Osmotic Pressures and Sedimentation Velocity of *Gastrophilus Methaemoglobin*

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In the preceding paper, Keilin & Wang (1946) have described the preparation and properties of the haemoglobin obtained from the tracheal cells of the larva of *Gastrophilus* (the bot fly). Samples from three of their preparations in the form of methaemoglobin were used for measurements of osmotic pressure. The amount of material available was small and the results recorded must be regarded as provisional.

METHODS

An account of the manufacture of collodion membranes and the method of measurement of osmotic pressures has been published elsewhere (Adair, 1925). Small cylindrical membranes of 0.6 cm. diameter were used, the permeability being about one-tenth that which had been found suitable for the serum proteins, as the more permeable membranes were found to adsorb *Gastrophilus* haemoglobin.

The equilibrating solution used was a sodium phosphate buffer mixture of ionic strength 0.1 and pH 7.4 (0.03 M- Na_2HPO_4 and 0.01 M- NaH_2PO_4). Previous investigations on sheep haemoglobin showed that an ionic strength of 0.1 is sufficient to reduce the pressure due to the unequal distribution of diffusible ions (symbolized p_i) to a very low value over the range from the isoelectric point to pH 7.4. The slightly alkaline reaction reduces the adsorption of protein by the membrane and the tendency of the protein to precipitate.

The protein concentrations were determined refractometrically. The determination of the specific refraction increments of the serum proteins in the presence and absence of salts has been described by Adair & Robinson (1930*a*). Unpublished experiments on CO-haemoglobin prepared from ox blood gave a specific refraction increment of 0.001932 for aqueous solutions and 0.001930 for solutions equilibrated with a sodium phosphate buffer mixture of ionic strength 0.1 and pH 7.4.

The quantity of *Gastrophilus* methaemoglobin available was insufficient for exact determinations of the refraction increment. A preliminary estimate of the value was made by measuring the difference between the refractive indices of water and of a dilute aqueous solution of the protein, and dividing the result obtained by the content of dry matter of the protein solution (0.48%) as determined by Keilin & Wang. A refraction increment of 0.00204 was obtained. In this paper, protein concentrations have been calculated by dividing the difference between the refractive indices of the protein solution and of the diffusate by the factor 0.00204. The estimation of protein content of the material used in Table 1, Exp. 6, agreed with a value calculated from a spectroscopic determination of the haematin content of the same solution made by Keilin & Wang.

If it be assumed that the relatively high value of 0.00204 is incorrect and the true value is 0.00193, the tabulated values for protein concentrations and molecular weights should be multiplied by the factor 1.057.

RESULTS

Ion-pressure difference and valence. In one experiment (Exp. 1, Table 1) the membrane potential was measured, and found to be -0.32 mV. The protein solution was negative. Formulae for calculating the valence of the protein (z_p) and the ion-pressure difference (p_i) for systems with very small membrane potentials have been given in a previous paper (Adair & Robinson, 1930*b*):

$$z_p = 0.0085 IM (E/C), \quad (1)$$

$$p_i = 30.76 I (E)^2, \quad (2)$$

where I denotes the ionic strength (0.10), M = molecular weight, E = membrane potential in mV. at 0° and p = pressure in mm. mercury at 0° .

The formula for the valence (1) gives the charge in equiv./mol. of protein, but to compare the valence of *Gastrophilus* haemoglobin with previous observations on mammalian haemoglobin at the same pH and ionic strength it is permissible to use the same formula to compute the valence/67,000 g. *Gastrophilus* haemoglobin. The value obtained, -9.5 , is more negative than the value -4.0 given by Adair & Adair (1940) for sheep haemoglobin, and it may be inferred that *Gastrophilus* haemoglobin is more acidic than mammalian haemoglobin. A difference of about 1.0 unit in isoelectric points would account for the difference in charge.

Molecular weights. The osmotic pressures can be correlated with the molecular weight of the protein by the formula

$$p = \phi (C/M) (170,330) (273 + t/273), \quad (3)$$

where C = g. dry protein/100 ml. solution, t = temperature in $^\circ\text{C}$. and ϕ = a factor which approaches unity when C approaches 0.

If the ratio p/C is known over a wide range of protein concentrations, an accurate value for zero concentration can be found by graphical methods as described by Adair & Robinson (1930*b*). In their work, approximately 1 g. protein was used for an estimate of molecular weight. In the case of

Gastrophilus haemoglobin, the quantity of material available (0.03 g.) was so small that only a very limited range of concentrations could be investigated, and it was not possible to evaluate molecular weights by extrapolation.

Unpublished observations made on CO-haemoglobin prepared from ox blood (molecular weight

to be suitable for the absorptiometric method; it was dissolved in buffer containing 0.2 M-NaCl, 0.02 M- Na_2HPO_4 and 0.005 M- KH_2PO_4 . The speed of rotation was 1000 rev./sec. Fig. 1 shows typical sedimentation diagrams.

The form of the boundary showed that the material was homogeneous and the rate of sedi-

Table 1. *Osmotic pressure of Gastrophilus methaemoglobin in equilibrium with 0.03 M- Na_2HPO_4 and 0.01 M- NaH_2PO_4 at 0.8°*

(Ionic strength of buffer, 0.1; pH, 7.4.)

Prep. no.	Purity from haematin content (%)	Exp. no.	Refraction	C_{204}	p (mm. Hg at 0.8°)	M/ϕ	$M (\phi_{ox}/\phi)$
1	—	1	0.00389	1.90	9.35	35,000	36,000
2	93	2	0.00373	1.83	8.77	35,000	37,000
2	93	3	0.00181	0.89	5.14	30,000	30,000
3	99	4	0.00345	1.69	9.32	31,000	32,000
3	99	5	0.00212	1.04	5.41	33,000	34,000
3	99	6	0.00149	0.73	3.82	32,000	33,000

For explanation of symbols see text.

66,000) showed that if the concentration of protein is less than 10 %, the factor ϕ_{ox} is given by the following formula

$$\phi_{ox} = 1/(1 - 0.0263 C). \quad (4)$$

The factor ϕ_{ox} increases from 1.02 to 1.05 when C increases from 0.73 to 1.90, the range covered in Table 1, which summarizes all the osmotic pressure measurements on *Gastrophilus* methaemoglobin.

In col. 4 of Table 1 refraction is given as the difference between the refractive indices of the protein solution and of the buffer with which it is in equilibrium, measured with an immersion refractometer at room temperature; col. 5 (C_{204}) gives provisional values for the protein concentrations in g. dry protein/100 ml. protein solution, computed from the measurements of refraction; col. 7 gives the ratio M/ϕ , computed by formula (1), and col. 8 the value of this ratio multiplied by ϕ_{ox} , the coefficient for ox haemoglobin computed by formula (4).

It will be noted that in the last three experiments, which were made on the purest preparation, the values for M , multiplied by (ϕ_{ox}/ϕ) , agree within the limits of experimental error.

Sedimentation in the ultracentrifuge

A sample of the methaemoglobin was examined in a Svedberg oil-turbine ultracentrifuge. Observations of the movement of the boundary were made alternately by the refractometric 'diagonalschlieren' method (Philpot, 1938) using light of wave-length 595–635 $m\mu$ and by the absorptiometric method (Philpot, 1939) with light of 525–555 $m\mu$. The concentration of the haemoglobin was 0.102 %, chosen

to be suitable for the absorptiometric method; it was dissolved in buffer containing 0.2 M-NaCl, 0.02 M- Na_2HPO_4 and 0.005 M- KH_2PO_4 . The speed of rotation was 1000 rev./sec. Fig. 1 shows typical sedimentation diagrams. The form of the boundary showed that the material was homogeneous and the rate of sedi-

Fig. 1. Sedimentation diagrams: (a) refractometric 60 min. after reaching full speed; (b) absorptiometric 80 min. after reaching full speed.

The sedimentation constant, corrected to solution in water at 20°, was 2.5×10^{-13} , measured from the refractive diagrams: the accuracy of this figure is probably $\pm 0.1 \times 10^{-13}$. The sedimentation constant

and the osmotically determined value of the molecular weight, together with an assumed value of 0.75 for the partial specific volume, allow the frictional ratio f/f_0 (Svedberg & Pedersen, 1940) to be calculated: the value obtained is 1.38. Comparison with the data for myoglobin (Theorell, 1932) shows a marked difference: myoglobin has a molecular weight of about 17,000, a sedimentation constant of 2.04×10^{-13} and is more nearly spherical having $f/f_0 = 1.1$.

SUMMARY

1. Preliminary measurements of the osmotic pressure of methaemoglobin prepared from the tracheal cells of the larva of *Gastrophilus* (the bot fly) yield a mean value of $34,000 \pm 3000$ for the molecular weight.

2. The material was found to sediment homogeneously in the ultracentrifuge, with sedimentation constant 2.5×10^{-13} .

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The Estimation of Free Choline in Plants

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Choline, being a constituent of lecithin, is probably present in every living cell. Schulze & Trier (1912*b*) and Klein & Zeller (1930) have examined more than 100 plants of widely different systematic origin and, with the exception of the three lichens examined, were able to detect free choline in all cases.

In the animal choline has lipotropic activity; it promotes phospholipin turn-over, prevents the development of fatty liver, protects against haemorrhagic kidney degeneration, and is concerned in the conversion of homocystine to methionine. Its vitamin-like action may be related to its activity as a methyl donator (du Vigneaud, Chandler, Moyer & Keppel, 1939; du Vigneaud, Chandler, Cohn & Brown, 1940; Chandler & du Vigneaud, 1940). The significance of choline in the methylation processes of higher plants has not hitherto been investigated. Klein & Linser (1932, 1933) have opened up the subject of choline metabolism in higher plants by their study of variations of lecithin and choline contents during germination of various seeds.

The present paper describes a method suitable for routine estimations of the free choline content of plant tissues in metabolic studies. Preliminary data on the distribution of free choline in the potato plant are presented.

Quantitative estimation of choline

Estimations of choline in animal tissues have been based upon the formation of the sparingly soluble choline periodide, bismuth-iodide, platinichloride, aurichloride or reineckate; upon the decomposition of choline into trimethylamine or upon acetylation followed by biological assay.

Roman (1930) has made a careful study of the conditions necessary for the quantitative precipitation of choline as periodide (ennea-iodide) and found that, taking all precautions, it was possible to estimate 0.005–5 mg. choline in pure aqueous solution with an error of less than $\pm 5\%$. The achievement of quantitative yields is, however, made a matter of considerable difficulty by variability in the composition of the precipitate, the extreme readiness with which it loses iodine and its ready solubility in potassium iodide. The application of the method to plant extracts encounters the additional difficulties that iodine will give precipitates with alkaloids and many other bases, a difficulty not satisfactorily prevented by addition of sodium bicarbonate (Schulze & Trier, 1912*a*; Vickery, 1925), and that the solubility of choline periodide is, in general, increased by the presence of salts. Methods involving potassium bismuth iodide (Jahns, 1885) as precipitant suffer from similar difficulties. Strack & Schwaneberg (1937) have noted that even pure bases can, under certain conditions, give an atypical gold salt and that from mixed solutions mixed salts are precipitated, so that gold chloride is unsuitable for the precipitation, identification or estimation of bases. Precipitation