CCCVIII. SOME PHYSICO-CHEMICAL CHARACTERISTICS OF THE YELLOW RESPIRATORY ENZYME.

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THE yellow respiratory enzyme isolated by Warburg & Christian [1933] is a substance of some considerable biological interest. By means of a technique involving electrophoresis, Theorell [1935] has recently purified the enzyme and reported its crystallization from solutions containing $(\text{NH}_4)_2\text{SO}_4$.

This paper is an account of some experiments carried out on a preparation supplied by Dr A. H. Theorell.

EXPERIMENTAL.

The stock material, a yellow paste consisting of enzyme and $(\text{NH}_4)_2\text{SO}_4$, was stored at approx. 0° .

For experimental purposes solutions of suitable concentration were made by dissolving small quantities of the paste in distilled water and dialysing against distilled water at 2° in cellophane tubes until no sulphate could be detected in the dialysate after 16 hours' contact with the solution.

The early stages of the dialysis were marked by the appearance of a yellow pigment in the dialysate, but this ceased to be detectable some time before a negative SO_4^- test was obtained. In the final stages of the dialysis a slight flocculent brownish precipitate usually formed; this was centrifuged off leaving a golden yellow supernatant solution.

The yellow pigment appearing in the dialysate was shown not to sediment in a centrifugal field of 180,000 times gravity and so was considered to consist of the flavin group derived by decomposition from some of the yellow enzyme, and probably previously associated with the small protein precipitate. Further slight decomposition tended to occur in the solution on standing.

For experiments in the velocity and equilibrium centrifuges quantities of the stock electrolyte-free yellow enzyme solution were diluted with buffers of suitable composition and the resulting solutions used immediately. For electrophoresis and diffusion experiments, however, the solution of yellow enzyme in buffer was dialysed against a large volume of buffer of the same electrolyte composition for 16 hours, to ensure equilibrium.

Sedimentation velocity measurements.

The sedimentation velocity experiments were carried out in a centrifugal field of approximately 180,000 times gravity, the movement of the sedimenting boundary being recorded by the absorption method [Svedberg, 1925; Svedberg & Eriksson, 1932]. A mercury vapour lamp was used as a source of light in conjunction with a nickel oxide filter ($\lambda=366 \text{ m}\mu$). A single experiment was made using the refractive index method [Lamm, 1928; 1929; McFarlane, 1935; Pedersen, 1936].

¹ Work carried out during the tenure of a Rockefeller Foundation Fellowship.

The results are given in Table I. In every case sufficient electrolyte was present in the solutions to suppress any charge effect on the sedimentation.

Table I. *The sedimentation constant of the yellow respiratory enzyme.*

Exp. no.	Buffer	Total salt conc. (M)	pH	$S_{20} \times 10^{13}$
15	HOAc, NaOAc, NaCl	0.180	4.46	5.94*†
13	HOAc, NaOAc, NaCl	0.180	4.95	5.79
10	KH ₂ PO ₄ , Na ₂ HPO ₄ , NaCl	0.300	5.72	5.72
17	KH ₂ PO ₄ , Na ₂ B ₄ O ₇ , NaCl	0.174	5.84	5.73
7	KH ₂ PO ₄ , Na ₂ HPO ₄ , NaCl	0.150	6.00	5.80
11	KH ₂ PO ₄ , Na ₂ HPO ₄ , NaCl	0.129	6.71	5.60
21	KH ₂ PO ₄ , Na ₂ HPO ₄ , NaCl	0.180	7.31	5.81
8	KH ₂ PO ₄ , Na ₂ HPO ₄ , NaCl	0.150	8.00	5.68
25	KH ₂ PO ₄ , Na ₂ B ₄ O ₇ , NaCl	0.150	8.65	5.82
27	Na ₂ B ₄ O ₇ , Na ₂ CO ₃ , NaCl	0.240	9.11	5.70
24	Na ₂ B ₄ O ₇ , Na ₂ CO ₃ , NaCl	0.240	9.65	5.93
26	Na ₂ B ₄ O ₇ , Na ₂ CO ₃ , NaCl	0.240	10.09	5.57†
22	KH ₂ PO ₄ , Na ₂ HPO ₄ , NaCl	0.242	11.48	5.63*†
19	HOAc, NaOAc, NaCl	0.183	4.46	6.14*†
20	Same solution as 19, but 24 hours later	—	—	6.38*†

Mean value of $S_{20} = 5.76 \pm 0.09 \times 10^{-13}$.

* Some decomposition.

† Omitted in calculating mean.

From the data of Table I it may be seen that the sedimentation constant, S_{20} , remains substantially constant over the pH range 5 to 9.5, which may be identified with the stability region of the enzyme. Below pH 4.5 there is a tendency for the enzyme to aggregate and for the flavin group to be liberated. At pH 4 this liberation occurs so rapidly that no sedimentation can be observed using light of wave-length 366 mμ.

The sedimentation constant $5.76 \pm 0.09 \times 10^{-13}$ is the mean of the values of S_{20} over the stability region.

In the case of Exp. 10, which was carried out by the refractive index method, the refractive increment due to the enzyme contained in the solution remained constant during the whole experiment. Moreover, the "spreading coefficient" showed no drift with time [McFarlane, 1935]. These two facts indicate that the preparation investigated was monodisperse, and this conclusion is supported by the curves obtained when the light absorption method was used.

*The diffusion coefficient.*¹

The diffusion coefficient was determined by the method of Lamm & Polson [1936] and the data were analysed by means of equation (2) of their paper and by comparison of the experimental curve with the normal dispersion curve. The data follow:

Buffer: 0.070 M KH₂PO₄, 0.010 M Na₂HPO₄, 0.1 M NaCl; pH = 5.86.

Time of diffusion hours	D cm. ² × 10 ⁻⁷ /sec.
17	6.07
27	6.01
41	6.13
Mean	6.07

$D_{\text{corr.}} = 6.28 \times 10^{-7}$ cm.²/sec.

¹ We are indebted to Mr A. G. Polson for carrying out this measurement.

The value of $D_{\text{corr.}}$ is for pure water at 20°, the correction being made by equation (10) of Lamm & Polson [1936].

Comparison with the normal dispersion curve showed the substance to be monodisperse, which is also indicated by the absence of drift in the diffusion constant with time.

The partial specific volume.¹

The partial specific volume occurs as a term in the equations used for calculating molecular weight from sedimentation data and was accordingly determined.

The determination was made on an electrolyte-free aqueous solution of the yellow enzyme at 20°. The value obtained was 0.731, which is low in comparison with most proteins, the usual range being 0.745–0.750.

The molecular weight.

It has been shown [Svedberg, 1925; 1927] that the molecular weight of a substance can be calculated from sedimentation and diffusion data by means of the equation

$$M = \frac{RTs}{D(1 - \bar{v}\rho)}$$

By substituting the values previously reported in this paper into the above equation, a value of 82,800 is obtained for the molecular weight of the yellow enzyme.

The molecular weight was also determined by sedimentation equilibrium, using the refractive index method [Lamm, 1929; Pedersen, 1936]. Owing to the small amount of material available, the refractive increment due to the enzyme in the solution used for the equilibrium experiment was estimated by carrying out a refractive index velocity experiment on the same solution. The molecular weight was then calculated from equations (12) and (15) of Pedersen's paper.

The actual determination was carried out at 20° using a phosphate buffer, pH 5.72. Two cells at different distances from the axis of rotation were used, and the equilibrium was measured for two speeds of rotation.

In Table II the data for one cell (cell A), at 120.9 rev./sec., is given in full; the remaining results are mean values. For the significance of the symbols see Pedersen [1936].

It will be observed that there is no drift in the M_{wx} values with distance from the centre of rotation. The M_{zx} values fluctuate considerably, and the observed molecular weight tends to fall as distance from the centre of rotation increases, which is the opposite of the behaviour expected for a polydisperse preparation. In general the data indicate that the substance is monodisperse.

The mean of all values obtained for the molecular weight from sedimentation equilibrium data is 77,500, which is rather lower than that calculated from the sedimentation and diffusion constants.

The mean of the values from sedimentation velocity and diffusion data and sedimentation equilibrium measurements is 80,000.

By means of a photoelectric determination of the flavin content of the yellow enzyme Theorell [1935] has estimated the molecular weight, on the assumption that the enzyme contains one flavin group in the molecule. The values obtained for several preparations vary from 70,000 to 75,000; these are in fairly good agreement with the figure just given.

¹ We are indebted to Dr A. H. Theorell for carrying out this determination.

Table II. *The molecular weight of the yellow respiratory enzyme by sedimentation equilibrium.*

Cell A; 120.9 rev./sec.					
x	$Z \mu$	$dn/dx \cdot 10^3$	$(n_1 - n_0) \cdot 10^3$	M_{wx}	M_{zx}
4.80	415	4.409	2.074	74,200	
					84,900
4.85	474	5.036	2.310	75,300	
					79,500
4.90	538	5.716	2.579	75,800	
					77,600
4.95	609	6.471	2.883	76,000	
					74,800
5.00	688	7.310	3.227	75,900	
					76,100
5.05	779	8.277	3.616	75,900	
					74,700
5.10	881	9.360	4.057	75,800	
			Mean	(M_w) 75,600	(M_z) 77,800
Cell	Rev./sec.		M_w		M_z
A	120.9		75,600		77,800
A	145.1		(64,800)		77,800
B	120.9		82,800		77,000
B	145.1		74,400		76,600
Final mean 77,500					

Electrophoretic mobility measurements.

A series of measurements of the electrophoretic mobility of the yellow enzyme was made, using the method of Tiselius [1930; *v. also* Pedersen, 1933], with the same recording conditions as for the sedimentation velocity experiments.

Measurements were made in acetate buffers (0.02 *M* NaOAc + *xM* HOAc), and phosphate buffers of constant ionic strength ($\mu = 0.02$), all the experiments being carried out at 20.0°.

Electrophoretic mobility of the yellow enzyme.

pH	Buffer	Mobility (u) cm./sec. $\cdot 10^{-5}$	Direction
4.60	Acetate	4.73	Cathodic
4.87	"	2.37	"
5.45	"	1.14	Anodic
5.41	Phosphate	1.53	"
6.14	"	4.72	"
7.45	"	8.06	"

Isoelectric point pH 5.22; slope of the mobility curve $\frac{du}{d(pH)} = 6.4 \times 10^{-5}$ at the isoelectric point.

The curves obtained showed no signs of inhomogeneity, and the value for the isoelectric point is in good agreement with that found by Theorell [1935], *viz.* 5.25, using another method.

DISCUSSION.

From a consideration of the data which have been presented it may be seen that the yellow enzyme preparation studied was monodisperse and electrochemically homogeneous. It therefore seems reasonable to conclude that the substance is a chemical entity of molecular weight 80,000.

The agreement between the molecular weight determination from ultracentrifugal data and by estimation of the flavin content of the molecule indicates that the molecule contains one flavin group.

SUMMARY.

1. Sedimentation, diffusion and electrophoresis data are presented for the yellow respiratory enzyme.

2. On the basis of the experimental results it is concluded that the preparation studied was a chemical entity of molecular weight 80,000.

3. The data indicate that the yellow enzyme contains one flavin group in the molecule.

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