CCXIV. THE OSMOTIC PRESSURE OF GLOBIN.

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THE present communication records a series of measurements of the osmotic pressure of globin, the protein constituent of the haemoglobin molecule. A number of derivatives of haemoglobin have been investigated by osmometric methods, and it appears that in the cases of reduced and oxygenated haemoglobin, methaemoglobin [Adair, 1924; 1928] and carbon monoxide haemoglobin, which has been studied also by centrifugal methods [Svedberg and Fåhræus, 1926; Svedberg and Nichols, 1927] the molecular weight is a constant, equal to about 67,000.

The preparation of denatured globin from haemoglobin was described by Schulz [1898]. More recently, several methods of obtaining native globin have been suggested [Hill and Holden, 1926; Hamsik, 1930; Anson and Mirsky, 1930 and others]. Most of these involve the treatment of haemoglobin with acids and some organic solvent, whereby the pigment haematin is removed. Roche [1930] has found that at least three different globins are formed when haemoglobin is treated with acids, namely, native globin, fully denatured globin and an intermediate substance, insoluble at the isoelectric point, which has been termed paraglobin. Some measurements of the osmotic pressures of paraglobin solutions are also recorded in this paper.

I. METHODS.

In this work, globin has been prepared by the methods of Hamsik [1930] and of Anson and Mirsky [1930]. A preparation of native globin (No. 6) was made by a slight modification of Hamsik's method, as follows. The haemoglobin of the ox, prepared by the ether and salt method of Adair [1925], was precipitated by the addition of acetone, crystals not being obtained. The precipitate was then treated with acetone containing oxalic acid, washed on a Büchner funnel with pure acetone and dissolved in water. The solution obtained was neutralised with 1 % ammonia, whereupon the paraglobin was precipitated, and the filtrate, containing native globin, was dialysed against distilled water. The preparation was carried out at 0° and yielded a slightly pigmented solution of globin.

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Other preparations were made by the methods described by Anson and Mirsky. 0.1 *N* hydrochloric acid was added to an equal volume of 10 % ox-haemoglobin, both solutions being ice-cold, and the globin was precipitated by acetone containing 1 % of *N* hydrochloric acid. After washing the precipitate with pure acetone and then removing as much acetone as possible by suction and pressing between filter-papers, the globin was dissolved in water without previous drying in air, and, in the case of preparations 7 and 9, the solutions were slowly neutralised with 0.1 *N* sodium hydroxide, whereby most of the paraglobin was precipitated; this was finally removed by subsequent addition of 0.4 % ammonium sulphate to the filtrate. The preparation of globin from crystallised and uncrystallised horse-haemoglobin is described in Section V.

In the case of preparations of ox-globin Nos. 1, 2, 3 and 5, and of sheep-globin, the solution of protein, obtained after dissolving the acetone precipitate in water, was dialysed against an acetate buffer of p_H 5.6. As will be described later, the majority of such protein solutions appeared to consist of paraglobin at the end of the period of dialysis. Additional preparations of paraglobin were made by redissolving in a minimum quantity of acid the precipitate formed on neutralisation of solutions containing mixtures of globin and paraglobin.

In the case of ox-globin, we were able to obtain by the method of Anson and Mirsky protein solutions in which the presence of haematin was undetectable. Attempts to prepare sheep-globin by this method yielded in general appreciably pigmented solutions. In our hands, the method of Anson and Mirsky gave large yields of native globin, but we found the native protein to be relatively unstable, even when kept rigorously at 0°.

Osmotic pressure measurements were obtained by the method of Adair [1925], the protein solutions in rigid collodion membranes being equilibrated with standard buffer solutions at 0°. Series of experiments were carried out at two hydrogen ion concentrations, namely, p_H 7.7 and p_H 5.6, the two buffers being of the same ionic strength, 0.1. With this concentration of electrolytes, the pressures are not appreciably influenced by traces of carbon dioxide. After equilibrium had been attained, estimations were made of the concentration of the protein and, in certain cases, of the density of the protein and of the membrane potential, by a slight modification of the method of Loeb [1922].

The concentration of the protein was calculated from refractometric measurements [Adair and Robinson, 1930, 1] supported by Kjeldahl nitrogen determinations. The percentage of nitrogen in globin was calculated by deducting four g. molecules or 2525 g. of haematin, containing 224 g. nitrogen from 1 g. mol. or 67,000 g. of haemoglobin. The percentage of nitrogen in the dried haemoglobin of the ox is stated to be 17.7, but a reference to the original paper [Hüfner, 1887] showed that this figure is apparently based on a single analysis. It was deemed advisable to redetermine the nitrogen content by the method of Kjeldahl, and from the result obtained, 16.75 ± 0.15 , it

was estimated that ox-globin contains 17.06 g. of nitrogen per 100 g. dry protein, and therefore the globin concentration is equal to the nitrogen multiplied by 5.862. The same factor has been used for sheep's globin. Schulz [1898] gives the value 16.89 % of nitrogen for horse-globin.

II. THE STABILITY OF NATIVE GLOBIN.

According to Hill and Holden [1926] and Haurowitz and Waelsch [1929], globin is an unstable protein. We have made a number of tests to determine whether the degree of stability of the protein under the conditions of our experiments is sufficient for the purpose of osmometric measurements, in which the protein is equilibrated with buffer solutions at p_H 7.7 or p_H 5.6 for periods varying from 7 to 27 days at 0°.

(i) No diffusible protein breakdown products could be detected when the dialysates were tested by the xanthoproteic and the biuret reactions at the end of equilibration.

(ii) Haematin was added to the globin solutions after equilibration at p_H 7.7 and 5.6, and it was found by spectroscopic observations that in all cases the protein had retained the property, characteristic of native globin, of forming methaemoglobin, and subsequently reduced and oxy-haemoglobin on reduction and oxygenation. Since very small traces of native globin are required for this reaction, the spectroscopic tests are of only qualitative significance, as a proof that a certain amount of globin remains unchanged after equilibration.

(iii) Solutions of globin after equilibration at p_H 5.6 at 0° were brought to the isoelectric point by addition of alkali or phosphate buffer. Since native globin is soluble and para- and denatured globins are insoluble at the isoelectric point, the formation of a precipitate is a proof of alteration in the globin. The results for different solutions are recorded in Sections IV and V. It may be noted that in cases where experiments were continued for 18 to 28 days after the date of preparation, considerable amounts of precipitate were found, but in a number of experiments on dilute solutions, which were completed within 10 or 12 days, only traces of protein were precipitated. Attempts were made to estimate the amount of paraglobin formed by determining the total nitrogen and the soluble nitrogen after neutralisation, but the results of these analyses have not been recorded in the Tables because it is not yet known whether the precipitate formed is paraglobin only, or a mixture containing occluded native protein. In a few experiments, marked "much paraglobin" in Table III, about 70 % of the protein was apparently precipitated at the isoelectric point, but in other experiments the proportion precipitated varied from 0 to about 30 % of the total protein.

(iv) Further evidence concerning the stability of globin was obtained by osmometric experiments, in which the pressures were observed daily for periods from 7 to 25 days, and the osmometers were reset, in order to see if the

pressures returned to their original values. These experiments indicated that the stability of globin depends upon the species, the method of preparation, and the concentration of protein and of electrolytes. The best results were obtained with native globin from ox-blood at p_H 7.7. In Exp. 517, the pressure remained constant within 0.1 mm. for 7 days. At p_H 5.6 there were greater variations, probably owing to the transformation of native globin into paraglobin; the manometer readings diminished, instead of remaining constant, in experiments made with solutions containing more than 6 % of protein. The native globin of the horse is less stable; almost all the protein precipitated at p_H 7.7, and no estimate of osmotic pressure could be made, but at p_H 5.6 the pressures were constant within about 0.2 mm. Hg for a few days. A similar degree of constancy was observed in experiments on the paraglobin of the ox and the horse, unless the protein gelatinised, when the pressure fell more rapidly.

The reproducibility of the final pressures, obtained in a series of experiments with membranes of varying permeability and with different times for equilibration, is an additional criterion not only for the existence of osmotic equilibrium but also for the stability of the protein. The degree of reproducibility attainable in the case of dilute solutions of a fairly stable protein, such as serum-albumin, is illustrated in a series of 27 experiments in which the deviations from the mean pressure range from 0 to 4 %, recorded by Adair and Robinson [1930, 2]. In the series of 19 experiments with ox-globin at p_H 5.6, given in Table III, in 5 experiments the deviations from the mean pressure are less than 1 %; in 8 experiments, the deviations range from 1 to 4 %; in 4 experiments there are deviations of 4 to 10 %, and in 2 experiments from 10.5 to 13.1 %. It seems therefore that the preparation of ox-globin must have a certain degree of stability at p_H 5.6 as the deviations are not excessive, although they are considerably greater than those observed for serum-albumin. The measurement of the osmotic pressure of globin presents greater difficulties and the range of error is increased because the time available for obtaining a rigorous proof of equilibrium is limited by the fact that the protein may change into paraglobin.

III. THE OSMOTIC PRESSURE OF GLOBIN SOLUTIONS AT p_H 7.7.

Investigations of the osmotic pressure of globin at hydrogen ion concentrations varying from p_H 6.74 to p_H 8.26 are of special interest, as it has been shown by Roche [1930] that paraglobin and denatured globin are insoluble within this range. The pressures obtained should therefore be unaffected by such degradation products of globin.

Considerable advantage is obtained by the use of a buffer solution at approximately the isoelectric point of the protein, as at such a concentration of hydrogen ions the partial pressure due to the unequal distribution of diffusible ions, designated p_i , is very small, and the observed pressure is

approximately equal to p_p , the partial pressure of the protein ions. The isoelectric point of native globin has been determined by Roche [1929; 1930] by the method of cataphoresis, and found to be p_H 7.5 at 18°. Adair, Cordero and Shen [1929] have found that the p_H of mixtures of haemoglobin and bicarbonates increases from 0.014 to 0.018 if the temperature be diminished by 1°; it was inferred therefore that the isoelectric point of native globin should be about p_H 7.7 to p_H 7.8 at 0°, the temperature at which our osmotic experiments were carried out. This inference was supported by measurements of the membrane potential, recorded below.

The initial concentration of the buffer used was KH_2PO_4 0.00357 *M* and Na_2HPO_4 0.03214 *M*. The buffer value of phosphates is not very great at this hydrogen ion concentration, but previous experiments on haemoglobin have shown that if the ionic strength of the buffer be 0.1 or more, the observed osmotic pressures of dilute solutions of the protein are practically constant over a considerable range of p_H values.

In most of the experiments referred to below, in which no exceptional precautions were taken to exclude CO_2 , the final percentage of phosphate ions in the bivalent form, calculated from hydrogen electrode measurements, was 85 % instead of 90 % as calculated from the original composition. Since one bivalent ion is equivalent to four univalent ions, the final value of the ionic strength of the buffers must have been from 0.1 to approximately 0.097.

Table I. *Native globin of the ox, osmotic pressures at p_H 7.71–7.74 and 0°.*

Dialysate Na_2HPO_4 , 0.03214 *M* + KH_2PO_4 , 0.00357 *M*; ionic strength 0.10.

Preparation No.	Exp. No.	<i>C</i>	<i>C_v</i>	<i>p</i>	π_v	<i>E</i>
9 F	528	0.92	0.92	1.77	1.92	—
9 G	520	0.96	0.97	1.68	1.74	—
9 F	527	1.58	1.61	2.38	1.48	–0.04
7	496	1.58	1.61	2.42	1.50	—
9 F	526	1.72	1.75	3.00	1.72	—
9 G	519	1.91	1.94	2.91	1.50	—
9 J	529	2.12	2.16	3.23	1.50	–0.02
9 J	530	2.32	2.37	3.72	1.57	+0.03
9 G	518	2.77	2.85	3.89	1.37	–0.10
9 K	536	3.13	3.23	4.58	1.42	–0.15
7	495	3.42	3.53	4.46	1.27	–0.12
7	494	3.45	3.57	4.48	1.26	+0.3
9 G	516	3.94	4.09	5.22	1.28	—
9 G	517	4.04	4.20	5.26	1.26	–0.25
9 K	539	5.52	5.83	6.49	1.11	–0.42

C = g. dry protein per 100 cc. solution; *C_v* = g. dry protein per 100 cc. solvent; *p* = osmotic pressure in mm. Hg at 0°; $\pi_v = p/C_v$; *E* = membrane potential in millivolts.

The results obtained from a series of experiments on native globin solutions of concentrations ranging from 0.915 % to 5.52 % are given in Table I. Comparative experiments, carried out on carbon monoxide haemoglobin, made from the ox-haemoglobin solution that had been used for the preparations of globin numbered 9 F to 9 K are recorded in Table II.

Table II. *Carbon monoxide haemoglobin of the ox, osmotic pressures and membrane potentials at p_H 7.75 and 0° .*Dialysate Na_2HPO_4 , 0.03214 *M* + KH_2PO_4 , 0.00357 *M*, ionic strength 0.10.

Exp. No.	<i>C</i>	<i>C_v</i>	<i>p</i>	π_v	π_p	<i>E</i> observed	<i>E</i> calculated
549	0.89	0.89	2.35	2.63	2.62	—	−0.07
548	1.80	1.83	4.89	2.67	2.63	—	−0.14
547	3.01	3.10	8.82	2.85	2.79	−0.18	−0.24
546	4.95	5.19	15.02	2.90	2.81	−0.42	−0.40
545	8.57	9.34	29.62	3.18	3.01	−0.76	−0.72
544	14.26	16.55	58.30	3.53	3.23	−1.33	−1.27
543	16.51	19.62	75.40	3.84	3.48	−1.43	−1.50

Two methods for the tabulation of the protein concentrations have been adopted in Table I. Under the heading *C*, the results are given in g. per 100 cc. of solution; the chief advantages of this method are that *C* is directly proportional to the refractive index of the protein solution and the nitrogen content determined by volumetric analysis, and that it involves no speculations concerning the hydration of the protein. The corrected concentration *C_v* is calculated from measurements of *C* by formula (1).

$$C_v = C \times 100 / (100 - V_1 C) \quad \dots\dots(1).$$

V₁ equals the volume occupied by 1 g. of dry protein *plus* the water of hydration combined with it. In the case of haemoglobin, in which about 0.2 g. of water appears to combine with 1 g. of protein [Adair and Robinson, 1931], the value of *V₁* is about 0.965, and for the purpose of provisional calculations, the same value has been used for globin. The corrected concentration, *C_v*, is of service in the theoretical interpretation of the osmotic pressures.

A number of measurements of the membrane potentials of globin and haemoglobin are recorded in Tables I and II. The potentials obtained with haemoglobin are directly proportional to the protein concentration, as shown by the agreement between the observed values of *E* and these calculated by the empirical formula $E = k_e C_v$, where *k_e* is a constant equal to −0.077. The negative sign shows that under these conditions the protein is acting as an anion.

In the experiments on globin, the conditions were not favourable for accurate measurements of membrane potentials. The observed potentials were small, and the available evidence is consistent with the assumption that at p_H 7.7 the protein is not far from the isoelectric point.

IV. THE OSMOTIC PRESSURES OF NATIVE GLOBIN AND PARAGLOBIN OF THE OX AT p_H 5.6.

One of the objects of this work was to compare the osmotic pressures of native globin and of paraglobin under the same conditions. Such a comparison is not possible at the isoelectric point, because paraglobin is insoluble. On the other hand, it is not possible to use solvents that are very far from neutrality, because native globin is then converted into paraglobin.

The determinations made previously by Roche [1930] indicated that at p_H 5.6 the solubility of paraglobin should be sufficient for the purpose of osmotic pressure determinations, and preliminary investigations made in this work showed that although native globin tends to change into paraglobin at p_H 5.6, the rate of change over a period of 7 to 14 days is not excessive, for in many of the experiments only a small fraction of the total protein was precipitated, when, at the end of equilibration, alkali was added to raise the p_H from 5.6 to the isoelectric point.

A list of the experiments made on preparations of native protein has been given in Table III. In the case of the preparations numbered 6 and 7, it appears that the pressure concentration ratio $p/C_v = \pi_v$ is a constant equal to 4.58, over the range of pressures from 4 to 50 mm. Of the 14 determina-

Table III. *Native globin of the ox, osmotic pressures at p_H 5.64 and 0° .*

Dialysate sodium acetate, 0.10 *M* + acetic acid, 0.0105 *M*.

Preparation 6: method of Hamsik; preparations 7 to 9 θ , method of Anson and Mirsky.

Preparation No.	Exp. No.	<i>C</i>	<i>C_v</i>	<i>p</i>	π_v	<i>E</i>	Test for paraglobin
6	483	3.09	3.19	14.62	4.59	—	None
6	481	1.53	1.56	7.02	4.51	—	Trace
6	480	1.49	1.51	6.88	4.57	—	None
6	485	1.06	1.07	4.46	4.18	—	"
6	484	0.95	0.96	4.51	4.72	—	"
6	482	0.86	0.87	3.96	4.60	—	"
7	486	10.18	11.30	48.80	4.32	+3.35	Some
7	487	10.18	11.30	53.30	4.72	+3.35	"
7	488	7.54	8.13	37.40	4.60	+2.55	"
7	489	5.79	6.13	28.60	4.66	+1.85	"
7	490	3.33	3.42	15.46	4.52	+1.05	Little
7	491	1.64	1.67	7.59	4.55	—	Trace
7	492	0.84	0.85	4.10	4.83	—	Little
7	493	0.80	0.81	4.25	5.26	—	Some
9 G	521	1.51	1.53	7.22	4.73	—	Much
9 G	522	1.01	1.02	4.86	4.75	+0.05	"
9 B	525	2.50	2.56	10.76	4.21	+0.57	Some
9 JA	531	2.08	2.13	9.63	4.52	+0.40	Much
9 θ	538	1.46	1.48	6.06	4.10	+0.45	"

tions, 10 come within about 3 % of 4.58, and 4 show larger deviations, ranging from 6 to 15 %, but it is doubtful whether any significance can be attached to these deviations, because it was deemed advisable to finish the series rapidly, owing to the risk of denaturation of globin at p_H 5.6, and it is possible that the time allotted for equilibration and for determinations of capillarity corrections was insufficient to exclude the risk of experimental errors.

In the cases of preparations 9 G and 9 JA, in which the protein was equilibrated with buffer at p_H 7.7 before it was brought to p_H 5.6, the values for π_v agree with 4.58 within the limits of experimental error, but in the cases of preparations 9 B and 9 θ , there are deviations of 8 and 10 % respectively, which are probably due to the presence of paraglobin. It is interesting to note that preparation 6, made by the method of Hamsik, gives the same result as the other preparations, made by the method of Anson and Mirsky.

Before discussing the theoretical significance of the formula $p = 4.58 C_v$ for the osmotic pressure of globin, it is necessary to estimate the pressure, symbolised p_i , due to the excess of diffusible ions inside the membrane at p_H 5.6.

In the case of ideal or infinitely dilute solutions, the pressure p_i can be calculated by Donnan's equations. A different method has been developed [Adair, 1929] and a formula (No. 2) has been obtained which can be applied to non-ideal systems in which the activities of the diffusible salts are kept constant at different protein concentrations by equilibration with buffer solutions of the same composition.

$$p_i = RT \int_0^u m_p n_p du \quad \dots\dots(2).$$

p_i = the pressure due to the excess of diffusible ions inside the membrane, expressed in mm. of mercury.

$$RT = 22.4 \times 760 = 17,033 \text{ at } 0^\circ.$$

$$u = E \times (F/1000RT) = E/23.5 \text{ at } 0^\circ,$$

where E = the membrane potential in millivolts.

$m_p n_p$ = the equivalent concentration of the protein acetate (the product of the molar concentration m_p and the valence n_p) in g. equivalents per litre of solvent.

The estimation of the equivalent concentration $m_p n_p$ has been discussed by Adair and Robinson [1930, 2], who have suggested that if E be small, under certain conditions the effects due to deviations from the ideal solution

Fig. 1. Membrane potentials of globin solutions equilibrated with acetate buffer at p_H 5.64. Circles, native globin of ox. Squares, unfractionated globin and paraglobin.

laws may cancel out, and $m_p n_p$ may be equal to $-uJ$, where J is the sum of the concentrations of the ions in the dialysate, multiplied by the squares of their valencies. The ionic strength is equal to $\frac{1}{2}J$. A simple formula for p_i (No. 3) can be derived from formula (2), on the assumption that $m_p n_p = -uJ$.

This assumption is consistent with the experiments recorded in Table III and Fig. 1, which show that membrane potentials are directly proportional to the protein concentrations over the range investigated.

$$p_i = \frac{1}{2}RTJu^2 = 15.4 JE^2 = 15.4 Jk^2c_v^2 \quad \text{.....(3).}$$

The term k in the formula numbered (3) is the ratio E/C_v , which must be a constant over the range of application of formula (3). In the observations recorded in Table III, $k = 0.3$ and $J = 0.2$.

It is probable that calculations of p_i based upon determinations of C_v and of the mean value of the ratio $E/C_v = k$ are more accurate than calculations based upon the individual values of the membrane potentials, which are subject to greater experimental errors than the measurements of protein concentration.

Fig. 2. Native globin and haemoglobin of ox. Pressure concentration ratios. Ordinate π_v and π_p in mm. of mercury at 0° . Abscissa C_v . Full lines, π_v ; broken lines, π_p = ratio of partial pressure of protein ions and protein concentration. π_p is equal to π_v in solutions of globin at $p_H 7.7$.

The results recorded in Fig. 2 show that although the ratio π_v is practically constant, the ratio $\pi_p = p_p/C_v = (p - p_i)/C_v$ tends to diminish as the protein concentration is increased. This diminution is discussed in Section VII.

The results of measurements of the osmotic pressure of paraglobin are recorded in Table IV. The pressures observed are smaller than those given by native globin.

Table IV. *Unfractionated globin and paraglobin of the ox, osmotic pressures at p_H 5.64 and 0°.*Dialysate sodium acetate, 0.10 *M* + acetic acid 0.0105 *M*.

Preparations 1 and 2: unfractionated globin not dried before dialysis; preparation 4: unfractionated globin stored in dry state before dialysis; preparations 9 C and 9 L paraglobin.

Preparation No.	Exp. No.	<i>C</i>	<i>C_v</i>	<i>p</i>	π_v	<i>E</i>
1	364	1.25	1.27	4.51	3.57	+0.35
2	369	1.07	1.08	3.28	3.03	+0.30
4	391	2.05	2.09	6.81	3.26	+0.60
4	392	2.08	2.12	7.05	3.32	+0.5
4	389	4.06	4.23	11.60	2.75	+1.3
4	390	4.10	4.27	10.45	2.45	+1.35
9 C	523*	5.22	—	6.79	—	+1.35
9 C	524	2.58	2.65	4.56	1.72	—
9 L	537	5.38	5.68	9.61	1.69	+1.75

* Exp. 523, some protein in gelatinous form.

The osmotic pressures of preparations of unfractionated globin are given in Table IV, and it will be observed that the results are intermediate between those recorded for native globin and for the paraglobin separated by precipitation.

The osmotic pressures of fractionated native globin and paraglobin of the sheep have not yet been determined, but a preliminary series of measurements on unfractionated globin has been made, the results of which are recorded in Table V (prep. 3), in which the values of π_v diminished from 1.38 to

Table V. *Unfractionated globin of the sheep, osmotic pressures at p_H 5.64 and 0°.*

Preparation No.	Exp. No.	<i>C</i>	<i>C_v</i>	<i>p</i>	π_v	α'
3	382	4.61	4.82	5.71	1.19	0.00185
3	383	2.28	2.33	2.89	1.24	0.00188
3	384	2.30	2.35	2.86	1.22	0.00187
3	385	3.35	3.47	4.28	1.23	0.00189
3	386	3.33	3.45	4.16	1.21	0.00189
3	387	1.15	1.16	1.56	1.34	0.00179
3	388	1.11	1.12	1.57	1.40	0.00187
5	433	3.31	3.42	5.70	1.67	0.00188

 α' = the specific refraction increment (ref. Section VI).

1.17 as the protein concentration was increased. It appears that the unfractionated globin of the sheep gives lower osmotic pressures than any of the other forms of globin studied in this work.

For comparison with the experiments on globin, determinations of the osmotic pressure of haemoglobin at p_H 5.65 have been recorded in Table VI. The carbon monoxide and methaemoglobin solutions were prepared from the stock solution of haemoglobin used for the globin preparations numbered 9 G to 9 θ . The osmotic pressures are discussed in Section VII. The observed membrane potentials agree with those calculated by the empirical formula $E = kC_v$, given in the last column of Table VI, assuming that *k* is + 0.089 for CO-haemoglobin and + 0.119 for methaemoglobin.

Table VI. *Haemoglobin of the ox, osmotic pressure and membrane potentials at p_H 5.65 and 0° .*Dialysate sodium acetate, 0.10 *M* + acetic acid, 0.0105 *M*.

Nos. 540*, 541*, 542* methaemoglobin. All other experiments CO-haemoglobin.

No.	<i>C</i>	<i>C_v</i>	<i>p</i>	π_v	π_p	<i>E</i> observed	<i>E</i> calculated
533	0.58	0.59	1.89	3.25	3.21	—	+0.05
534	0.95	0.96	3.01	3.15	3.13	—	+0.09
514	1.44	1.46	4.80	3.30	3.27	—	+0.13
513	1.86	1.90	6.52	3.44	3.39	+0.10	+0.17
512	2.72	2.79	9.36	3.36	3.29	+0.28	+0.25
511	5.46	5.62	21.2	3.77	3.63	+0.63	+0.50
510	8.07	8.76	33.7	3.85	3.63	—	+0.80
509	12.34	14.00	63.4	4.53	4.18	+0.90	+1.25
535	12.97	14.82	67.5	4.55	4.19	+1.33	+1.32
515	15.38	18.05	92.8	5.14	4.69	+1.70	+1.61
508	17.88	21.60	120.2	5.56	5.03	+1.75	+1.93
532	20.90	26.20	161.9	6.18	5.53	+2.67	+2.34
540*	3.30	3.41	11.36	3.33	3.19	+0.32	+0.41
542*	3.39	3.50	12.10	3.46	3.31	+0.47	+0.42
541*	3.77	3.91	13.39	3.42	3.25	+0.47	+0.47

V. THE OSMOTIC PRESSURES OF NATIVE GLOBIN AND
PARAGLOBIN OF THE HORSE.

In order to compare the osmotic pressures of globins prepared from crystallised and from uncrystallised haemoglobins, experiments were carried out on the globin of the horse, the haemoglobin of which can be crystallised without difficulty.

In the preparation 8 *b*, referred to below, crystals of the carbon monoxide haemoglobin of the horse were obtained by the addition at 0° of an equal volume of saturated ammonium sulphate to a solution of haemoglobin which had been separated from the stroma by the addition of ether and salt. The mother-liquor was removed on a Büchner filter, and the crystals were redissolved and recrystallised and freed from impurities by dialysis.

Preparations of globin were made by the method of Anson and Mirsky from this material and from uncrystallised haemoglobin. Unfortunately, the globin of the horse appears to be less stable than that of the ox, and most of the protein precipitated during the removal of ammonium sulphate by dialysis. Attempts to measure the osmotic pressure at p_H 7.7 were moreover unsuccessful, as most of the protein precipitated. Six determinations obtained at p_H 5.64 are recorded in Table VII.

The solutions were so dilute that the difference between the concentration *C* and the corrected concentration *C_v* is within the limits of experimental error. The calculation of the protein concentration from nitrogen determinations is not recorded, because the amounts of material available were insufficient for adequate analyses, and provisionally all the calculations have been based on refractometric measurements, assuming that the specific refraction increment for horse-globin is 0.00183, the same as that determined for ox-globin (ref.

Table VII. *Native globin and paraglobin of the horse, osmotic pressures at p_H 5.6 and 0° .*

Preparation 8 a, native globin from uncrystallised haemoglobin; preparation 8 b, native globin from recrystallised haemoglobin; preparation 8 c paraglobin.

Preparation No.	Exp. No.	C	p	E	π^*	Test for paraglobin
8 b	497	0.68	3.94	+0.23	5.8	Trace
8 b	498	0.68	3.85	+0.36	5.7	Some
8 b	499	0.45	2.80	—	6.2	Trace
8 b	500	0.47	2.66	+0.28	5.7	Some
8 a	501	1.11	6.43	+0.50	5.8	Much
8 a	502	0.60	3.63	—	6.1	Trace
8 c	505	1.89	5.00	+0.85	2.7	—
8 c	506	0.94	2.54	—	2.7	—
8 c	507	0.95	2.72	—	2.9	—

$$\pi^* = p/C.$$

Section VI). In the solutions containing less than 0.5 g. of protein per 100 cc., the error in the refractometric determinations may be about 5 %.

It appears that the mean value for π is 5.88, whereas the value obtained for ox-globin is about 4.58. The difference is about four times as great as the maximum observed deviation from the mean value 5.88, and it seems probable that the difference is a real phenomenon. In the second place, it appears that the osmotic pressures of the globins prepared from recrystallised and from uncrystallised haemoglobin are the same, within the limits of experimental error. As in the case of ox-globins, the osmotic pressure of the paraglobin of the horse is less than half the pressure given by the native globin.

VI. A COMPARISON OF THE DENSITIES AND THE SPECIFIC REFRACTION INCREMENTS OF GLOBIN AND HAEMOGLOBIN.

In a number of the experiments made in this research, the measurements of osmotic pressure were supplemented by determinations of the specific refraction increments and the densities of the protein solutions.

The measurements of densities are recorded in Table VIII. It seems that for both proteins, the density of the protein solution, D' , is directly proportional to the concentration C expressed in g. per 100 cc. of solution, as stated in formula (4).

$$D' = D'' + Ck_a \quad \dots\dots(4).$$

D'' = density of the dialysate; k_a = an empirical coefficient. This formula has been used in calculations of osmotic pressures of dilute solutions, in which the observed heights of the columns in cm. of protein solution must be reduced to mm. mercury. The values of k_a have been calculated to five places, but the fifth figure is uncertain, and for the purposes of this work it is sufficient to determine k_a approximately to four places. The values of k_a for haemoglobin and for globin in both the solvents used are 0.00256 ± 0.0001 . It is not yet possible to say whether the slight deviations from 0.00256 are of any significance.

Table VIII. *Densities of solutions of globin and haemoglobin of α , equilibrated with buffer mixtures at 0°.*Buffer 7.7. Na_2HPO_4 , 0.03214 *M* + KH_2PO_4 , 0.00357 *M*.Buffer 5.6. CH_3COONa , 0.10 *M* + CH_3COOH , 0.0105 *M*.

No.	Protein	p_{H}	Density of dialysate D''	Density of protein D'	C	k_d
544	CO-haemoglobin	7.7	1.0047	1.0404	14.26	0.00250
543	"	7.7	1.0050	1.0467	16.51	0.00253
515	"	5.6	1.0045	1.0435	15.41	0.00253
508	"	5.6	1.0045	1.0496	17.89	0.00252
532	"	5.6	1.0046	1.0561	20.90	0.00247
540	Methaemoglobin	5.6	1.0043	1.0126	3.30	0.00250
527	Globin	7.7	1.0051	1.0093	1.58	0.00266
518	"	7.7	1.0046	1.0119	2.77	0.00263
516	"	7.7	1.0053	1.0152	3.95	0.00252
525	"	5.6	1.0045	1.0108	2.49	0.00250
488	"	5.6	1.0041	1.0238	7.54	0.00261

Measurements of the specific refraction increments of haemoglobin and of globin are recorded in Table IX. The numbers of the experiments have been stated and other data concerning the protein concentration and conditions in these experiments are given in Tables I, II, III and VI. In accordance with previous work on the serum-proteins [Adair and Robinson, 1930, 1] it was found that the value of α' , defined by formula (5), is independent of the protein concentration, C , expressed in g. per 100 cc. of solution.

$$R' - R'' = \alpha' C \quad \dots\dots(5).$$

R' = refractive index of protein solution; R'' = refractive index of dialysate. All dialyses were carried out at 0° but the measurements of R' and R'' were made at about 15°.

Table IX. *Specific refraction increments of carbon monoxide haemoglobin and of globin of the α , equilibrated with phosphate and acetate buffers at 0°.*

Haemoglobin			Globin p_{H} 7.7		Globin p_{H} 5.6	
Exp. No	Buffer	α'	Exp. No.	α'	Exp. No.	α'
543	7.7	0.001955	516	0.00180	481	0.00183
544	7.7	0.001935	526	0.00179	486	0.00182
545	7.7	0.001965	527	0.00179	488	0.00185
546	7.7	0.001941	528	0.00176	489	0.00182
508	5.6	0.001955	530	0.00175	525	0.00181
513	5.6	0.001988	536	0.00178	531	0.00194
515	5.6	0.001960	539	0.00178	538	0.00189
534	5.6	0.001960	—	—	—	—

Theoretically, there may be appreciable differences between α' and α , the specific refraction increment of the protein in water, because $R' - R''$ is affected by the unequal distribution of salts across the membrane. The refraction increments of the salts in the dialysates were estimated by deducting the refractive index of water, and the results ranged from 0.0011 to 0.0012 for the acetate buffer at p_{H} 5.6 and from 0.00095 to 0.00099 for the phosphate buffer at p_{H} 7.7. In the case of globin at p_{H} 5.6, the membrane potentials

indicate that there is an appreciable excess of acetate ions inside the membrane, and it is possible that the refraction due to the diffusible ions is greater than 0.0012. In the systems containing haemoglobin and globin at p_H 7.7 the potentials are small, and it is unlikely that there is much difference between the refractions due to salts on both sides of the membrane.

In the case of human oxyhaemoglobin, α is 0.001942 [Stoddard and Adair, 1923], and we have obtained a similar value of α , namely 0.001952 for the haemoglobin of the ox. The values of α' recorded in Table IX agree with 0.00195 within the limits of experimental error.

In the case of the native globin of the ox at p_H 7.7, the mean value of α' is 0.00178. The earlier experiments on native globin at p_H 5.6 gave a mean value for α' of approximately 0.00183, but some of the experiments equilibrated for longer periods gave higher values and in the case of unfractionated globin (preparation 4, Table III) the following figures were obtained: 0.00190, 0.00188, 0.00188, 0.00190. It seems possible that the higher values are due to the presence of paraglobin, but the problem requires further investigation.

The values of α' for native globin at p_H 7.7 are similar to those obtained for egg-albumin and serum-albumin. Haurowitz and Waelsch [1929] have published lower values for globin, ranging from 0.00146 to 0.00154, but from the account of their experiment it seems unlikely that the concentration term C in their paper is the same as C in formula (5), which is calculated from nitrogen determinations, assuming that anhydrous globin contains 17.06 % of nitrogen. It is not improbable that the powdered globin used by Haurowitz and Waelsch was a hydrate containing less than 17 % of nitrogen. Schulz's [1898] preparations of globin, dried at a higher temperature (100°) contained about 16.89 % of nitrogen. Robertson [1912] prepared globin by Schulz's method and obtained values for α of 0.00169 ± 0.00005 for his purest preparation.

VII. DISCUSSION.

In the formation of globin from haemoglobin by the action of acid acetone, 1 g. mol. or 67,000 g. of haemoglobin yields 4 g. mols or about 2525 g. haematin ($C_{34}H_{30}O_4N_4FeOH$) and the difference of about 64,475 must represent the mass of globin. The ideal osmotic pressure of a substance of this molecular weight is given by the formula $p/C_v = 2.64$ at 0°.

The observed pressure ratios differ from 2.64. In discussing these differences, there are three factors which must be considered. These factors are represented by the symbols p_i , g_p and M in the general formula for the osmotic pressure of a protein, restated below.

$$p = p_i + p_p = p_i + RTg_p \cdot 10C_v/M \quad \dots\dots(6).$$

p = observed osmotic pressure; p_i = ion pressure difference; p_p = partial osmotic pressure of protein ions; RT , the product of the pressure and volume of an ideal gas, = $22.4 \times 760 = 17,033$ at 0°; g_p = the osmotic coefficient of the protein ions, which represents the effects of attractive and repulsive forces

causing deviations from the ideal solution laws, M = the molecular weight, or the mean molecular weight if the protein is a mixture of aggregates. M may be a variable affected by the protein concentration if the aggregates can dissociate into smaller particles in accordance with the law of mass action.

The factor p_i can be calculated from membrane potential measurements, as described in Section IV, and the number of variables reduced to 2 by the calculation of the ratio π_p defined below.

$$\pi_p = p_p/C_v = 10RTg_p/M \quad \text{.....(7).}$$

The results of calculations of π_p for the native globin of the ox are recorded in Fig. 2, in which π_p is plotted against C_v .

For comparison with the experiments on globin, a number of determinations of the osmotic pressure of haemoglobin in the same solvents, at p_H 7.7 and at p_H 5.6, have been made using carbon monoxide haemoglobin and methaemoglobin prepared from the stock solution of ox-haemoglobin utilised in the preparation of globins numbered 9 B to 9 K.

The osmotic pressures of CO-haemoglobin at p_H 7.7 may be considered first. Fig. 2 shows that π_p is about 2.6 in dilute solutions, and it increases as the protein concentration is increased. In both respects, this curve resembles the results obtained for the haemoglobins of the horse, the sheep, and of man, in solvents with p_H values ranging from 6.8 to 8.0, and with salt concentrations ranging from 0.01 to 0.23 mols per litre, recorded in previous papers [Adair, 1924; 1928].

It is almost certain that in the systems referred to above, the molecular weights of the proteins are constant, and that the changes in π_p are due to variations in the osmotic coefficients. If this condition is fulfilled it is legitimate to extrapolate the curve to determine the value of π_p when C_v is 0, as described by Adair and Robinson [1930, 2], and from the result obtained, namely 2.55 mm., it appears that the molecular weight of haemoglobin is about 67,000 in the case of the haemoglobin of the horse, sheep and man and the preparation of ox-haemoglobin used in this work.

The measurements on CO-haemoglobin and on methaemoglobin at p_H 5.6 show that π_p increases with increasing protein concentrations, an observation which proves that g_p must increase under these conditions, for any changes in aggregation which may occur tend to diminish π_p . In addition, the measurements indicate that π_p approximates to 3.15 in dilute solutions. Theoretically an effect of this order could be obtained if a certain proportion, not exceeding about 24 %, of the molecules dissociate into smaller units at p_H 5.6¹. Svedberg and Nichols [1927] have shown that at p_H 5.4, the diffusion constant and the sedimentation velocity of haemoglobin differ by about 20 % from the normal

¹ Spectroscopic observations, for help with which we are greatly indebted to Prof. D. Keilin, revealed no denatured derivatives of haemoglobin in the material equilibrated for about 2 weeks at p_H 5.6 (in experiments numbered 540-542) but traces could be detected in material stored for longer periods.

values; their measurements being made on the CO-haemoglobin of the horse, at about 30°.

The results recorded in Fig. 2 show that there are remarkable differences between the osmotic pressures of globin and of haemoglobin. The osmotic pressure of globin is much more sensitive to changes in p_H , and the ratio π_p diminishes as the protein concentration is increased, even in the isoelectric region where π_p is equal to π_o .

Theoretically, the diminution in π_p must be due to a diminution of the osmotic coefficient, or a polymerisation of the protein, or to both of these factors acting in the same direction. There are two arguments in favour of the hypothesis that changes in polymerisation play an important part in the diminution of π_p . Firstly, this hypothesis agrees with the observation that, in dilute solutions, π_p at p_H 5.6 is two or three times as great as at p_H 7.7, for it is not improbable that an increase in acidity should favour the dissociation of the larger particles. Secondly, it appears to be a general rule that in the region of the isoelectric point, the osmotic coefficients increase rather than diminish. Since no exceptions to this rule have been found in previous investigations on the osmotic coefficients of the proteins, it seems reasonable to attribute the fall in π_p at the isoelectric point to changes in polymerisation rather than to a fall in the osmotic coefficient.

The arguments in favour of changes in the osmotic coefficient or of aggregation are more evenly balanced in the case of the ionised globin at p_H 5.6. The measurements of membrane potentials show that globin is more highly charged than haemoglobin, and according to the inter-ionic attraction theory of Debye and Hückel [1923] the osmotic coefficient should be diminished by an increase in the concentration of protein ions of high valency. On the other hand, the fact that the osmotic coefficient of haemoglobin in the same solvent fails to diminish is evidence in favour of the aggregation hypothesis. Nevertheless, as globin is more highly charged than haemoglobin at p_H 5.6 the diminution in π_p cannot be regarded as a definite proof of aggregation changes.

The calculation of the molecular weight of globin is complicated by the possibility that the protein undergoes polymerisation. The methods for the elimination of the effects due to the osmotic coefficient by extrapolation [Adair and Robinson, 1930, 2] cannot be applied to a system in which both M and g_p vary with the protein concentration and it will be seen that no attempt has been made to extrapolate the values of π_p for globin in Fig. 2. Although exact values cannot be obtained, rough estimates of the value of M for globin may throw light on the changes in aggregation which take place, and it is probable, though not certain, that in some of the systems studied in this work, the "uncorrected molecular weight" M/g , calculated by formula (7), is not greatly different from the corrected value. The results for native ox-globin at p_H 7.7 are given in Table X.

If M be a function of the protein concentration, the globin must be a mixture of polymerides. Svedberg and his colleagues have shown that there

Table X. *Effect of protein concentration on the uncorrected value of M for ox-globin at p_H 7.7. (Subject to corrections of unknown magnitude.)*

(g. per 100 cc. solvent)	1.0	2.0	3.0	4.0	5.0	6.0
M (uncorrected)	94,000	110,000	123,000	139,000	150,000	155,000

are mixtures of aggregates in certain proteins, including their preparations of eu- and pseudo-globulins [Svedberg and Sjögren, 1930; Mutzenbecher, 1931]. Svedberg [1930] has included globin in a list of proteins which are polydisperse. It is not improbable that the variations demonstrated by the centrifugal method are mainly due to the existence of simple physical mixtures of aggregates. Variations in M demonstrated by osmotic methods cannot be attributed to the existence of a simple physical mixture, and therefore it seems probable that in solutions of globin there is a dynamic equilibrium between the larger polymerides and the products of their dissociation.

The interpretation of the results obtained at p_H 5.6 presents greater difficulties. The measurements on native globins of the ox and the horse show that the ratio π_v is practically independent of the protein concentration over a considerable range. Since π_v and π_p approximate in dilute solutions, some provisional calculations of the value of M in dilute solutions have been made by using the observed values of π_v for comparisons with the results obtained for paraglobin.

Table XI. *Comparison of uncorrected molecular weights of different globins and paraglobins at p_H 5.6 and approximately the same protein concentration (about 1 %).*

Ox native globin	...	...	...	...	37,000
Horse native globin	...	...	...	...	29,000
Ox paraglobin	...	...	...	...	99,000
Horse paraglobin	...	...	...	...	63,000
Ox unfractionated globin	...	...	...	...	52,000
Sheep unfractionated globin	...	...	...	...	125,000

The values of M for native globin are about one-half of the molecular weight of haemoglobin, but no conclusions can be drawn from this observation because M may vary with the protein concentration. It is clear that the degree of polymerisation is lower in native globin than in paraglobin or in unfractionated globin, and it may be that there is some correlation between the tendency for paraglobin to form larger aggregates and the fact that it can be precipitated more easily than native globin.

SUMMARY.

The stability of globin at 0° in solutions buffered by phosphates and acetates has been investigated. In general globin is much less stable than haemoglobin, but preparations of the native globin of the ox have been made by the methods of Hamsik and of Anson and Mirsky, which give reproducible results in osmotic pressure measurements. The globin of the horse appears to be less stable than that of the ox.

In the case of ox-globin, the relationship between osmotic pressure and protein concentration has been studied at the isoelectric point, p_H 7.7, and at p_H 5.6, and for comparison with globin, experiments have been made on ox-haemoglobin, equilibrated with the same buffer mixtures. The partial pressure of the protein and the pressure due to the unequal distribution of ions have been estimated from measurements of membrane potential.

There is a striking difference between results obtained from globin and haemoglobin. In the case of globin, the ratio of partial pressure of protein and its concentration, π_v , tends to diminish as the protein concentration is increased. It is unlikely that this decrease is wholly due to deviations from the ideal solution laws, particularly in the case of isoelectric globin, and it seems probable that there is a dynamic equilibrium between polymerides of different sizes in solutions of globin, so that the average molecular weight is a function of the concentration of the protein, of hydrogen ion concentration and of other variables. At p_H 7.7, the mean molecular weight may be greater than that of haemoglobin.

Comparison of measurements of the osmotic pressure of unfractionated globin and of paraglobin with that of native globin at the same hydrogen ion concentration (p_H 5.6) indicates that paraglobin is more highly aggregated than native globin.

Preliminary investigations have been made on the osmotic pressures of the globins of the horse and the sheep.

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