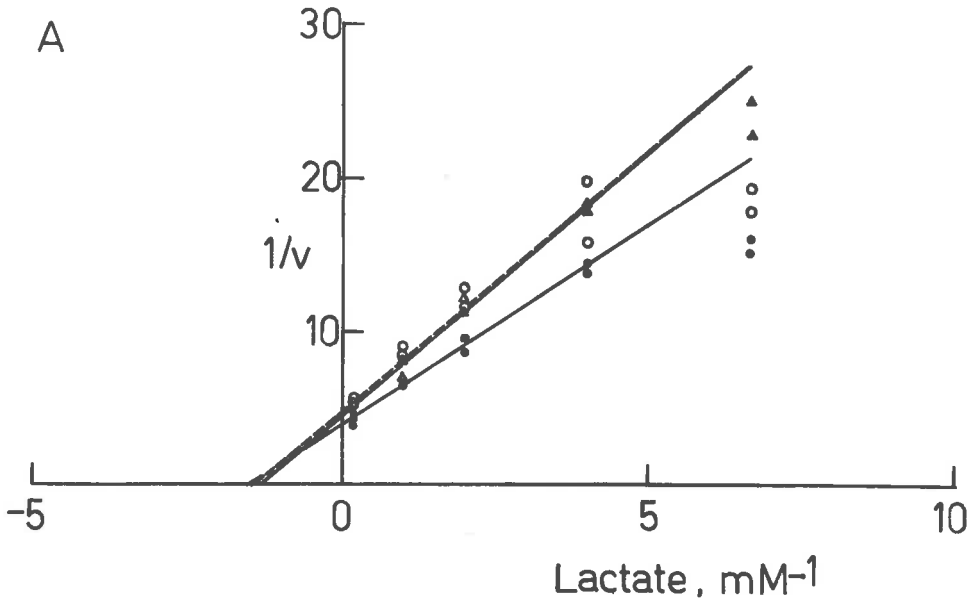
The rate of ferricyanide reduction at 30° was measured at 420 mμ with a recording spectrophotometer in 1 cm. cells against appropriate blanks (water for 0.72 mM-and 0.255 mM-ferricyanide and 1.0 mM-ferricyanide for 1.574 mM-ferricyanide), in 0.033 M-pyrophosphate buffer, pH 8.0, 10^{-4}M- EDTA. The reaction was started by the addition of YLD, final concentration 2.42×10^{-8} M.

A. "Least squares" plots of $1/v_{obs.}$ vs. 1/ [lactate] . Ordinate, min./$\Delta E^{1\ cm.}_{420\ m\mu}$; abscissa, 1./ mmole of L(+)-lactate (from racemic mixture).

Δ———Δ 1.574 mM-ferricyanide, ●———● 0.720 mM-ferricyanide, O———O 0.255 mM-ferricyanide.

B. Plots of $1/v_{obs.}$ vs. 1/ [ferricyanide]. Ordinate, min./$\Delta E^{1\ cm.}_{420\ m\mu}$, abscissa, 1./ mmole of ferricyanide.

The points used are the values obtained from the "least squares" plots in A. Lactate concentration from 5 mM-L(+)-lactate (bottom line) to 0.15 mM-L(+)-lactate (top line).


A
30
20
1/v
10
-5
0
5
10
Lactate , mM-1

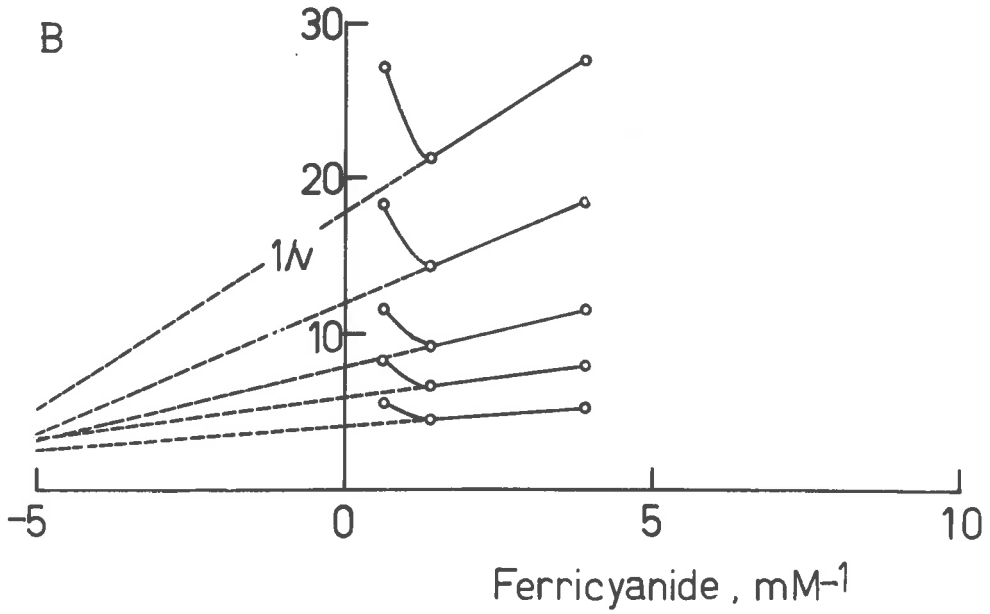

B
30
20
1/v
10
-5
0
5
10
Ferricyanide , mM-1

Fig. 19. "Double reciprocal" plots of apparent Michaelis-Menten parameters for YLD with ferricyanide

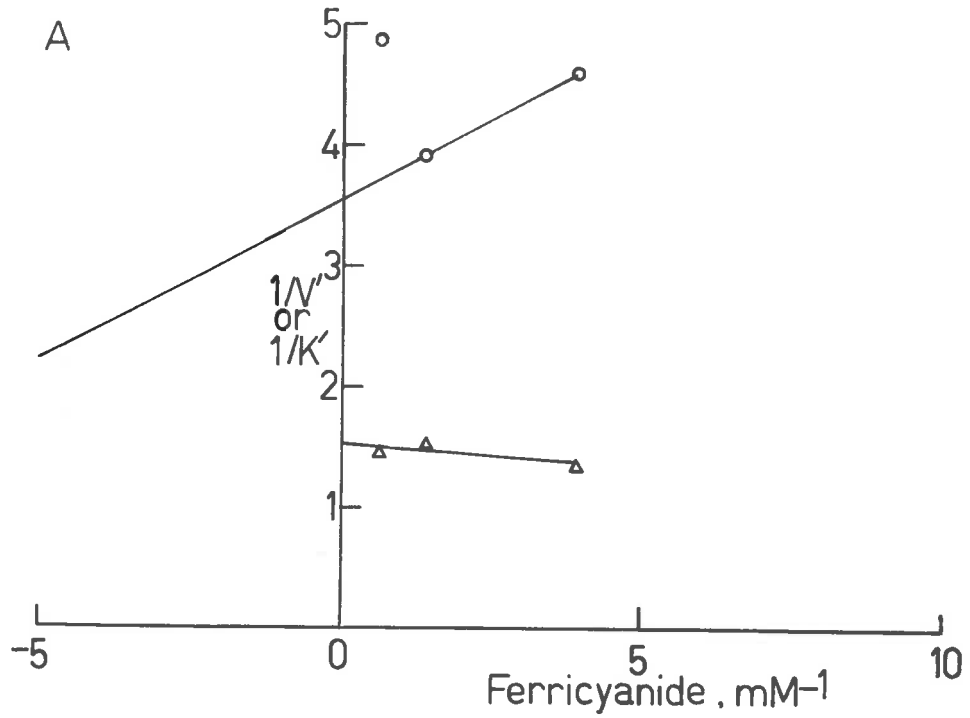
A. Apparent Michaelis-Menten parameters V', $K'_{lactate}$, obtained from intercepts in Fig. 18 A.

O——O $1/V'$, min./$\Delta E^{1\,cm.}_{420\,m\mu}$, Δ——Δ, $1/K'_{lactate}$, mM^{-1}.

B. Apparent Michaelis-Menten parameters V', $K'_{ferricyanide}$, obtained from intercepts in Fig. 18 B.

O——O $1/V'$, min./$\Delta E^{1\,cm.}_{420\,m\mu}$, intercept on ordinate = $1/V$, on abscissa = $1/K_{lactate}$.

Δ——Δ $1/K'_{ferricyanide}$, mM^{-1}.


A
5
4
3
2
1
1/V'
or
1/K'
-5
0
5
10
Ferricyanide , mM-1

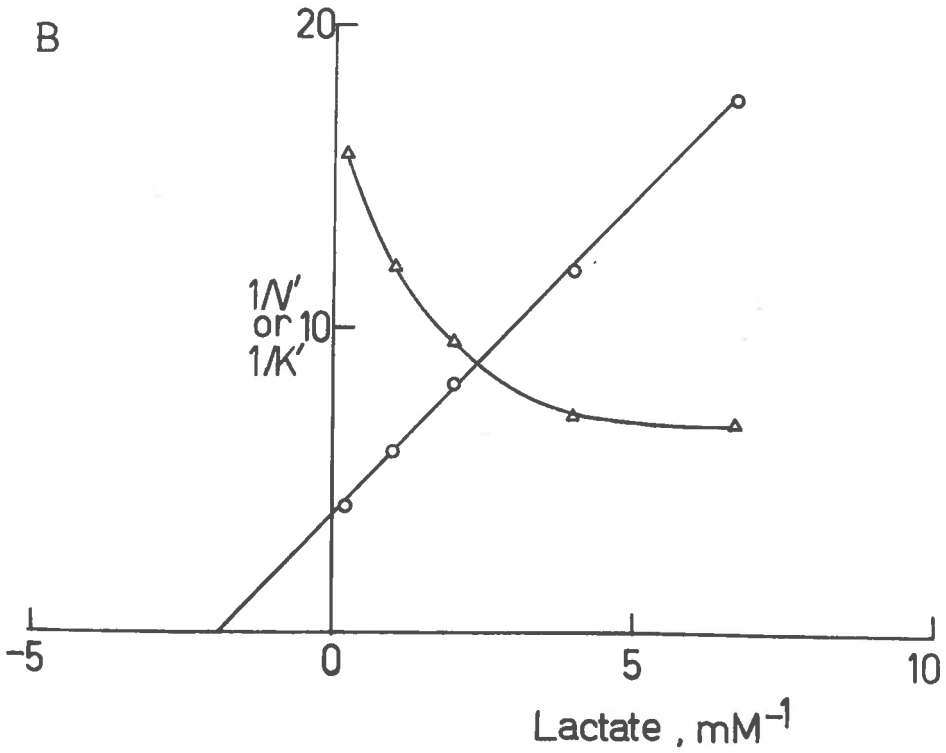

B
20
10
1/V'
or
1/K'
-5
0
5
10
Lactate , mM-1

Table 16.

Apparent Michaelis-Menten parameters for lactate-ferricyanide reductase activity of YLD.

In 0.033 M-pyrophosphate buffer, 10^{-4}M-EDTA, pH 8.0 and 30°.
YLD 2.42×10^{-8}M

L(+)lactate (0.15 mM to 5 mM)

Ferricyanide mM	0.255	0.720	1.574
$K'_{lactate} \times 10^{4}$ (M)	7.43	6.61	6.89
$V'_{lactate} \times 10^{6}$ (M/min.)	209	246	197

Ferricyanide (0.255 mM and 0.720 mM)

L(+)lactate (mM)	0.15	0.25	0.5	1.0	5.0
$K'_{FeCN} \times 10^{3}$ (M)	1.44_0	1.40_5	1.04_8	0.83_4	0.63_5
$V'_{FeCN} \times 10^{6}$ (M/min)	55	78	118	162	234

p. 116), and for some of the mechanisms, this is also true of K'_m. However, some of the mechanisms have rate equations of the form

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A][B]}}$$

where $K'_A = \dfrac{K_A + \frac{K_{AB}}{[B]}}{1 + \frac{K_B}{[B]}}$, and $V'_A = \dfrac{V}{1 + \frac{K_B}{[B]}}$

It is therefore possible to obtain K_B from $1/V_A$ vs. $1/B$.

Now

$$K'_A\left(1 + \frac{K_B}{[B]}\right) = K_A + \frac{K_{AB}}{[B]},$$

i.e., the left-hand side of the equation is a linear function of $1/[B]$. Fig. 20 is a plot of this form for the apparent Michaelis constants for ferricyanide. Although the apparent Michaelis-Menten parameters for ferricyanide are obtained from rather inadequate data, the agreement between the behaviour of these parameters and the predicted behaviour for the rate equation given above can scarcely be fortuitous, and it seems probable that the lactate-ferricyanide reductase activity of YLD can be described in terms of this rate equation. It is clear from Fig. 18 that the enzyme-substrate complex does not break down to form reduced enzyme and product prior to the reaction with the acceptor. For a two substrate reaction, a mechanism of the form corresponding to the above rate equation is therefore highly likely; Table 17 lists the rate parameters obtained by assuming the rate equation given above.

Hasegawa and Ogura (1961) have obtained a value for $K'_{lactate}$ at pH 7.2 and 31° of 2.3×10^{-4} M and a corresponding value of $V'/[E]$ of 180 $sec.^{-1}$. No value for K'_{FeCN} was reported. Boeri and Tosi (1956) found

Table 17.

Steady state rate parameters for lactate-ferricyanide reductase activity of YLD.

In 0.033 M-pyrophosphate buffer, 10^{-4}M-EDTA, pH 8.0, at 30°.

YLD, 2.42 x 10^{-8}M.

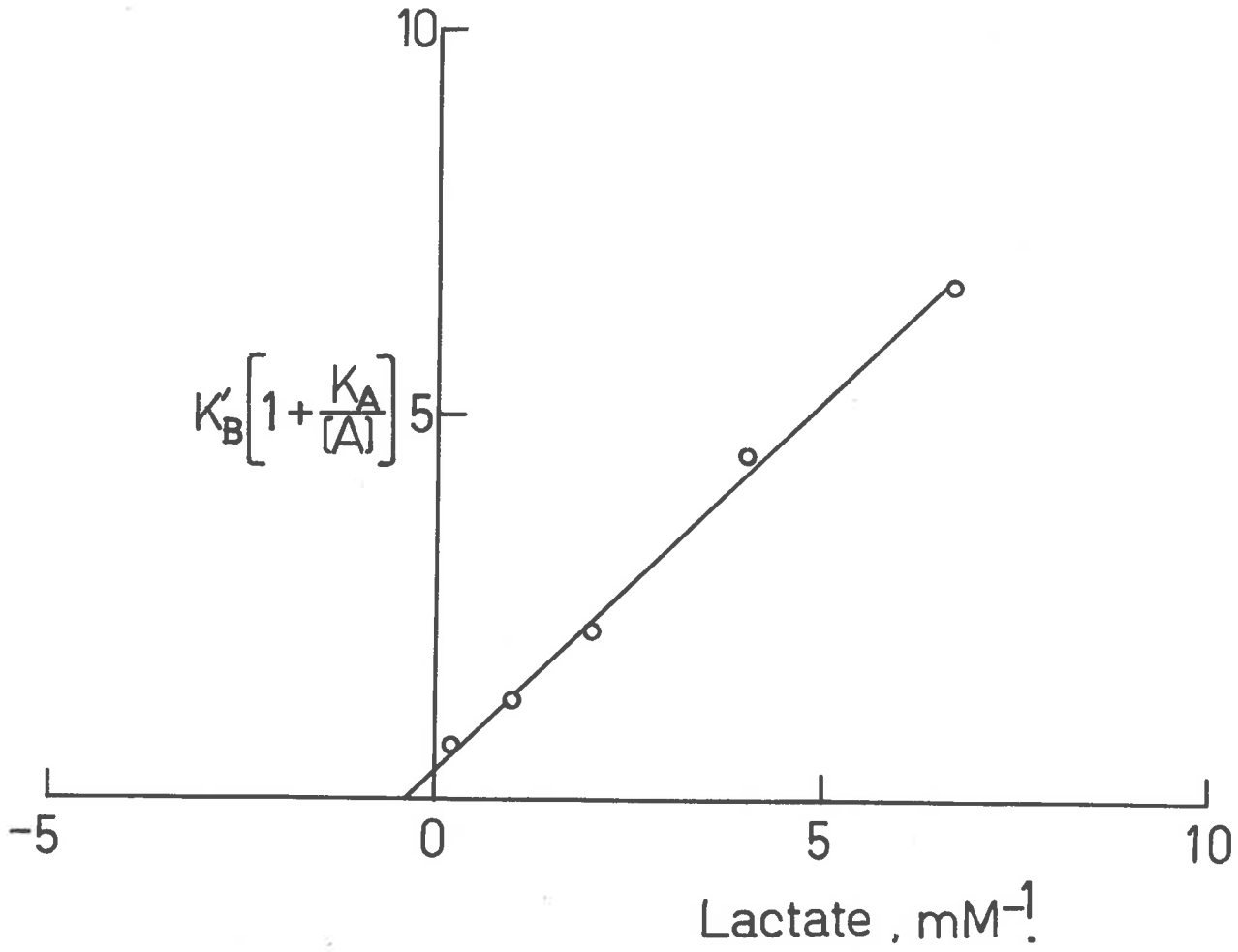
Calculated assuming a rate equation of form $v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A][B]}}$

$K_{lactate}$	5.46 x 10^{-4}M
K_{FeCN}	3.10 x 10^{-4}M
K_{AB}	9.8 x $10^{-4}M^2$
V	254 µM ferricyanide/min.
$V/_{[E]}$ (lactate)	87.5 sec.$^{-1}$

Fig. 20. Special plot of apparent Michaelis-Menten constant for ferricyanide

The values of K'_B were taken from intercept on abscissa in Fig. 18.

The value of K_A was obtained from Fig. 19B. [A]is the concentration of L(+)-lactate for which the particular value of K'_B was obtained. $K'_B(1 + K_A/[A])$ has units of mM.
Intercept on ordinate = K_B; intercept on abscissa = $\frac{-K_B}{K_{AB}}$


10
$K'_B\left[1+\frac{K_A}{[A]}\right]$ 5
−5
0
5
10
Lactate , mM^{-1}

found that $V'/[E]$(lactate) was 115 $sec.^{-1}$ at pH 7.4 and 20° with ferricyanide as acceptor.

ii. Lactate-cytochrome c reductase activity. In accordance with the results obtained by Appleby and Morton (1959b), and Beeri (1955), it was found that the reduction of cytochrome c by lactate in the presence of YLD followed first order reaction kinetics over the concentration range of cytochrome c studied. Although the optimum conditions for cytochrome c reduction are rather different from those for ferricyanide reduction, the experiments reported here were carried out under the same conditions as used for ferricyanide, so that a more direct comparison of the two acceptors can be made. Lineweaver-Burk plots for lactate and for ferricyanide are shown in Fig. 21, and double reciprocal plots of the apparent Michaelis-Menten parameters are also presented (Fig. 22). It is immediately obvious that the reduction of cytochrome c must occur by a different mechanism to ferricyanide reduction (cf. Fig. 18, p.153a). The results for cytochrome c reduction by lactate and YLD show the predicted behaviour for a mechanism in which the enzyme-substrate complex dissociates to form reduced enzyme and product, prior to the reaction with the acceptor. The general significance of these results in relation to electron transfer by YLD will be discussed later.

Table 18 gives the steady state rate parameters for the lactate-cytochrome c reductase activity of YLD. Hasegawa and Ogura (1961) reported values for $K_{lactate}$ and $V/[E]$ at 22° and pH 7.2 of 1.8×10^{-4} M and 140 $sec.^{-1}$ respectively, calculated for the same rate equation.

Table 18.

Steady State Rate Parameters for Lactate-Cytochrome c Reductase Activity of YLD.

The reduction of cytochrome c by lactate in the presence of YLD was measured in a recording spectrophotometer at 550 mμ. The reactant concentrations were ; sodium pyrophosphate buffer, pH 8.0, 0.033 M, EDTA, 10^{-4}M, DL- lithium lactate, 0.2 mM to 10 mM, cytochrome c , 0.5 μM to 170 μM, YLD, 2.52×10^{-8}M. Temperature, 30°.

Lineweaver- Burk plots of $1/v_{init.}$ vs. $1/[A]$ were used to obtain apparent parameters (see Fig. 21); the steady state parameters were obtained from the variation of the apparent parameters with the concentration of reactant B, as in Fig. 22, assuming a rate equation of the form

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]}}$$

	lactate as reactant A	cytochrome c as reactant A
$K_{lactate}$	5.52×10^{-4}M	5.20×10^{-4}M
$K_{cytochrome\ c}$	6.54×10^{-5}M	6.41×10^{-5}M
V	336 M cytochrome c / min.	322 M cytochrome c / min.
V/ [E] (lactate)	110 $sec.^{-1}$	107 $sec.^{-1}$

Fig. 21. Lineweaver-Burk plots for lactate-cytochrome c reductase activity of YLD.

The rate of reduction of ferri-cytochrome c at 30° was measured at 550 mμ with a recording spectrophotometer, in 0.5 cm. cells (0.171 mM-cytochrome c) and 1 cm. cells (0.082 mM; 0.015 mM-and 0.005 mM-cytochrome c) against appropriate blanks (0.072 mM-cytochrome c for 0,171-mM cytochrome c, 0.037 mM-cytochrome c for 0.082 mM-cytochrome c and water for the two lower concentrations). These blanks were used to obtain suitable initial optical density readings. The measurements were carried out in 0.033 M-pyrophosphate buffer, pH 8.0, 10^{-4}M-EDTA. The reaction was started by the addition of YLD, final concentration 2.52×10^{-8}M.

A. $^1/v_{init.}$ vs. $^1/$[lactate]; ordinate, min./ $\Delta E \, ^{1cm.}_{550}$; abscissa, L(+)-lactate (in racemic mixture) $mM.^{-1}$.

O——O 0.171 mM-cytochrome c Δ——Δ 0.082 mM-cytochrome c,

●——● 0.015 mM-cytochrome c, ▲——▲ 0.005 mM-cytochrome c,

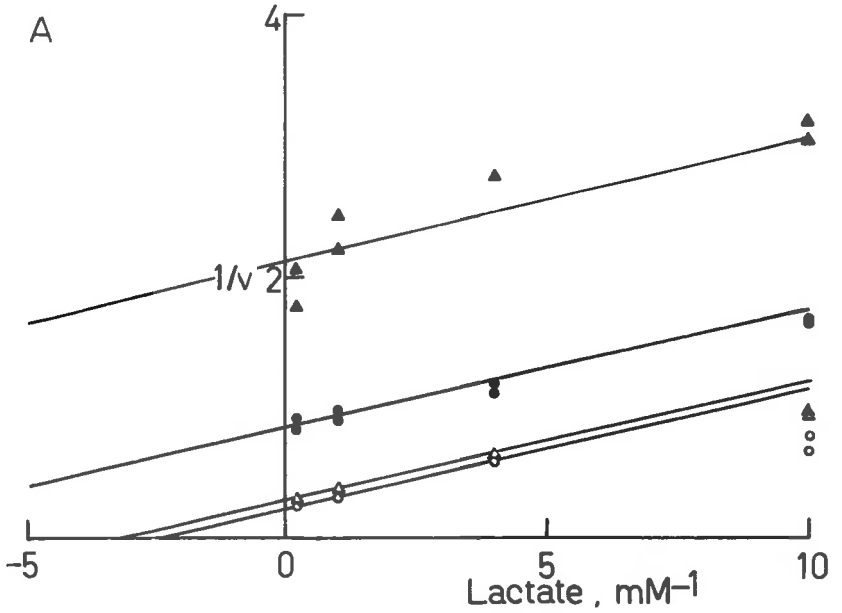
line fitted to be parallel to other lines.

B. $^1/v_{init.}$ vs. $^1/$[cytochrome c]; ordinate, min/$\Delta E \, ^{1cm.}_{550\ m\mu}$;

O——O 5 mM-L(+)-lactate; Δ——Δ 1 mM-L(+)-lactate;

●——● 0.25mM-L(+)-lactate; ▲——▲ 0.1 mM-L(+)-lactate;

□——□ 0.1 mM-L (+)-lactate, $^1/v_{init.}$ obtained from extrapolation of lines in A.


A
4
1/v 2
-5
0
5
10
Lactate , mM-1

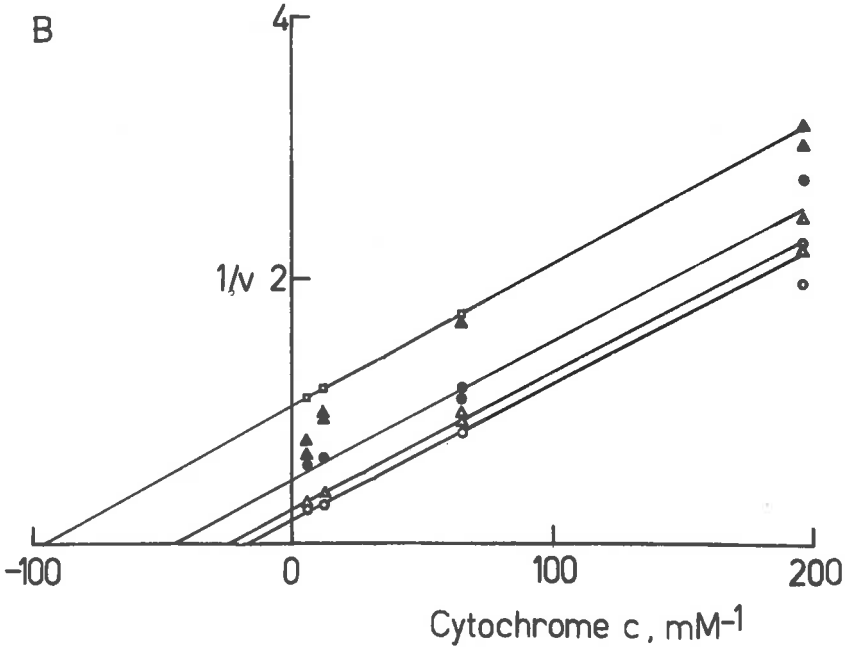
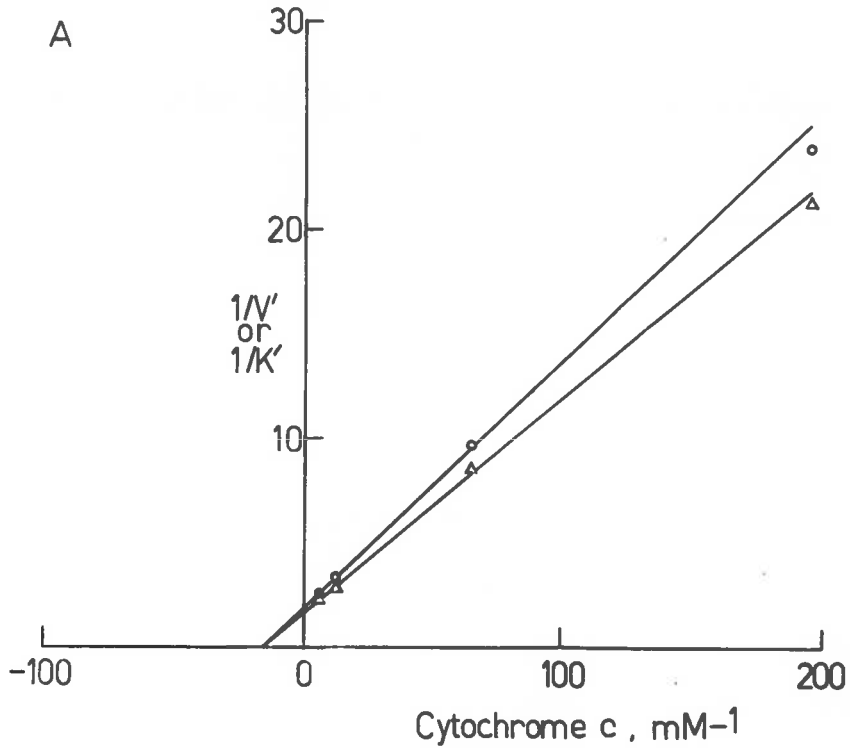
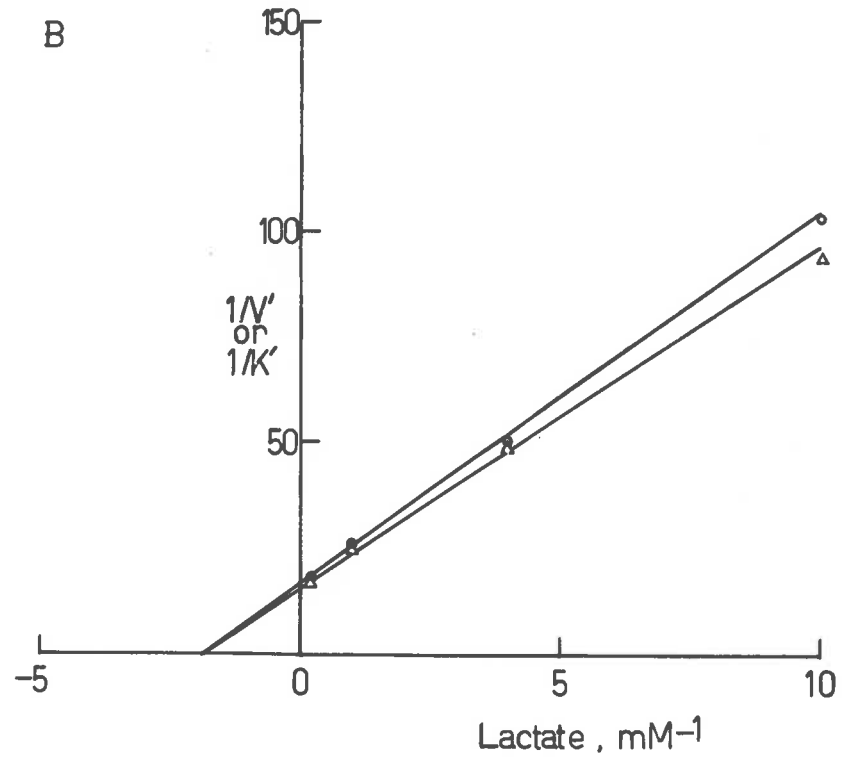

B
4
1/v 2
-100
0
100
200
Cytochrome c , mM-1

Fig. 22. "Double reciprocal" plots of apparent Michaelis-Menten parameters for YLD with cytochrome c

A. Intercepts from Fig. 21 A. Ordinate; $(1/K')_{lactate}$, mM^{-1}, O——O; $(1/V')_{lactate}$, min/ $\Delta E^{1cm.}_{550\ m\mu}$ x 10, Δ——Δ
Intercepts: ordinate $1/K_{lactate}$ $1/V$; abscissa. - $1/K_c$.

B. Intercepts from Fig. 21 B. Ordinate: $(1/K')_c$ mM^{-1}, Δ——Δ ; $(1/V')_c$, min/ $\Delta E^{1cm.}_{550\ m\mu}$ x 10^2, O——O.
Intercepts: ordinate $1/V$, $1/K_c$; abscissa - $1/K_{lactate}$.


A
30
20
1/V'
or
1/K'
10
-100
0
100
200
Cytochrome c , mM-1


B
150
100
1/V'
or
1/K'
50
-5
0
5
10
Lactate , mM-1

Boeri et al. (1955) found that $K'_{lactate}$ was 3.5×10^{-4}M at pH 7.4 and 20°; the corresponding values of $K'_{cyt.\ c}$ and $V'/[E]$ (lactate) were 1.85×10^{-5}M and 36 $sec.^{-1}$. Boeri and Tosi (1956) subsequently obtained a value for $V'/[E]$ of 115 $sec.^{-1}$

iii. Lactate-indophenol reductase activity. The reduction of dichlorophenol indophenol (DCPIP) was measured at pH 8.0 and 30° in 0.033M-pyrophosphate buffer, 0.1 M-DL-lactate, 10^{-5}M-EDTA, over the range 0.027-0.223 mM-DCPIP, Zero order kinetics of dye disappearance were followed over this concentration range. Table 19 gives the apparent Michaelis-Menten parameters for DCPIP (Fig. 23). The maximum velocity of lactate oxidation is considerably lower than the maximum velocity attained with ferricyanide or cytochrome c as acceptor. Hasegawa and Ogura (1961) found values for $K'_{lactate}$, 1.6×10^{-4}M, and $V'/[E]_2$ 220 $sec.^{-1}$ at 33° and pH 7.2 with DCPIP as acceptor.

iv. Ionic strength and rate of ferricyanide reduction by yeast lactate dehydrogenase. Boeri and Tosi (1956) found that the rate of reduction of cytochrome c by YLD was considerably decreased as the ionic strength was increased, whereas the rate of reduction of ferricyanide was virtually unaffected by ionic strength. These workers used sodium chloride to increase the ionic strength. Some observations in the present work (see p.201) suggested that YLD was less stable in the presence of sodium chloride, and therefore the effect of increasing ionic strength on the rate of reduction of ferricyanide by lactate and YLD has been examined, using both sodium pyrophosphate and sodium chloride to increase the ionic

Table 19.

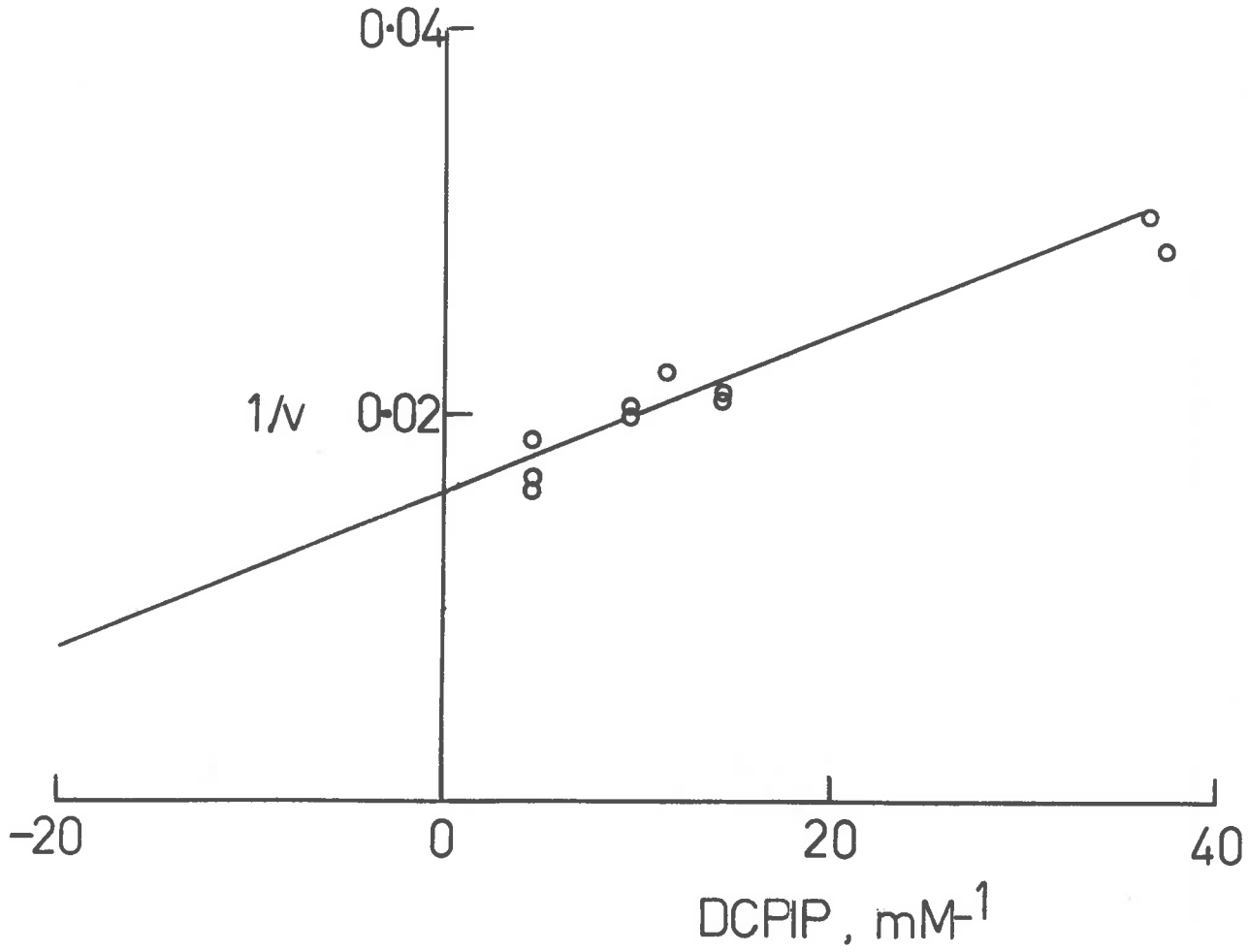
Apparent Michaelis-Menten parameters for dichlorophenol indophenol with YLD.

The reduction of the dye was followed spectrophotometrically at 600 mμ in a reaction mixture containing 0.1 M-M-lithium lactate, 0.033M-pyrophosphate buffer, pH 8.0, 10^{-5}M-EDTA, and DCPIP over the range 0.027 mM to 0.223 mM. The reaction was started by addition of YLD.
Temperature 30°

K'_{DCPIP}	2.35×10^{-5}M
V'	61.9 μM/min.

Fig. 23. Lineweaver-Burk plot for dichlorophenol indophenol reduction with YLD

The rate of reduction of 2,6-dichlorophenol indophenol at 30° was measured with a recording spectrophotometer at 600 mμ in 0.033M - pyrophosphate buffer, pH 8.0, 10^{-4}M-EDTA, 0.1M lithium lactate. For 0.223 mM-DCPIP, 0.5cm. cells and a blank of 0.158 mM-DCPIP was used, for 0.104 mM-DCPIP 0.5 cm. cells and a blank of 0.047 mM-DCPIP, for 0.087 mM-DCPIP 0.5 cm. cells and a blank of water, and for 0.027 mM-DCPIP, 1 cm. cells and a blank of water. The reaction was started by the addition of YLD. Ordinate min/μM. DCPIP. abscissa, DCPIP, mM^{-1}.


0·04
1/v
0·02
-20
0
20
40
DCPIP , mM-1

strength (Table 20). The total ionic strength was calculated for the entire reaction mixture, lactate and ferricyanide included. The solution of YLD used had an ionic strength of 0.11 and since the final dilution was 1 in 151, the ionic strength on addition of enzyme was increased by 7×10^{-4}. This has been allowed for. All solutions were carefully adjusted to pH 8.0 before use.

It may be seen that increasing the ionic strength increased the initial velocity of ferricyanide reduction, whether sodium pyrophosphate or sodium chloride was used to increase the ionic strength, and that a maximum effect is obtained with sodium chloride. It will also be noted that the rate of ferricyanide reduction decreased with time in the presence of sodium chloride to a considerably greater extent than pyrophosphate. Boeri and Tosi (1956) found that chloride ion of the corresponding ionic strength caused greater decrease in lactate-cytochrome c reductase activity than phosphate ions.

A similar experiment to that described in Table 20 was carried out, but the YLD solution was incubated at 0° for various lengths of time in a solution containing all the reactants but ferricyanide prior to the determination of enzyme activity.

Activities were measured 2 mins, 22 mins. and 65 mins. after addition of YLD to the incubation mixture. The activity of YLD at various ionic strengths in lithium lactate / sodium pyrophosphate or lithium lactate / sodium chloride did not change during the period of the experiment. The state of YLD in the two experiments differs in that in the presence of ferricyanide, there is a steady state concentration of oxidised YLD, whereas in the absence of ferricyanide, YLD is completely reduced. It is therefore

Table 20

Effect of Ionic Strength on the Lactate-Ferricyanide Reductase Activity of YLD.

The following conditions were used: DL-lactate, 5 mM, ferricyanide, 0.80 mM, pyrophosphate/HCl, pH 8.0, at approx. 24°. Pyrophosphate/HCl or NaCl added as indicated; the enzyme was in a solution of the following composition; 0.03 M-pyrophosphate/HCl, 0.09 M-sodium lactate, 1.5×10^{-5} M-EDTA, pH 8.0. The reaction was started by addition of YLD; mean of two activity determinations.

Pyrophosphate (mM)	NaCl (mM)	$T_{1/2}$	$\Delta E^{1cm.}_{420m\mu}$ / min.		
			1st min.	2nd min.	2nd min. (% of 1st min.)
0.518	-	0.0145	0.202	0.201	99.5
1.295	-	0.0245	0.220	0.220	100
5.698	-	0.0545	0.236	0.210	89
13.485	-	0.1145	0.243	0.240	99
25.933	-	0.2105	0.263	0.252	96
0.518	10	0.0245	0.224	0.224	100
0.518	40	0.0545	0.269	0.252	93
0.518	100	0.1145	0.298	0.225	75.5
0.518	196	0.2105	0.258	0.218	84

possible that the decrease in rate of ferricyanide reduction in the presence of chloride ion is due to an effect of Cl^- on oxidised YLD. The dissociation of FMN from old yellow enzyme is considerably increased by Cl^- and other anions of strong acids (Theorell and Nygaard, 1954). Similarly, the complexes of yeast alcohol dehydrogenase with DPN and DPNH are considerably affected by Cl^-. It is also known that the dissociation of FMN from old yellow enzyme is 170 times greater for the oxidised form of the enzyme than for the reduced form of the enzyme (Vestling, 1955).

v. Interpretation of kinetic results. The studies on the mechanism of action of YLD lead to the conclusion that the oxidation of lactate by ferricyanide in the presence of YLD differs markedly from the oxidation of lactate where cytochrome c is the acceptor. The steady state kinetics of ferricyanide satisfactorily fit a rate equation of the form

$$v = \frac{V}{1 + \frac{K_{lactate}}{[lactate]} + \frac{K_{FeCN}}{[FeCN]} + \frac{K_{AB}}{[lactate]\,[FeCN]}}$$

This rate equation can be derived for mechanisms in which a ternary complex or several different binary complexes of enzyme, first reactant and second reactant are formed; for the case of flavoproteins, as discussed earlier (p. 150), this requires that the acceptor reacts with the enzyme-substrate complex prior to the dissociation of the product. Were ferricyanide to oxidise the haem of YLD, then a different form of rate equation would be required. This rate equation is satisfied by the results obtained for cytochrome c and has the form

$$v = \frac{V}{1 + \frac{K_{lactate}}{[lactate]} + \frac{K_{cytochrome\ c}}{[cytochrome\ c]}}$$

which, for flavoproteins, requires the formation of reduced enzyme and product from the enzyme-substrate complex prior to reduction of the acceptor. It therefore seems highly unlikely that ferricyanide and cytochrome c are accepting electrons from the same site. Since a number of reactions catalysed by flavoproteins are known to conform to either one or the other of the above steady state rate equations, it can probably be concluded that the oxidation of YLD by ferricyanide takes place at the flavin prosthetic group, as suggested by the rate equation found for the reduction of ferricyanide. It is therefore suggested that cytochrome c oxidised the haem prosthetic group of YLD. This group would satisfy the requirements for the reduced form of the enzyme required by the second rate equation. Chance and Boeri (1961) concluded that the haem of YLD was not involved in the electron transport process of the enzyme, but, as discussed earlier, their results could be explained by the presence of enzymically inactive YLD. Hasegawa and Ogura (1961) found that the kinetics of ferricyanide reduction followed those obtained for cytochrome c and for various dyes (i.e. corresponding to the second rate equation). This finding disagrees with the present work; these workers carried out their studies at very low concentrations of ferricyanide (0.02 - 0.08 mM FeCN). It is possible that at low concentrations, ferricyanide reacts at the same site as cytochrome c, and at a different site at higher concentrations. In terms of the present interpretation, low concentrations of ferricyanide would oxidise the haem prosthetic group, while high concentrations would compete with the haem group for the electrons from the flavin

prosthetic group. This competition would result in all reduction of ferricyanide occurring at the flavin site at high enough concentrations of ferricyanide. Since concentrations over the range 0.35 to 1.5 mM-ferricyanide gave a satisfactory Lineweaver-Burk plot and a value of K'_{FeCN} consistent with those obtained in the studies on the mechanism of ferricyanide reduction (p.152a), it seems unlikely that two sites for the steady state reduction of ferricyanide by the YLD and lactate exist, since a very abrupt transition from one site to the other would be necessary. It is clear, however, that in reaching the steady state, the haem group of YLD is oxidised by ferricyanide (Hasegawa and Ogura, 1961).

There is no simple explanation of the effect of ionic strength on the rate of reduction of ferricyanide. Theorell et al. (1954) studied the effect of ionic strength and specific ions on the individual rate constants of the reaction catalysed by yeast alcohol dehydrogenase and found that some were increased and others decreased. The apparent effect of chloride ion in decreasing the rate of ferricyanide reduction only when the enzyme is turning over can probably be best explained by an effect on the oxidised YLD present during the steady state, thereby reducing the enzyme concentrations. The greater decrease caused by chloride in the rate of cytochrome c reduction, as compared with phosphate (Boari and Tosi, 1956) confirms the results obtained here. The observed effectiveness of various anions in increasing the dissociation of FMN from old yellow enzyme was $Br' > NO_3' > Cl' \gg SO_4'' > PO_4'''$ > acetate (Theorell and Nygaard, 1954) suggests that YLD is probably affected in a similar manner. On this basis, pyrophosphate might be expected to have rather less effect on ferricyanide reduction by YLD than chloride.

IV. SUBSTRATE SPECIFICITY AND INHIBITION OF YEAST LACTATE DEHYDROGENASE

1. Materials.

i. Chemicals. A number of compounds were obtained commercially and used without further purification. The sources of these compounds were: Sigma Chemical Company, St. Louis, Missouri.
DL-α-hydroxy-n-butyrate, (Ba salt), DL-α-hydroxy-iso-butyric acid, DL-α-hydroxy-iso-valeric acid, DL-α-hydroxy-n- and -iso-capreic acids, L(+)-tartaric acid and DL-β-phenyl lactic acid.

California Corporation for Biochemical Research, Los Angeles, California.

L (+)-lactate, Ca salt, D(-)-lactate, Ca salt, L(+)-malic acid, D(-)-malic acid.

British Drug Houses (B.D.H.)

DL-malic acid.

DL-mandelic acid (Light Chemicals, Bucks.) was recrystallised from hot aqueous solution after treatment with activated charcoal. Glycollic acid (B.D.H.) was recrystallised from hot ether. Lithium lactate (B.D.H.) was recrystallised from ethanolic solution (Barker, 1957).
Hydroxymalonic acid was prepared from racemic tartaric acid by decarboxylation and oxidation with fuming nitric acid/P_2O_5 (Behrend and Osten, 1905; Prusse, 1918).

Several methods for the preparation of DL-glyceric acid were tested, and that described in Beilstein (1921) was finally used. In this method, fuming nitric acid is layered under a 50% aqueous solution of glycerol, and

the two are permitted to diffuse, without disturbance. It was not found possible to layer the nitric acid without a violent evolution of nitrous oxides in the interface, so the nitric acid was frozen in the long tubular reaction vessel, and the glycerol solution was poured over the frozen acid. The glyceric acid was isolated as the calcium salt, converted into the free acid by treatment with Dowex-50-(H^+), adsorbed onto a column of Dowex-2-acetate, and eluted with 0,2M-ammonium acetate buffer, pH 4.0. The collected fractions of the eluate were tested for glyceric acid with naphthoresorcinol/H_2SO_4 (Rapoport, 1937) and for non-volatile acids by spotting onto filter paper, which was dried at 100° for 2 hours, and sprayed with an alkaline solution of bromphenol blue (1% in ethanol). Acidic, naphthoresorcinol-positive compounds were obtained in fractions 18-38 (36-76 cc of eluate). Samples from these fractions were chromatographed on Whatman No.1 paper with methyl ethyl ketone: acetone: water: formic acid:: (90:4:2:2 v/v). An authentic sample of D(-)-glyceric acid, prepared by hydrolysis of D(-)-phosphoglyceric acid with alkaline phosphatase, was used as a reference. The chromatogram was dried at 100° for 2 hours and sprayed with bromphenol blue. Glyceric acid was present in fractions 20 to 35. Fractions 19-37 were treated with Dowex-50-H^+ to remove NH_4^+, and concentrated under vacuum, treated again with Dowex-50-H^+ to remove a trace of NH_4^+, and further concentrated on a steam bath by blowing a stream of nitrogen onto the syrupy solution. The weight of syrup was 190 mg. This was dissolved in a small volume of water, titrated with sodium hydroxide, and adjusted to 0.3M.

L-(-)α-hydroxy-glutaric acid was prepared by deamination of L(+)-glutamic acid with nitrous acid (Winitz et al., 1956). A trace of nitrite

present after recrystallisation was removed by treatment with HCl and ethylamine. The acid was isolated as before, yield 3.8g (39%), $[\alpha]_D^{22°}$ = - 6.95°.

Barium α -hydroxy-n-butyrate was converted to the sodium salt by dissolving in very dilute acetic acid; the barium was removed as barium sulphate with a slight excess of sodium sulphate, and the supernatant after centrifuging was neutralised with sodium hydroxide, and adjusted to 0.36M.

The calcium salts of lactic acid were converted to the sodium salts by passage of the solution through a small column of Dowex-50-(H^+) and neutralisation.

ii. Enzyme. Since YLD was normally stored in a solution 0.5M in sodium lactate, the diluted samples used for these studies contained sufficient lactate to produce an appreciable rate of ferricyanide reduction without any added substrate. Dialysis against 0.5M sodium chloride overnight at 2° under anaerobic conditions decreased this blank reduction rate considerably.

The activity of the enzyme solution was determined from time to time with lactate as substrate during any series of experiments, and corrections were made where necessary. Very little loss of activity was observed.

2. Studies of Substrate and Inhibition Specificity of Yeast Lactate Dehydrogenase.

As discussed previously, there has been some controversy about

the substrate specificity of YLD, in particular concerning the ability of the enzyme to oxidise substrates other than α-hydroxy acids. Various possible substrates have been tested for the ability to reduce ferricyanide in the presence of crystalline YLD (Table 21). Several α-hydroxy acids are substrates for the enzyme; the maximum rate of ferricyanide reduction is obtained with L(+)-lactate, and decreases as the chain length increases in the homologous series of aliphatic α-hydroxy acids. It is to be noted, however, that the rate of oxidation of glycollate is very low. This was also observed by Appleby and Morton (1959a); a different sample of glycollic acid was used by these workers. The effect could possibly be due to the highly polar nature of glycollate, since Dikstein (1959) has obtained results which suggest that the transfer of substrate from the aqueous phase to the surface of YLD is favoured when the substrate is lipophilic (see p.166).

Since α-hydroxy-iso- butyrate has no α hydrogen atom it is not surprising that it is not oxidised byYLD, as Marcus and Vennesland (1956) found that the α hydrogen atom, rather than a β hydrogen atom, was removed from lactate in the oxidation to pyruvic acid by YLD.

The failure of DL-α-hydroxy-iso-valerate to act as a substrate for YLD is interesting, especially as DL-α-hydroxy-iso- caproate was oxidised by YLD at the same rate as DL-α-hydroxy-n-caproate. It was found, however, that the rate of lactate oxidation was markedly decreased in the presence of α-hydroxy-iso-valerate (Table 22). Therefore, the failure of the latter compound to act as a substrate for YLD is not due to its inability to combine with YLD. If models of lactate and α-hydroxy-iso-valerate are

Table 21.

Substrate specificity of YLD.

The rate of ferricyanide reduction in the presence of YLD and the listed compounds was determined spectrophotometrically at room temperature in 0.033 M. - sodium pyrophosphate/HCl buffer, pH 8.0, containing 10^{-5}M-EDTA. The rate of ferricyanide reduction by the enzyme sample alone (4% of rate with lactate) has been subtracted.

Compound	M	Relative rate of reduction of $K_3 Fe(CN)_6$
DL-lactate	0.1	100
DL-α-hydroxy-n-butyrate	0.12	30
DL-β-hydroxy-n-butyrate	0.1	0
DL-α-hydroxy-iso-butyrate	0.1	0
DL-α-hydroxy-iso-valerate	0.1	0
DL-α-hydroxy-n-caproate	0.1	18
DL-α-hydroxy-iso-caproate	0.1	17
glycollate	0.1	3
DL-glycerate	0.1	0
hydroxy-malonate	0.1	0
DL-malate	0.1	0
D(-)-malate	0.05	0
L(+)-tartrate	0.05	5
L(-)-α-hydroxy glutarate	0.05	0
DL-β-phenyl-lactate	0.1	5
DL-mandelate	0.1	0
TPNH	0.00054	0

compared, it is found that the methyl groups on the β carbon atom of iso valerate come very close to the hydrogen atom on the α carbon atom and that rotation about the α carbon is restricted. It is possible that this prevents a satisfactorily close approach of the enzyme to the α carbon atom. Rotation about the α carbon atom is also restricted for mandelic acid, which is not oxidised by YLD (Dikstein, 1959).

Neither TPNH nor malate were substrates for crystalline YLD. The TPNH - and malate - cytochrome c and - "cytochrome b_2" reductase activities observed by other workers (Yamashita et al., 1958; Yamanaka et al., 1958), for impure YLD may probably be attributed to the presence of contaminating enzymes.

The effect of these various compounds on lactate-ferricyanide reductase activity was measured (Table 22). With the exception of TPNH, the rate of ferricyanide reduction was depressed by the addition of these compounds. The inhibition caused by α-hydroxy-iso-valerate was large, and since this substance is not oxidised by YLD, this suggests that the enzyme can form a stable complex with α-hydroxy-iso-valerate, and that the affinity of YLD for this acid is higher than for lactate.

The inhibition found on addition of α-hydroxy-n and -iso-caproate is less than that obtained with α-hydroxy-iso-valerate but is greater than that obtained by the addition of α-hydroxy-n-butyrate. Dikstein (1959) found that the inhibition of YLD lactate-ferricyanide reductase activity by aliphatic acids increased as the number of methylene carbons increased, so that the series glycollate, α-hydroxy-iso-butyrate, α-hydroxy-n-butyrate,

Table 22.

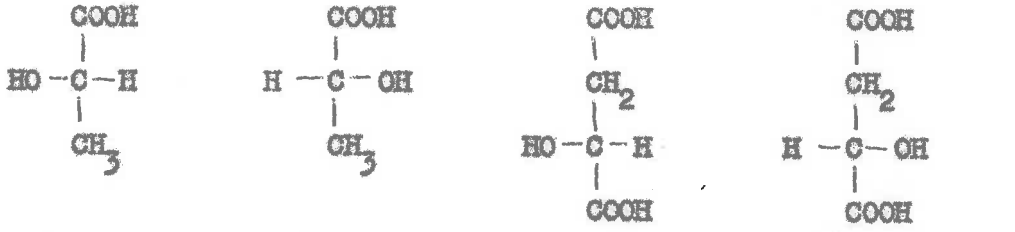
Inhibition of lactate-ferricyanide reductase activity by analogues.

Conditions as in Table 21; the rate of ferricyanide reduction was determined for the mixture of the specified compound and 0.1M DL-lactate.

Compound	M	Inhibition Relative decrease of rate (%)
DL-α-hydroxy-n-butyrate	0.12	45
DL-β-hydroxy-butyrate	0.1	15
DL-α-hydroxy-iso-butyrate	0.1	32
DL-α-hydroxy-iso-valerate	0.1	76
DL-α-hydroxy-n-capruate	0.1	65
DL-α-hydroxy-iso-caproate	0.1	51
glycollate	0.1	21
DL-glycerate	0.1	40
hydroxy-malonate	0.1	8
DL-malate	0.1	95
L(+)-malate	0.01	86
D(-)-malate	0.05	53
L(+)-tartrate	0.05	2
L(+)-α-hydroxy-glutarate	0.05	7
DL-β-phenyl-lactate	0.1	74
DL-mandelate	0.1	72
TPNH	0.00054	0

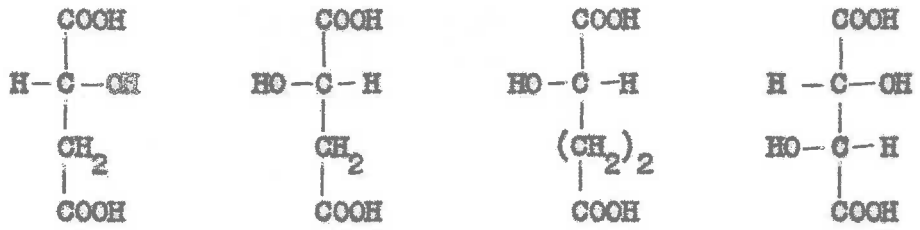
α-hydroxy-iso-caproate, α-hydroxy-n-caproate is consistent with this finding, while α-hydroxy-iso-valerate is anomalous. The small inhibition obtained with β-hydroxy-n-butyrate suggests that the α configuration causes a specifically higher inhibition. The effect of chain length on inhibition was attributed by Dikstein to the favourable free energy change associated with transfer of methylene groups from the aqueous phase to the protein surface. The strong inhibition found with mandelate and β-phenyl lactate is probably related to a similar phenomenon.

The inhibitions obtained in the presence of dicarboxylic acids are difficult to explain. Thus, while hydroxy-malonate, L(+)-tartrate and L(-)-α-hydroxy-glutarate inhibit to only a small extent, malate produces a very large inhibition. In order that this may be discussed, it is necessary to comment on the absolute configuration about the α carbon atom in these compounds. The Fischer projections for these compounds are



L(+)-lactic acid | D(-)-lactic acid | L(+)-malic acid (natural) | D(-)-malic acid (unnatural)

D(+)-α-hydroxy-glutaric acid has the same configuration as L(+)-lactic acid. If however malic acid and α-hydroxy-glutaric acid are written so that they are α-hydroxy acids (rather than β-and γ-hydroxy acids, as above) then the situation is as follows:



L(+)-malic acid D(-)-malic acid L(-)-hydroxy glutaric acid L(+)-tartaric acid

($\bar{=}$ D(-)-lactate) ($\bar{=}$ L(+)lactate) ($\equiv$ L(+)lactate) ($\equiv$ D(-)lactate)

It becomes quite clear that L(+)-malic acid is quite anomalous and that both isomers of malate occupy a special position in relation to their ability to inhibit the reduction of ferricyanide by lactate in the presence YLD. This is unlikely to be due to the presence of two carboxyl groups separated by two carbon atoms, since L(+)-tartrate has very little effect. Similarly, the possibility that the inhibition is due to a β-hydroxy-configuration seems unlikely because of the small inhibition obtained with tartrate and with β-hydroxy-butyrate. Since L(+)-malate and L(+)-tartrate differ markedly in their ability to cause inhibition, it is unlikely that the inhibition observed with L(+)-malate is due to its D(-) configuration as an α-hydroxy acid.

3. Stereospecificity of yeast lactose dehydrogenase.

Huennekins and Mahler (1953) and Boeri et al. (1955) reported that YLD was specific for L(+)-lactate, and Boeri et al. were unable to find any inhibition in the rate of lactate oxidation when using racemic lactate. Dikstein (1959) also observed that the enzyme was specific for L(+)-lactate, but found that D(-)-lactate was a competitive inhibitor of L(+)-lactate oxidation. This he attributed, at least in part, to the fact that lactate

oxidation by YLD is inhibited by a number of carboxylic acids.

The stereospecificity of crystalline YLD towards lactate was investigated. In order that there should be very little lactate present in the sample of YLD used, the crystal pellet obtained after recrystallisation of YLD in 0.05M-lactate was washed with pyrophosphate buffer, pH 8.0 (0.004M) and the washed crystals dissolved in the same buffer (0.10M). The blank rate for ferricyanide reduction was extremely small. Table 23 shows that crystalline YLD is specific for L(+)-lactate, and that the rate of ferricyanide reduction is lower with racemic lactate, either natural, or prepared by mixing equimolar amounts of D(-)-and L(+)-lactate. Both natural and artificial racemic lactate give identical rates of ferricyanide reduction.

From results given elsewhere in this thesis it is possible to calculate the constant, K_i, for the competitive inhibition of L(+)-lactate oxidation by D(-)-lactate. The apparent Michaelis constant for DL-lactate with 0.72mM-ferricyanide is known. The steady state rate equation may be written

$$v_i = \frac{V'}{1 + \frac{K'_s}{[S]} + \frac{K'_s}{K_i} \cdot \frac{[I]}{[S]}} = \frac{V'}{1 + \frac{K'_s}{[S]} + \frac{K'_s}{K_i}} \quad \text{(Since (S) = (I))}$$

The equivalent rate equation when L(+)-lactate is used as the substrate is

$$v = \frac{V'}{1 + \frac{K'_s}{[S]}}$$

The intercept for the plot of $1/v_i$ vs. $1/[S]$ (as L(+)-lactate in the racemic mixture) for $1/v_i = 0$ is $-1/[S] = 1/K^*_s$

Table 23

Stereospecificity of YLD.

The rate of ferricyanide reduction in 0.033M-sodium pyrophosphate /HCl, pH 8.0, 10^{-4}M-EDTA, 0.75 mM-ferricyanide at room temperature (24°) was followed spectrophotometrically at 420 mμ in 1 cm. cells for 2.0 minutes.

Substrate	Lactate (mM)	Initial Velocity* ($\Delta E_{420m\mu}$/min)	(μ moles/min).
None	-	0.008	0.023
L(+)-lactate	2.5	0.434 ± 0.004	1.252 ± 0.012
D(-)-lactate	2.5	0.005 ± 0.001	0.014 ± 0.003
DL-lactate (Natural racemic)	5.0	0.374 ± 0.003	1.080 ± 0.008
DL-lactate (Artificial racemic)	5.0	0.371 ± 0.003	1.072 ± 0.009

* Rate of ferricyanide reduction virtually linear under these conditions from 0-45 secs.

where $K_s^* = \frac{K'_s}{1 + \frac{K'_s}{K_i}}$, whence $K'_s = \frac{K_s^*}{1 - \frac{K_s^*}{K_i}}$.

K_s^* is the value of the apparent Michaelis constant for lactate in the racemic mixture.

It may be shown that

$$K'_s = \frac{(1 - {}^{v_i}/_{v})(S)}{{}^{v_i}/_{v}(1 + (S)/K_i) - 1}$$

and therefore

$$K_i = \frac{K_s^* \cdot (S)}{\frac{(1-v_i)}{v}(K_s^* + (S))}$$

While K_i is independent of the ferricyanide concentration, K'_s is the value of the Michaelis constant for a specified ferricyanide concentration.

The value of the K_i obtained from the results presented in Table 24 is smaller than that calculated from Dikstein's (1959) data. The value of K'_s (3.5×10^{-4}M L(+)-lactate) used by Dikstein was obtained by Boeri *et al.* (1955) for cytochrome *c* reduction in phosphate buffer at pH 7.4, and is not applicable when ferricyanide is used as acceptor. It is unlikely that the discrepancy is due to experimental error alone, so that it is possible that the constants K'_s and K_i are affected by the composition of the reaction mixture. In the present work, pyrophosphate buffer, pH 8.0, was used, whereas Dikstein carried out his experiments at pH 7.5 in borate buffer.

Since the L(+)-lactate-ferricyanide reductase activity of YLD is inhibited by D(-)-lactate at lower concentrations (one sixth) than required for propionate (Dikstein, 1959), the α-hydroxyl group must be involved in the binding of D(-)-lactate at the active centre of YLD.

Table 24.

Inhibition Constant for D(-) lactate with YLD.

Calculated from data in Table 23; for comparison, the data presented by Dikstein are calculated in a similar way.

K_s^* (racemic lactate) (L(+)-lactate, M)	K_s' (L(+) lactate, M)	K_i (D(-) lactate, M)
6.60×10^{-4} **	$8.2 + 0.2 \times 10^{-4}$ *	$3.8 \pm 0.4 \times 10^{-3}$ *
—	—	—
6.45×10^{-4} +	6.9×10^{-4} +	9.4×10^{-3} ‡
—	3.5×10^{-4} ≠	5.0×10^{-3} ‡

** Calculated from this thesis, table 16 (p.153a) and table 23 (p. 168a) for 0.72 mM ferricyanide, pyrophosphate buffer pH 8.0.

\+ From this thesis, table 16 (p153a) by interpolation for 0.92 mM ferricyanide.

‡ From Dikstein (1959), I_{50} D(-) lactate = 0.15M in 10.2 mM L(+) lactate, 0.92 mM ferricyanide, borate buffer pH 7.5.

≠ Used by Dikstein (1959), obtained by Boeri et al. (1955) for L(+) lactate-cytochrome c reductase in phosphate buffer, pH 7.4.

$$K_i = \frac{K_s^* ((I)_{50} + (S))}{K_s^* + (S)}$$

4. Inhibition of yeast lactate dehydrogenase by sulphydryl reagents.

Appleby and Morton (1954, 1959b), Boeri et al. (1955) and Nygaard (1961) found that YLD was inhibited by sulphydryl reagents, particularly by PCMB. As discussed elsewhere (Appleby and Morton, 1959b; Armstrong, Coates and Morton, 1960; this thesis, p. 201) it is believed that these reagents inhibit YLD by displacement of FMN from a sulphydryl group to which it is bound; a strong yellow fluorescence slowly appears on the addition of such reagents to solutions of YLD. Table 25 shows the results of some experiments on the inhibition of YLD by N-ethyl-maleimide (NEM) PCMS and iodoacetamide (IAM). Both NEM and PCMS inhibit the enzymic activity of YLD, the latter much more rapidly. Appleby and Morton (1959b) obtained immediate 90% inhibition of YLD activity immediately after addition of YLD to the standard ferricyanide assay system containing 0.01 M PCMB. Since the percentage inhibition by PCMS obtained in the present study did not alter with time, it seems probable that insufficient PCMS was present to give complete inhibition, although it was present in four times the concentration of YLD haem. This may be due to the binding of PCMS by other sulphydryl groups present in YLD, and possibly to residual EDTA. If it is assumed that all the PCMS added is bound by the enzyme, then the results suggest that there is more than one sulphydryl group present; with the additional assumption that the remaining uninhibited YLD has no bound PCMS, it may be calculated that YLD has at least 6 sulphydryl groups per haem. This compares favourably with the 4 sulphydryl groups/haem found by direct analysis.

Table 25

Inhibition of lactate-ferricyanide reductase activity of YLD by sulphydryl reagents.

YLD was incubated with the reagents at the concentrations specified in 0.033 M pyrophosphate pH 8.0, 50 μM sodium lactate and samples were assayed at the times indicated. NEM 1.8×10^{-4}M, YLD 1.5×10^{-5}M, EDTA 10^{-5}M; IAM 9.3×10^{-4}M, YLD 1.5×10^{-5}M, EDTA 10^{-5}M; PCMS 1×10^{-4}M, YLD 2.5×10^{-5}M, EDTA $< 10^{-6}$M.

NEM		IAM		PCMS	
Time (mins.)	Inhibition (%)	Time (mins.)	Inhibition (%)	Time (mins.)	Inhibition (%)
1.5	12.8	2.5	2	5.0	64.2
6.5	19.0	21.0	0	16.0	63.0
41.5	30.8	43	0	22	65.1

The failure of IAM to inhibit YLD at pH 8.0 is in agreement with the finding that mono-iodoacetate does not inhibit YLD at neutral pH (Boeri et al., 1955) and suggests that the sensitive sulphydryl group (s) of YLD is inaccessible to this reagent. This has been interpreted as the masking of a sulphydryl group by FMN (Appleby and Morton, 1959b).

V. PHYSICAL PROPERTIES OF YEAST LACTATE DEHYDROGENASE AND DERIVED COMPOUNDS.

A. Behaviour of Solutions in the Ultracentrifuge.

To facilitate interpretation of the experimental studies on the sedimentation behaviour of YLD, an outline of the principles underlying the use of the ultracentrifuge is given here. This review is largely based on the works of Svedberg and Pederson (1940) and of Schachman (1959).

The rate of sedimentation of solute molecules in a solution subjected to a centrifugal field may be expressed in terms of the sedimentation coefficient, s, which is defined as follows;

$$s = \frac{dx/dt}{\omega^2 x} = \frac{d \ln x/dt}{\omega^2} \qquad (1)$$

where $\frac{dx}{dt}$ is the velocity of the solute molecules at a distance x from the axis of rotation, and the angular velocity is ω. Thus, s represents the hypothetical rate of sedimentation under unit centrifugal field, and has the units cm/sec/dyne/g, (i.e. sec.). The unit employed is the svedberg (S) and one svedberg equals 10^{-13} sec. The sedimentation of a solute is observed by centrifuging a solution at constant angular velocity and temperature in a sector-shaped cell designed so as to permit optical observation of some function of the concentration distribution of the solute throughout the cell. Several methods of observation utilise the changes in refractive index at different points in the solution brought about by changes in concentration of the solute caused by the sedimentation process, since the refractive index of a solution is usually a linear function of

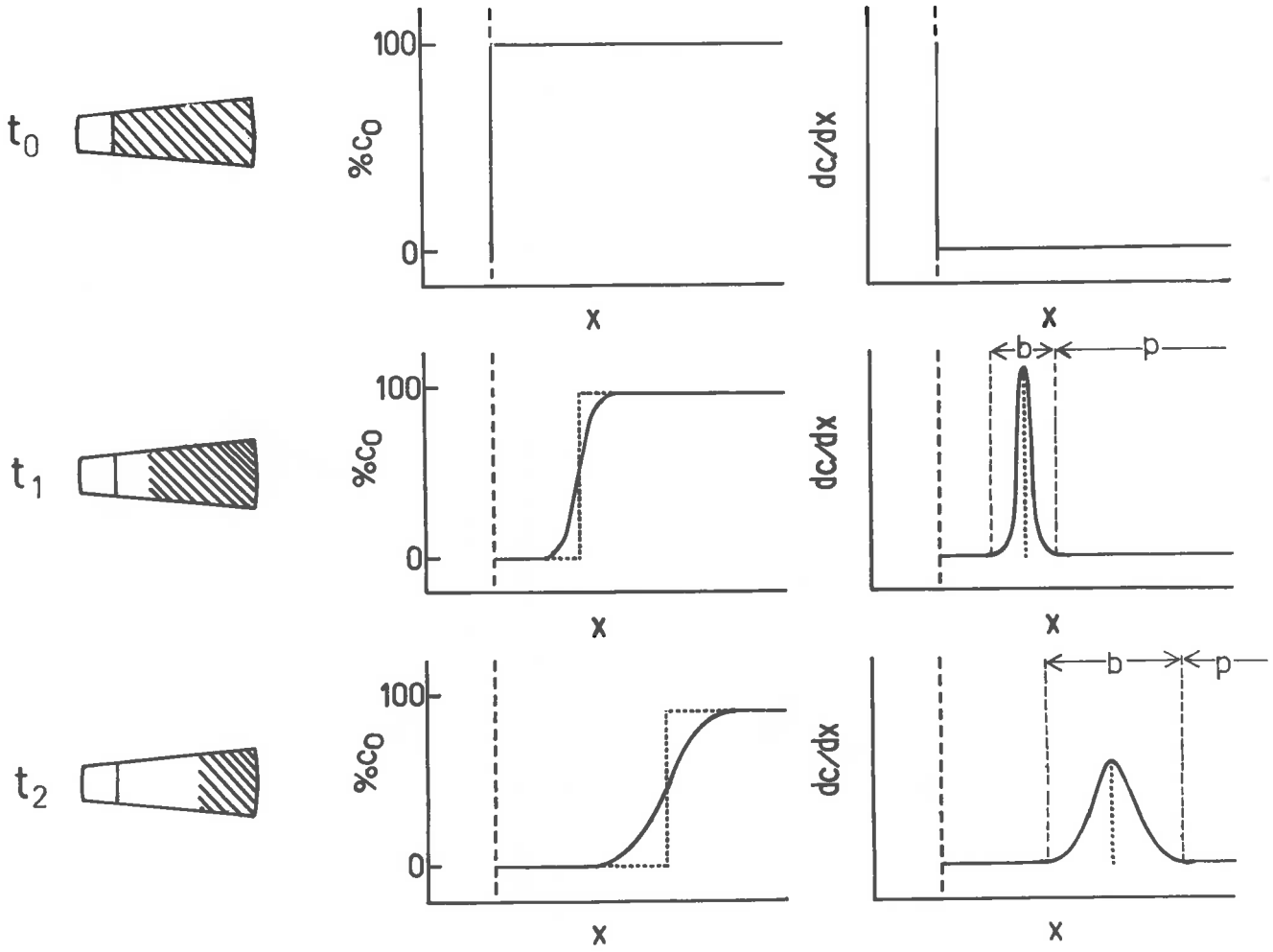
solute concentration for any particular solvent. The most commonly-used optical system for observing refractive index changes in the cell employs the schlieren principle; in which the displacement of a light ray by a refractive index gradient in the solution is converted into a horizontal displacement by an inclined edge (the "bar") and a cylindrical lens. This system is known as the Philpot-Svensonn astigmatic schlieren optical system, and records the refractive index gradient, $\frac{dn}{dx}$, as a function of radial distance in the cell. Another commonly-used method of observing the distribution of solute in the cell relies on the specific absorption by the solute of light of an appropriate wavelength. The concentration of the solute at any point in the cell is related to the light absorption of the solution at that point by the Beer-Lambert law. (Svedberg and Pedersen, 1940; Shooter and Butler, 1956; Schumaker and Schachman, 1957).

1. <u>The Moving Boundary Method for the Determination of Sedimentation Coefficients</u>.

If the rate of sedimentation is sufficiently large, the solute molecules will sediment towards the cell bottom (the greatest radial limit of the cell) and leave behind a layer of pure solvent. This creates a boundary between solution and solvent (see Fig. 24). If diffusion of the solute back into the solvent layer were negligible, the boundary would be infinitely sharp and would therefore move at the same velocity as the solute molecules ahead of the boundary (i.e. in the plateau region). Therefore the sedimentation coefficient could be measured in terms of the rate of movement of the boundary.

Fig. 24. Boundary formation in the ultracentrifuge

See text for explanation. At times t_o (=0) $< t_1 < t_2$. The boundary region is indicated by b, and the plateau region by p.


t_0
t_1
t_2
100
0
%c_0
x
dc/dx
b
p

Since, however, solute is being transported by diffusion in the opposite sense to the transport of solute by sedimentation, the boundary becomes progressively more diffuse as the experiment progresses. The problem is therefore to determine in what way the movement of the boundary is related to the movement of the solute molecules in the plateau region, and whether there is any concentration level in the boundary which moves at the same velocity as the bulk of the sedimenting solute molecules (i.e. in the plateau region). Goldberg (1953) has shown that the square root of the second moment of the gradient curve ($\sqrt{\mu_2}$) about the centre of rotation moves at the velocity of the solute molecules in the plateau region. The evaluation of μ_2 is rather laborious, and for symmetrical boundaries the position of the maximum ordinate (x_H) of the gradient curve is sufficiently close to $\sqrt{\mu_2}$ for the calculation of the sedimentation coefficient in most cases. The slope of the plot of $\ln x_H$ (or $\ln \sqrt{\mu_2}$) against time, $d \ln x/dt$ may be substituted into equation (1) to obtain the sedimentation coefficient of the solute.

2. Transport Methods for Determination of Sedimentation Coefficients.

Where application of the moving boundary method for the determination of S is precluded for any reason, it is possible to measure S in terms of the transport of solute across an arbitrarily chosen cylindrical surface within the plateau region of the cell. While it is possible to employ a cell which separates the solution into an upper and lower portion (e.g. Yphantis & Waugh, 1956) and determine, by any appropriate method, the concentration changes brought about by sedimentation, the necessary

equations for application to optical systems have been developed. The equations arise from a consideration of the conservation of mass for the system as a whole. The basic equation for use with optical systems is

$$S = -\frac{1}{2\omega^2 t} \ln \left\{ \frac{2\int_m^p cx\,dx}{x_p^2\, Co} + \frac{x_m^2}{x_p^2} \right\}$$

Where this equation is to be applied to refractive index gradient curves, the integral is best evaluted by integration by parts and by use of the equations relating the plateau concentration at any time to the initial concentration. This leads to (Baldwin, 1953)

$$s = -\frac{1}{2\omega^2 t} \ln \left\{ 1 + \frac{x_m^2 \int_m^p \frac{\partial c}{\partial x}\,dx - \int_m^p x^2 \frac{\partial c}{\partial x}\,dx}{x_m^2\, Co} \right\}$$

i.e. $$s = \frac{1}{2\omega^2} \frac{-d \ln f(c)}{dt}$$

where f (c) is the term in square brackets.

3. Correction of the Sedimentation Coefficient to Standard Conditions.

Since the rate of sedimentation is affected by the viscosity of the solution, sedimentation coefficients are usually corrected for viscosity to standard conditions and for temperature, by the following relationship. (Svedberg & Pedersen, 1940).

$$s_{20°,w} = s_{obs.} \cdot \frac{\eta}{\eta_{20°,w}} \cdot \frac{(1 - \bar{v}\rho_{20°,w})}{1 - \bar{v}\rho} \qquad (2)$$

where $s_{20°,w}$ is the hypothetical sedimentation coefficient which would be obtained in a solution with the viscosity and density of water at 20°,

$s_{obs.}$ is the observed sedimentation coefficient in a solution of viscosity η and density ρ at the particular temperature of the experiment, and $\eta_{20,w}$ and $\rho_{20,w}$ are the viscosity and density, respectively, of water at 20°, for a solute of partial specific volume $\bar{V}$. The second term is usually small in relation to the viscosity correction, and is often neglected in the calculation of $s_{20,w}$.

4. The Svedberg Equation.

The sedimentation coefficient of a solute is related to the molecular weight (M) of the solute by the following relationship, which is known as the Svedberg equation.

$$M = \frac{RT}{(1-\bar{V}\rho)} \cdot \frac{S}{D} \left[1 + \frac{d\ln Y}{d\ln c}\right] \qquad (3)$$

where R is the gas constant, T is the absolute temperature, D the diffusion coefficient of the solute, and Y and c are the activity coefficient and concentration, respectively, of the solute. For ideal solutes, the "thermodynamic" term (in square brackets) equals unity. It is generally accepted that the thermodynamic term becomes unity for non-ideal solutions at infinite dilution of the solute.

Therefore, the values of S and D used for the determination of molecular weight using the Svedberg equation, are obtained by extrapolation of plots of S and D vs. concentration to zero concentration.

The Svedberg equation has been derived in a number of ways, by a kinetic approach (Svedberg, 1925), from thermodynamic considerations (Svedberg and Pedersen, 1940; Lamm, 1953; Goldberg, 1953; Williams, 1954).

and by application of the theory of irreversible thermodynamics (Hooyman, 1956).

5. Concentration Dependence of Sedimentation.

The observed value of s for most solutes depends on the concentration of the solute. Attempts to explain this concentration dependence have been made, and all the factors suggested decrease s as the concentration increases. Such negative concentration dependence of s is characteristic of most proteins. The concentration dependence of s can often be described by equations of the form

$$s = s_o (1 - kc) \quad \text{or} \quad s = \frac{s_o}{1 + kc} \qquad (4)$$

where s_o is the sedimentation coefficient at zero concentration of solute, k is a constant and c the concentration of the solute. Negative concentration dependence usually results in a sharpening of the boundary. This can be explained in the following way. Since the concentration of solute is lower at the trailing edge of the boundary, the trailing edge of the boundary will sediment faster than the leading edge of the boundary. Therefore the trailing edge will tend to catch up with the leading edge of the boundary and will thus tend to offset the effect of diffusion in spreading the boundary. In fact, there will be a continuous distribution of rates of sedimentation throughout the boundary region, decreasing with the continuous increase in concentration of solute across the boundary from the region of pure solvent to the plateau region. For this reason,

determination of apparent diffusion coefficients from the spreading of a sedimenting boundary results in errors in the value of $D^{apparent}$, and can lead to deviations from the Boltzmann assumption that the spreading of the boundary is proportional to $\sqrt{t}$, unless correction is made for the concentration dependence of sedimentation (Gosting, 1956; Fujita, 1956; Baldwin, 1957a).

In spite of the hydrodynamic effects which tend to decrease s as solute concentration increases, for some protein systems in dilute solutions, s increases as the protein concentration increases. This has been attributed to reversible association-dissociation reactions of the protein, i.e.

$$nA \rightleftharpoons A_n$$

At low concentrations, the non-associated form, with low sedimentation coefficient predominates, while high concentrations favour the associated form, because of the change in the number of molecules brought about by the reaction.

If the rate of adjustment of the equilibrium is rapid as compared with the rate of sedimentation, then separation of the associated and dissociated forms to give two boundaries will not necessarily occur, and in this case a single sedimenting boundary will be observed. The rate of movement of $\sqrt{\mu_2}$ for such a boundary will give the weight average sedimentation coefficient of the molecular species in the plateau region. Gilbert (1955, 1956) has derived a theory for such reversibly aggregating systems by analogy with chromatographic separation. This theory predicts that when aggregation proceeds beyond dimerisation, (i.e. $n > 2$), two

boundaries will be observed, regardless of the rate at which the equilibrium is adjusted. If the aggregation is a monomer-dimer equilibrium then only a single boundary will be observed. Rao & Kegeles (1958) have shown that Gilbert's theory is not directly applicable to the aggregation observed with α-chymotrypsin, but by an extension of Gilbert's theory found that a single boundary would be observed for the case where monomer, dimer and trimer are in equilibrium

$$2A \rightleftharpoons (A)_2 \qquad (A)_2 + A \rightleftharpoons (A)_3$$

The observed data for α-chymotrypsin fitted the predicted behaviour for this latter system well.

Reversible association-dissociation has been observed for haemoglobin (Field and O'Brien, 1955) and insulin (Oncley, Ellenbogen, Gitlin and Gurd, 1952) in mildly acidic solutions and for α-chymotrypsin (Schwert, 1949; Kegeles and Rao, 1958), chymotrypsinogen (Massey, harrington and Hartley, 1955) and trypsin (Cunningham, Tietze, Green and Neurath, 1953).

6. <u>The Ultracentrifuge Differential Equation for a Sector-shaped Cell</u>

This equation was derived by Lamm (1929) and describes the change in concentration of solute with time at any radial position in the cell during centrifugation. It is derived by consideration of the nett transport of solute, by diffusion and by sedimentation, into a volume element bounded by two cylindrical surfaces in the cell, at distances x and x + dx from the centre of rotation.

The general equation has the form

$$\frac{\partial c}{\partial t} = \frac{1}{x}\frac{\partial c}{\partial x}\left[\left(D\frac{\partial c}{\partial x} - \omega^2 s x c\right)x\right] \qquad (5)$$

If it is assumed that s and D are not concentration dependent, equation 5 may be written

$$\frac{\partial c}{\partial t} = D\left[\frac{\partial^2 c}{\partial x^2} + \frac{1}{x}\cdot\frac{\partial c}{\partial x}\right] - \omega^2 s\left[\frac{x\partial c}{\partial x} + 2c\right] \qquad (5a)$$

For the plateau region, since there is no concentration gradient, equation (5a) leads to equation (6), which expresses the concentration change due to centrifugal and sectorial dilution in terms of the initial concentration (C_o) and the concentration (C_t) in the plateau region at time t.

$$\frac{dc}{dt} = -2\omega^2 sc \qquad (6)$$

or

$$c_t = c_o e^{-2\omega^2 st} \qquad (6a)$$

This concentration change may be also described in the following way, where c_o is the concentration at time o, c_t is the concentration at time t and x_m is the position of the meniscus.

$$\frac{c_t}{c_o} = \left(\frac{x_m}{(\sqrt{\mu_2})_t}\right)^2 \qquad (7)$$

This arises from equations (1) and (6) by integration and is valid even if s changes markedly during the experiment because of concentration dependence of sedimentation.

It is desirable to have equation (7) in a form suitable for use with concentration gradient curves. By substituting for the second moment

$$\left\{\mu_2 = \frac{\int_{x_m}^{x_p} x^2 \frac{\partial c}{\partial x}\, dx}{\int_{x_m}^{x_p} \frac{\partial c}{\partial x}\, dx}\right\}$$

and for c_t ($= \int_{x_m}^{x_p} \frac{\partial c}{\partial x}\, dx$) in equation (7)

$$c_o = \frac{1}{x_m^2} \int_{x_m}^{x_p} x^2 \frac{\partial c}{\partial x}\, dx \qquad (8).$$

7. Sedimentation Equilibrium and "Approach to Equilibrium".

If a solution is centrifuged for a sufficiently long time, the solute distribution in the cell reaches an equilibrium condition, where the net transport of solute in every region in the cell is zero, that is, the sedimentation of solute is balanced by the diffusion of solute in the opposite direction. The equilibrium distribution of solute is characteristic of the molecular weight of the solute, and is also governed by the temperature, the partial specific volume of the solute, the density of the solution, the length of the solution column and the magnitude of the centrifugal field. Unfortunately, the determination of molecular weight by this method requires long times of centrifuging, even for short columns of solution, and there is always some uncertainty as to whether equilibrium has been reached.

Archibald (1947) pointed out that there were two positions in the cell which at all times fulfilled the equilibrium condition of no solute flow, these positions being the solution meniscus and the cell bottom.

From the same considerations used in the derivation of the ultracentrifuge differential equation, he derived the following expressions, relating the concentration of the solute at the meniscus (c_m) and cell bottom (c_b) to the concentration gradient of the solute at these positions in the cell at any particular time during an experiment.

$$c_m \omega^2 s x_m = D \left(\frac{dc}{dx} \right)_m \qquad (9a)$$

$$c_b \omega^2 s x_b = D \left(\frac{dc}{dx} \right)_b \qquad (9b) .$$

For a homogeneous solute, rearrangement of these equations leads to

$$\frac{s}{D} = \frac{\left(\frac{dc}{dx} \right)_m}{\omega^2 x_m c_m} = \frac{\left(\frac{dc}{dx} \right)_b}{\omega^2 x_b c_b} \qquad (9c).$$

Substituting equation (9c) into the Svedberg equation for an ideal solute,

$$M = \frac{RT}{\omega^2 (1 - \bar{v}\rho)} \cdot \frac{\left(\frac{dc}{dx} \right)_m}{x_m c_m} \qquad (10a)$$

$$= \frac{RT}{\omega^2 (1 - \bar{v}\rho)} \cdot \frac{\left(\frac{dc}{dx} \right)_b}{x_b c_b} \qquad (10b)$$

The terms $\frac{\left(\frac{dc}{dx} \right)_m}{x_m c_m}$ and $\frac{\left(\frac{dc}{dx} \right)_b}{x_b c_b}$ are equal and do not vary with time for a homogeneous solute at a particular angular velocity and temperature, and at equilibrium, for any cylindrical surface at a distance r from the axis of rotation, $\frac{\left(\frac{dc}{dx} \right)_r}{x_r c_r}$ will be constant throughout the cell.

If the solute is heterogeneous, then $M^{app.}_{meniscus}$ will decrease and $M^{app.}_{bottom}$ will increase with time, since the apparent molecular weights at

these positions for any particular time give the apparent weight average molecular weight for particular proportions of the molecular species present at that time. The relative proportions of the various molecular species at the menisus and cell bottom will alter with time, because of the partial separation affected by sedimentation, and will vary with position in the cell. Since the solution is uniform in composition at zero time, the extrapolated zero-time molecular weight will be equal at the meniscus and cell bottom and will give the weight average molecular weight of the solution. These equations can therefore be applied for the detection of heterogeneity in molecular weight.

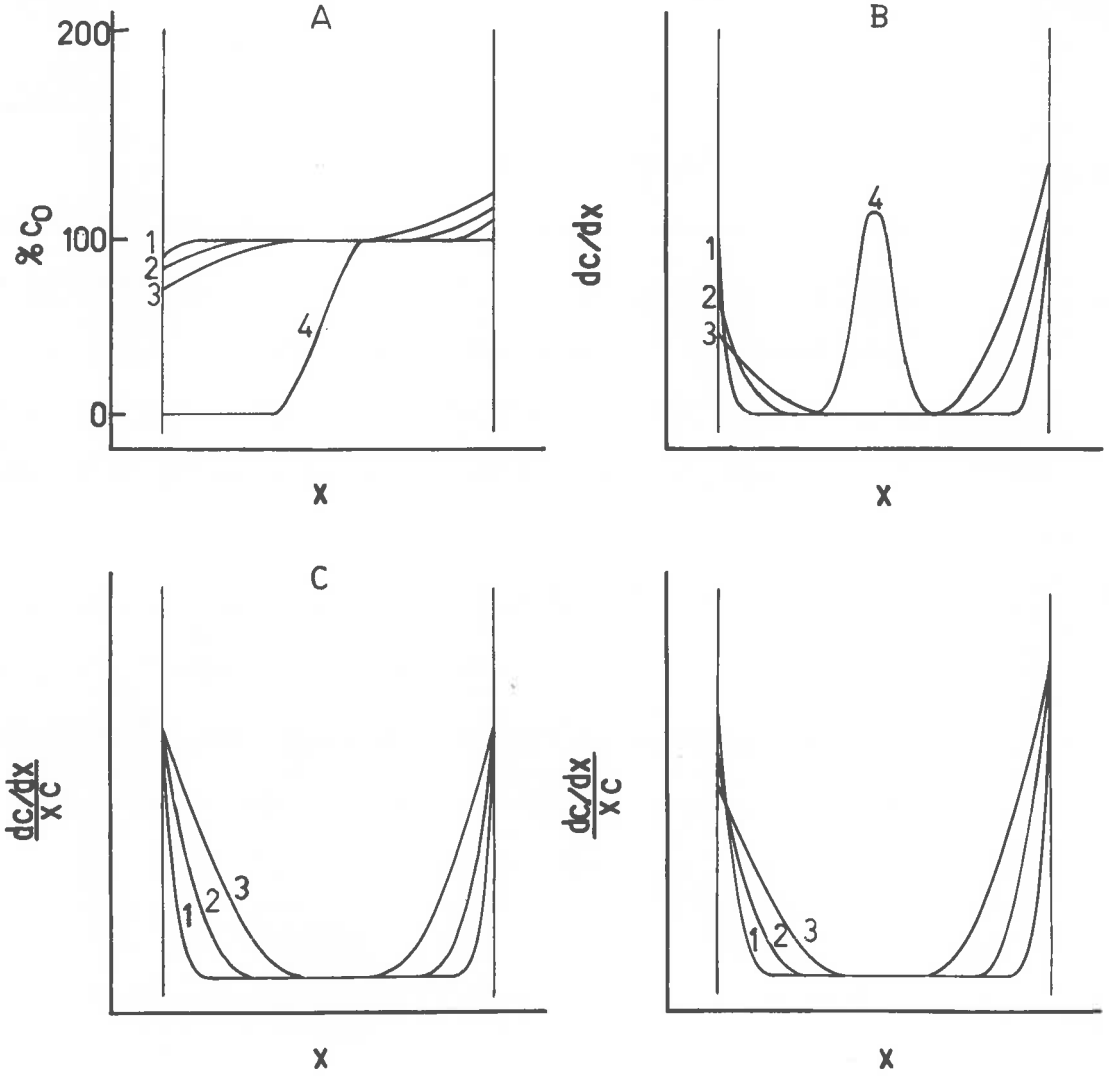
Experimental procedures for the determination of molecular weight based on the foregoing principles are known as Archibald or "approach to equilibrium" methods, although the latter term is misleading, since the system need not be any where near its equilibrium state. Most of these procedures are designed for use with schlieren optical systems, and the general procedure for such experiments is now described.

The solution is centrifuged at some selected angular velocity and a series of photographs is taken at various time intervals. The change in concentration and in concentration gradient for a typical experiment are shown in Fig. 25. The angular velocity is selected rather empirically. It depends largely on the duration of the experiment which can be permitted, and on the particular method of evalution of the data which is to be used. It is desirable to use the lowest possible angular velocity, since the concentration gradients obtained become very large at high angular velocities.

Fig. 25. Concentration distribution functions of solute in approach to equilibrium.

A and B show respectively the concentration and concentration gradient of solute in the cell from the meniscus (left) to the cell bottom, at increasingly greater times 1, 2 and 3.
Curve 4 shows how the initial concentration, C_0, may be determined in the same arbitrary units, by use of a synthetic boundary cell.

C and D show the function $\frac{dc}{dx} / ox$, which is proportional to S/D, for a homogeneous (C) and a heterogeneous (D) solute, at increasingly greater times 1, 2 and 3. The intercepts at the meniscus and cell bottom are identical and independent of time for C, but differ and depend on time for D.


A
200
100
0
% c_0
1
2
3
4
x
B
dc/dx
1
2
3
4
x
C
$\frac{dc/dx}{xc}$
1
2
3
x
$\frac{dc/dx}{xc}$
1
2
3
x

It is then necessary to obtain values of $\left(\frac{dc}{dx}\right)_m$ and c_m (and the corresponding values for the cell bottom if required) from the gradient curves. The values of c_m and c_b are most easily obtained while a plateau region exists in the cell (Klainer and Kegeles, 1955). The concentration in the plateau region is (equation 6a)

$$c_p = c_o e^{-2\omega^2 st}$$

Since the area under the gradient curve between the meniscus and plateau is equal to the difference in concentration at the meniscus and the plateau region

$$\text{i.e.} \quad c_m = c_p - \int_{x_m}^{x_p} \frac{\partial c}{\partial x}\, dx \qquad (11),$$

combination of these two expressions, using the continuity equation leads to

$$c_m = c_o - \frac{1}{x_m^2} \int_{x_m}^{x_p} x^2 \frac{\partial c}{\partial x}\, dx \qquad (12).$$

The integral in equation (12) is calculated by trapezoidal integration. The corresponding equation for the bottom of the cell is

$$c_b = c_o + \frac{1}{x_b^2} \int_{x_p}^{x_b} x^2 \frac{\partial c}{\partial x}\, dx. \qquad (12a)$$

It is therefore necessary to have a value for c_o in terms of the same units of concentration. Although this can be obtained from the optical constants of the ultracentrifuge (i.e. optical lever arm, cell thickness, cylindrical lens magnification and inclination of the schlieren diaphragm), the specific

refractive index increment of the solute and the solute concentration, evaluation of the individual terms in the integrals is then very laborious, and it is now common practice to use the ultracentrifuge as a refractometer. Provided the same cell thickness and "bar" angle is used, direct comparison is then possible. The following method is used. The original solution is loaded into a special cell, known as a synthetic boundary cell, which has a reservoir for solvent. The solvent will not drain from the reservoir until a certain centrifugal force is reached. The solvent in the reservoir then layers over the solution, convective disturbances being minimised by the density difference between solvent and solution. In this way a boundary is created. The boundary spreads by diffusion, and after it is resolved, photographs of the schlieren pattern are taken. If the photographs are taken as soon as possible, and the angular velocity is small, sectorial dilution can be neglected, and the concentration, c_o, is evaluated by the following integral

$$c_o = \int_{x_m}^{x_p} \frac{\partial c}{\partial x}\, dx. \qquad (2b)$$

The evaluation of $\left(\frac{dc}{dx}\right)_m$ and $\left(\frac{dc}{dx}\right)_b$ is somewhat subjective. Even if the optical system has been critically adjusted, interference between the gradient curve for the solute and the infinite gradient at the air-solution meniscus prevents precise evaluation of $\left(\frac{dc}{dx}\right)_m$. Extrapolation procedures are therefore used. Several methods are available; values of $\frac{dc}{dx}$ near the meniscus may be extrapolated to the meniscus or alternatively $\frac{1}{x_r c_r}$. $\left(\frac{dc}{dx}\right)_r$ vs. r may be extrapolated to the meniscus (see Fig. 25). For a

homogeneous solute, this latter procedure has the advantage that $\frac{1}{x_m c_m} \cdot \left(\frac{dc}{x}\right)_m$ is time invariant, so that for a homogeneous solute, $\frac{1}{x_r c_r} \cdot \left(\frac{dc}{dx}\right)_r$ should extrapolate to the same value for successive photographs. Ehrenberg (1957) recommends the use of a high angular velocity so that almost complete boundary is formed. If this is carried out so that the gradient curve is almost horizontal in the region of the meniscus, then $\left(\frac{dc}{dx}\right)_m$ can be evaluated with considerable precision. However, since this makes c_m rather small, some loss of precision occurs. In order to reduce the labour involved in the measurement of c_o and c_m, Ehrenberg (1957) developed a procedure whereby sectorial dilution corrections are made automatically. In this procedure, the synthetic boundary experiment is carried out under identical conditions to the "approach to equilibrium" experiment, and photographs of the schlieren pattern are taken at identical times. Thus the plateau region in the synthetic boundary experiment undergoes the same dilution as in the equilibrium experiment. The area under the gradient curves is measured by mechanical integration, using a planimeter and enlarged tracings of the gradient curves.

8. Detection of Heterogeneity in Sedimentation Velocity Experiments.

Although the appearance of two or more discrete sedimenting boundaries is evidence of the heterogeneity of the solute with respect to sedimentation coefficient, a single sharp symmetrical sedimenting boundary does not necessarily indicate homogeneity of the solute. For a homogeneous solute, the sedimentation velocity will be expressed in terms of a single value of s, while for a heterogeneous solute there will be either a range

of values of s, or two or more well-separated values of s corresponding to the various sedimenting species present in the solute. There are several factors which will affect the shape of a boundary in a sedimentation velocity experiment. Of these, the most important are (a) spreading of the boundary due to diffusion, (b) spreading of the boundary due to heterogeneity in s, and (c) sharpening of the boundary due to concentration dependence of sedimentation. It is therefore necessary to analyse the shape of the sedimenting boundary and to eliminate the effects of diffusion and concentration dependence of s before information can be obtained on the homogeneity or heterogeneity of the solute. Such methods measure the distribution of sedimentation coefficients, g(s), for the solute, and it is implicit in these methods that the distribution be continuous. At finite times, the apparent distribution $g^*(s)$ is affected by diffusion, and the distribution, free from diffusion effects, is obtained only at infinite time. Various extrapolations have been tested however, they are either empirical, or the theoretical justification for the approximations involved in making the extrapolation is not consistent with the practical limitations imposed by the resolving power of the ultracentrifuge. The importance of such methods is that they permit an estimate of the extent of heterogeneity of the solute with respect to s to be made.

It may be seen that spreading of the boundary due to heterogeneity in s is proportional to the first power of time, whereas boundary spreading due to diffusion is proportional to the square root of time. Thus heterogeneity in s will result in upward curvature of plots of boundary area/

boundary height vs. $\sqrt{t}$. In effect this method uses the ultracentrifuge differential equation (Lamm equation) to predict boundary shape for a homogeneous solute. Faxen's (1929) approximate solution for the continuity equation is valid only for systems where s and D are independent of solute concentration. Fujita (1956) obtained an approximate solution to the continuity equation for systems showing a linear dependence of s on c of the form

$$s = s_o (1 - kc),$$

by assuming that s and D were independent of pressure and that D was independent of concentration. This latter assumption is not serious since the variation of D with concentration across a boundary is likely to be much smaller than that found for s. For the solution to be valid it is necessary that the equivalent time of centrifugation, τ ($= 2\omega^2 st$) be small ($\ll 1$). By suitable manipulations, the basic equation is transformed to the following relationship, for use with refractive index gradient curves.

$$\frac{\left\{A\,F(\xi_m)\right\}^2}{H} = \left\{\frac{2D}{\omega^2 s_o}\,(e^{\tau}-1)\right\} \left\{1 + \sqrt{(1-\lambda)}\right\}^2$$

where $\xi_m = \left\{\frac{\omega^2 s_o x_m k c_o}{2D}\right\}^{\frac{1}{2}} \left\{\frac{\sqrt{\lambda}}{1 + \sqrt{(1-\lambda)}}\right\}$,

$$F(\xi_m) = \frac{\phi'(\xi_m)}{1 + \phi(\xi_m)} + 2\xi_m,$$

$\phi(\xi_m)$ is the equation of the Gaussian curve

$$\phi(\xi_m) = \frac{2}{\sqrt{\pi}} \int_0^{\xi_m} e^{-y^2}\, dy \quad ,$$

$\phi^1(\xi_m)$ is the derivative of the above equation.

$$\lambda = kc_o\,(1 - e^{-\tau}), \quad \tau = 2w^2 st$$

and A and H are the area under the gradient curve and the maximum ordinate of the boundary, respectively.

This method has been applied to the major sedimenting component in bovine serum albumin. This component was homogeneous in that boundary spreading could be accounted for entirely in terms of diffusion, and the diffusion coefficient calculated by this method agreed well with the diffusion coefficient as measured in a Gouy diffusiometer (Baldwin, 1957). Cod myosin was heterogeneous by this method as $D^{app.}$ increased markedly with time. An interesting result of this latter study was that the spreading of the boundary due to heterogeneity was balanced by the self sharpening of the boundary due to concentration dependence of sedimentation, which is large for myosin. Thus, where the calculation of $D^{app.}$ was made without correction for concentration dependence (i.e. using Faxen's solution to the continuity equation), $D^{app.}$ was independent of time, although lower than $(D^{app.})_{t=o.}$ for the calculation from Fujita's equation (Connell, 1958). Baldwin (1957b) states that this procedure is a more sensitive test for heterogeneity in s than any of the available g(s) methods. However, it cannot provide an estimate of the extent of heterogeneity,

In the light of the foregoing discussion, it is now possible to comment on whether or not a single symmetrical sedimenting boundary implies homogeneity.

For a homogeneous solute, the apparent diffusion coefficient measured from the spreading of a sedimenting boundary will be independent of time, and will agree well with the diffusion coefficient measured by static free diffusion, provided corrections are made for the effects of concentration dependence of s on the spreading of the boundary. If these corrections are not made, $D^{app.}$ may vary with time and will tend to decrease. For a heterogeneous solute, $D^{app.}$ will tend to increase with time. The standard deviation of the distribution of sedimentation coefficients (p) should be zero (within the precision of the measurements) for a homogeneous solute, and the distribution, g (s), of sedimentation coefficients at infinite time should be very small, provided an adequate extrapolation procedure to infinite time is used. Such behaviour has been observed with a sample of β-lactoglobulin at about 25° (Baldwin, 1957a, b).

Where a continuous distribution of sedimenting species is present in the solution, $D^{app.}$ will increase with time, and heterogeneity in s will be indicated by $p > o$ and by g (s). Such heterogeneity has been observed in bovine serum albumin (Baldwin, 1957b) and in DNA (Schumaker and Schachman, 1956; Coates and Jordan, 1960).

Where heterogeneity in s is observed for what is believed to be a pure protein, examination of the concentration dependence of s should reveal whether this heterogeneity is due to reversible association of the protein. Reversibly associating systems for which boundary analysis has been carried out are haemoglobin in mildly acidic solution (Field & O'Brien, 1955) and cod myosin (Connell, 1958).

B. Sedimentation Behaviour of Yeast Lactate Dehydrogenase and Derived Compounds.

1. Materials and Methods.

(i) Centrifuge. The experiments in the following section were carried out with a "Spinco" Model E analytical ultracentrifuge, as described for cytochrome c. However, the light source was positioned on the optic axis of the system, using a half silvered cell window. (Trautman, 1958). Otherwise the optical system was aligned as recommended by the manufacturers. Standard 12 mm. analytical cells, single or double sector, were used. The rotor and rotor chamber were precooled to approximately 1°. Where the ultraviolet absorption optical system was used, lenses and cell windows were carefully cleaned, and Kodak transparency film was used, since a ghost image was obtained when the recommended Kodak commercial ortho film was used. This ghost image was due to the chromatic aberration of the optical system, since the Br_2/Cl_2 filter used to isolate the 248 mμ, 254 mμ and 265 mμ emission lines of a low pressure mercury arc also transmitted light above 550 mμ. The transparency film was not sensitive to green, yellow or red light, whereas commercial ortho film was. The film was developed with Kodak DK 50 developer under standardised conditions of time and temperature. Adenylic acid was used to check the linearity of film blackening with concentration.

(ii) Syringes. Since YLD is readily inactivated by heavy metals, all-glass syringes were used. Special needles were constructed from polythene

blocks and small diameter (22-24 guage) stainless steel tubing, since commercially available needles used plated brass for the female coupling. A blind hole was drilled into the polythene, a smaller hole was then drilled through to the opposite surface of the block and the tubing was inserted in the smaller hole. The diameter of the holes was chosen to give a tight push fit for the syringe and tubing.

(iii) Experimental techniques. Sedimentation velocity experiments were carried out at 59,780 rev./min. with single sector cells, and 44,770 rev./min. with double sector cells.

The most suitable speed for approach to equilibrium experiments was found to be 5227 rev./min., using the 6.4:1 reduction system for speed control. Some experiments were carried out at 7447 and at 7928 rev./min.

Some preliminary experiments indicated the necessity of excluding oxygen from the solutions of YLD, so all manipulations in handling YLD solutions were carried out in an atmosphere of "oxygen-free" nitrogen ($<$8 p.p.m.) obtained from Commonwealth Industrial Gases, Thebarton, South Australia). For the determination of sedimentation coefficients by the moving boundary method, the position of the apparent maximal ordinate of the boundary was measured, using a Beck measuring microscope, as described elsewhere (p. 15). The boundary analysis (p.197) was carried out with enlarged tracings of the schlieren patterns, the starting time of centrifuging being determined from the extrapolation of log x vs. time to the position of the meniscus. The overall magnification of the tracing in relation to the cell was determined from the distance between the reference edges on the

tracing. Some of the approach to equilibrium experiments were measured from tracings, others directly from the schlieren patterns with the measuring microscope. An "Analytrol" model RA recording densitometer (Spinco Division, Beckman Instruments) was used to follow boundary movement, for experiments in which absorption optics were employed. The position of the 50% concentration level of the boundary was used in the calculation of sedimentation coefficients.

(iv) Yeast lactate dehydrogenase. A stock solution of freshly recrystallised YLD was prepared for each series of experiments. This solution was stored at -15° under nitrogen, and was usually recrystallised for further experiments after one week. The YLD was dissolved in 0.3M-sodium lactate/0.05M-tetra sodium pyrophosphate/10^{-4}M-EDTA, adjusted to pH 6.80 with HCl. This buffer has an ionic strength of 0.63, which is necessary to obtain reasonable concentrations of YLD in solution.

(v) Nucleotide-free yeast lactate dehydrogenase. (AS-YLD.) This was prepared from YLD as described elsewhere in this thesis (p.93).

(vi) Viscosity. The viscosity of the standard buffer relative to water was measured with an Ostwald viscometer (B.S.S.188/1936), having a flow time of 397 seconds for water at 20°. The relative viscosity (1.128) was determined at 2°, 3°, 4°, 18° and 19°, and was found to be virtually independent of temperature over the range examined. The relative viscosity of the buffer containing 2.67 urea was determined at 19.5°.

(vii) Density. The density of the standard buffer, relative to water, was determined at 2°, 3° and 4°; a value of 1.030 was found. The density of 2.67 M-urea in buffer was calculated from values in International Critical Tables and was found to be 1.071 $\pm$ 0.002 from 14° to 20°.

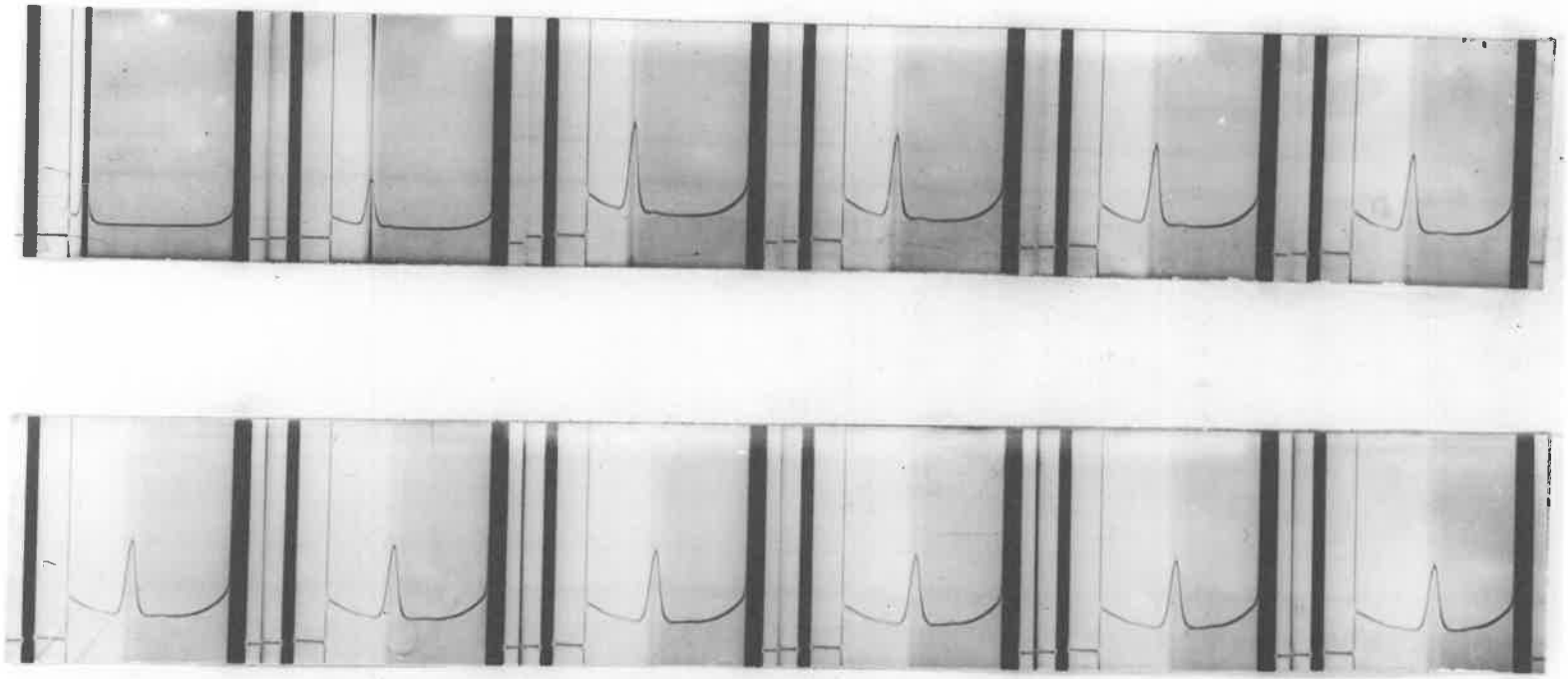
(viii) Calculation of sedimentation coefficients. Usually, six photographs taken at uniform intervals were used to calculate the slope $\Delta \log x/\min$. The average angular velocity of the rotor over the period of the experiment was determined from the difference in odometer readings, using an accurate stop watch to measure the elapsed time. Sedimentation coefficients were corrected to $s_{20,w}$ from the viscosity of the buffer and water at the temperature of the experiment. The buoyancy correction was not made; if $\bar{V}$ lies between 0.70 and 0.75, the sedimentation coefficients reported in this thesis are approximately 10% smaller than the correct values.

2. Sedimentation Velocity Studies

(i) Unmodified yeast lactate dehydrogenase. In general, YLD sedimented with a single symmetrical boundary, although occasionally a minor, faster sedimenting boundary was also observed. Fig. 26 shows typical schlieren patterns obtained with YLD; the minor component is a very small fraction of the total sedimenting material. The red colour of the solution sedimented with the boundary, leaving a a colourless solvent layer. The schlieren patterns in Fig. 26 show very sharp gradients gradually extending from the bottom of the cell towards the meniscus as the time of

Fig. 26. Sedimentation patterns of unmodified YLD

Sedimentation is from left to right. The photographs were taken at 36 minutes, 44 minutes, 52 minutes after reaching full speed and thereafter at 4 minute intervals. Concentration of YlH, 109 μM in YLD haem (~0.8% protein), in 0.05 M-sodium pyrophosphate-0.3 M-sodium lactate-10^{-4}M-EDTA, adjusted to pH 6.84 with HCl, $\Gamma/2 = 0.63$. Temperature 2.5°, 59,780 rev./min.



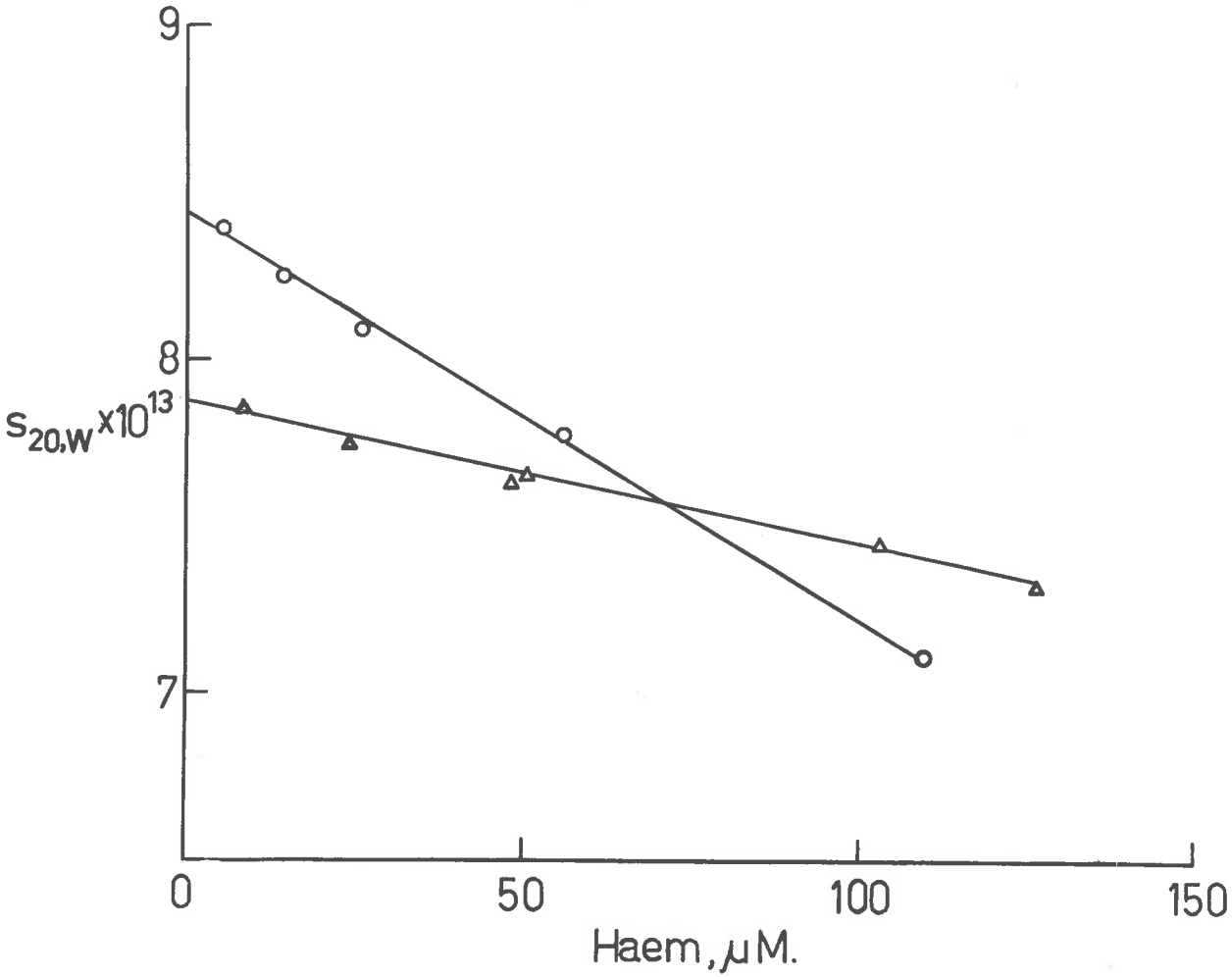
centrifugation increases. Although such patterns are typical of convective disturbances, it seems unlikely that they are caused by convection, since they have been observed in all the sedimentation velocity experiments carried out with unmodified YLD, regardless of the cell centre section used, also, the rotor temperature did not change rapidly during the course of these experiments, in general not increasing by more than about 0.7° over a one hour period. Such gradients have not been observed with other proteins which have been examined under similar conditions in the same ultracentrifuge. Schachman (1959) describes a similar apparent convective disturbance for serum albumin treated with reducing agents and iodoacetate; he suggests that this is due to self-interaction of the sedimenting protein. The mean value of $s_{20,w}$ obtained for the minor component which was sometimes observed was $10.96 \pm 0.67 \times 10^{-13}$ sec.

Fig. 27 shows the dependence of sedimentation coefficient on YLD concentration. A linear relationship of the form $s = s_o(1-kc_o)$ is obtained over a twenty-fold concentration range ($\sim$ 0.04 - 0.8% YLD). Since there is no evidence for a biphasic curve of s vs. [YLD], it seems unlikely that YLD is a reversibly associating system under these conditions unless the equilibrium constant is very much in favour of the associated form. Extrapolation to zero concentration gives $S^o_{20,w} = 8.44$ svedbergs, and k has the value $1.40_5 \times 10^{-3}$ μM^{-1} at 20°, when YLD concentration is estimated spectrophotometrically in μ moles of YLD per litre. The concentration determined, c_o, is the concentration of the initial solution, i.e. c_o is the meniscus concentration before any sedimentation has occurred. According to Schachman (1959), this is not the correct concentration, and the mean of the

Fig. 27. Concentration dependence of sedimentation of YLD and AS-YLD.

Sedimentation was carried out in lactate pyrophosphate buffer, pH 6.8. Concentrations of YLD were determined from $E_{423\,m\mu}$, and expressed as μmoles YLD haem/ l.

O——O YLD, Δ——Δ AS/YLD.


9
8
7
$s_{20,w} \times 10^{13}$
0
50
100
150
Haem, μM.

concentrations in the plateau region, at the times of the first and last photographs, should be used. However, the concentration dependence is not large enough to produce any noticeable departure from linearity in the plot of log x vs. time for any of the YLD concentrations used, and therefore this correction was not made.

The high salt concentration in the buffer resulted in a marked gradient due to buffer ion sedimentation. Since it was possible that this gradient was (a) concealing a slower sedimenting component in YLD or (b) affecting the calculation of sedimentation coefficient, a sedimentation velocity experiment was carried out with a double-sector centre section in the analytical cell to superimpose the base line on the schlieren patterns. Buffer was placed in one sector, and a solution of YLD in the same buffer in the other sector. The schlieren patterns obtained showed only one sedimentating boundary. The refractive index gradient at various levels in the boundary region was measured, and the position of the maximum ordinate due to YLD was obtained by subtraction of the buffer gradient (base line) from the sum gradient of buffer and YLD. This corrected maximum ordinate was shifted slightly in a centrifugal direction when compared with the apparent maximum ordinate (Table 26). The second moment of the gradient curve was also determined and is slightly more shifted in a centrifugal direction. The values of $s_{20,w}$ for the apparent and corrected maximum ordinates and for the square root of the second moment of the gradient curve have been calculated (Table 26). The second moment sedimentation coefficient is the smallest, however the difference resulting from the use of the apparent maximum ordinate is sufficiently small to justify its use in the calculation of the sedimentation coefficient, rather than the much

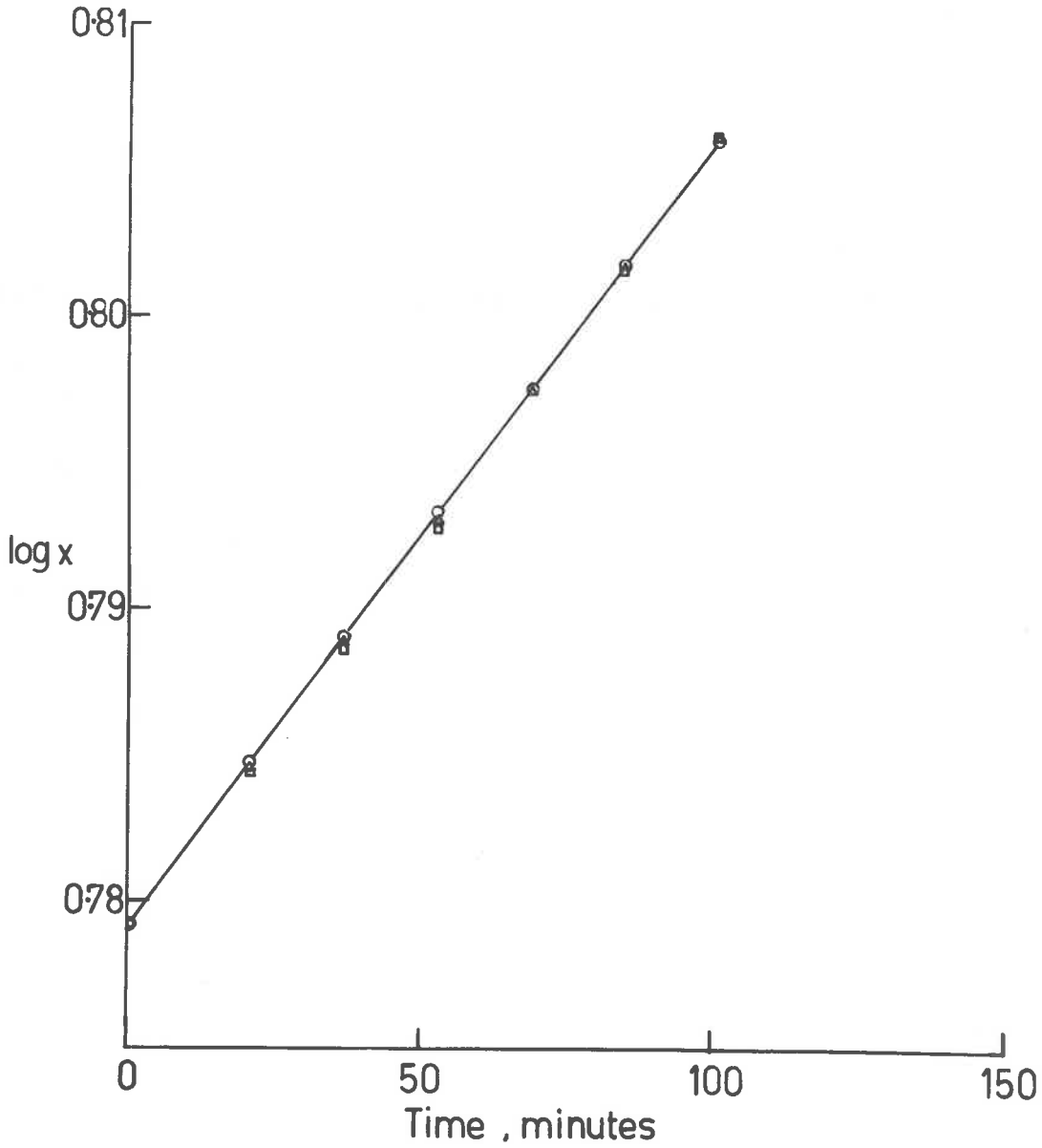
Table 26

Comparison of Apparent and Protein Maximum Ordinates with μ_1 and $\sqrt{\mu_2}$ of the Gradient Curve for XLD.

Time (mins.)	$x_H^{app.}$ cm	$x_H^{protein}$ cm	μ_1 cm	$\sqrt{\mu_2}$ cm
21	6.087	6.089	6.0908	6.0909
37	6.145	6.149	6.1524	6.1525
53	6.205	6.209	6.2120	6.2122
69	6.271	6.272	6.2723	6.2725
85	6.332	6.333	6.3340	6.3345
101	6.398	6.400	6.3970	6.3972
$s_{20,w} \times 10^{13}$ (sec.)	8.00	7.96	-	7.84
$\frac{100(s-s_{\sqrt{\mu_2}})}{s_{\sqrt{\mu_2}}}$ (%)	2.04	1.53	-	0

Fig. 28. Plot of boundary position vs. time during sedimentation of YLD

YLD (74 μM) in lactate pyrophosphate buffer, pH 6.8, was centrifuged at 44,770 rev./min. in a 12 mm., 2½°, double sector epoxy cell at 5.1°, with YLD solution in one sector and buffer in the other. The log of apparent (Δ——Δ) and corrected (□——□) maximal ordinates of the boundary and the square root of the second moment (O——O) are plotted as a function of time.


0·81
0·80
0·79
0·78
log x
0
50
100
150
Time , minutes

more laborious computation of the second moment. Figure 28 shows that the best fit of log x vs. time is obtained for the square root of the second moment.

(a) The effect of temperature on the sedimentation coefficient of yeast lactate dehydrogenase. Most of the experiments with YLD were carried out at temperatures of 1° to 6°, and corrected to $s_{20,w}$. Because of the large difference between the temperature of the experiment and 20°, it was decided to investigate the effect of temperature on the value of $s_{20,w}$. A freshly prepared sample of YLD, inadvertently adjusted to pH 8.8, was centrifuged at 4°, 10° and 20°, and $s_{20,w}$ was calculated for each experiment (Table 27). No buoyancy correction was made. It may be seen that $s_{20,w}$ is independent of the temperature at which it was measured, and therefore the viscosity correction may be used with confidence.

(b) Boundary analysis for sedimentation of yeast lactate dehydrogenase. A boundary analysis was carried out with the schlieren patterns shown in Fig. 26, as described by Baldwin (1957a). The minor rapidly sedimenting component was neglected, and the boundary was analysed only for the major component. Fig. 29 shows the results of this analysis in terms of the apparent diffusion coefficient for each photograph. It will be seen that there is no definite trend in $D_{app.}$ with time, which indicates that the major component is homogeneous with respect to sedimentation coefficient. Table 28 gives the area under the gradient curve, corrected for sectorial dilution for each photograph; this indicates that the measurements of $\frac{dc}{dx}$ from the tracings are consistent. The value of $(D_{app.})_{2.5°}$ buffer obtained in this way was 3.61×10^{-7} cm.^{2}sec.$^{-1}$.

Table 27

The effect of temperature on the sedimentation coefficient of YLD.

YLD in lactate pyrophosphate buffer at pH 8.8 was centrifuged at 59,780 rev./min. at approximately 4°, 10° and 20°. A fresh sample was used for each experiment. The relative viscosity of the buffer was 1.128, and corrections have been made for viscosity only.

Temperature °C.	$s_{20,w} \times 10^{13}$ sec.
4.19	7.85
10.30	8.01
20.80	7.94
Mean	7.93
Variation ±	1%

Table 28.

Area under gradient curve in boundary analysis experiment.

Time mins.	$\sum_{xm}^{xp} \frac{dn}{dx}$	$(\frac{\mu 1}{xm})^2 \sum_{xm}^{xp} \frac{dn}{dx}$	$(\frac{xm}{xH})^2 \cdot (\frac{\mu 1}{xm})^2 \sum_{xm}^{xp} \frac{dn}{dx}$
60	0.904	1.023	0.900
68	0.885	1.000	0.899
72	0.902	1.028	0.895
76	0.897	1.030	0.887
80	0.890	1.038	0.880
84	0.874	1.017	0.875
88	0.852	0.999	0.868
	mean	1.0193 ± 0.0148	

Footnote:

$\sum_{x_m}^{x_p} \frac{dn}{dx}$: is area under refractive index gradient curve;

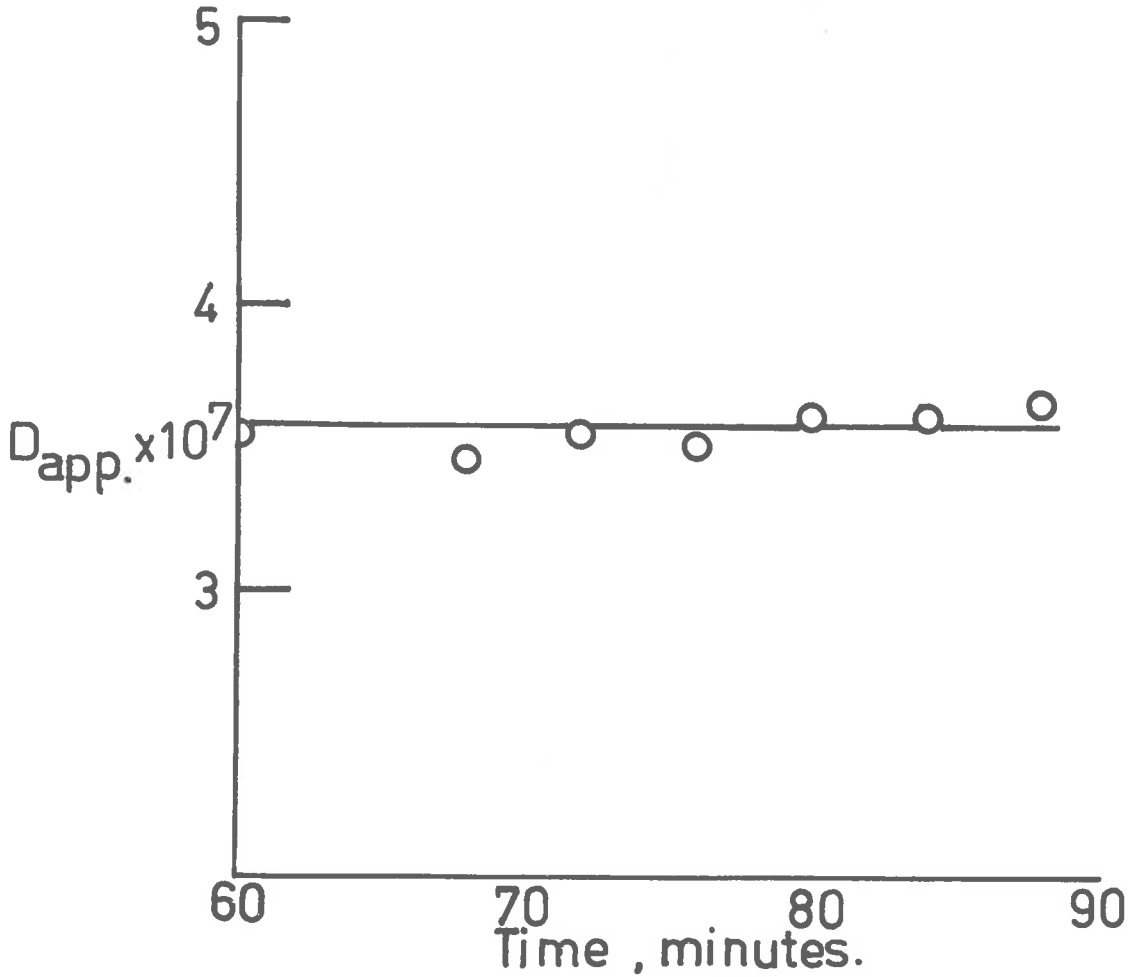
$x_m x_p$ is distance from the axis of rotation to meniscus/plateau region;

$\mu 1$ is the first moment of the gradient curve;

xH is the maximal ordinate of the gradient curve.

Fig. 29. Variation of the apparent diffusion coefficient for YLD with time.

The diffusion coefficient for YLD was calculated from the boundary spreading in the photographs in Fig. 26, as described in the text.


5
4
3
$D_{app.} \times 10^7$
60
70
80
90
Time , minutes.

ii. Nucleotide-free yeast lactate dehydrogenase. Appleby and Morton (1960) found that precipitation of the protein from solutions of YLD with ammonium sulphate caused the dissociation of the polydeoxyribonucleotide associated with the crystalline enzyme, and that the nucleotide-free cytochrome b_2 (AS-YLD) would no longer crystallise under the conditions which resulted in crystallisation of unmodified YLD. This suggests that the polynucleotide has a marked effect on the properties of YLD.

Provided that suitable precautions in excluding oxygen were taken, AS-YLD sedimented with a single symmetrical boundary in the standard buffer (Fig. 33). The concentration dependence of sedimentation (Fig. 27) could be described by the equation $s = s^{\circ}(1-kc_o)$; $S^{\circ}_{20,w} = 7.87$ svedbergs, $k = 5.33_6 \times 10^{-4}\ \mu M^{-1}$ and c_o is the initial concentration of AS-YLD in μ moles of cytochrome/litre, assuming that AS-YLD and YLD have the same extinction coefficients. A sample of YLD was centrifuged before and after incubation with DNA-ase (Table 29). The degradation of the polynucleotide by DNA-ase which was observed by Appleby and Morton (1960) is reflected in the decrease of the sedimentation coefficient following incubation with DNA-ase. The decrease in sedimentation coefficient is rather greater than would be expected if the DNA-ase treated enzyme were similar to AS-YLD, and suggests that ammonium sulphate precipitation brings about some additional change in the properties of the protein.

The removal of the polynucleotide from YLD has two effects on the sedimentation behaviour of the cytochrome. First, as might be expected, the intrinsic sedimentation coefficient is decreased, although at concentrations $> 80\ \mu M$, YLD has a smaller sedimentation coefficient than AS/YLD. The

Table 29.

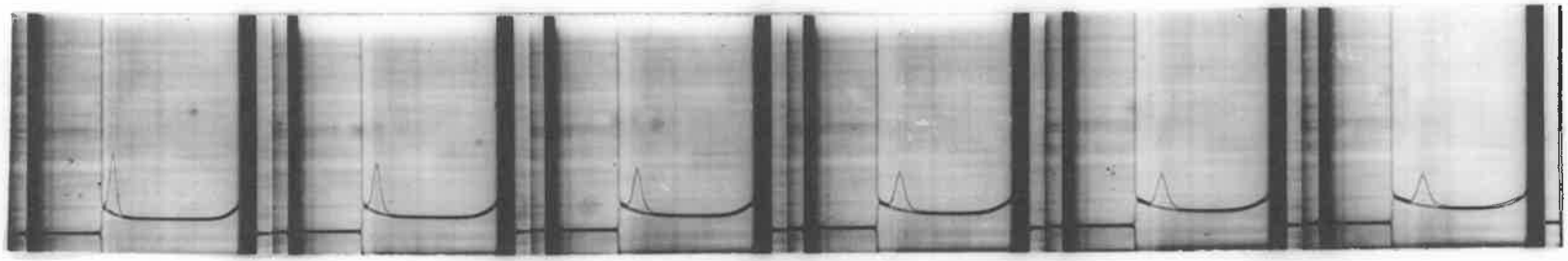
Sedimentation coefficient of YLD before and after incubation with DNA.ase.

The sedimentation coefficient of 60 μM YLD in standard buffer was determined before incubation with DNA-ase (Worthington Ltd., 3 x crystallised, proteinase-free, pancreatic deoxyribonucleotidase), and after incubation of 0.4 ml. of YLD with 0.05 ml. of crystalline DNA-ase (5 mg./ml.) for 24 hours at 2° to 4° under nitrogen.

Condition	$s_{20,w} \times 10^{13}$ (sec.)
Before DNA-ase	7.80
After DNA-ase	7.09
Predicted for AS/YLD	7.62

Fig. 33. Sedimentation patterns of AS-YLD

Sedimentation patterns of AS-YLD (51 μM-YLD haem, ~0.4% protein) in lactate pyrophosphate buffer, pH 6.8. Sedimentation from left to right. First photograph 16 minutes after reaching full speed, thereafter at 8 minute intervals, bar angle 70°; temperature 3.0°, 44,770 rev./min.



change in sedimentation coefficient seems rather large, since the polynucleotide constitutes about 5% of the molecular weight of YLD. However, the contribution of the polynucleotide to the density of YLD will be proportionally greater than this, because of the greater density of polynucleotides. Doty (1955) has found that the partial specific volume of highly polymerised thymus DNA is 0.55 $cm.^3/g$; by comparison, most proteins have partial specific volumes in the region of 0.75 $cm.^3/g$.

The second effect of the polynucleotide is to increase the concentration dependence of the sedimentation coefficient; $ks^{\circ}_{20,w}$ for the YLD and AS-YLD are, respectively, 1.18×10^{-15} and 4.20×10^{-16} sec. μM^{-1}. The approximately 3-fold difference between YLD and AS-YLD shows that the polynucleotide increases the concentration dependence of sedimentation markedly.

Current theories of concentration dependence of sedimentation do not provide satisfactory explanations for this phenomenon. Hence no adequate explanation of the difference between the concentration dependence of sedimentation for YLD and AS-YLD can be offered, although it is clear enough that the difference can be attributed to the presence or absence of the polynucleotide. It is possible that the macroscopic viscosity of YLD solutions is higher than that for AS-YLD, and this could possible account for the greater concentration dependence observed for YLD. It is difficult to accept that the backflow of solvent would be very different for the two forms of the enzyme, although it is possible that differences in charge would affect the size of the ionic envelope surrounding the protein molecules, and would therefore affect the backflow of solvent. It is known from electrophoretic studies that YLD is negatively charged in the standard

buffer, although some of this negative charge can be attributed to the interaction of YLD with pyrophosphate ions (p. 214). Removal of the negatively charged polynucleotide would decrease the negative charge on the protein, and it is possible that the net charge of AS-YLD, whether negative or positive, might be less than the net charge of YLD. Since YLD possesses charge in the system studied, it is possible that primary charge effects may influence the sedimentation of the enzyme, even though the buffer used has a high ionic strength.

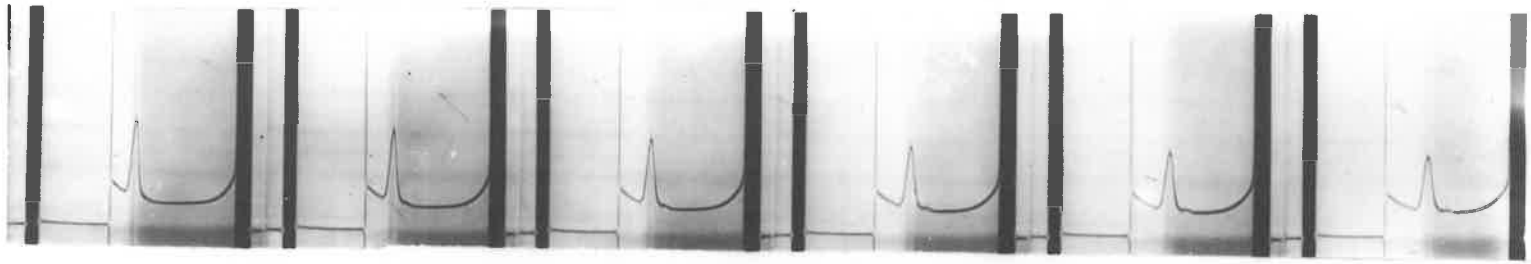
Thus there is as yet no certainty as to the explanation of the marked effect of the polynucleotide on the sedimentation behaviour of YLD.

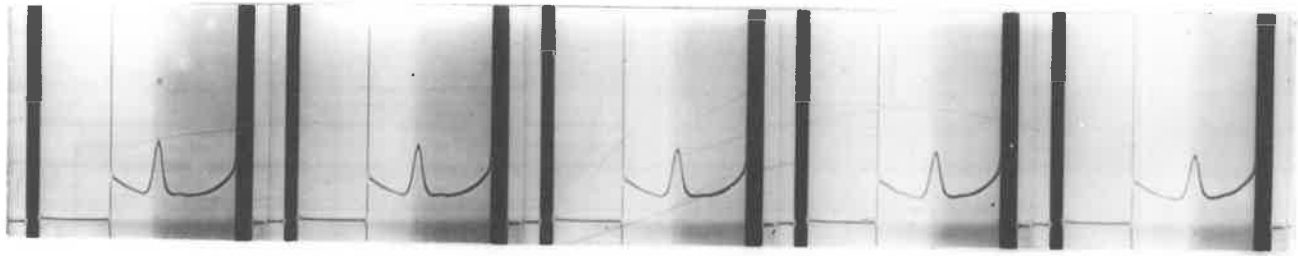
iii. Yeast lactate dehydrogenase after treatment with p-chloromercuriphenylsulphonate. When p-chloromercuriphenylsulphonate (PCMS) was added to YLD under anaerobic conditions, the solution slowly became fluorescent. The fluorescence was yellow and the solution changed to a brownish pink colour. Only one sedimenting component was observed (Fig. 30), but the supernatant above the red plateau and boundary region was yellow and fluorescent. The implications of this in relation to the bonding of the flavin prosthetic group of YLD are discussed elsewhere (p. 203).

It was found that the sedimentation coefficient of PCMS-treated YLD increased as the protein concentration decreased. However, the results were rather too scattered to obtain a linear relationship between concentration and sedimentation coefficient (Table 30). A possible explanation of this is the effect of storage on PCMS-treated YLD (Table 31). It will be noted that the sedimentation coefficient of PCMS-treated-YLD slowly

Fig. 30. Sedimentation pattern of YLD after treatment with p-chloromercuri-phenyl sulphonate

The sedimentation of YLD in lactate pyrophosphate buffer following addition of PCMS to a final concentration of 10^{-4}M: top and second row. 73 µM-YLD, under nitrogen; 1st photograph 30 minutes after starting, thereafter 4 minute intervals, bar angle 70°, temperature 2.85°. 59,780 rev./min. Bottom row 54 µM-YLD, exposed to oxygen; 1st photograph 20 minutes after starting, thereafter 4 minute intervals, bar angle 70°, temperature 2.75°, 59,780 rev./min.





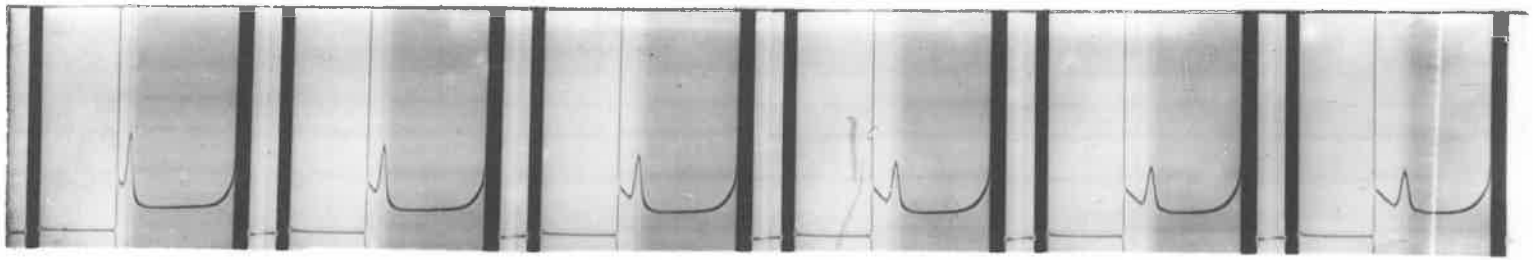


Table 30

Variation of sedimentation coefficient with concentration for PCMS-treated YLD

YLD in lactate pyrophosphate buffer, pH 6.8, containing 10^{-3}M PCMS. Measurements were made on the same sample over several days.

Concentration μM	$s_{20,w} \times 10^{13}$
6.8	8.20
26.9	8.05
54.0	7.79
73.0	7.87

Table 31.

The effect of storage on the sedimentation coefficient of PCMS-treated YLD.

To YLD, in lactate-pyrophosphate buffer, pH 6.8, was added PCMS in the same buffer to a final concentration of 10^{-3} M-PCMS. The solution was centrifuged after 1 hour. The cell was removed from the rotor, gently shaken by slowly rolling and inverting it, and stored at 0-2°. The same solution was centrifuged after 24 hours, 5 days and 13 days.

Conditions	α $(s_{20,w}) \times 10^{13}$	β $(s_{20,w}) \times 10^{13}$
Prior to addition of PCMS	7.69	11.06
1 hour after addition of PCMS	8.43	12.34
24 hours after addition of PCMS	7.99	14.37
5 days after addition of PCMS	7.80	no longer present
13 days after addition of PCMS	7.20	" " "

decreases with storage. A small amount of a second, more rapidly sedimenting component was observed at first, but this disappeared gradually with storage. It therefore seems possible that PCMS may be reacting slowly with disulphide bonds. This reaction is known to occur at elevated temperatures (Cunningham, Muenke and Strayhorn, 1957). Such a reaction might be expected to affect the shape and therefore the sedimentation behaviour of YLD. It has been found that addition of PCMS increases the sedimentation coefficient and this increase is greater than might be expected on the basis of dilution (Table 32). This difference is presumably due to the replacement of FMN by PCMS. An obvious difference between these two compounds is in their charge at pH 7; FMN having about 1.6 negative charges as compared with one for PCMS. A primary or secondary charge effect on the sedimentation of YLD might be expected to have the observed effect.

iv. Flavin dissociation and aggregation of yeast lactate dehydrogenase. Some preliminary experiments were carried out in 0.4M-sodium lactate/ 0.15M-NaCl/ 10^{-4}M-EDTA, pH 6.80, which was used by Appleby and Morton (1960). No special precautions were taken to exclude air. It was observed that the enzyme solution in the cell changed from its original pink colour to a brownish pink, and three components were observed in sedimentation velocity experiments (Fig. 31). These three components have also been observed for YLD and AS-YLD in the standard lactate pyrophosphate buffer, although they were formed less readily, and only appeared after storage following exposure to air. They have also been observed for AS-YLD. The sedimentation

Table 32

Effect of PCMS on the sedimentation coefficient of YLD.

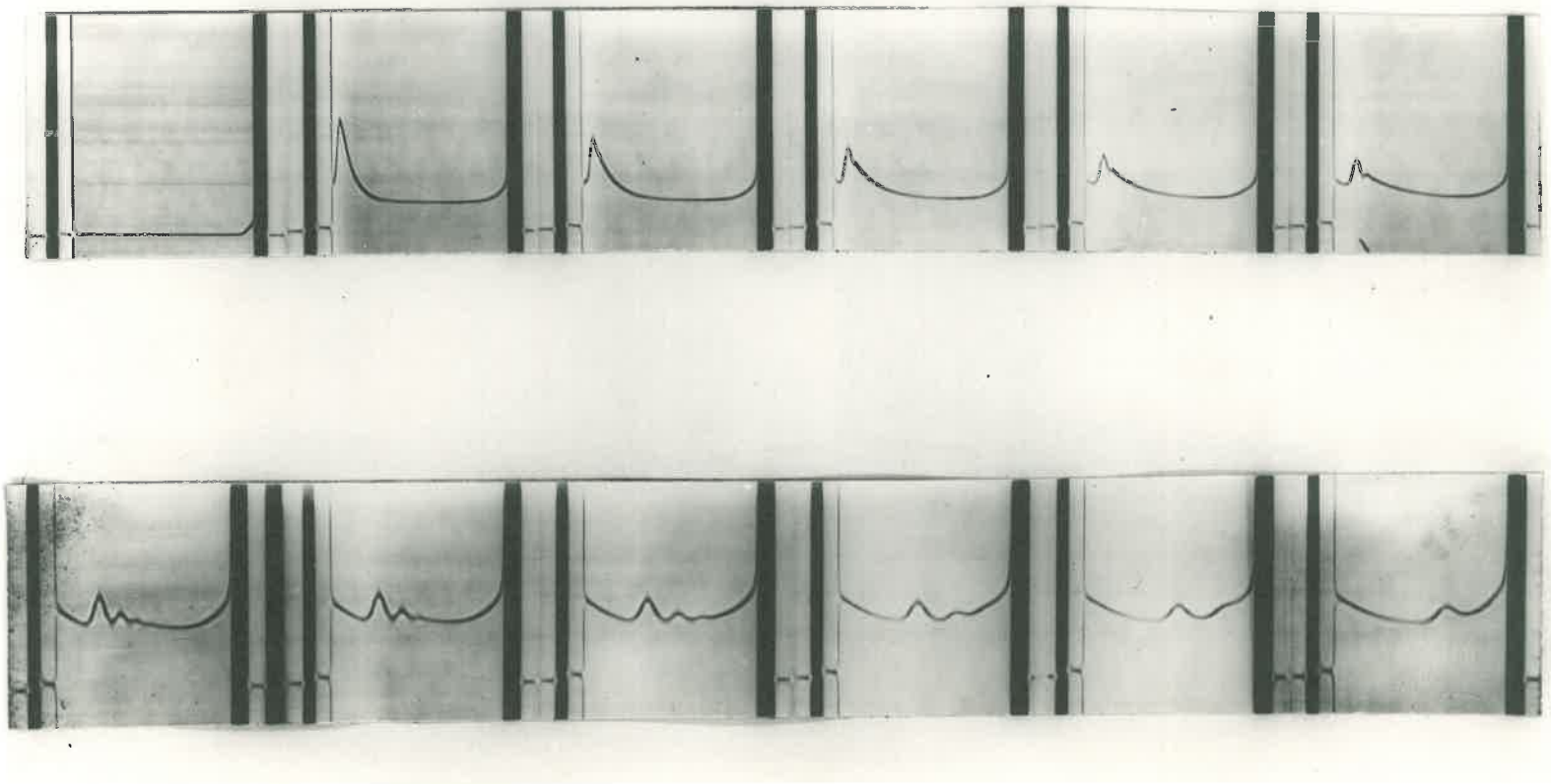
Sedimentation velocity experiments were carried out within 2 hours after addition of PCMS (final concentration, 10^{-4}M) to solutions of YLD in standard lactate-pyrophosphate (final concentration of YLD, $4-7 \times 10^{-5}$M).

$s_{20,w} \times 10^{-13}$ sec.		
without PCMS	predicted on dilution	with PCMS
7.05	7.50	7.85
7.09*	7.55*	8.67*
7.67	7.93	8.42

* Sample of YLD containing 3 components; $s_{20,w}$ of other two components prior to addition of PCMS were 8.75×10^{-13} sec. and 11.94×10^{-13} sec.; after addition of PCMS, $s_{20,w} = 11.97 \times 10^{-13}$ sec. and 13.91×10^{-13} sec. respectively.

Fig. 31. Sedimentation pattern of YLD after exposure to oxygen

Sedimentation of YLD (78 μM) in 0.15 M- sodium chloride, 0.4 M-sodium lactate, 10^{-4} M-EDTA, pH 6.8, after exposure to air. Photographs at 4 minute intervals. Bar angle 70° (Top row), 60° (bottom row), temperature 7.0°, 59,780 rev./min.



coefficients for these components are given in Table 33.

The appearance of these components was always accompanied by the colour change to brownish pink, and the YLD solution exhibited a yellow fluorescence. Furthermore, the red colour sedimented with the boundary, leaving a yellow supernatant solution. This yellow colour is attributed to FMN which has dissociated from YLD by an irreversible process. Appleby and Morton (1959) had concluded that the instability of YLD in the presence of oxygen was due to the oxidation of a sulphydryl group which was involved in the binding of FMN to the protein.

$$E - S - FMN \rightleftharpoons E - SH + FMN$$

$$E - SH + \text{oxidant} \longrightarrow (ES)_{ox.} + \text{reduced oxidant.}$$

Appleby and Morton (1954) and Boeri, Cutolo, Luzzati and Tosi (1955) found that the enzymic activity of YLD was markedly inhibited by p-chloromercuribenzoate, although monoiodoacetate had little effect on the activity. When p-chloromercuriphenylsulphonate (PCMS) was added to YLD, the enzyme solution slowly became fluorescent; only a single sedimenting component was observed (Fig. 30), the red colour moving with the boundary and leaving a yellow supernatant. Exposure of PCMS-treated YLD to air did not result in the formation of additional sedimenting components, and the sedimentation coefficient was not very different from that for "native" YLD. If PCMS was added to YLD which had been exposed to air and which contained the three sedimenting components, no marked change was observed, in the sedimentation behaviour of the solution (Table 32). These experiments are interpreted in the following way.

Table 33.

Sedimentation coefficients of the components present in YLD after exposure to oxygen.

YLD	$s_{20,w} \times 10^{13}$ Boundary 1	Boundary 2	Boundary 3
crystalline (a)	7.88	11.22	14.54
crystalline (b)	6.97	9.8	19.4
nucleotide free	8.18	11.77	15.39
crystalline (b) + PCMS	8.09	11.40	13.83

(a) and (b) refer to different samples of YLD.

The equilibrium

$$E - S - FMN \rightleftharpoons \text{"E-SH"} + FMN$$

is very much in favour of the bound form of the flavin. In the presence of PCMS, "E-SH" forms a derivative with the mercurial, and the reaction proceeds to virtual completion, completely displacing the FMN.

If oxygen is present, then "E-SH" is oxidised to form intermolecular disulphide bonds. Since more than one new species if formed, it appears that YLD has more than one binding site for FMN, and that higher polymeric forms of "E-SH" than the dimer can be formed, i.e.

$$2\ \text{"HS - E - SH"} \longrightarrow \begin{matrix} S - E - SH \\ | \\ S - E - SH \end{matrix} \longrightarrow \begin{matrix} S - E - S \\ | \quad | \\ S - E - S \end{matrix}$$

$$\begin{matrix} S - E - SH \\ | \\ S - E - SH \end{matrix} + HS - E - SH \longrightarrow \begin{matrix} S - E - S - S - E - SH \\ | \\ S - E - SH \end{matrix}$$

etc.

The very minor sedimenting component sometimes observed with intact YLD has a sedimentation coefficient very close to that of one of the aggregated species observed after exposure to oxygen, and it is believed that the minor component is probably aggregated YLD which has been carried along in the crystallisation of YLD.

3. Molecular Weight of Yeast Lactate Dehydrogenase.

Ogura (1961) has shown that the flavin prosthetic group of YLD is fully reduced by lactate. Since the haem group is also reduced, complete

reduction of YLD requires 1½ moles of lactate per mole of haem. This raises the question as to whether YLD exists as a monomer or as a polymer. A dimer of YLD would permit the reduction to occur by intramolecular reaction, whereas the monomeric form apparently requires intermolecular electron transfer, for complete reduction. It seems probable that the reaction mechanism, and therefore the kinetic behaviour of YLD, would be very much dependent on whether YLD existed as a monomer or as a polymer. Intra molecular electron transfer implies that the active centres of YLD in polymeric forms of YLD would not be independent of one another.

For this reason, a considerable amount of effort was directed towards the determination of the molecular weight of YLD. Because YLD is not particularly stable, the approach to equilibrium method for the determination of molecular weights was used, since this method permits the determination of molecular weight to be performed in relatively short experiments. A number of such determinations have been carried out, and widely varying values of the molecular weight of YLD were obtained (Table 34).

There appears to be no reasonable explanation for the threefold variation found, either in terms of concentration or temperature. Since the values of the sedimentation coefficients calculated from the approach to equilibrium experiments by a transport method are unsatisfactory, it may be concluded that the approach to equilibrium method is not applicable to the measurement of the molecular weight of YLD, at least under these conditions. It seems unlikely that the extrapolation of the gradient curves to the meniscus can be at fault, in view of the agreement between the various

procedures, particularly as this is reduced, to a considerable extent, in the Archibald calculation. Since consistent behaviour has been observed in sedimentation velocity experiments, it seems reasonable to conclude that the variation is not associated with a variable extent of aggregation of YLD, and that the results of the approach to equilibrium experiments are in error, for some unknown reason. Because the plots of -log F (c) vs. time in the transport method for calculation of s are linear, this suggests that any error would be systematic. It is therefore possible that the anomalous results presented in Table 34 may be due to some fault in the measurement of the initial concentration, c_o. This is also suggested by the variability of $D_{20,w}$ calculated from the synthetic boundary experiments.

Trautman (1959) describes an approach to equilibrium method for determining molecular weight, in which the measurement of c_o is avoided, and which permits an estimate of c_o to be made also. This method has been applied to the results obtained with YLD, but was no more satisfactory than the other procedures used for the determination of molecular weight.

With the same centrifuge and with the same experimental procedures, reasonable values for the molecular weights of yeast and horse heart cytochrome c (p.34), and for yeast alcohol dehydrogenase (YADH) were obtained. A single experiment, using approach to equilibrium, gave a value of 129,000, for the molecular weight of YADH. Hayes and Velick (1954) obtained a molecular weight of 150,000 for YADH from sedimentation and diffusion experiments. These workers stated that the value for the diffusion coefficient which they used was probably not reliable, since it was obtained from one experiment, under conditions in which denaturation of YADH occurred readily. Therefore it seems likely that the procedures used

Table 34.

Molecular Weight of YLD by Approach to Equilibrium.

In standard buffer; all values are calculated for the meniscus unless the cell bottom (b) is specified.

Experiment Number	Speed (revs./min.)	Temperature (°C)	Time from full speed (min.)	Concentration (μM)	Method of Calculation	Number of Photographs	Molecular Weight	$D_{20,w}$ x 10^7 [a]	$S_{20,w}$ x 10^{13} Transport [c]	$S_{20,w}$ x 10^{13} Moving boundary [d]
W/31	5227	0.8	32-192	109.6	Ehrenberg	5	343,700 ± 18,600		9.65	7.16
					Archibald	5	315,600 ± 3,600			
		1.5				5		2.08		
W/68	5227	3.4	16-192	190.5	Ehrenberg	8	246,000 ± 4,800		12.70	7.09
					Archibald	5	247,800 ± 3,500			
		5.9				8		2.53		
W/70 [e]	7928	2.2	64-128	90.0	Archibald	5	65,200 ± 2,600 [g]		variable	-
			224-384		Ehrenberg	8	481,900 ± 44,500			
		2.7				21		3.08		
W/75	5227	0.7	32	110.3	Klainer and Kegeles	1	450,900	-	-	-
W/77 [f]	5227 then 7447	2.5	0-80 80-144	110.3	Klainer and Kegeles	3	412,200 ± 32,500	- [h]	19.1	-
W/43 [i] YLD + PCMS	5227	2.5	32-160	73.0	Ehrenberg	5	165,800 ± 7,900		6.68	7.85
		4.4				5		7.16		
W/86 AS/YLD	7928	6.4	32-224	126.3	Klainer and Kegeles	7	305,500 ± 16,500		7.94	7.33
						7	179,800 ± 9,400 (b)			
					Archibald	7	219,500 ± 9,000 (b)	-		
W/92 AS/YLD	7928	3.2	32-192	103.0	Archibald	6	194,000 ± 2,500		6.40	7.43
		5.5				5		5.98		

a From boundary spreading in synthetic boundary experiment. b Calculated for cell bottom. c Calculated from approach to equilibrium results. d Measured on same solution in a velocity experiment. e Same batch as used in W/68. g Possibly caused by incorrect extrapolation to the meniscus; very steep refractive index gradient curves present at this period; f Same solution as used in W/75.

h Boundary badly disturbed during formation. i Same batch as used in W/31.

in carrying out the experiments and in calculation were satisfactory, and the variation in molecular weight for YLD is related to the properties of this particular system. The sedimentation coefficient of the sample used in W 68 is higher than would be predicted, from Fig. 27, but such deviations at high concentrations of protein are not uncommon, and frequently are allowed for by inclusion of a 'square-term' in concentration, i.e. $s = s_{20,w} (1-k_1c + k_2c^2)$ where $k_2 \ll k_1$.

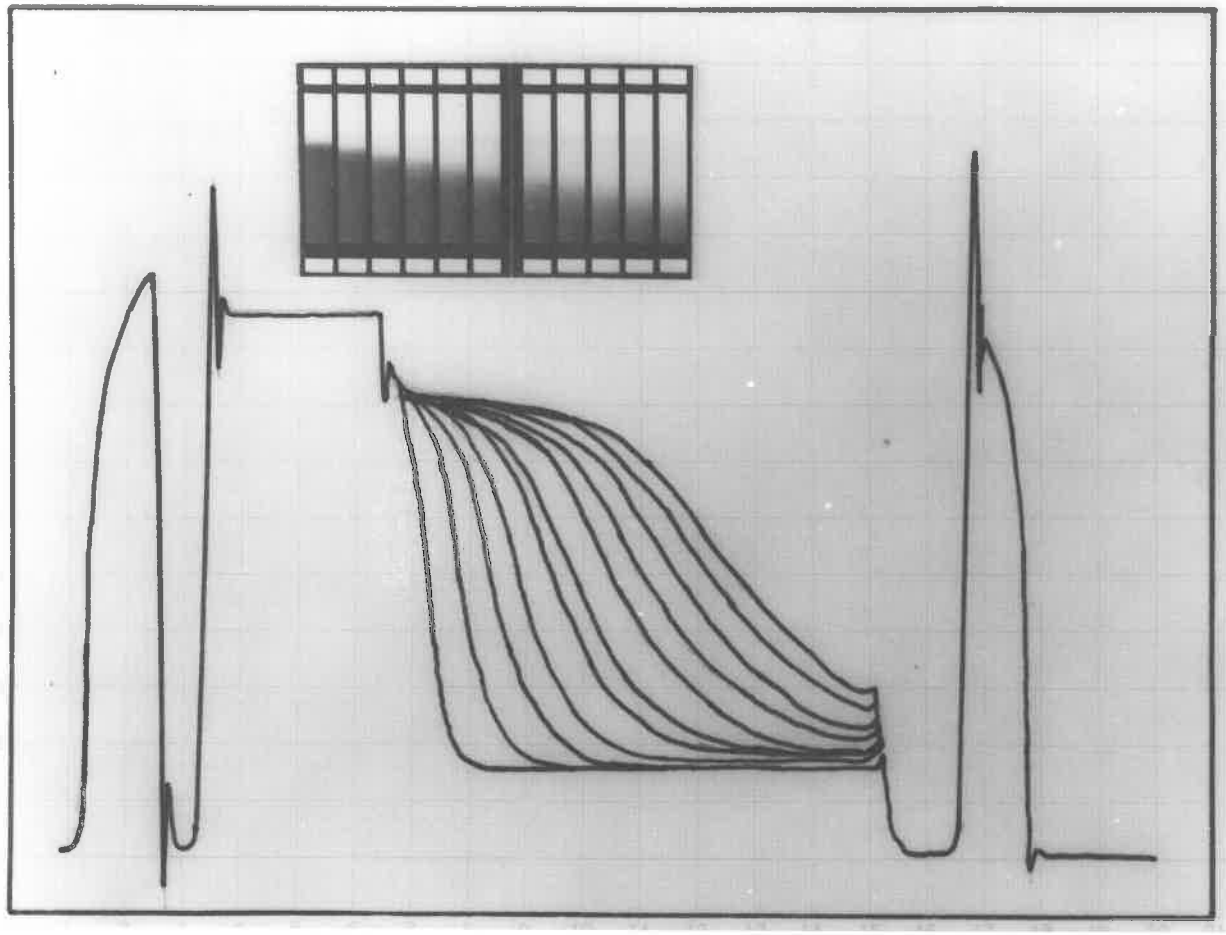
If the value of $D^{apparent}$ obtained in the boundary analysis (p.197) is used for the calculation of M by the Svedberg equation, a value of 123,000 is obtained with $D_{2.5,\ Buffer} = 3.61_6 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ and $s^{\circ}_{2.5,\ Buffer} = 4.55_5 \times 10^{-13}$ sec.

4. Studies on the Polynucleotide from Yeast Lactate Dehydrogenase

The polydeoxyribonucleotide from YLD was prepared as a chromatographically homogeneous fraction by the method described by Montague and Morton (1960). A solution of the nucleotide, in 0.01M-potassium phosphate, 0.078 M NaCl, pH 7.0, $\Gamma/2 = 0.10$, was centrifuged, and the movement of the boundary was followed by use of the ultraviolet absorption optical system fitted to the centrifuge. The concentration of the polynucleotide was 0.003%; at this concentration none of the hypersharpening of the boundary which occurs with DNA at higher concentrations (Schumaker et al., 1956) would be expected. Fig. 32 shows the microdensitometer tracings of the photographs. It is apparent that there is no gross heterogeneity in sedimentation coefficient for the sedimenting, ultraviolet-absorbing material. However a continuous distribution of sedimenting species cannot be ruled out without

Fig. 32. Sedimentation behaviour of polynucleotide from YLD

Sedimentation of polynucleotide (0.003%) in 0.01 M-potassium phosphate, 0.078 M-sodium chloride, pH 7.0, $\Gamma/2 = 0.1$. Microdensitometer tracings obtained from successive photographs at 8 minute intervals. Sedimentation is from left to right. Temperature 16.3°, 59,780 rev./min.



an analysis of the boundary. The mean value of the sedimentation coefficient for two experiments was $s_{20,w} = 5.8 \pm 0.2 \times 10^{-13}$ sec. This can be taken as being the intrinsic sedimentation coefficient (Schumaker and Schachman, 1957).

The diffusion coefficient of the polynucleotide was measured from the spreading of the boundary at low angular velocity with a synthetic boundary cell and with the u.v. absorption optical system of the centrifuge (Schumaker and Schachman, 1957). The first experiment was carried out by layering the equilibrium buffer over the dialysed polynucleotide solution. When the separation of the 25% and 75% concentration levels in the boundary was plotted against the square root of time, the line intercepted the abscissa some minutes before the formation of the boundary. Since the density difference between the buffer and the solution is probably much too small to prevent convective disturbances during the formation of the boundary, sodium chloride was added to the dialysed DNA solution to give a sodium chloride concentration 4 mM in excess of the buffer, and the experiment was repeated. The formation of the boundary was observed with the schlieren optical system. A rapidly diffusing boundary was formed and this was attributed to the diffusion of sodium chloride. This boundary was very sharp on formation and did not appear to be disturbed. The gradient due to the sodium chloride had disappeared by the time photographs were taken. The value of $D_{20,w}$ for this experiment was $4.4 \times 10^{-7} cm^2 sec^{-1}$. Once again the intercept of this curve with the time axis is substantially negative. While this value is probably rather inaccurate, it is unlikely that the error would be as large as one order of magnitude.

The molecular weight calculated from the sedimentation and diffusion coefficients, assuming a value of 0.635 for the partial specific volume, obtained for DNA by Coates (1957), is 96,000. This is very much larger than the molecular weight obtained from end group analysis of the polynucleotide. Montague and Morton (1960) found a molecular weight of 10,000 to 12,000 by this method. Provided that any reliance can be placed on the physically determined molecular weight, it seems likely that intermolecular interaction of the polynucleotide has occurred. This would affect the sedimentation and diffusion coefficients while the end groups would still be accessible for chemical analysis. A possible form of interaction is the exchange of intramolecular hydrogen bonds to form intermolecular bonds.

Oth (1955) found that the molecular weight of DNA was proportional to the third power of the sedimentation coefficient. Provided that this relationship holds for the polynucleotide from YLD, the molecular weight of the polynucleotide would be in the range 60,000 to 150,000, assuming that DNA has a weight average molecular weight of 5 to 8 million and weight average $s_{20,w}$ 22 to 25 x 10^{-13} sec.

5. Effect of Urea on Yeast Lactate Dehydrogenase.

It has been suggested by various workers that YLD might be a complex of two proteins, a flavoprotein and a haemoprotein. α-Keto-glutarate dehydrogenase is known to be a complex of two proteins, and is dissociated by urea into the two proteins*. It was therefore decided to investigate the effect of urea on the sedimentation behaviour of YLD. The initial

* Massey (1960c)

experiment was carried out in 5 M-urea; after 120 minutes the boundary had not separated from the meniscus (Fig. 34). This behaviour has been observed with several samples of YLD, and the patterns obtained resemble those for an approach to equilibrium experiment with native protein. Other experiments were carried out at a lower urea concentration, and only a single sedimenting boundary was observed (Fig. 34). Immediately urea was added to solutions of YLD, enzymic activity was lost, the solutions became orange brown at the higher urea concentration and brown-pink at the lower urea concentration , and a very strong yellow fluorescence was observed; the supernatant above the sedimenting boundary was yellow. After dialysis of urea treated YLD the solutions were re-examined in the ultracentrifuge. Once again a single sedimenting boundary was observed for YLD treated with 2.67 M-urea, while YLD treated with 5 M-urea seemed much the same before and after dialysis. Although the buoyancy correction for solution density was not made for untreated YLD, a correction $\frac{1 - \bar{V}\rho_{buffer}}{1 - \bar{V}\rho_{urea}}$ was made to the sedimentation coefficient for YLD in urea, so that the values were comparable for "native" and urea treated YLD. (Table 35).

Provided that viscosity and buoyancy corrections are possible in the presence of urea (Wetlaufer and Edsall, 1960), the addition of 2.67 M-urea to lactate-reduced YLD causes a reversible change in the sedimentation properties of YLD. The decrease in sedimentation coefficient in the presence of urea is probably due to a change in the frictional coefficient of YLD, resulting in some loss of the tertiary structure of native YLD. The dissociation of flavin by urea can either imply a direct effect of the added urea on

Table 35

Sedimentation Coefficients of YLD treated with 2.67M-urea.

Sedimentation in lactate/pyrophosphate/EDTA buffer, pH 7.2 - pH 7.5, with and without 2.67M-urea. Dialysis of urea treated YLD was carried out against the buffer for 24 hours at 2° under anaerobic conditions. No precipitate formed during dialysis. The buoyancy correction has been made as in the text, for $\bar{V}$ = 0.70 and 0.75 cm^3/gm.

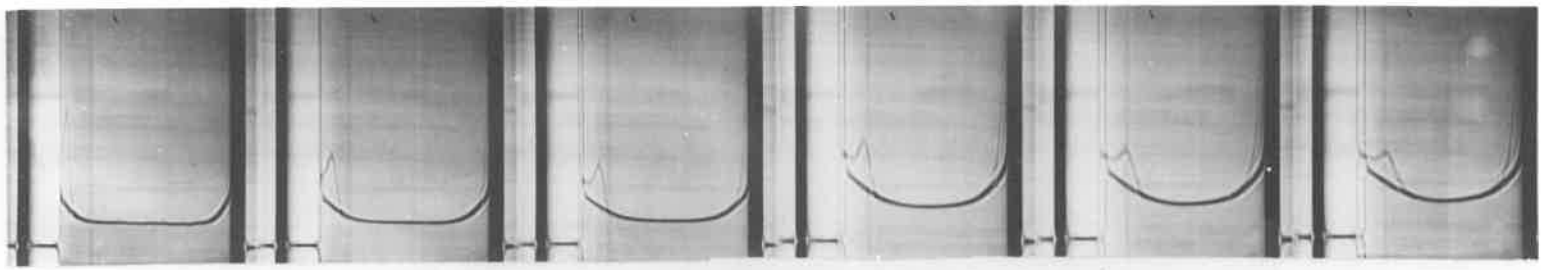
$s_{20,w} \times 10^{-13}$ (sec.)		
untreated	2.67M urea	dialysed.
-	5.59 - 5.78	-
7.25	4.35 - 4.46	7.47
7.25	6.08 - 6.29	-
oxidised YLD		
7.08	6.94 - 7.12	-
pH 8.8, reduced		
7.94	3.12 - 3.24	-

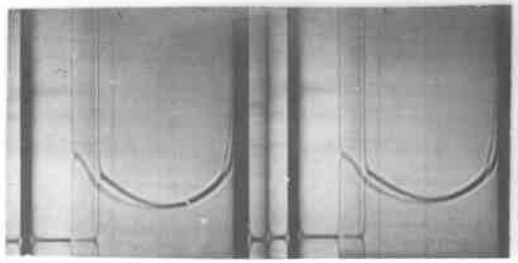
Fig. 34. Sedimentation patterns of YLD after treatment with urea.

Sedimentation of YLD (0.3% protein) in lactate pyrophosphate buffer, pH 7.2. Sedimentation is from left to right; 44,770 rev./min.

Top row. YLD in the presence of 2.67 M-urea; bar angle 60° (1 to 3), 50° (4 to 6). Photographs at 16 minute intervals. Temperature 7.7°.

Bottom row. YLD in the presence of 5M-urea; bar angle 55°. 1st photograph, 90 minutes after reaching full speed; second photograph after a further 27 minutes. Temperature 6.7°.





the bonding of the flavin, or more probably on an indirect effect due to the loss of the tertiary structure required for flavin bonding. If the flavin is bound to the protein by a number of weak forces, which have a cumulative effect in binding the flavin to the protein, a loss of structure in the region of the flavin binding site, caused by urea, would probably be sufficient to produce dissociation of the flavin. The effect of 5M-urea on YLD is apparently considerably greater than 2.67M-urea. Because of the variability of the values of s for YLD obtained by the transport method (p.204), it was felt that no reliance could be placed on data obtained in this way for YLD plus 5M-urea. From a qualitative point of view, the sedimentation coefficient of YLD in 5M-urea must be very much smaller than that of "native" YLD or untreated YLD, and the effect of 5M-urea is probably irreversible. This would suggest that in 5M-urea, YLD loses most, if not all of its tertiary structure and possibly even some of its secondary structure, becoming more like a linear polymer.

A series of experiments on the sedimentation behaviour of YLD in urea was inadvertently carried out at approximately pH 8.8. In both 2.67M-urea and 5M-urea, a boundary was formed by sedimentation (Figs. 35,36). Unfortunately, the sedimentation coefficient in 5M-urea could not be measured, but sedimentation was slower than in 2.67M-urea. After dialysis, YLD treated with 2.67M-urea and with 5M-urea showed at least two sedimenting components (Figs. 35, 36). From the rate at which the sedimenting boundaries spread, it would appear that the boundaries represent a number of molecular species with a range of sedimentation coefficients. These probably differ in the manner in which new hydrogen bonds are formed after the removal of urea.

Fig. 35. Sedimentation pattern of YLD after treatment with 2.67 M-urea at pH 8.8.

Sedimentation of YLD (~0.4% protein) in lactate pyrophosphate buffer, pH 8.8, containing 2.67-M urea. 44,770 rev./min.

Top row. After dialysis of urea treated YLD against a large volume of lactate pyrophosphate buffer, pH 8.8, for 11 hrs. at 2°. Bar angle 50°; first photograph 6 minutes after reaching full speed, subsequent photographs at 8 minute intervals. Temperature 21.1°.

Bottom row. Sample used above, before dialysis. Bar angle 50°. first photograph 30 minutes after reaching full speed, thereafter at 8 minute intervals. Temperature 22.0°.

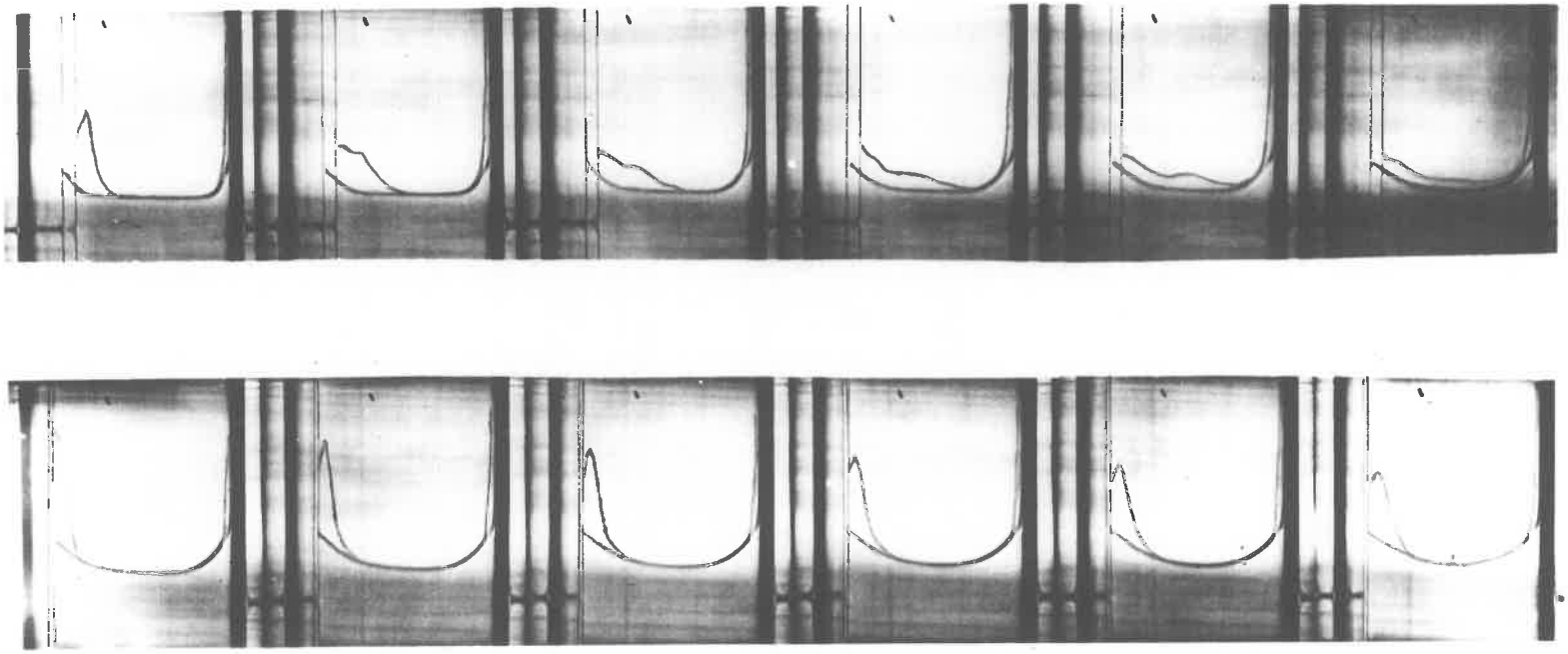
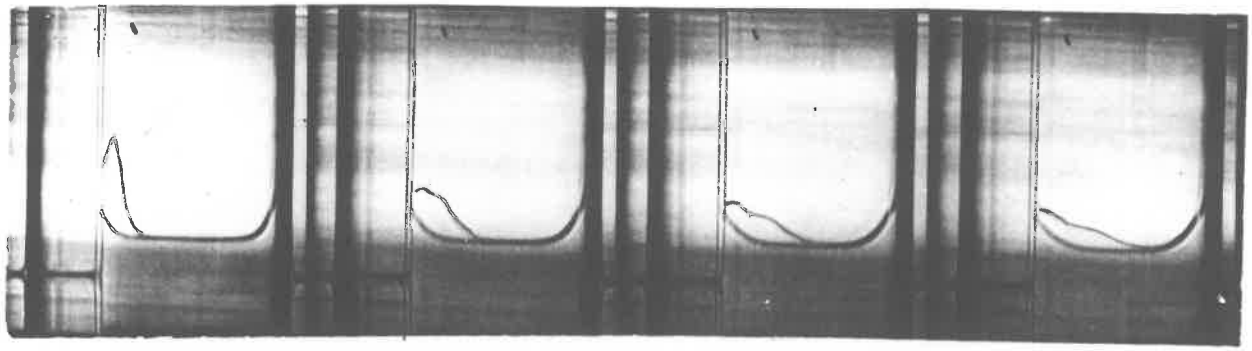


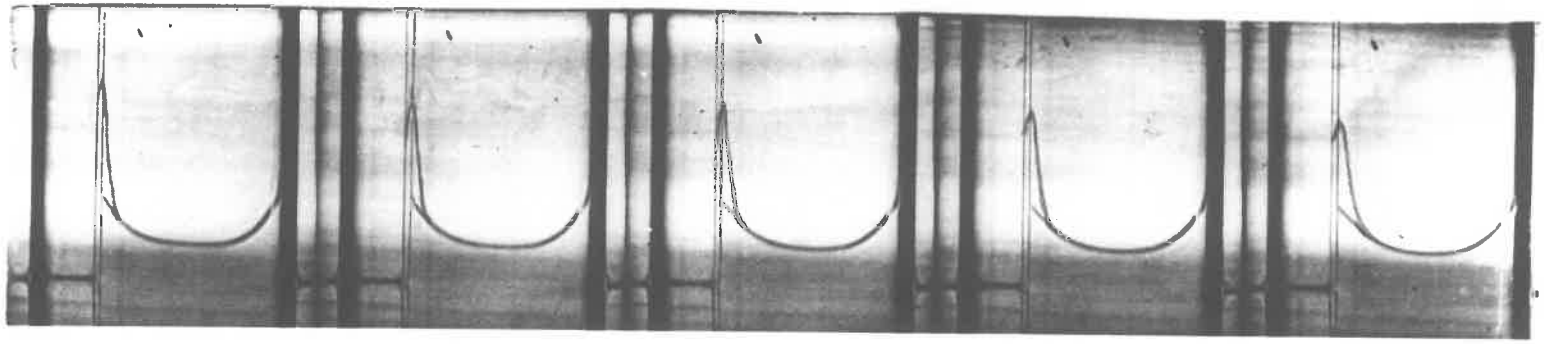
Fig. 36. Sedimentation of YLD after treatment with 5 M-urea at pH 8.8

Sedimentation of YLD (~0.4% protein) in lactate-pyrophosphate buffer, pH 8.8, containing 5 M-urea. 44,770 rev./min.

Top row. After dialysis of urea treated YLD against a large volume of lactate-pyrophosphate buffer, pH 8.8, for 14 hours at 2°. Bar angle 50°, first photograph 5 minutes after reaching full speed, thereafter at 8 minute intervals. Temperature, 21.7°.

Bottom row. Sample used above, before dialysis. Bar angle 50°, first photograph 21 minutes after reaching full speed, thereafter at 4 minute intervals. Temperature, 22.5°.





The presence of urea had virtually no effect on YLD in a buffer when sodium acetate was substituted for sodium lactate, however a possible minor rapidly sedimenting component was observed (Fig. 37). Since the enzyme was in its oxidised state, it is possible that the oxidation level of the prosthetic groups has a marked effect on the stability of the tertiary structure of YLD. It is also possible that the difference in behaviour is due to differences in the effect of lactate and acetate on the system, apart from differences in the oxidation state of the prosthetic groups. The failure of urea to dissociate YLD into two or more sedimenting components seems good evidence for the existence of YLD as a single protein with two prosthetic groups.

6. Diffusion of Yeast Lactate Dehydrogenase.

Because of the anomalous results obtained with approach to equilibrium methods for the determination of the molecular weight of YLD, an attempt to obtain the diffusion coefficient of YLD by static free diffusion was made. It was found that interference methods for following boundary spreading were unsuitable, even using monochromatic light of longer wavelength than the α-band absorption maximum of reduced YLD. This is because YLD still has appreciable absorption at these higher wavelengths, and satisfactory interference cannot be obtained. It was therefore necessary to use a schlieren optical system. The diffusion of sucrose in water was used to check the suitability of the Perkin-Elmer electrophoresis apparatus for diffusion studies. The results obtained were most unsatisfactory, since the spreading of the boundary was not a linear function of $\sqrt{t}$, and it is believed that the

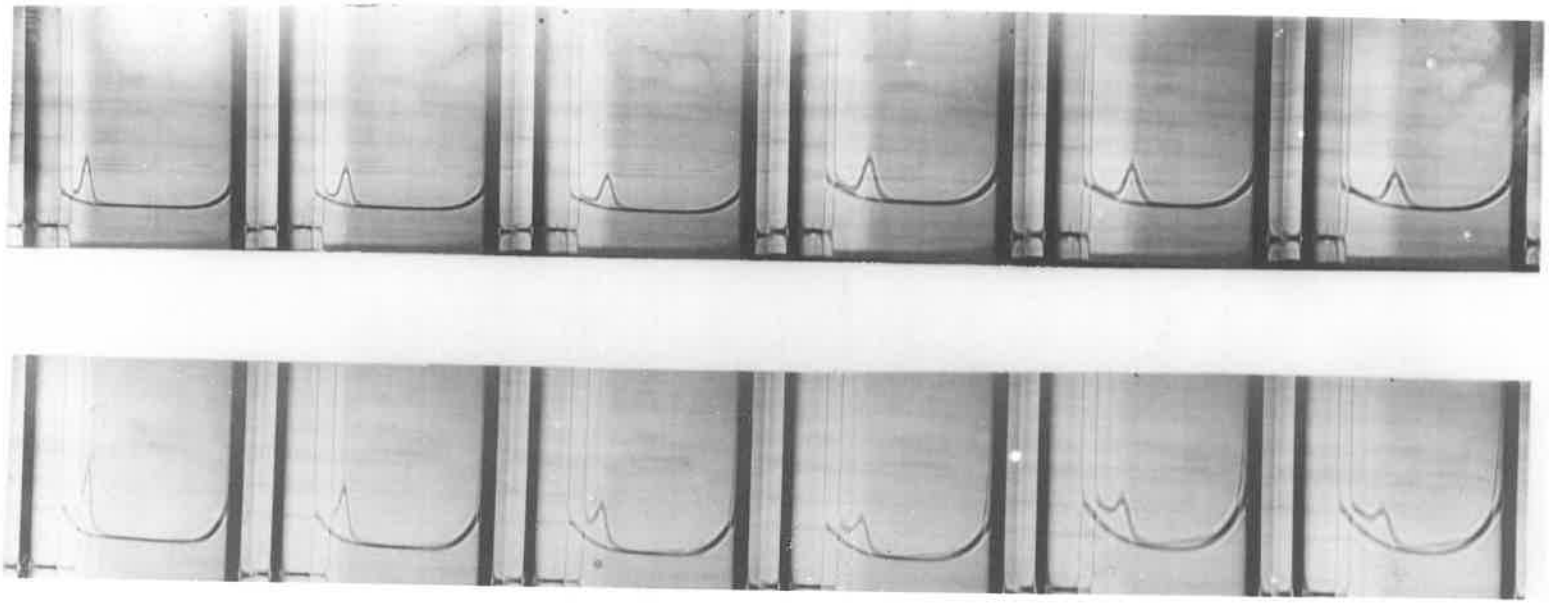
Fig. 37. Sedimentation pattern for oxidised YLD before and after treatment with 2.67 M urea

Sedimentation of YLD in 0.3M-sodium acetate, 0.05M-sodium pyrophosphate buffer, pH 7.4, 44,770 rev./min.

Top row. Before treatment with urea (≈0.35% protein).

Bar angle 60° (1-3), 50° (4-6). First photograph 20 minutes after reaching full speed, thereafter at 8 minute intervals. Temperature 16.0°.

Bottom row. After addition of urea to 2.67 M (0.23% protein). Bar angle 50°, first photographed 16 minutes after reaching full speed, thereafter at 8 minute intervals. Temperature 16.4°.



temperature control of the apparatus is not sufficiently good to permit diffusion studies to be carried out. Therefore, arrangements were made to carry out a diffusion experiment with a "Spinco" Model H diffusiometer. The schlieren optical system of this apparatus was checked by measuring the diffusion of sucrose against water; the diffusion coefficient so obtained was in satisfactory agreement with published values of the diffusion coefficient of sucrose. The diffusion of YLD against the standard lactate/pyrophosphate buffer at pH 6.8 under anaerobic conditions was followed at 1°; it was found that the area under the gradient curve decreased as the experiment proceeded, so that a value of the diffusion coefficient of YLD could not be obtained in this experiment. Unfortunately, further access to the instrument has not been possible, because it has been required for other purposes. It is hoped that further experiments with YLD can be performed at a later date.

C. Electrophoresis of Yeast Lactate Dehydrogenase.

Appleby and Morton (1959b) carried out electrophoresis of YLD in buffers having ionic strength of 0.25. These experiments were carried out with low concentrations of YLD, ($\sim$ 0.2%) except for one experiment at pH 8.8, where flavin and polynucleotide dissociation occurred. It was therefore decided to repeat the electrophoresis of YLD, using high ionic strength buffers. Electrophoresis was carried out as described for cytochrome c (p. 14). In order to prevent the access of oxygen to the YLD solutions during electrophoresis, the cell and buffer vessels were filled under nitrogen, and the buffer in the buffer vessels and top section was layered over with liquid paraffin.

A number of experiments were carried out in the standard lactate/pyrophosphate used in the sedimentation studies; one experiment was carried out in veronal/lactate/EDTA buffer at pH 8.0. The results of these experiments are reported in Table 36. In all of the experiments, the major component was negatively charged. In some of the experiments, a highly mobile, negatively charged minor component was observed (Fig. 38). The identity of this component is unknown, but since no colour was observed migrating with this boundary, it seems unlikely that it was due to FMN (Appleby and Morton, 1960), and it possibly arises from the presence of a small amount of non-dialysable polynucleotide present as a contaminant in the preparations (Appleby and Morton, 1960).

Since YLD has less negative charge in lactate/veronal/EDTA buffer at pH 8.0 than in lactate/pyrophosphate/EDTA buffer from pH 6.8 to pH 7.30,

Table 36.

Electrophoretic Mobility of YLD.

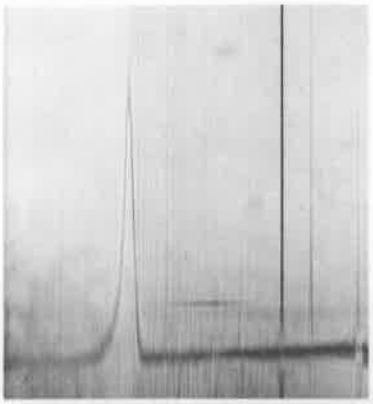
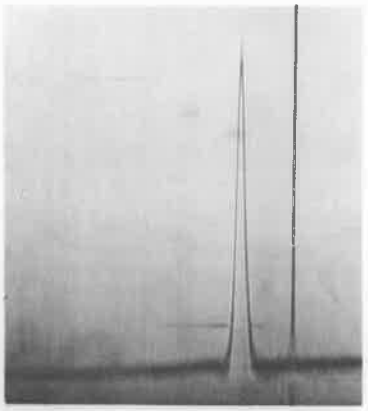
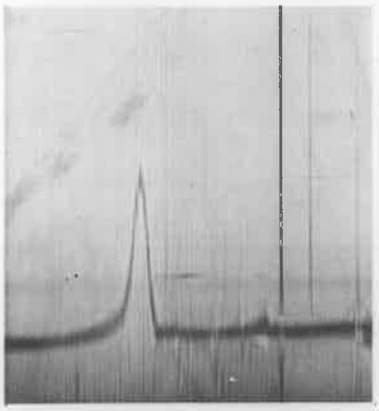
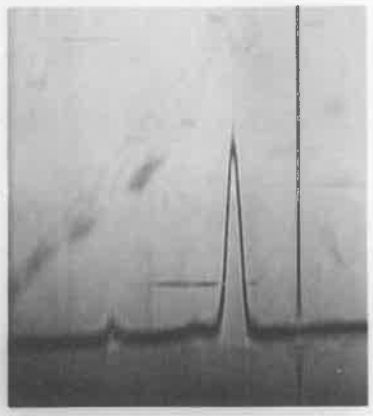
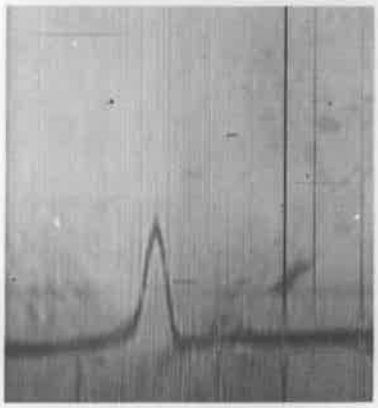
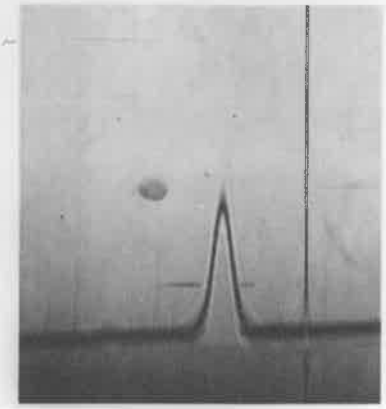
Experiment	Temperature	Buffer	pH	$T/_2$	Current (mA)	Duration (hr.)	Mobility x 10^6 ($cm.^2$ $sec.^{-1}$ $volt^{-1}$)
EW62	2.4°	lactate-pyrophosphate-EDTA	6.75	0.60	15	3.3	-8.7_1 (A)*
EW69	2.2	lactate-pyrophosphate-EDTA	6.82	0.63	15	2.5	-9.3_2 (A) -9.4_3 (D)
EW72	2.2	lactate-veronal-EDTA	8.06	0.52	15	3.5	-7.9_5 (A) -6.2_7 (D)
EW75	2.0	lactate-pyrophosphate-EDTA	6.85	0.64	10	6.7	-9.3_2 (A), - 100.1 (A) -10.4_5 (D), - 97.6 (D)
EW85	2.0	lactate-pyrophosphate-EDTA	7.30	0.63	9	6.0	-10.2_2 (A), - 122.3 (A) -10.2_5 (D), - 117.8 (D)
EW86 sample exposed to oxygen, until about 50% in aggregated form.	2.0	lactate-pyrophosphate EDTA	7.2	0.62	9	4.0	-10.0 (A) -12.2 (D)

* A and D refer to the ascending and descending boundaries.

Fig. 38. Electrophoretic patterns of YLD at pH 7.3

Freshly prepared, twice crystallised YLD, after dialysis for 18 hours at 2° against 0.3 M-sodium lactate-0.05 M-sodium pyrophosphate- 10^{-4}M-EDTA, adjusted to pH 7.3 with HCl. The cell was filled under nitrogen as described in the text. Current 9 mA, temperature 2.0°.

⟶	⟵
Descending (cathodic)	Ascending (anodic)
0 mins.	0 mins.
120 mins.	120 mins.
240 mins.	240 mins.

the higher negative charge of YLD in the pyrophosphate buffer is probably caused by a specific interaction of the enzyme with the pyrophosphate ion. It is difficult to reconcile the negative charge which YLD has in either buffer with the amino acid composition of the protein (Appleby, Morton and Simmonds, 1960). Table 37 shows that YLD has a considerable excess of basic amino acids. A possible cause of the discrepancy is that the amide nitrogen has been overestimated.

A sample of YLD which had been exposed to oxygen and stored was highly fluorescent. Two sedimenting components were found to be present after 24 hours, and after a further 12 hours, the amounts of each were approximately equal. When this solution was subjected to electrophoresis, only one component was observed (Fig. 39). Nicol (1960) found that solutions of urease which contained several sedimenting components corresponding to urease in various states of aggregation still migrated as a single boundary on electrophoresis. It seems probable that the charge/volume ratio of aggregated and unaggregated YLD and urease is sufficiently similar to prevent their separation by electrophoresis.

No accurate estimate of the iso-electric pH of YLD could be made, however, if extrapolation of mobility vs. pH for lactate/ pyrophosphate buffer is carried out, zero mobility of YLD occurs at approximately pH 3.8. Appleby (1957) found that the turbidity of a YLD solution was maximum at approximately pH 4.0.

Table 37.

Net Charge of YLD at pH 6.8.

(From Appleby, Morton and Simmonds, 1960).

At pH 6.8 it is assumed that the acidic and basic residues are fully ionised. For histidine (pK 6.4), 30% of the residues are ionised at pH 6.8; the polynucleotide is assumed to have one primary phosphate dissociation; the secondary phosphate dissociation is about 50% complete at pH 6.8. The same consideration applies to FMN.

Negatively charged residues / YLD haem

Component	Total residues	Charged at pH 6.8
Aspartic acid	59.5	59.5
Glutamic acid	59.1	59.1
Amide nitrogen	81	
(Aspartic + glutamic acids) - amide nitrogen		37.6
Polynucleotide (15 residues)		
primary	1	1
secondary	15	7.5
FMN		
primary	1	1
secondary	1	0.5

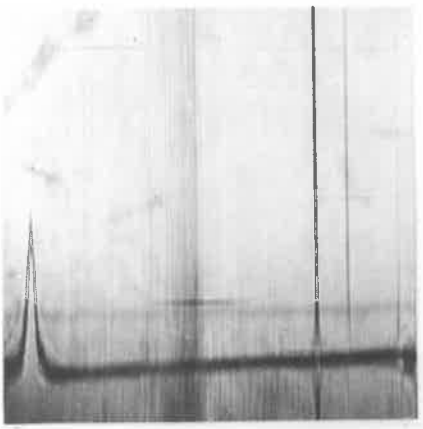
Positively charged residues / YLD haem

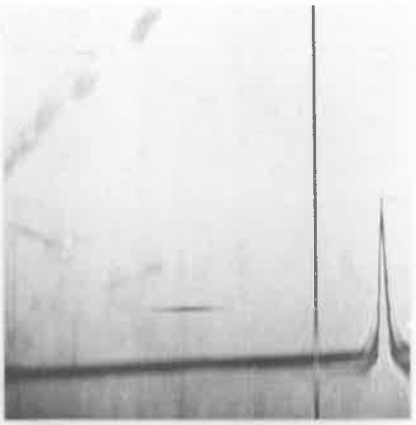
Component	Total residues	Charged at pH 6.8
Lysine	45.7	45.7
Arginine	21.0	21.0
Histidine	6.2	1.9
Total positive charge		68.6
Total negative charge		47.6
Excess positive charge		21

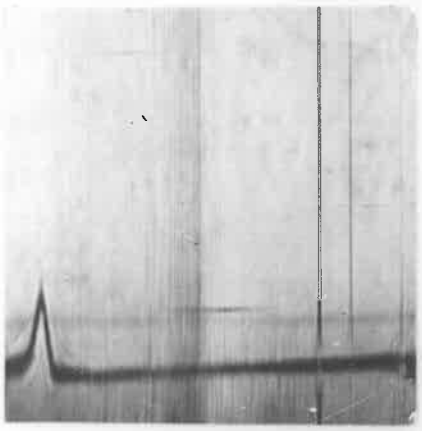
Fig. 39. Electrophoretic patterns of YLD after exposure to oxygen

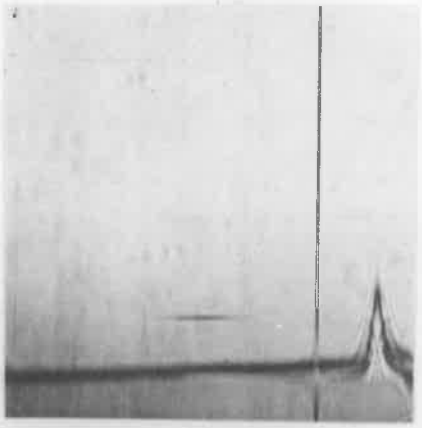
Freshly prepared YLD, after dialysis for 18 hours at 2° against lactate pyrophosphate buffer, pH 7.2, was exposed to air and stored at 2° until a considerable amount of aggregation had occurred (see text). Electrophoresis was then carried out at 2.0° with a current of 9 mA.

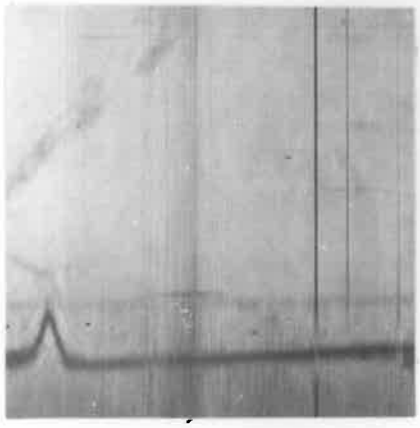
⟶	⟵
Descending (cathodic)	Ascending (anodic)
10 mins.	10 mins.
70 mins.	70 mins.
160 mins.	160 mins.

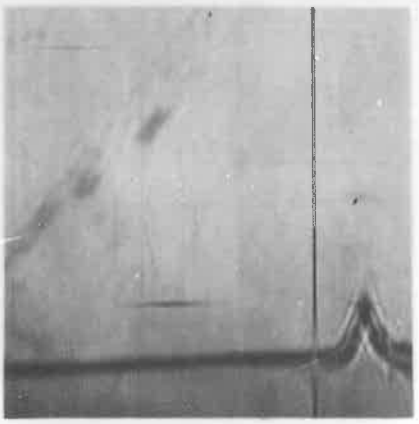












VI. GENERAL DISCUSSION.

1. Homogeneity of Yeast Lactate Dehydrogenase.

The behaviour of YLD on electrophoresis (p.213) and sedimentation (p. 194) provides no evidence that the crystalline YLD preparations used here contain more than a single protein; this is confirmed by boundary analysis (p. 197) and by the effect of urea on YLD (p.208). It therefore appears that crystalline YLD is a nucleoprotein having two prosthetic groups, and that it is not a complex of a flavoprotein and a haemoprotein. It is apparent that the polydeoxyribonucleotide must be firmly associated with the protein, and that it is present in every molecule of the crystalline enzyme, since a random association of the protein with large DNA molecules would result in heterogeneity of the system.

2. Molecular Weight of Yeast Lactate Dehydrogenase.

The molecular weight of YLD remains uncertain. It is clear that approach to sedimentation equilibrium is not a suitable method for the determination of the molecular weight of YLD, although the reason for this is unknown. The molecular weight calculated from the sedimentation coefficient and the diffusion coefficient lies unsatisfactorily between a molecular weight of 80,000, corresponding to one flavin and one haem prosthetic group per molecule, and 160,000, corresponding to a dimer of the minimum molecular weight. The determination of the diffusion coefficient of YLD by static free diffusion has not been successful as yet, and awaits further experimentation. Probably the best indication that YLD exists as

the dimer (molec. wt. 160,000) comes from the end group determination of the molecular weight of the polynucleotide from YLD (Montague and Morton, 1960). As was pointed out earlier, the association between the protein and the polynucleotide cannot be random, so that the molecular weight of the polynucleotide must be related to the molecular weight of the enzyme; it is possible for the molecular weight of the polynucleotide in relation to the analytically determined composition of YLD to be equivalent to the molecular weight of YLD or to be a sub multiple of it. The molecular weight of the polynucleotide by end group analysis was approximately twice the minimum molecular weight obtained from the polynucleotide composition. Therefore the minimum molecular weight of YLD must be 160,000.

From the viewpoint of stoichiometry, the presence of two justaposed flavin-haem units per molecule would appear to be a preferable arrangement for the reduction of YLD by lactate. Three reducing equivalents ($1\frac{1}{2}$ moles of lactate) are required to completely reduce one haem and one flavin prosthetic group for oxidised YLD (p. 203). A monomeric form of YLD (one flavin-haem unit) requires a complex series of intermolecular electron transfers, as does a dimer in which the flavin-haem units are well separated and effectively independent of one another. A dimer of YLD in which electronic interaction between the flavin-haem units may occur provides the simplest minimum hypothesis for the successful alternation of YLD between oxidised and reduced states.

3. <u>The Active Site of Yeast Lactate Dehydrogenase</u>.

The results reported in this thesis are supported by the observations of Dikstein (1959), who has shown that the reaction of α-hydroxy acids

with YLD involves the carboxyl group of the acids. Dikstein (1959) also showed that the transfer of carboxylic acids from the bulk phase to the active site of YLD was more favourable for highly lipophilic acids. Comparison of Table 38 , which shows Dikstein's observations, with Table 21 (p. 164) indicates that there is substantial agreement between the two studies, and that the lack of agreement as to whether glycerate and L-malate are substrates may possibly arise because of the blank ferricyanide reduction rate in the present studies.

The absolute specificity of YLD towards L-α-hydroxyl groups seems to be somewhat in doubt, since L(+)-tartrate is slowly oxidised by YLD and L(+)-malate is an extremely potent inhibitor of lactate oxidation by YLD. Both these compounds have D-α or L-β configurations. Malate occupies an anomalous position in its ability to inhibit lactate oxidation by YLD, both with respect to its optical configuration, and to the presence of two carboxyl groups.

The oxidation of lactate by YLD and ferricyanide is affected by ionic strength and by the nature of the anion present (p.157). This can either be brought about by a direct effect on the active site of YLD or by an effect which favours the transfer of lactate from the bulk phase to the active surface of the enzyme. Since specific ions and ionic strength affect the stability of complexes of the apo-enzyme with the appropriate nucleotides in old yellow enzyme and liver and yeast alcohol dehydrogenases (Theorell and Nygaard, 1954; Theorell, Nygaard and Bonnichsen, 1955), it appears likely that a similar effect might explain the results obtained with YLD.

Table 38.

Substrates and Inhibitors of YLD.

(From Dikstein, 1959).

Substances that are substrates for YLD.	Relative maximum velocity.	Substances that are not substrates but inhibitors of YLD.	I_{50}	Substances that are neither substrates for nor inhibitors of YLD.
L-lactate	1.00	D-lactate	0.15 M	iso-propanol
DL-chlorolactate	0.35	DL-mandelate	2×10^{-3} M	DL-glyceraldehyde
DL-Fluorlactate	0.10	DL-phenylpyruvate	10^{-2} M	glycerol
DL-α-hydroxydecanoate	0.30	DL-phenylalanine	1.0 M	propylene glycol
glycollate	0.04	p-toluenesulphonate	0.1 M	glucose
DL-glycerate	0.03	formate	6 M	serine
DL-iso-citrate	0.01	acetate	2.3 M	alanine
L-malate	0.01	propionate	0.8 M	ethyl lactate
DL-tartrate	0.01	butyrate	0.3 M	
		valerate	0.12 M	
		caprylate	0.01	
		β-hydroxy butyrate	Not determined	
		DL-citrate	Not determined	

The kinetic results suggest that the enzyme-substrate complex directly involves the flavin prosthetic group of YLD. Hasegawa and Ogura (1961) demonstrated that the flavin prosthetic group of YLD is fully reduced in reduced YLD. Since ferricyanide is reduced by YLD prior to the dissociation of pyruvate, it seems probable that the reduced flavin prosthetic group is an enzyme-lactate complex of the type discussed earlier (p. 143).

For efficient transfer of reducing equivalents from the flavin-substrate complex to the haem prosthetic group, it is essential that the haem group must be sufficiently close to the flavin group to permit intramolecular interaction of the two prosthetic groups, since it is clear that intermolecular transfer of reducing equivalents takes place far too slowly to be of any significance in the reduction of cytochrome c by the enzyme (Yamashita et al. 1958, see p. 51). For reasons discussed earlier (p. 216), it seems probable that two such flavin-haem pairs are present in close juxtaposition at the active site of YLD.

4. Binding of the Prosthetic Groups of Yeast Lactate Dehydrogenase.

The splitting of the prosthetic group of YLD from the protein by acidified acetone and by acidified methyl ethyl ketone suggests that the nature of the binding between the haem and the protein is similar to that found in other proteins such as haemoglobin, peroxidase, catalase and cytochrome b_5, and that most probably only the ligands from the protein to the 5 and 6 coordination valencies of the haem iron are involved (p. 103).

Because of the similarity of the spectrum of reduced YLD with the spectra of the haemochromes of protohaem and heterocyclic bases, it seems likely that strongly basic ligands occupy the 5 and 6 coordination valencies of the haem of YLD; the most probable ligands are histidine residues, of which YLD has six per haem. The spectrum of the haem prosthetic group in methyl ethyl ketone/HCl is further evidence that it is protohaem.

The binding between FMN and protein differs from that in old yellow enzyme, in that a direct interaction between a sulphydryl group and the iso-alloxazine nucleus apparently occurs (Armstrong, Coates and Morton, 1960). Such a linkage was suggested by Appleby and Morton (1959a) from studies on the inactivation of YLD by oxygen and from the inhibition of the enzyme by PCMB but not by iodoacetate. The displacement of the flavin prosthetic group by PCMS and by oxygen, the formation of heavier sedimenting components in the presence of oxygen but not in the presence of PCMS (p.201) and the irreversible dissociation of flavin (Appleby and Morton, 1959a) are evidence that a thiol group is involved in the binding of FMN by YLD, and that the irreversible flavin dissociation is due to the formation of intermolecular disulphide bonds. It has been demonstrated by kinetic studies (Yagi and Ozawa, 1959) that the combination of FAD with the apo-protein of D-amino acid oxidase involves a sulphydryl group and the adenine moiety of FAD. Since the prosthetic group of D-amino acid oxidase can be reversibly dissociated, it is reasonable to assume that the thiol group is protected against oxidation to an intermolecular disulphide bridge. This protection may be steric or may be due to the influence of other groups on the reactivity of the sulphydryl group; for example,

the difference in the rate of autoxidation between cysteine and glutathione may be cited. Although the adenine moiety of FAD is involved in the bonding between protein and flavin nucleotide, the similarity between this situation and that observed with YLD is close. Moreover, it is highly probable that the electronic systems of the flavin and adenine moieties are in mutual interaction, because of the differences in spectrum and fluorescence intensity between FMN and FAD. It is also most likely that the adenine moiety of FAD is closest to the protein surface in D-amino acid oxidase, or for any FAD protein, so that the flavin moiety, which presumably lies in a parallel plane, is accessible to the substrate. At present there is no information available on the role of sulphydryl groups in the binding of flavin in other flavoproteins, and in old yellow enzyme such a binding is not possible since there are no free sulphydryl groups in old yellow enzyme.

The nature of the interaction between flavin and sulphydryl group is not known. It has been suggested by Appleby and Morton (1959b) that a hydrogen bond between the sulphydryl group and the imino nitrogen of the isoalloxazine nucleus might be formed, by analogy with the proposed hydrogen bond between tyrosine hydroxyl and imino nitrogen in old yellow enzyme (Theorell and Nygaard, 1954). An alternative possibility is that the sulphydryl group is dissociated to the negatively charged sulphydryl ion, and that an ion dipole interaction occurs between the positive imino dipole of isoalloxazine and the sulphydryl ion. It also seems probable that phosphate-amino ion pair formation is involved in the bonding of FMN to YLD protein, as is the case in old yellow enzyme. Such weak forces as these

would account for the pH lability of YLD, which is stable only over the range pH 5 to pH 9 (Appleby and Morton, 1959; Horio, Yamashita and Okunuki, 1959). Below pH 5 both the sulphydryl group of the protein and the phosphate of the FMN would exist largely in the undissociated forms, and above pH 9 the protein amino groups would become uncharged and the flavin imino nitrogen would react with hydroxyl ion. Although no critical experiments have been carried out to confirm the following hypothesis, it was observed in a number of experiments that YLD seemed much less stable in the presence of oxygen when the concentration of chloride ion was high. It is believed that this may be due to a specific effect of Cl^- in increasing the dissociation of FMN from YLD. This suggestion receives some support from the experiments on the variation of the rate of ferricyanide reduction with ionic strength (p.157), where, although a stimulation of the initial rate of ferricyanide reduction by Cl^- was observed, the rate of reduction decreased with time. Boeri and Tosi (1956) found that Cl^- had a greater effect on decreasing the lactate-cytochrome c reductase activity of YLD than phosphate ions.

5. Mechanism of Acceptor Reduction.

It is immediately apparent from the Lineweaver-Birk plots of reciprocal initial velocity against reciprocal lactate concentration at different acceptor concentrations that the reduction of ferricyanide and cytochrome c by YLD involve different mechanisms (see p.146a). It is reasonable to assume that this implies that these two acceptors are reduced at different sites on YLD. By analogy with the microsomal DPNH cytochrome

c reductase system of animals and with the intact mitochondrial respiratory chain, it was suggested by Appleby and Morton (1954) that cytochrome c oxidised YLD at the haem prosthetic group, and that although ferricyanide is a non-specific oxidising agent, that it reacted with the lower potential flavin prosthetic group. This hypothesis seems to be supported by the detailed kinetic analysis of mechanism for the general form of the steady state rate equations. The steady state rate equation for ferricyanide reduction corresponds to one of the two general forms which might be expected for a flavoprotein system (see p.150) and shows that ferricyanide is oxidising an enzyme-lactate complex. The steady state rate equation for cytochrome c reduction shows that the product (pyruvate) is dissociated prior to the reduction of cytochrome c, which reacts with a reduced form of the enzyme. It is suggested that this reduced form of the enzyme is the haem prosthetic group of YLD; this seems more probable than a reduced form of the flavin, since ferricyanide oxidised a flavin-lactate complex. The reduction of ferricyanide by YLD resembles the acceptor reduction observed with soluble DPNH-cytochrome c reductase from mitochondria (Frieden, 1957), glucose oxidase (Ogura, 1952) and notatin (Laser, 1952). It must be admitted that cytochrome c reduction by YLD is similar to the reduction of acceptor observed with two flavoproteins, D-amino acid oxidase and lipoyl dehydrogenase, but with these two enzymes there is evidence which suggests that intramolecular oxidation of the enzyme-substrate complex by another group occurs. In the case of lipoyl dehydrogenase, this second group seems to be a disulphide bond, and in D-amino acid oxidase, another flavin group. The analogy between these two enzymes and YLD is further evidence that the haem is involved in electron transport to cytochrome c in YLD.

Although YLD is the only well characterised enzyme in which two prosthetic groups appear to be associated with a single protein, there have been preliminary reports which suggest that there are other flavohaemoproteins than YLD. Hauge (1960) has reported a glucose dehydrogenase which he believes is a flavohaemoprotein, and Linnane (1960) has suggested that the formate dehydrogenase of E. coli is a flavohaemoprotein. A more critical appraisal of these two reports must await publication of detailed studies.

6. Significance of Yeast Lactate Dehydrogenase in Relation to Physiological Electron Transport.

The analogy between YLD and the insoluble electron transport systems of mitochondria and microsomes is obvious. Although these latter systems involve a number of separate components, in the cell they may be regarded as single functional units. Microsomal DPNH-cytochrome c reductase and YLD have marked similarities in that the mediation of a haem group between flavin and cytochrome c is necessary for electron transport. The similarity between YLD and the respiratory chain is not so strong. Available evidence suggests that ubiquinone acts as an intermediate in electron transport between flavoprotein dehydrogenases and cytochrome b, which is however, directly linked to cytochrome c_1. The respiratory chain is blocked by antimycin A, the inhibition occurring between ubiquinone and cytochrome b. This may possibly explain the insensitivity of YLD (Appleby and Morton, 1959) and microsomal DPNH cytochrome c reductase (Strittmatter and Velick, 1956; Martin and Morton, 1955) to antimycin A, since the coupling between

flaveprotein dehydrogenase and cytochrome b_5 does not require an intermediate such as ubiquinone, while an intramolecular electron transfer from the flavin to haem prosthetic group of YLD is indicated by the present study. The importance of YLD in relation to the insoluble electron transport systems is that it provides a soluble system in which a particular steric relationship between flavin and haem groups is maintained, and therefore provides a model system for the study of the behaviour of electron transport systems.

VII. GENERAL CONCLUSION.

This thesis describes some of the properties of two haemoproteins from baker's yeast. Both were obtained in true solution and were extensively purified. One of these haemoproteins, cytochrome *c*, has been regarded as the reference protein when considering other cytochromes. Cytochrome *c* from heart-muscle in particular has been well described by other workers. The present studies show some of the differences between cytochrome *c* of heart-muscle and cytochrome *c* of baker's yeast.

Since the finding by Appleby and Morton (1954) that crystalline yeast lactate dehydrogenase had both a flavin and a haem prosthetic group, there has been considerable controversy as to whether this enzyme should be regarded as a true cytochrome. As indicated earlier (p. 47), Bach, Dixon and Keilin (1942) gave the name 'cytochrome b_2' to a haemoprotein associated with yeast lactate dehydrogenase. The subsequent studies of Appleby and Morton (1954, 1959, 1960) and of Beeri and Tosi (1956), and as described in this thesis, establish that YLD is a haemoprotein with a flavin prosthetic group. The haem prosthetic group is firmly bound to the protein, as in other cytochromes; however, the flavin prosthetic group is bound by relatively weak forces. This raises the question of nomenclature discussed earlier (p.49) as to whether yeast lactate dehydrogenase is a cytochrome. The present work confirms the viewpoint that yeast lactate dehydrogenase must be regarded as a true cytochrome. The special feature of cytochrome b_2 is that it has enzymic properties conferred by the flavin prosthetic group and that these properties are distinct from, but related to the electron-transferring property conferred by the haem prosthetic group. The ability

to activate a specific substrate (lactate) should not be regarded as anomalous behaviour for a cytochrome; the alternative viewpoint may have been due to the extensive use of cytochrome c as a model for the properties of other cytochromes.

APPENDIX I.

Detailed Steady State Rate Equations.

The equations presented are deliberately given for the simplest possible mechanism of each type, however the same generalisations hold for more elaborate forms of the mechanisms, although the composition of the Michaelis parameters is different. Apparent rate parameters are denoted by a prime.

1. i. Simple Binary Complex.

Two electron acceptor (Chance (1943).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \; ; \quad ES + A \xrightarrow{k_3} E + P + H_2A$$

$$v = \frac{V}{1 + \frac{K_S}{[S]}}$$

$$V = k_3 [E] [A] = V'_S \, , \quad V'_A = k_1 [E] [S] \, , \quad K'_S = \frac{k_2 + k_3 [A]}{k_1} = \frac{k_2}{k_1} + \frac{V'_S}{k_1 [E]}$$

$$K'_A = \frac{k_1 [S] + k_2}{k_3} = \frac{k_2}{k_3} + \frac{V'_A}{k_3 [E]}$$

V'_S V'_A, K'_S and K'_A approach ∞ as $[A]$ or $[S]$ approach ∞ and are linear functions of $[A]$ or $[S]$.

One electron acceptor (Original).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \; ; \quad ES + A \xrightarrow{k_3} E^* + \text{"HA"} \; ; \quad E^* + A \xrightarrow{k_5} E + \text{"HA"}$$

E^* may be ES^ϕ or E^ϕ (P has dissociated).

$$v = \frac{V}{1 + K + \frac{K_s}{[S]}}$$

$V = k_3[E][A]$ (or $k_5[E][A]$), $K = \frac{k_3}{k_5}$ (or $\frac{k_5}{k_3}$), $V'_S = \frac{k_3 k_5 [E][A]}{k_3 + k_5}$,

$V'_A = k_1[E][S]$, $K_S = \frac{k_2 + k_3[A]}{k_1}$ (or $\frac{k_5(k_2 + k_3[A])}{k_1 k_3}$),

$K'_S = \frac{k_5(k_2 + k_3[A])}{k_1(k_3 + k_5)} = \frac{k_2 k_5}{k_1(k_3 + k_5)} + \frac{V'_S}{k_1[E]}$,

$K'_A = \frac{k_1[S](k_3 + k_5) + k_2 k_5}{k_3 k_5} = \frac{V'_A(k_3 + k_5)}{k_3 k_5 [E]} + \frac{k_2}{k_3}$.

V, K_S, V'_S, V'_A, K'_S and K'_A are linear functions of $[A]$ or $[S]$ and have no finite limit value. $\frac{K'_S}{V'_S}$ (or $\frac{K'_A}{V'_A}$) is a function of $[A]$ (or $[S]$).

Although $[A]$ appears only in K_S and V in the rate equations, Michaelis Menten kinetics are followed for A at fixed concentrations of S.

ii. Two Binary Complexes. (Theorell and Chance, 1951).

Two electron acceptor.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES;\ ES + A \overset{k_3}{\rightleftharpoons} E^* + B;\ E^* \xrightarrow{k_5} E + C$$

(B or C may be either P or H_2A; however, if the breakdown of E^* is rate limiting, then the steady state equation will have to allow for the effect of $[B]$ on the velocity).

$$v = \frac{V}{1 + \frac{K_S}{[S]} + \frac{K_A}{[A]} + \frac{K_{AS}}{[S][A]}}$$

where $V = k_5 [E]$; $K_S = \frac{k_5}{k_1}$; $K_A = \frac{k_5}{k_3}$; $K_{AS} = \frac{k_2 k_5}{k_1 k_3}$.

$$V'_S = \frac{V}{1 + \frac{K_A}{[A]}} = \frac{k_5 [E]}{1 + \frac{k_5}{k_3 [A]}} ; \quad V'_A = \frac{V}{1 + \frac{K_S}{[S]}} = \frac{k_5 [E]}{1 + \frac{k_5}{k_1 [S]}}$$

$$K'_S = \frac{K_S + \frac{K_{AS}}{[A]}}{1 + \frac{K_A}{[A]}}$$

$$K'_A = \frac{K_A + \frac{K_{AS}}{[S]}}{1 + \frac{K_S}{[S]}}$$

By Slater's method, the plot of K'_s vs. V'_s has a slope $\frac{1}{k_1 [E]}$ and intercept $\frac{k_2}{k_1}$ while K'_A vs. V'_A has a slope $\frac{1}{k_3 [E]}$ and intercept $\frac{k_2}{k_3}$. It should be noted that the term $\frac{K_{AS}}{[A] \text{ or } [S]}$ tends to keep K'_S and K'_A large at low concentrations of A and S, respectively, and to modify the hyperbolic term in [A] or [S] considerably at low concentrations.

<u>One-electron acceptor</u> (requires 3 binary complexes to avoid terms in $[A]^2$).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \; ; \quad ES + A \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EB + HA \; ; \quad EB + A \xrightarrow{k_5} ED + HA$$

$$ED \xrightarrow{k_7} E + P.$$

$$v = \frac{V}{1 + \frac{K_S}{[S]} + \frac{K_A}{[A]} + \frac{K_{AS}}{[A][S]}}$$

$$V = k_7 [E] ; \quad K_S = \frac{k_7}{k_1} ; \quad K_A = \frac{k_7 (k_3 + k_5)}{k_3 k_5} ; \quad K_{AS} = \frac{k_2 k_7}{k_1 k_3} .$$

$$V'_S = \frac{V}{1 + \frac{K_A}{[A]}} \qquad V'_A = \frac{V}{1 + \frac{K_S}{[S]}}$$

$$K'_S = \frac{K_S + \frac{K_{AS}}{[A]}}{1 + \frac{K_A}{[A]}} \qquad K'_A = \frac{K_A + \frac{K_{AS}}{[S]}}{1 + \frac{K_S}{[S]}}$$

Slater Plots K'_S vs. V'_S : slope = $\frac{1}{k_1 [E]}$, intercept = $\frac{(k_2 + k_5)}{k_1 (k_3 + k_5)}$

K'_A vs. V'_A : slope = $\frac{k_3 + k_5}{k_3 k_5 [E]}$ intercept = $\frac{k_2}{k_3}$

iii. Ternary Complex (Ogura, 1952).

Two Electron Acceptor.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES ; \quad ES + A \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ESA \xrightarrow{k_5} E + P + H_2A$$

$$v = \frac{V}{1 + \frac{K_S}{[S]} + \frac{K_A}{[A]} + \frac{K_{AS}}{[A][S]}}$$

$$V = k_5 [E] ; \quad K_S = \frac{k_5}{k_1} ; \quad K_A = \frac{k_4 + k_5}{k_3} ; \quad K_{AS} = \frac{k_2 (k_4 + k_5)}{k_1 k_3} .$$

$$V'_S = \frac{V}{1 + \frac{K_A}{[A]}} \; ; \quad V'_A = \frac{V}{1 + \frac{K_S}{[S]}} + \quad V'_{lim.} = V$$

$$K'_S = \frac{K_S + \frac{K_{AS}}{[A]}}{1 + \frac{K_A}{[A]}} \qquad (K'_S)_{lim.} = K_S$$

$$K'_A = \frac{K_A + \frac{K_{AS}}{[S]}}{1 + \frac{K_S}{[S]}} \qquad (K'_A)_{lim.} = K_A$$

Slater Plots

K'_S vs. V'_S : slope $\frac{1}{k_1 [E]}$, intercept = $\frac{k_2}{k_1}$

K'_A vs. V'_A : slope $\frac{1}{k_3 [E]}$, intercept = $\frac{k_2 k_4 + k_5 (k_2 + k_4)}{k_3 k_5}$

$\frac{K'_S}{V'_S}$ and $\frac{K'_A}{V'_A}$ are functions of $\frac{1}{[A]}$ and $\frac{1}{[S]}$ respectively.

<u>One Electron Acceptor (original).</u>

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \; ; \quad ES + A \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ESA \xrightarrow{k_5} E^* + \text{"HA"}; \quad E^* + A \xrightarrow{k_7} E + \text{"HA"}$$

$$v = \frac{V}{1 + \frac{K_S}{[S]} + \frac{K_A}{[A]} + \frac{K_{AS}}{[A][S]}}$$

$$V = k_5 [E] : \quad K_S = \frac{k_2}{k_1} ; \quad K_A = \frac{k_5 (k_3 + k_7) + k_4 k_7}{k_3 k_7} ; \quad K_{AS} = \frac{k_2 (k_4 + k_5)}{k_1 k_3}$$

$$V'_S = \frac{V}{1 + \frac{K_A}{[A]}} \; ; \quad V'_A = \frac{V}{1 + \frac{K_S}{[S]}} \qquad (V')_{lim.} = V$$

$$K'_S = \frac{K_S + \frac{K_{AS}}{[A]}}{1 + \frac{K_A}{[A]}}, \quad (K'_S)_{lim.} = K_S$$

$$K'_A = \frac{K_A + \frac{K_{AS}}{[S]}}{1 + \frac{K_S}{[S]}}, \quad (K'_A)_{lim.} = K_A$$

$\frac{K'_s}{V'_s}$ is a function of $\frac{1}{[A]}$; $\frac{K'_A}{V'_A}$ is a function of $\frac{1}{[S]}$

Slater plots.

K'_S vs. V'_S : slope $= \frac{1}{k_1[E]}$, intercept $= \frac{k_2 k_7 (k_4 + k_5)}{k_1 \{k_4 k_7 + k_5 (k_3 + k_7)\}}$

K'_A vs. V'_A : slope $= \frac{k_3 + k_7}{k_3 k_7 [E]}$, intercept $= \frac{k_2 (k_4 + k_5)}{k_3 k_5}$

2. Product Dissociates before Reaction with Acceptor.

Two electron acceptor (Goldman, 1953, Alberty, 1956).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E'' + P ; \qquad E'' + A \xrightarrow{k_5} E + H_2A$$

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_S}{[S]}}$$

$V = k_3[E]$; $K_A = \frac{k_3}{k_5}$; $K_S = \frac{k_2 + k_3}{k_1}$.

$$V'_S = \frac{V}{1 + \frac{K_A}{[A]}} ; \quad V'_A = \frac{V}{1 + \frac{K_S}{[S]}} ; \quad (V')_{lim.} = V .$$

$$K'_S = \frac{K_S}{1 + \frac{K_A}{[A]}}, \qquad (K'_S)_{\text{lim.}} = K_S .$$

$$K'_A = \frac{K_A}{1 + \frac{K_S}{[S]}}, \qquad (K'_A)_{\text{lim.}} = K_A .$$

$\frac{K'_S}{V'_S}$ and $\frac{K'_A}{V'_A}$ are independent of [A] and [S] respectively.

Slater Plots.

K'_S vs. V'_S : slope = $\frac{1}{k_1 [E]}$ intercept = o.

K'_A vs. V'_A : slope = $\frac{1}{k_5 [E]}$ intercept = o.

A mechanism allowing for a binary complex between E" + A has been developed (Alberty, 1956) and gives a rate equation of the same form.

One electron acceptor (original).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E" + P ; \quad E" + A \xrightarrow{k_5} E' + "HA" ; \quad E' + A \xrightarrow{k_7} E + "HA"$$

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_S}{[S]}}$$

$$V = k_3[E]; \quad K_A = \frac{k_3 (k_5 + k_7)}{k_5 k_7} ; \quad K_S = \frac{k_2 + k_3}{k_1}$$

$$V'_S = \frac{V}{1 + \frac{K_A}{[A]}}, \quad V'_A = \frac{V}{1 + \frac{K_S}{[S]}}, \quad (V')_{\text{lim.}} = = V .$$

$$K'_S = \frac{K_S}{1 + \frac{K_A}{[A]}} ; \qquad (K'_S)_{lim.} = K_S .$$

$$K'_A = \frac{K_A}{1 + \frac{K_S}{[S]}} ; \qquad (K'_A)_{lim.} = K_A .$$

$\frac{K'_S}{V'_S}$ and $\frac{K'_A}{V'_A}$ are independent of [A] and [S] respectively.

Slater Plots.

K'_S vs. V'_S : slope = $\frac{1}{k_1 [E]}$ intercept = o

K'_A vs. V'_A : slope = $\frac{(k_5 + k_7)}{k_5 k_7 [E]}$ intercept = o

APPENDIX II.

The Extinction Coefficient of 2,6 dichlorophenol-indophenol.

There are considerable discrepancies in the values for the molar extinction coefficient dichlorophenol-indophenol (DCPIP) at 600 mμ. (Merrill, Spencer and Getty, 1948; Singer and Kearney, 1957, Basford and Huennekens, 1954; Crane, Mii, Hauge, Green and Beinert, 1956; Savage, 1957; Steyn-Parve and Beinert, 1959). It was therefore necessary to determine the extinction coefficient of this compound for the kinetic studies presented elsewhere in this thesis. Initially the measurements were carried out with DCPIP dissolved in water, however somewhat variable results were obtained, and it was realised that since DCPIP is a pH indicator that control of pH was necessary.

Materials and Methods.

DCPIP. A sample of BDH DCPIP (Na salt) was dried for 24 hours at 80° and 20 mm over P_2O_5 and kept over P_2O_5. It was assumed that this removed any water of crystallisation. A stock solution, $3.39_6 \times 10^{-4}$M was made up in water. This was diluted 1 in 10 in buffers of ionic strength 0.1.

Buffers. Phosphate buffers were used for pH 3.0, pH 5.7, pH 6.2, pH 7.2 and pH 7.9. Acetate buffers were used for pH 3.9, pH 4.7 and pH 5.2. Pyrophosphate/HCl buffer was used for pH 8.4, and carbonate buffers were used for pH 10.4 and pH 11.0.

Spectrophotometry.

This was carried out in 1 cm. matched quartz cuvettes in an Optica CF4 grating spectrophotometer. The wavelength and optical density scales of the spectrophotometer had been calibrated. All measurements were made at 26 ± 0.2°.

pH. The pH of the solutions was measured with a Cambridge null point pH meter, having a universal glass electrode and sleeve-type, saturated calomel electrode. The glass electrode was checked against standard potassium hydrogen phthalate and sodium borate solutions, and was found to be satisfactory. All measurements were made at 26°.

Results.

Figure 40 shows the variation of extinction coefficient with pH for DCPIP. Below pH 3.0, rapid destruction of DCPIP took place. It was therefore not possible to obtain a value for the extinction coefficient of unionised DCPIP. (ε_{HA}). The increase in optical density above pH 10 suggests that hydroxyl attack on DCPIP ion takes place. Within the range pH 4 to pH 10, the optical density did not change with time. The optical density at pH 3 was obtained by extrapolation of a series of readings to zero time.

Calculation of ε_{HA}.

An approximate value of pK' (= 5.9) was obtained by inspection of Figure 40. The extinction coefficients at pH 3.90, pH 4.66 and pH 8.37 were used. Calculation from the assumed pK and the extinction coefficients at pH 3.9 and pH 8.37 gave a value of 2.56×10^3 cm.$^{-1}$ M^{-1} of ε_{HA}, and for pH 4.66 and pH 8.37, 2.44×10^3 cm.$^{-1}$ M^{-1}. The extinction coefficient at pH 8.37 was assumed to be that of DCPIP ion. A variation in pK' of ± 0.3

Table 39.

Spectrophotometric determination of pK' for dichlorophenol-indophenol.

Calculated from $(\varepsilon_{600m\mu})_{pH}$ for $(\varepsilon_{600m\mu})_{HA} = 2.50 \pm 0.11 \times 10^3$ cm.$^{-1}$ M^{-1}, and $(\varepsilon_{600m\mu})_{A'} = 20.88 \pm 0.13 \times 10^3$ cm.$^{-1}$ M^{-1} using $pK' = pH - \log \frac{\varepsilon - \varepsilon_{HA}}{\varepsilon_{A'} - \varepsilon}$ (Flexser, Hammett and Dingwall, 1935).

pH	pK'
3.91	5.86
4.66	5.92
5.16	5.88
5.69	5.91
6.20	5.89
6.65	5.91
7.26	6.09
7.93	5.99
8.37	6.17

$\overline{pK'}$ (pH 5.16 to pH 6.65) = $5.89 \pm 0.02_5$

affects $\varepsilon_{A'}$ by 0.6% and ε_{HA} by 4.7%.

Extinction Coefficient of DCPIP ion.

The extinction coefficient for DCPIP ion was found to be 20.88 ± 0.13 x 10^3 $cm.^{-1}$ M^{-1}. At pH 8.0, the extinction coefficient was 20.74 ± 0.12 x 10^3 $cm.^{-1}$ M^{-1}. This figure was used in calculating the molarity and rate of reduction of DCPIP by YLD and lactate (p. 156).

Calculation of pK'.

This was calculated from the extinction coefficients according to Flexner, Hammett and Dingwall (1935). Table 39 gives the calculated values of pK' for ε_{HA} = 2.50 ± 0.11 x 10^3 $cm.^{-1}$ M^{-1}, and $\varepsilon_{A'}$ = 20.88 ± 0.13 x 10^3 $cm.^{-1}$ M^{-1}. Since errors in ε_{HA} and $\varepsilon_{A'}$ have a large effect on pK' when the differences between these values and ε_{pH} are small, only the values within about 0.7 pH units of pK' have less than 1% variation due to errors in ε_{HA} and $\varepsilon_{A'}$. Using these values pK' = $5.89 \pm 0.02_5$.

If the activity corrections given by Edwards (1953) are used, then pK may be calculated; a value of 6.02 ± 0.03 is obtained.

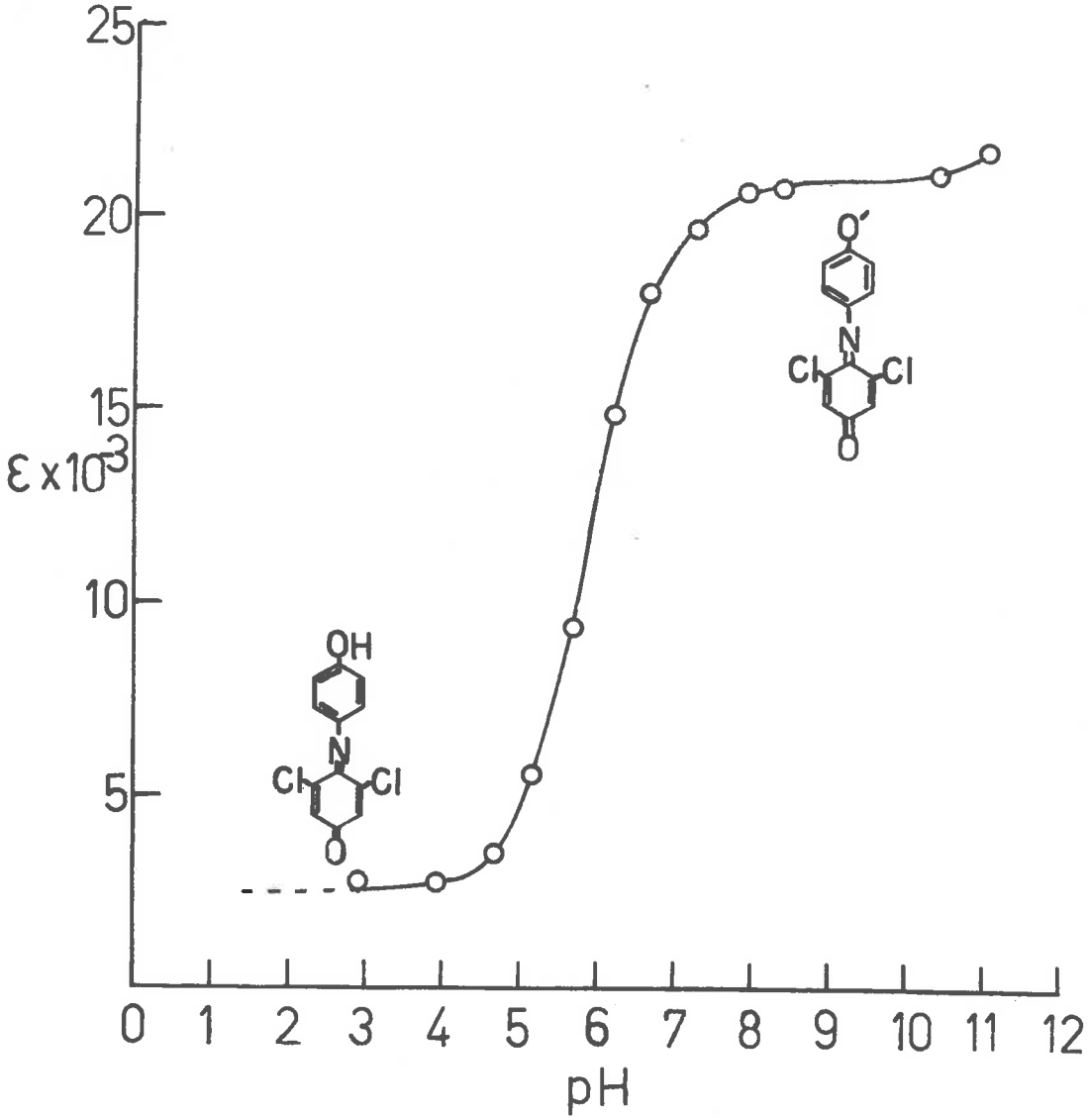
Discussion.

The range of values reported in the literature for the extinction coefficient of DCPIP at 600 mμ extends from 16 x 10^3 $cm.^{-1}$ M^{-1} to 21 x 10^3 $cm.^{-1}$ M^{-1} over pH values ranging from pH 6.5 to pH 8.0. The very careful measurements of Stayn-Parve and Deinert (1959) give a value of $\varepsilon_{600m\mu}$ = 21.0 ± 0.3 x 10^3 $cm.^{-1}$ M^{-1} at pH 6.98 and 20° to 30°. Savage (1957) found that $\varepsilon_{600m\mu}$ was 21 x 10^3 $cm.^{-1}$ M^{-1} at pH 8.0 for a carefully purified and standardised sample of DCPIP. This value is very close to that predicted

Fig. 40. Spectrophotometric titration curve for 2,6-dichlorophenol indophenol at 600 mμ

Ordinate, molar extinction coefficient, $cm.^{-1}$ $mole^{-1}$.

Abscissa, pH of 0.1 ionic strength buffers.


25
20
15
ε x10³
10
5
OH
N
Cl
Cl
O
O
N
Cl
Cl
O
0 1 2 3 4 5 6 7 8 9 10 11 12
pH

in the present study for pH 8.0 ($20.73 \pm 0.13 \times 10^3$ cm.$^{-1}$ M^{-1}). The predicted value for pH 6.98 is $19.14 \pm 0.12 \times 10^3$ cm.$^{-1}$ M^{-1}. Rafter and Colowick (1957) reported $\varepsilon_{525m\mu} = 6.8 \times 10^3$ cm.$^{-1}$ M^{-1}, as compared with $\varepsilon_{522m\mu} = 7.69 \pm 0.05 \times 10^3$ cm.$^{-1}$ M^{-1} obtained in the present study for the pH independent isosbestic point in the spectrum of DCPIP.

Cohen, Gibbs and Clark (1924) found pK' = 5.7 for DCPIP by electrometric titration as compared with pK' = 5.89 in the present study.

Appendix III

The Molecular Weight of Cytochrome b_2

by J. McD. Armstrong, J. H. Coates and R. K. Morton

It was indicated earlier in this thesis that attempts to establish the molecular weight of cytochrome b_2 by methods using the principle of approach to sedimentation equilibrium were unsuccessful. It was therefore decided to estimate the molecular weight by separate determination of the static diffusion coefficient and the sedimentation coefficient. The first attempt to determine the static diffusion coefficient was unsucessful (p. 212); further determinations are reported here.

The determination of the static diffusion coefficient was made by Dr. J. H. Coates and Prof. R. K. Morton. Three times recrystallised cytochrome b_2 (containing the DNA component) was dialysed against 0.3 M-sodium lactate-0.05 M-tetrasodium pyrophosphate- 10^{-4} M-EDTA, pH 6.8, under nitrogen and in the absence of oxygen, at about 0°C on a rocking dialyser. The static diffusion coefficient was determined using a modified Spinco Model H electrophoresis-diffusion apparatus. Because of the high light absorption in the green region of the spectrum exhibited by cytochrome b_2 , the mercury arc light source 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. Rayleigh interference optics could not be used because of the high light absorption of cytochrome b_2 even at the wavelength of the sodium D line. The accuracy of the optical system was verified by measuring the diffusion coefficient (D_A) of a sucrose solution

concentration 0.750 gm. per 100 ml. of water, at a temperature of 1.44°C. The value obtained was 2.37×10^{-6} cm.^{2}sec.$^{-1}$ when corrected to 1°C in water. This may be compared with a value of 2.408×10^{-6} for D_{c-} derived from the results of Gosting and Akeley (1952)* for the same concentration increment and a temperature of 1°C. The cell was loaded with cytochrome b_2 in an atmosphere of oxygen-free nitrogen to prevent oxidative modification of the enzyme. Sedimentation coefficients were determined as previously described. The diffusion coefficient was also estimated by the procedure of Baldwin (1957a) from the sedimentation diagrams, as described elsewhere in this thesis.

The results are shown in the accompanying table. It is seen that the latter two experiments gave values which were in good agreement with one another. From these, the molecular weight is estimated as 172,000. Since the minimum molecular weight, from analysis of flavin and of haem is 80,000, it is clear that there are two flavin and two haem groups per molecule of enzyme.

It will be observed that the first experiment in this series gave an elevated value for the molecular weight (210,000). This preparation was dialysed for only 30 hours; it is rejected from the mean value since there was discrepancy in the extrapolation to zero time in the estimation of the static diffusion coefficient.

The values of the diffusion coefficient, calculated by Armstrong from the sedimentation data for the same samples of cytochrome b_2 used in the determination of the static diffusion coefficient, do not agree with the latter values. The explanation of this discrepancy is not known.

* Gosting, L. J., and Akeley, D. F. (1952). J. Amer. Chem. Soc. 74, 2058.

It would appear that the time of dialysis is a critical factor in the estimation of the diffusion coefficient of cytochrome b_2. A minimum of 48 hours, and preferably longer, is desirable. In the early studies reported in this thesis, long periods of dialysis were avoided to prevent loss of enzymic activity. In fact, as shown by the work reported here, the enzyme may be dialysed for these long periods without loss of activity provided that oxygen is rigidly excluded from the system.

Table (Appendix III)

Determination No.	Activity (µmoles ferricyanide/ hour/ mg. at 25°)	Protein concn. (mg./ml.)	$S_{20,w}$ x 10^{13} (sec.)	$D_{20,w}$* x 10^{7} ($cm.^2 sec.^{-1}$)	Molecular** Weight	$D_{20,w}$ ‡ x 10^{7} ($cm.^2 sec.^{-1}$)	Hours of dialysis
1	8480	4.39	7.74	3.09	210,600	3.60	30
2	6135	4.40	7.72	3.86	168,000	5,51	72
3	8800	4.20	7.86	3.75	176,200	-	108
				Mean ♭	172,100		

* Static diffusion; diffusion of sucrose gave $D_{1°,w}$ = 2.37 x 10^{6} $cm.^2 sec.^{-1}$.

** Estimated using a partial specific volume of 0.71, calculated from composition.

‡ From sedimentation; previously determined value (p. 197) 6.60 x 10^{-7} $cm.^2 sec.^{-1}$.

♭ Calculated from 2 and 3. The discrepancy in values for the first determination is probably due to incomplete dialysis, since zero time in diffusion was unreasonably large.

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