XVII. ULTRAFILTRATION STUDIES WITH NORMAL HORSE SERUM.

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THE purpose of this investigation has been the acquisition of further evidence upon the important biological question as to the state of dispersion of the proteins in normal serum. Do the albumin and globulin fractions obtained from serum by salting-out processes exist as chemical individuals in the native state, or are they the artificially resolved constituents of a natural albumin-globulin complex? The method of ultrafiltration analysis has been applied in this study.

Previous filtration work.

The numerous references in the literature relating to ultrafiltration of various kinds of sera are mostly concerned with the distribution of inorganic salts between the colloidal and crystalloidal components in serum. The paucity of detailed investigations of the state of dispersion of the serum proteins is no doubt due largely to the lack of suitably graded membranes. Collodion sacs, although useful for the separation of colloidal and crystalloidal fractions, as in dialysis operations, have not the reproducibility and uniformity of pore size which is required when systematic analysis over a range of graded porosities is proposed. Bendien and Snapper [1933] filtered normal and pathological human sera through collodion sacs; the proportion of albumin to globulin in the ultrafiltrate was two to three times that in the original serum. This evidence that the albumin filtered more readily than the globulin made the existence of an albumin-globulin complex in serum very improbable.

EXPERIMENTAL.

The graded series of flat collodion membranes described by Elford [1931] is very suitable for such an investigation as that here considered and has been employed in all the analyses of sera described in this paper. These membranes [Elford, 1931; Elford and Ferry, 1935] are characterised by their excellent reproducibility and uniformity and the wide range of porosities available. The serum has been filtered under positive pressures of air or nitrogen employing the form of ultrafilter described by Barnard and Elford [1932]. In each experiment successive samples (each 1 ml. approx.) of filtrate have been collected in calibrated glass receiving tubes, and then from the subsequent analyses filtration curves have been constructed [*cf.* Elford and Ferry, 1934, on the filtration of serum proteins].

Analysis of filtrates.

The amounts of protein in the filtrates were determined refractometrically with a Zeiss dipping refractometer. Precipitation tests were also made, half-saturated ammonium sulphate being used to indicate the globulin, whilst

salicylsulphonic acid served to indicate the albumin remaining in the solution after filtering or centrifuging off the globulin precipitate. Comparisons were made with a control series of dilutions of the unfiltered serum.

Preparation of serum.

Several different batches of normal horse serum have been studied. The blood was collected aseptically and allowed to clot spontaneously, unless otherwise stated. The serum was pipetted from the clot and centrifuged for 15 min. at 2000 r.p.m. It was then filtered through a membrane of average pore diameter (A.P.D.) 0.45μ to remove any chance contaminating organism and to furnish the serum in a condition suitable for the subsequent ultrafiltration analyses. This filtered serum was kept in a conical resistance glass flask having a tight-fitting cotton-wool plug and was stored in the cold at 0° . Before using the serum in any particular experiment the flask was placed in a water-bath at 25° for at least 20 min. so that the equilibria of the colloidal system could become readjusted for room temperature. Frequently after storage in the cold a sediment formed in the serum but this quickly dissolved on warming. The sensitiveness of colloidal equilibria to changes of temperature and salt concentration makes it very necessary to adopt a uniform treatment for all samples of serum studied in order that reliable comparisons of results may be made.

Filtration curves of normal horse serum.

When normal horse serum prepared in the manner outlined was filtered through graded membranes having progressively lower porosities an interesting series of filtration curves was obtained (Fig. 1). Membranes of A.P.D. $> 90 m\mu$

Fig. 1. Filtration curves of normal horse serum. A.P.D. values of membranes inserted against the appropriate curves.

permitted the serum to pass with undiminished protein content, and the curves were normal in type, *i.e.* when the adsorption capacity of the membranes had been satisfied the serum passed in entirety with little evidence of subsequent blocking. Over the range of porosities $80 m\mu$ to $35 m\mu$ the curves became abnormal in type,

the concentration of protein in the filtrate reaching a maximum value and then falling off rapidly as blocking of the pores set in. However, in the region of $25\text{ m}\mu$ reversion to the normal type of filtration curve occurred. These facts suggest that all the protein of normal serum may traverse pores having diameters $> 90\text{ m}\mu$ almost unhindered when the adsorption capacity has been saturated. However, with porosities $< 90\text{ m}\mu$ a portion at least of the protein has increasing difficulty in passing the protein-coated pores and the membrane becomes choked. The curves indicate that the blocking is never complete but tends towards a limiting value which still permits a small fraction of the protein to pass. It is significant that this corresponds roughly to the maximum reached in the normal filtration through even less porous membranes. These normal curves obtained with porosities $< 30\text{ m}\mu$ suggest that a fraction of the serum protein, some 20 to 30 %, is able to pass fairly readily in spite of the initial surface adsorption. We are thus led to the conclusion that in normal serum we are not dealing with a homo-disperse colloidal protein system.

End-point curve for the protein of normal horse serum.

The end-point curve for the total proteins was obtained by plotting the values of the maximum relative protein concentration ($u_{\max}$), furnished by the filtration curves, against either the A.P.D. or the log (A.P.D.) of the respective membranes. The maximum relative concentration of protein in the filtrate yielded by any particular membrane was given by

$$u_{\max} = \frac{(u_{\text{filtrate max}} - K)}{(u_{\text{serum}} - K)},$$

where

$u_{\text{filtrate max}}$ = maximum refractometer reading for the filtrate, read from the filtration curve.

u_{serum} = refractometer reading for whole serum.

$K = u_{\text{water}} + u_{\text{non-protein}}$ = refractometer reading of the filtrate from a $9\text{ m}\mu$ membrane, which is the most porous membrane to retain all the protein of serum. This value is characteristic for each particular serum.

Fig. 2. Filtration end-point curves for total proteins of normal horse serum, corresponding to three independent series of observations on different batches of horse serum. Zero protein given by $9\text{ m}\mu$ filtrate in each case.

Curve (a) anticipated curve for monodisperse system; curve (b) established curve for normal horse serum, $u_{\max}$ plotted against A.P.D.; curve (c) corresponding curve to (b) for $u_{\max}$ plotted against log (A.P.D.).

The end-point curve based on three independent series of observations for different batches of normal horse sera is given in Fig. 2, plotted in curve *b* as $u_{\max}$ against A.P.D. and in curve *c* as $u_{\max}$ against log A.P.D. It is seen that membranes of A.P.D. $< 10\text{ m}\mu$ retained all the protein. This is significant, for it agrees with the end-points found by Elford and Ferry [1934] for serum albumin and pseudoglobulin separately, these being 9 to $10\text{ m}\mu$ and 11 to $12\text{ m}\mu$ respectively. This strongly suggests that these proteins do exist independently in serum, for were they present only in an associated form a rather higher end-point value indicating a greater particle size would be expected. A further interesting and very suggestive feature characterises the end-point curve. A definite flattening of the curve, varying slightly among different batches of serum, is situated in the region of porosity 20 to $30\text{ m}\mu$. The curve appears to be the resultant for a system containing protein in two broad states of dispersion and cannot be explained purely in terms of variable adsorption effects. Without analysing the interpretation further at this juncture it will be helpful to adopt as a provisional hypothesis that the section of the end-point curve in the region of porosity 30 to $90\text{ m}\mu$ corresponds to the filtration of a complex protein, whilst the portion from 20 to $10\text{ m}\mu$ corresponds to the filtration of molecular protein.

Filtration of diluted serum.

The filtration and end-point curves were determined for serum which had been diluted in physiological saline and also in Hartley's broth at $p_H\ 7.6$. The proportions in each case were one part of serum to nine parts by volume of the diluent. The results given in the end-point curves of Fig. 3 show that dilution

Fig. 3. Filtration end-point curves for diluted serum compared with that for whole serum. Dotted curve, whole serum; o, serum diluted 1:9 in saline; x, serum diluted 1:9 in broth.

has a pronounced effect upon the filterability of the serum proteins. Whilst the end-point for the complete retention of all the protein remains essentially what it was for undiluted serum, the form of the curve for the saline dilution now corresponds to that of a homodisperse system and this is supported by the fact that the filtration curves were all normal in type. The filterability of the broth-serum system suggests that in this case the proportion of complex protein has been reduced and that of the molecular fraction increased compared with undiluted serum. These results indicate that the postulated complex protein readily tends

to dissociate when the system is diluted in an appropriate medium, saline being much more favourable than broth. Comparison of the curves for saline and broth dilutions shows that the end-point for the saline system is slightly higher than that for the broth system, being 13 $m\mu$ instead of 9 to 10 $m\mu$. This is probably to be explained by the increased adsorption of the saline system [cf. filterability of proteins in different media, Elford and Ferry, 1934].

Filtration of aged serum.

Serum stored at 0° in the manner already indicated for periods of from 6 to 20 months exhibited definite differences in filtration behaviour compared with fresh serum. The end-point curves of Fig. 4 illustrate how the filtration of serum

Fig. 4. Filtration end-point curves for aged serum. (a) Dotted curve, normal serum about 2 weeks old. (b) $\circ$, normal serum 15 months old. (c) $\times$, normal serum 22 months old. (d) Δ , dilution 1 : 9 in broth of the 22 months-old serum.

proteins depends upon the age of the serum. Comparing curve (a) for normal serum about 2 weeks old with curve (b) for the same serum after 15 months at 0° it appears that aggregation of the protein has proceeded further and that the proportion of molecularly dispersed protein has decreased. Curve (c) for a still older serum (22 months at 0°), but of a different batch from (a) and (b), further emphasises the effect. The gradual slope of the curve indicating partial retention of protein over a greater range of porosity than for fresh serum suggests that particles of varying sizes have been formed through aggregation of protein units. In order to ascertain whether this secondary aggregation was also of a reversible type some of the 22 months-old serum was diluted 1 : 9 in broth and filtered. The resulting curve (d) shows again that the aggregates have been resolved, for it closely resembles the curve for fresh serum similarly diluted.

Filtration of Type I pneumococcus antiserum.

Filtration experiments were made with some Type I antipneumococcus horse serum. Unfortunately freshly collected serum was not available, and the preparation used was an oxalated serum 30 months old and had been stored after filtration without preservative in a waxed stoppered bottle. The potencies of the undiluted antiserum and of the filtrates were estimated by the optimum

proportions test. We are much indebted to Dr Wilson Smith for his advice concerning the technique of this method. It was estimated that 75 % of the antibody passed a 140 $m\mu$ membrane, rather less than 1 % passed an 80 $m\mu$ membrane, whilst the filtrate from a 54 $m\mu$ membrane showed no trace of antibody activity. Although these experiments are not strictly comparable with those on the normal sera previously described, since no adequate control on the treatment to which the serum had been subjected was possible, nevertheless the result suggests very strongly that the antibody activity is associated with the larger complex protein in the serum. Further, the possibility is presented of concentrating the antibody above a relatively porous membrane.

DISCUSSION.

The complete retention of the proteins of normal horse serum by all membranes of A.P.D. < 10 $m\mu$, whether the serum is whole or diluted, fresh or old, indicates that the smallest unit of the protein has a particle size of about 5 $m\mu$ [Elford, 1933]. The limiting porosity coincides with the end-point found for horse serum albumin when solutions of the crystallised protein are studied, and the particle size compares well with the findings of Svedberg and Sjogren [1930] in their centrifugal studies with this protein. Within narrow limits of porosity, 10 to 13 $m\mu$, filtrates of normal serum have frequently given positive tests for albumin but no evidence of globulin. This again accords well with the observations on individual proteins [Elford and Ferry, 1934], the end-point for pseudoglobulin having been found to be slightly higher than that for serum albumin, viz. 11 to 12 $m\mu$ as compared with 9 to 10 $m\mu$. These facts have led us to the conclusion that the albumin and pseudoglobulin fractions of serum do co-exist as individual molecular species in the native state. However, the observations recorded in the foregoing paragraphs would further suggest that the proteins of serum are not homodisperse. Experience with the membranes used in this study has led us to expect an end-point curve of the form (a) in Fig. 2 for a homodisperse suspension. The experimentally established curve (b), Fig. 2, for undiluted serum suggests that the native protein exists broadly in two different states of aggregation, i.e. besides the molecularly dispersed albumin and pseudoglobulin whose end-points are quite close together, there is an additional protein unit having a particle size approximately twice that of molecular serum albumin. Precipitation tests and values of the albumin/globulin ratio, given by nitrogen estimations for which a modification of the method of Howe [1921] was used, have indicated that the more readily retained fraction of the serum protein consists principally of globulin, whilst the major portion of the more filterable fraction passing membranes below 30 $m\mu$ is albumin. The fact that only 20 to 30 % of the total albumin is accounted for in these filtrates, and even a smaller proportion of the total globulin, would be explained either by the presence of associated forms of these proteins or by a statistical effect of sieving, through modification of the membrane porosity consequent upon retention of the gross globulin fraction. Maybe both these factors operate. Studies, in continuation of those recorded here, on the filtration of the euglobulin fraction of horse serum by one of us [Grabar, 1934] have shown that this protein is very definitely less filterable than either serum albumin or pseudoglobulin. Also Elford and Ferry [1934] found that, in concentrations comparable with that of the protein of serum, solutions of the individual proteins, more particularly pseudoglobulin, filtered less readily than did solutions containing only 0.5 % protein, suggesting some degree of association in higher concentrations. It seems probable therefore

that the less filterable fraction of the protein in normal serum is an associated complex of molecular protein, predominantly globulin, maybe the euglobulin. We have not followed the distribution of lipid, but in view of the observations of Chick [1914] on the phosphorus content of the various protein fractions of serum the possibility that lipid may also be associated with the complex must not be overlooked. The filtration experiments with diluted serum indicate strongly that the associated protein can dissociate readily under suitable conditions with the result that the system then behaves as might be expected of a mixture of the molecular proteins.

The observations on the filterability of the type I pneumococcus antiserum (horse) are in keeping with the above interpretation, since the antibody found in our experiments to be associated with the more readily retained fraction of the serum is well known to be contained in the globulin portion.

Recent ultracentrifugal studies have furnished several extremely significant facts regarding the molecular complexity of the proteins of serum. Thus Mutzenbecher [1933] found that the albumin and globulin sedimented at characteristic rates in serum, and concluded that the proteins existed as separate individuals in the native state. There was evidence that the globulin was not monodisperse. Extended studies of both normal and pathological sera by McFarlane [1935] revealed a more complex state of affairs. Normal serum was found to contain two components possessing sedimentation constants comparable with those of serum albumin and serum globulin. The lighter of these fractions however was found not to be homogeneous, but to consist of two substances, one of which (*X* substance) had a molecular weight only slightly greater than that of serum albumin, which was in the same fraction. A further interesting effect was found by McFarlane, *viz.* that on dilution the proportion of globulin increases whilst that of the two lighter constituents decreases. It becomes necessary then to postulate an equilibrium between albumin and globulin in which the compound product is of intermediate molecular weight, but rather nearer to that of albumin. The evidence at present available is insufficient to permit the formulation of the type of interaction responsible for this.

It will be seen that there is in general a large measure of agreement between the filtration results and those of sedimentation analysis. The protein found to accompany the albumin in filtrates furnished by membranes of A.P.D. $< 30\text{ m}\mu$ which we have found to behave in precipitation tests as globulin and in its filtration end-point as pseudoglobulin, may correspond to McFarlane's *X* component. The larger protein fraction which we have regarded as a globulin aggregate, possibly with lipid and some albumin loosely associated, would correspond to the more readily sedimented globulin fraction.

Hartley [1914] presented analytical evidence that serum albumin differs chemically from serum globulin, both the eu- and pseudo-fractions of which were identical. The physical evidence of the present study supports Hartley's conclusions, for undoubtedly the albumin and globulin behave as distinct individuals in serum, the former existing mainly in the molecularly disperse condition whilst the globulin appears to be partly in an associated form and partly molecularly dispersed.

SUMMARY.

The analysis of the state of aggregation of the proteins in normal horse serum by the method of ultrafiltration through graded collodion membranes has indicated (1) that the simplest protein units present correspond in particle size to molecular serum albumin and pseudoglobulin as studied individually in

dilute solutions of the pure proteins, and (2) that in addition to the molecularly dispersed protein there is a fraction more readily retained by membranes and in particle size about twice that of serum albumin. This is essentially a globulin aggregate, maybe with some lipid and albumin loosely associated, and in view of its poor filterability it may correspond with the euglobulin fraction of serum. The associated globulin complex appears to dissociate readily under favourable conditions, particularly upon dilution, when the filtration behaviour of the serum proteins resembles that of a mixture of the molecularly dispersed individual constituents.

The antibody of an aged sample of type I pneumococcus antiserum was retained by membranes simultaneously with the more complex globulin fraction of the serum protein.

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REFERENCES.

- Barnard and Elford (1932). *Proc. Roy. Soc. Lond.* B **109**, 360.
Bendien and Snapper (1933). *Biochem. Z.* **260**, 105.
Chick (1914). *Biochem. J.* **8**, 404.
Elford (1931). *J. Path. Bact.* **34**, 505.
—— (1933). *Proc. Roy. Soc. Lond.* B **112**, 384.
—— and Ferry (1934). *Biochem. J.* **28**, 650.
—— — (1935). *Brit. J. Exp. Path.* **16**, 1.
Grabar (1934). *Compt. Rend. Soc. Biol.* **116**, 72.
Hartley (1914). *Biochem. J.* **8**, 541.
Howe (1921). *J. Biol. Chem.* **49**, 93.
McFarlane (1935). *Biochem. J.* **29**, 407, 660, 1175, 1209.
Mutzenbecher (1933). *Biochem. Z.* **266**, 226.
Svedberg and Sjogren (1930). *J. Amer. Chem. Soc.* **52**, 2855.