XVI. THE ULTRAFILTRATION OF PROTEINS THROUGH GRADED COLLODION MEMBRANES.

II. HAEMOCYANIN (*HELIX*), EDESTIN, AND EGG ALBUMIN.

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THE behaviours of protein solutions when filtered through membranes of graded porosities under different conditions of p_H and dispersion medium have been further studied in the case of haemocyanin (*Helix pomatia*), edestin and egg albumin. The particle sizes of these proteins in dilute solution have been determined by Svedberg and his co-workers by the method of ultracentrifugal analysis, the respective values being 24, 8 and $4.34 m\mu$. The purpose of our present study has been to ascertain the conditions most favourable for the filtration of these proteins in a manner similar to that followed for the serum proteins [Elford and Ferry, 1934], and to establish for each the filtration end-point. A means is then provided of checking further the value of the empirical correction factor adopted by Elford [1933] for deducing the particle size of a suspension from the pore diameter of the limiting membrane by which it is just completely retained.

The experimental ultrafiltration technique has been exactly as described in the earlier paper dealing with the serum proteins [1934]. The analyses of filtrates have been based upon refractometric measurements and controlled precipitation tests with salicylsulphonic acid. The stock protein-solutions were always sterilised by filtration through a suitable membrane.

Haemocyanin (Helix pomatia).

Three separate batches of crystalline haemocyanin were used. We are greatly indebted to Dr G. A. Millikan for his valued advice and assistance in preparing the crystalline protein. The procedure followed was essentially that outlined by Svedberg and Chirnoaga [1928].

Influence of medium on filterability. The filtration curves have been established for haemocyanin dispersed in the same concentration at p_H 7.3 in each of the four media: water, 1% saline, $M/15$ phosphate buffer and Hartley's broth. 0.1 N NaOH was used to adjust the p_H when necessary. The curves for membranes of average pore diameters (A.P.D.) $125 m\mu$ are given in Fig. 1. Broth is clearly the best medium for the filtration of haemocyanin but it is very interesting to find that for small volumes of solution (1–2 ml.) the relative filtrate concentration, u , for water as medium may exceed that for broth. However, considerable mechanical blocking of the membrane occurs in the later stages suggesting that whilst the initial adsorption of haemocyanin may be only slight the system is relatively sensitive to influences favouring aggregation. The initial adsorption appears to be considerable in the presence of 1% saline and the buffer salts, but the broth medium ensures a high value of $u_{\max}$ and its stabilising influence minimises the degree of blocking of the pores.

Influence of p_H . Over a wide range of p_H , 5 to 8, the values of $u_{\max}$ for 0.5% solutions haemocyanin in broth medium when filtered at 76 cm. mercury pressure

through membranes of A.P.D. 0.16μ were consistently within 95 to 100%. Below p_H 6.0 the initial adsorption zone increased progressively, but the value of $u_{\max}$ was not affected until p_H values below 5.0 were reached, when an abrupt

Fig. 1. Filtration curves of 0.75% haemocyanin in different media. (a) water p_H 7.30; (b) broth p_H 7.31; (c) 1% saline p_H 7.28; (d) $M/15$ phosphate buffer p_H 7.33. Membrane characteristics A.P.D. $125m\mu$; thickness 0.13 mm . Filtration pressure 76 cm. mercury.

falling off was observed. It was evident that for purposes of establishing the filtration end-point curve, the most favourable medium would be broth at a p_H between 7 and 8.

Filtration end-point data. Table I contains a summary of filtration data for membranes of various porosities enabling the end-point for haemocyanin to be deduced.

Table I.

Haemo- cyanin batch	Medium	Concen- tration %	p_H	Pressure atmos.	A.P.D. $m\mu$	$u_{\max}$ %
A	XD broth	1	7.0	3	84	1-5
	"	"	"	3	55	Nil
	Stock broth	1	6.8	2	115	80
	"	1	6.8	2	84	20
	"	"	7.0	2	70	15
B*	"	1	7.3	1	115	75
	"	"	"	"	75	10
	"	"	"	"	64	1
	"	"	"	"	57	? trace < 1
	"	"	"	"	50	Nil
	Phosphate	1	7.3	1	75	Nil
C	Stock broth	0.5	7.31	1	125	85
	"	"	"	"	85	60
	"	"	"	"	74	55
	"	"	"	"	64	10
	"	"	"	"	57	? trace
	"	"	"	"	54	Nil
	"	"	"	"	50	Nil

* This batch showed great reluctance to crystallise; in experiments with the dialysed solution all membranes of $70m\mu$ and less retained the haemocyanin completely and the filtration curves were markedly abnormal. The stock was re-dialysed against distilled water and a white precipitate formed and collected mainly as a surface layer on centrifuging. The blue opalescent solution of haemocyanin was pipetted into another sac and dialysis continued against acetate buffer, p_H 4.8. Crystallisation of haemocyanin occurred. The results in the table are those given by the recrystallised preparation.

Membranes of A.P.D. below $55m\mu$ retain the protein completely even in broth medium under conditions most favourable for filtration, whilst more porous membranes permit the protein to pass. The filtration end-point for haemocyanin (*Helix*) is therefore $55m\mu$, from which we deduce [Elford, 1933] that the protein has a particle size of 18 to $28m\mu$. Svedberg and Chirnoaga [1928] and also Svedberg and Heyroth [1929], by the method of sedimentation analysis, concluded that the protein particles were spherical and $24m\mu$ in diameter. Thus the ratio of the particle diameter (by sedimentation analysis) to the average pore diameter of the limiting membrane for complete retention of the protein is 0.44.

Edestin.

The edestin was prepared from hemp-seed by extraction with neutral salt solution according to the method of Osborne [1909].

Special difficulties were encountered in the filtration studies with edestin solutions. A high concentration of salt was necessary to dissolve the protein at all, and even so the solubility was small. Further, the edestin solutions became turbid on standing and a precipitate settled out, a process which continued for a month or two until about half the protein had become insoluble. Contact with a collodion membrane appeared to accelerate this process. In the presence of broth the solution was relatively stable, and most of our filtration studies were made in this medium.

Influence of p_H . Edestin may be dissolved in acidulated water directly, in the absence of salt, and then adjusted, by adding alkali, nearly to the isoelectric point before precipitation occurs. Experiments made with solutions in dilute HCl at p_H 2.7 showed that the protein filtered very readily under these conditions. The filtration end-point was $15m\mu$, which is slightly lower than that established for solutions in broth at p_H 7.6. It is interesting to note that the reaction p_H 2.7 is outside the range of stability for monodispersion, p_H 5.5 to 9.7, determined by Svedberg and Stamm [1929]. They found edestin to be quite unstable on the acid side of its isoelectric point (p_H 5.5). The filtration results with acid edestin solutions are in accord with this view.

Fig. 2. Filtration end-point curves for edestin. (a) 0.5% edestin in 1.5 *M* sodium chloride at p_H 7.2; (b) 0.4% edestin in broth at p_H 7.4.

Filtration end-point curves. The end-point curves were established for edestin dissolved in (a) 1.5 *M* sodium chloride at p_H 7.2, and (b) Hartley's broth at p_H 7.4 (see Fig. 2). The filterability of edestin was much improved by the pres-

ence of broth, but the filtration curves for edestin solutions were in general of the abnormal type, and in this respect they resembled those of serum globulin. A trace of protein in broth solution passed a $20m\mu$ membrane on one occasion, but membranes of porosities below $18m\mu$ have always retained the edestin. This filtration end-point $18m\mu$ indicates a particle diameter $6-9m\mu$, in good agreement with the value $8m\mu$ established by Svedberg and Stamm [1929]. Thus the ratio of the particle diameter, $8m\mu$, given by sedimentation analysis, to the limiting pore diameter for the complete retention of edestin is 0.44.

Egg albumin.

The egg albumin was prepared from a Schering-Kahlbaum product which originally contained globulin. After refractionation by means of half-saturated ammonium sulphate, the albumin was three times recrystallised from solution at p_H 4.7 in the presence of ammonium sulphate and then dialysed against distilled water. The final product yielded aqueous solutions which were quite colourless and contained only a very slight trace of sulphate.

The filtration end-point for egg albumin had previously been found by Elford [1933] to be $6m\mu$, a value confirmed by Bauer and Hughes [1934]. In the present study the influences of filtration conditions upon the filterability of the protein have been more extensively investigated.

Fig. 3. Variation of u_{max} for 0.5% egg albumin solutions of different p_H values when filtered at 3 atmos. pressure through $\Delta = 9m\mu$ membranes; $\bullet = 11m\mu$ membranes; $\square = 23m\mu$ membranes.

Influence of p_H . Curves showing the variation of u_{max} with p_H for three different grades of membrane are given in Fig. 3. In all cases adjustments of p_H were made by adding 0.1 *N* HCl or 0.1 *N* NaOH and the final protein concentration was kept at 0.5%. A well-defined zone of minimum filterability occurs in the region of p_H 5.0-5.5, which is slightly more alkaline than the usually accepted isoelectric point, p_H 4.6. The surface activity of the protein is maximum in this region, as shown by a minimum value in the surface tension of its solutions and a maximum degree of adsorption on collodion particles (see Fig. 5).

Influence of sodium chloride. The presence of salt in the solution may exert a profound influence upon the filtration behaviour of a protein. Egg albumin is a protein which is readily soluble in water in contrast to the relatively insoluble

globulins typified by edestin in the previous section. The influence of varying salt concentrations upon the filterability of egg albumin is shown in Fig. 4.

Fig. 4. Variation of $u_{\max}$ for 0.5% egg albumin solutions containing varying amounts of NaCl. Curves "a" and "b" are for 11 $m\mu$ membranes, and curve "c" for 19 $m\mu$ membranes. The figures against the points are the respective p_H values.

Fig. 5. Adsorption of egg albumin on collodion particles. A: from 0.5% solutions at different p_H values. B: from 0.5% solutions with different concentrations of NaCl at same p_H 5.05. The actual measured p_H values are inserted against the points in the graph.

Curves (a) and (b) give the variation of $u_{\max}$ with the percentage concentration of NaCl for 0.5% protein solutions at p_H 7.0 and p_H 5.05 respectively, filtered through 11 $m\mu$ membranes. Curve (c) is for p_H 4.93 and more porous membranes,

19 $m\mu$. The p_H value of each solution is inserted near its appropriate point on the graph. The initial decrease in filterability shown in the early portion of curve (a) is fully accounted for by the change in p_H (cf. Fig. 3), and it is apparent that the presence of NaCl in concentrations up to 1 % slightly improves the filterability of egg albumin at p_H 7.0. Considering curve (b) for p_H 5.05 the effect of the salt concentration is here more pronounced. Initially there is a definite improvement in filterability, $u_{\max}$ increasing until the concentration of sodium chloride reaches 0.2–0.3 %, when a decrease commences and continues until for 1 % salt the value of $u_{\max}$ is slightly lower than that for the salt-free solution. The direction of the effect is similar in curve (c) but the maximum is less pronounced with the more porous membrane. Studies of the adsorption of egg albumin at p_H 5.05 on to collodion particles have demonstrated that for small additions of salt a region of minimum adsorption exists (see Fig. 5) which corresponds with the zones of maximum filterability in curve (b), Fig. 4. The change in filtration behaviour may thus be correlated with the modified surface properties of the protein in the presence of added electrolyte. Further, and largely in consequence of such modified surface properties, the state of dispersion of the colloidal protein units may be expected to be affected, particularly in more concentrated solutions.

Comparison of different media at p_H 7.2. The filterabilities of 0.5 % solutions of egg albumin in water, 1 % sodium chloride, $M/15$ phosphate buffer (Sørensen), Hartley's broth and extra-digested broth (no protein detectable), each at p_H 7.2, were compared. The values of $u_{\max}$ are shown in Table II.

Table II.

Medium	$u_{\max}$	Remarks
Water	0.05	A.P.D. of membranes used 9 $m\mu$
1 % NaCl	0.12	
$M/15$ phosphate buffer	0.15	Filtration pressure = 3 atmos.
Extra-digested broth	0.15	
Stock Hartley's broth	0.45	

Conforming with our previous studies, Hartley's broth is seen to be the best medium for filtering egg albumin. However, considerable variation is found among different batches of broth. The value of the broth from the filtration standpoint is determined largely by the stage at which the digesting process is arrested in its preparation [Elford and Ferry, 1934]. A good broth enables as much as 75 % of the egg albumin in a 1 % solution to be obtained in the filtrate through a 9 $m\mu$ membrane [Elford, 1933], whilst in the other extreme case of the extra-digested broth containing no surface-active constituent, the percentage of protein passing is much lower, comparable with that in 1 % saline and $M/15$ phosphate buffer media—viz. 10 to 15 % only. This represents a slight improvement upon the filterability of the protein in water, although a different result might be expected at a p_H nearer the isoelectric zone (see curve (b), Fig. 4).

DISCUSSION.

The studies with the serum proteins [Elford and Ferry, 1934], haemocyanin, edestin and egg albumin, have revealed a general uniformity in filtration behaviour among the proteins, particularly in respect of the influence of medium and hydrogen ion concentration. The optimum conditions may be said to be within the limits of p_H 7 to 8, whilst of the several media investigated, Hartley's

broth has invariably facilitated filtration most. For serum albumin, egg albumin and edestin, a zone of good filterability was found in each case for aqueous solutions more acid than the isoelectric point of the protein, but the prevailing conditions of p_H were somewhat outside the p_H range of molecular stability found by Svedberg, whose sedimentation data suggest some degradation of the molecule in extreme acid solutions.

It is of particular importance that the optimum conditions for the filtration of proteins coincide with those already established for viruses and bacteriophages. This enables comparisons of their relative filterabilities and particle sizes to be made on a reliable common basis.

Elford [1933], as the result of experiments with suspensions of known particle size, concluded that under comparable optimum conditions of filtration, the particle sizes of suspensions might be deduced from the relationship $d = (\text{factor}) p$, where d = particle diameter, p = end-point pore diameter. The value of the factor adopted for the range of membrane porosities 10 to 100 $m\mu$, within which our present studies have been mainly conducted was 0.33 to 0.50. The probable particle size is thus expressed as lying between the limits 0.33 p and 0.5 p . The data for the proteins whose molecular particle sizes have been independently determined by the method of ultracentrifugal sedimentation analysis enable a check to be made on this value of the "factor". In Table III the relevant data are summarised.

Table III.

Protein	D_s $m\mu$	p $m\mu$	D_s/p
Haemocyanin (<i>Helix</i>)	24	55	0.44
Edestin	8	18	0.44
Serum pseudoglobulin (horse)	6.9	11-12	0.57-0.63
Serum albumin (horse)	5.4	9-11	0.54-0.60
Egg albumin	4.34	6	0.72

D_s = particle diameter by sedimentation analysis. p = end-point pore diameter.

The values of D_s/p , except that for egg albumin, are seen to lie between 0.4 and 0.6, thus affording good agreement with the adopted value of the "factor", for membranes of A.P.D. greater than 10 $m\mu$. Provided that the optimum conditions for filtration do prevail when the end-point is determined, the protein data suggest that the true particle size will correspond more closely with the upper rather than the lower value deduced from the pore diameter, *i.e.* the true conversion factor will be nearer 0.5 than 0.33.

The closer approximation of the limiting pore size to that of the retained particle indicated by the figures for egg albumin for membranes of porosities less than 10 $m\mu$ has been previously discussed [Elford, 1933].

SUMMARY.

1. The proteins haemocyanin (*Helix*), edestin and egg albumin, like the serum proteins, animal viruses and bacteriophages, have been found to filter best when dissolved in Hartley's broth at p_H 7 to 8. The influences of various media, of p_H and of the concentration of neutral salt on filterability have been studied.

2. The filtration end-points have been established for haemocyanin (*Helix*) 55 $m\mu$ and edestin 18 $m\mu$, indicating particle sizes 18-28 $m\mu$ and 6-9 $m\mu$ respectively.

3. The results are shown to provide further confirmation of the value of the factor employed in deducing the particle size of a suspension from the porosity of the limiting membrane which just completely retains the disperse phase.

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