CXLVIII. THE ULTRACENTRIFUGAL ANALYSIS OF NORMAL AND PATHOLOGICAL SERUM FRACTIONS.

BY ARTHUR SPROUL McFARLANE[1].

From the Institute of Physical Chemistry, Upsala, Sweden.

(*Received April 1st, 1935.*)

IN this paper, which is the concluding one of the series, we are concerned with the fractionation, by means of a neutral electrolyte and electrodialysis, of a few sera which have already been investigated in the ultracentrifuge [McFarlane, 1935, 1, 2, 3]. The particular objects in view have been (1) to investigate the composition of the globulin fractions obtained by a single precipitation with ammonium sulphate; (2) to compare the analytical results of the fractionation procedures with the results of ultracentrifugal analysis of the same serum; and (3) to discover in the case of certain sera whether new molecular types which have been shown to be present are precipitated or remain in solution on half saturation with ammonium sulphate.

For several reasons we have relied on ammonium sulphate as protein precipitant. It was advisable to use only one neutral electrolyte in order to be able to compare the results for one serum with those for another. Ammonium sulphate has been the most widely used electrolyte in the past in the fractionation of sera, although sodium sulphate is becoming increasingly used as a result of the work of Howe [1923]. It may be noted, however, that Howe finds the values for the albumin and globulin concentrations in cow sera obtained by a single precipitation with ammonium sulphate to agree with those obtained with sodium sulphate. Wu [1922] obtains the same analytical results with saturated magnesium sulphate as with half-saturated ammonium sulphate. Cullen and Van Slyke [1920] and Sørensen [1925; 1930] and others have used ammonium sulphate extensively in fractionating serum proteins.

It has been shown [1935, 1] that when horse serum albumin and globulin, prepared by ammonium sulphate fractionation, are mixed together in concentrated solution and the mixture is analysed in the ultracentrifuge, the concentration of the albumin fraction is found to be abnormally high and that of the globulin fraction correspondingly low. These proteins exist in concentrated solution in a form of equilibrium with each other. The same has been shown indirectly to be true for cow and human serum proteins by ultracentrifugal analysis of the concentrated and diluted sera. Since we know very little about the action of ammonium sulphate as a protein precipitant and in view of this difference in the state of the proteins in concentrated and diluted sera, it was decided in the first place to investigate the composition of the globulin precipitates obtained by half saturating a concentrated and a diluted cow serum with ammonium sulphate.

It is not proposed to repeat here the numerous details of the technique of an ultracentrifugal experiment which will be found in the earlier papers referred to. In the following tables we show the concentrations of the various protein fractions in g./100 ml. calculated from measurements of protein refraction in-

[1] Beit Memorial Research Fellow.

crement using the values given in Table I of a previous paper [1935, 2] for the specific refraction increments (α) of the proteins. As already pointed out some of these α values cannot be regarded as generally established, and our concentration values may therefore be subject to errors. They are the best available α values and it serves a useful purpose here to record even approximate values for the protein concentrations.

Exp. 1. Total globulins from normal cow and horse sera by precipitation with ammonium sulphate.

We employed in this experiment a portion of the cow serum used in Exps. 1–11, and a portion of the horse serum used in Exps. 12, 13, 15 and 16 of a previous paper [1935, 2]. The cow serum was treated in the following ways.

(*a*) 4 ml. of serum were diluted with 40 ml. of 1 % sodium chloride in a stoppered centrifuge-tube. 44 ml. of saturated ammonium sulphate were then added slowly and with constant shaking. The mixture was left for 2 hours during which time the globulin precipitate settled to the bottom. The tube was then centrifuged until the precipitate was well packed down on the bottom and the supernatant fluid could be poured off completely and the precipitate drained. This globulin precipitate was dissolved in water and dialysed against 1 % sodium chloride under pressure until equilibrium was reached. The solution (1·0 ml.) was transferred to a small graduated cylinder and the small collodion sac washed out with drops of dialysate which were transferred to the cylinder to make a final volume of 2·2 ml.

Results.

Refraction increment of globulin	0·01060
Therefore refraction increment of globulin in original serum ...	0·000583
Total protein refraction increment of original serum	0·01414
Therefore proportion of total protein refraction increment due to globulin fraction	41·2 %

(*b*) To 4 ml. of serum were added slowly and with shaking 4 ml. of saturated ammonium sulphate solution. The mixture was left for 2 hours and then centrifuged. The supernatant fluid was poured off and the precipitate drained and then stirred up with 8 ml. of saturated ammonium sulphate. After one hour the precipitate was again separated by centrifuging, suspended in a little water and dialysed against 1 % sodium chloride. The volume of combined protein solution and washings from the dialysis sac was in this case 3·0 ml.

Results.

Refraction increment of globulin	0·00818
Therefore refraction increment of globulin in original serum ...	0·00614
And proportion of total protein refraction increment due to globulin fraction	43·4 %

It is thus seen that the difference in the analytical results by these two procedures is very small.

The horse serum was treated as described under method (*a*) and the results were:

Volume of globulin solution	2·50 ml.
Refraction increment of globulin	0·01021
Therefore refraction increment of globulin in original serum ...	0·00638
Total protein refraction increment of original serum	0·01310
Therefore proportion of total protein refraction increment due to globulin fraction	48·7 %

Table I shows the results of the ultracentrifugal experiments on these three specimens of globulin. Fig. 1 *a* shows the curve of cow serum globulin precipitated from dilute solution (exposure taken 57½ minutes after reaching full speed), and Fig. 1 *b* shows the curve of horse serum globulin prepared in a similar

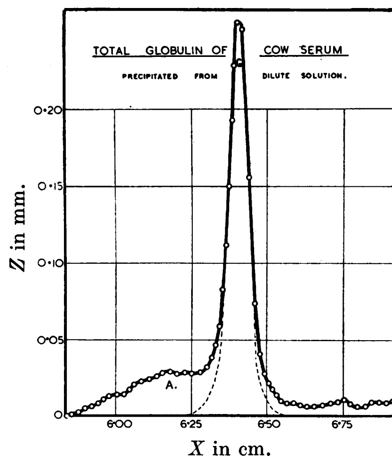


Fig. 1 *a*.

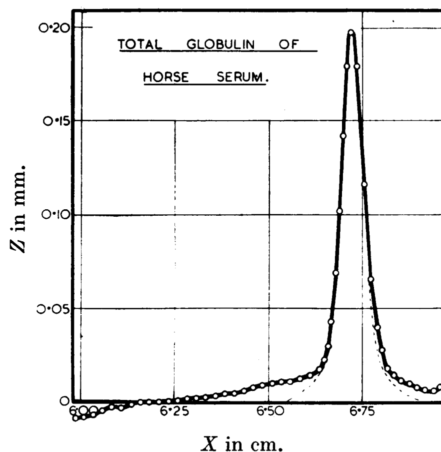


Fig. 1 *b*.

Table I. *Results obtained in the investigation of total globulin from cow and horse sera.*

		Cow serum globulin precipitated from dilute solution	Cow serum globulin precipitated from concentrated solution	Horse serum globulin precipitated from dilute solution
Cell thickness (mm.)		2·00	2·00	2·00
Optical scale distance (cm.)		2·58	2·44	1·09
Cell temperature (first exposure)		33·8°	32·4°	31·5°
Cell temperature (last exposure)		34·3°	35·5°	34·3°
Approximate duration of run (hours)		2	2	2
Mean centrifugal force used (times gravity)		255,000	250,000	230,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\,m\mu$		0·0106	0·00818	0·0102
Corresponding total protein concentration (g./100 ml.)		5·65	4·42	5·48
Total $n_1 - n_0$ corrected to $\lambda = 366\,m\mu$		0·0114	0·00884	0·0110
Average $S_{W,20} . 10^{13}$ of globulin		6·23	6·18	5·31
Average $n_1 - n_0$ from sedimentation diagram, $\lambda = 366\,m\mu$	Globulin	0·00560	0·00560	0·00833
	Other molecules	0·00309	0·00254	0·00089
	Total	0·00869	0·00814	0·00922
"% diff." total $n_1 - n_0$		−24 %	−8 %	−16 %
Proportion of total calculated $n_1 - n_0$ due to globulin fractions		64·5 %	69 %	90 %

way (exposure taken 116½ minutes after reaching full speed). The curves at corresponding times for cow serum globulin precipitated from concentrated and dilute solutions are all closely similar and differ markedly from the curves for horse serum globulin precipitated from dilute solution. In both specimens of cow serum globulin 30–35 % of other molecules (mainly *A* fraction) are present. All of this proportion, of course, does not represent albumin which we might hope to recover by repeated fractionation under ideal circumstances because the globulin was examined in concentrated solution. In such circumstances the

concentration of the A fraction in a mixture is greater than corresponds to the albumin added. We have examined concentrated globulin solutions because these afford better possibilities of detecting traces of albumin.

Although the horse globulin was examined in more concentrated solution than the two cow globulin preparations the curves (*e.g.* Fig. 1*b*) show the presence of only some 10 % of an A fraction. From our experience with artificial mixtures of horse serum albumin and globulin [1935, 1] in various concentrations we estimate that at least 5 % of "recoverable" albumin is present in this preparation. Similarly, it is probable that some 15–20 % of recoverable albumin is present in both cow globulin preparations.

It is interesting to find so little difference in the two cow globulin preparations. We deduce from this experiment that when ammonium sulphate precipitates globulin from solution it is a matter of little importance whether all the globulin is present with the usual molecular dimensions, or is present, as in native sera, in a low molecular form probably in some way associated with albumin. Presumably, immediately the G fraction is precipitated from a concentrated mixture the equilibrium between A and G fractions results in the production of more G fraction which is precipitated, and this goes on until only albumin remains in the solution.

The preparation of horse serum globulin is surprisingly homogeneous for a single precipitation, in view of the fact that a preparation from another serum [*cf.* 1935, 1, Fig. 2*d*] after repeated reprecipitation contained nearly 20 % of an A fraction.

Exp. 2. Total globulins from normal human sera by precipitation with ammonium sulphate.

In this experiment we used the sera from O.D.B. and C.A. [1935, 2, Table XVII] and fractionated in accordance with method (*a*) above.

Results.

4 ml. of serum from O.D.B. gave 3·13 ml. of globulin solution.	
Refraction increment of globulin	0·00615
Therefore refraction increment of globulin in original serum ...	0·00482
And proportion of total protein refraction increment due to globulin fraction...	33 %
4 ml. of serum from C.A. gave 2 ml. of globulin solution.	
Refraction increment of globulin	0·00815
Therefore refraction increment of globulin in original serum ...	0·00407
And proportion of total protein refraction increment due to globulin fraction...	25·5 %

The results are shown in Table II and Fig. 2*a* shows the curve of an exposure taken 65 minutes after reaching full speed in the experiment on O.D.B.'s globulin, and Fig. 2*b* that of an exposure taken 69½ minutes after reaching full speed in the experiment on C.A.'s globulin. It is clear from these curves that both globulin preparations contain considerable amounts of albumin. It is calculated from the area of the curves that O.D.B.'s globulin contains no less than 43 % of an A fraction and C.A.'s contains 22 % of an A fraction.

It would clearly have been informative to examine the globulin preparations in a highly diluted state in order to determine how much of the A fraction represents theoretically recoverable albumin. This we have not done because the graphical analysis of the curves of very dilute mixtures is not accurate, particularly when we are concerned with a mixture containing excess of globulin. The data from the examination of these concentrated globulin solutions suffice

to show, however, that the results of serum analysis which depend on a single precipitation with ammonium sulphate are not consistent. Heterogeneous globulin fractions result from the precipitation, and the degree of heterogeneity appears to vary not only between sera of different species but also between normal members of the same species. Results given later in this paper lend further support to these conclusions.

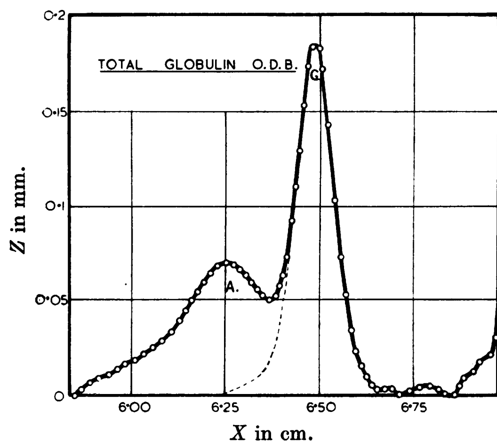


Fig. 2*a*.

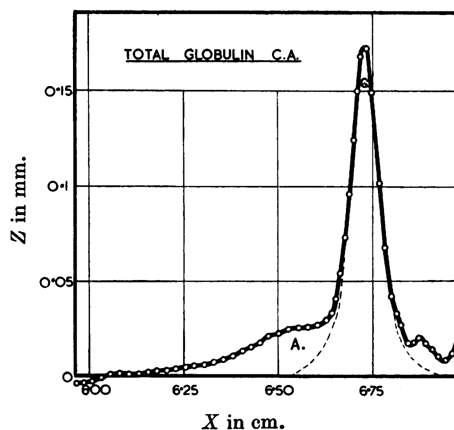


Fig. 2*b*.

Table II. *Results obtained in the investigation of the total globulins from the sera of O.D.B. and C.A.*

		Total globulin from serum of O.D.B.	Total globulin from serum of C.A.
Cell thickness (mm.)		2·00	2·00
Optical scale distance (cm.)		5·28	1·98
Cell temperature (first exposure)		34·1°	32·4°
Cell temperature (last exposure)		34·5°	36·0°
Approximate duration of run (hours)		2	2
Mean centrifugal force used (times gravity)		255,000	255,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\,m\mu$		0·00615	0·00815
Corresponding total protein concentration (g./100 ml.)		2·81	3·72
Total $n_1 - n_0$ corrected to $\lambda = 366\,m\mu$		0·00664	0·00880
Average $S_{W,20} \cdot 10^{13}$ of globulin		6·22	5·87
Average $n_1 - n_0$ from sedimentation diagram, $\lambda = 366\,m\mu$	Globulin	0·00306	0·00546
	Other molecules	0·00229	0·00152
	Total	0·00535	0·00698
"% diff." total $n_1 - n_0$		-19·5 %	-21 %
Proportion of total calculated $n_1 - n_0$ due to globulin		57 %	78 %

It appears that some additional factor or factors other than the concentrations of the fractions determine whether ammonium sulphate produces a globulin precipitate which is free from albumin. It is well known that the results of the precipitation analysis of sera vary with the hydrogen ion concentration of the mixture. No doubt this is due to variable quantities of albumin which are precipitated with the globulin. It is also noteworthy in connection with the gross heterogeneity of the above normal human globulin preparations that Roche and Bracco [1934] found much lower molecular weights (103,000) for human globulin than for horse globulin (150,000) by osmotic pressure measurements.

Exp. 3. Serum fractions after drying.

We have described previously [1935, 2] the investigation of a human serum which had been dried by the Hardy-Gardner procedure using ether and alcohol at low temperatures. Much work has been done using albumin and globulin preparations obtained from this redissolved powder, and it seemed to us that by repeated fractionation it might be possible to obtain albumin and globulin fractions which were free from the polydisperse matter which we have shown to contaminate the dried serum. A dilute solution of the serum powder was therefore treated with an equal volume of saturated ammonium sulphate and the globulin precipitate separated off and reprecipitated twice from dilute solution. The precipitate was also washed once with half saturated ammonium sulphate solution between each precipitation. The albumin fraction was precipitated three times in all using saturated ammonium sulphate. The albumin and globulin solutions after dialysis against 1 % sodium chloride were examined in the ultracentrifuge and the results are shown in Table III.

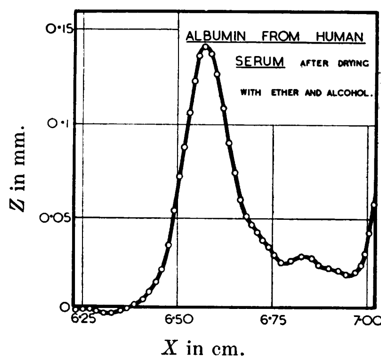


Fig. 3*a*.

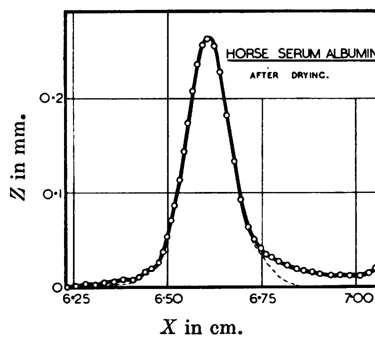


Fig. 3*b*.

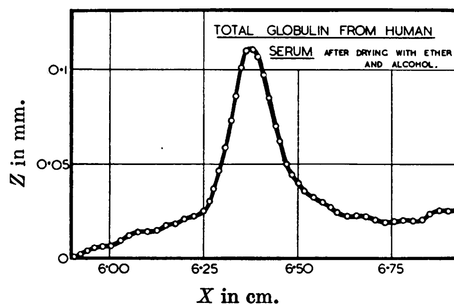


Fig. 3*c*.

Fig. 3*a* shows the curve of an exposure from the albumin experiment obtained 37½ minutes after reaching full speed. It is clear that considerable amounts of polydisperse protein still contaminate this protein. The curves of the globulin preparation show the presence of even greater amounts of polydisperse protein. We show a curve from this experiment in Fig. 3*c* obtained from an exposure taken 30 minutes after reaching full speed. From the curves it is calculated that less than 30 % of the total protein may be regarded as homogeneous globulin.

Table III. *Results obtained in the investigation of serum fractions which had been subjected to certain drying procedures.*

	Prepared from human serum which had been dried with ether and alcohol		Horse serum albumin after drying by simple evaporation
	Albumin	Globulin	
Cell thickness (mm.)	2·00	3·00	4·00
Optical scale distance (cm.)	4·44	5·64	3·64
Cell temperature (first exposure)	32·9°	32·1°	31·4°
Cell temperature (last exposure)	34·9°	34·9°	34·3°
Approximate duration of run (hours)	2	2	1¾
Mean centrifugal force used (times gravity)	255,000	255,000	250,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\, m\mu$	0·00457	0·00381	0·00339
Corresponding total protein concentration (g./100 ml.)	2·28	1·74	1·85
A. Total $n_1 - n_0$ corrected to $\lambda = 366\, m\mu$	0·00494	0·00411	0·00366
Average $S_{W,20} . 10^{13}$	4·18	6·66	4·23
B. Average $n_1 - n_0$ from curve of sedimentation diagram, $\lambda = 366\, m\mu$	0·00283	0·00127	0·00319
$\left(\frac{A-B}{B}\right) \times 100$	43 %	69 %	15 %
Boundary spreading coefficient (cm.²/sec. 10⁷)	5·9	9·2	4·8

We show in parallel (Table III) the results of an experiment on a recrystallised preparation of horse serum albumin which had been dried by simple evaporation under reduced pressure and then redissolved. The dried albumin, unlike the serum dried with ether and alcohol, did not redissolve completely. The experiment is also not an exact parallel with the earlier one because we have used horse instead of human albumin, and because in one case we are dealing with a dried albumin preparation whereas in the other we are dealing with an albumin fraction from dried whole serum. The results, however, together with the curve of Fig. 3*b* (from an exposure taken 40 minutes after reaching full speed) serve to illustrate an important fact which we have already recorded for dried cow serum, *viz.* that the soluble protein after drying by this technique has all the normal characteristics of the original protein.

Exp. 4. Fractions arising in the cataphoresis of normal human serum.

We have not obtained by any fractionation process a specimen of human serum albumin comparable in homogeneity with specimens of recrystallised horse serum albumin. Nor have we obtained a specimen of human globulin so nearly homogeneous as the best horse globulin preparations obtained by repeated precipitation [1935, 1] although these are not perfect in this respect. We therefore decided to try the method of transport cataphoresis and in this experiment we used an apparatus described by Theorell [1934][1]. About 100 ml. of fresh serum from a healthy student volunteer were placed in the bend of the special U-tube, the limbs of which are built up of short glass sections. By sliding shutters it is possible to close off the ends of each section without leakage so that each limb becomes subdivided into a number of closed cylindrical compartments and the fluid may be withdrawn from each compartment. Liquid junctions in the two limbs between the serum below and a phosphate buffer of the same p_H (7·21) and conductivity above were carefully made and cataphoretic movement of the serum protein induced in the usual way by means of a high potential applied through silver/silver chloride electrodes. The serum pigment migrated with the

[1] I am indebted to Med. Kand. O. Mellander for assistance in this experiment.

more rapidly moving albumin boundary. After 12 hours the various compartments were shut off and the solutions withdrawn, and a portion of each was tested for albumin and globulin. The solution from the upper compartment in the positive limb of the U-tube gave only an albumin reaction. The solutions from the upper three compartments in the negative limb gave no protein reactions, and that from the fourth gave only a globulin reaction. These albumin and globulin solutions were then dialysed against 1 % sodium chloride to determine the protein refraction increments and were then examined in the ultracentrifuge.

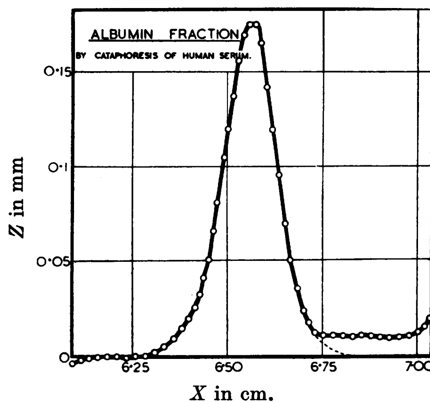


Fig. 4a.

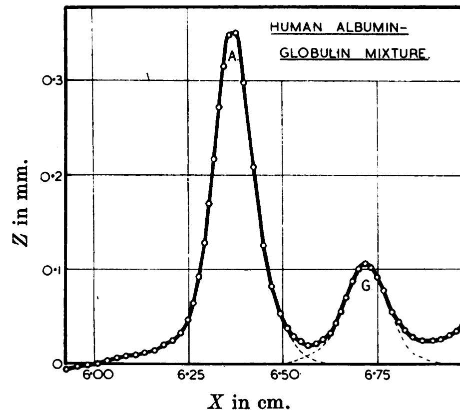


Fig. 4b.

Table IV. *Results obtained in the investigation of fractions arising in the cataphoresis of human serum.*

	Prepared from normal human serum by transport cataphoresis	
	Albumin	Globulin
Cell thickness (mm.)	4·00	3·00
Optical scale distance (cm.)	4·94	3·64
Cell temperature (first exposure)	32·8°	31·7°
Cell temperature (last exposure)	35·1°	34·1°
Approximate duration of run (hours)	2	$1\frac{3}{4}$
Mean centrifugal force used (times gravity)	250,000	255,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\, m\mu$	0·00189	0·00371
Corresponding total protein concentration (g./100 ml.)	0·95	1·70
Total $n_1 - n_0$ corrected to $\lambda = 366\, m\mu$	0·00204	0·00401
Average $S_{W,\,20} \cdot 10^{13}$	4·39	6·75
Average $n_1 - n_0$ from curve of sedimentation diagram $\lambda = 366\, m\mu$	0·00184	0·00289
"% diff." $n_1 - n_0$	-10 %	-28 %
Spreading coefficient of boundary (cm.^{2}/sec. 10^7)	6·3	4·9

Table IV shows the results of the two experiments, and Fig. 4a a curve from the albumin experiment from an exposure taken 55 minutes after reaching full speed. The curves show that the albumin contains only very small amounts of globulin, if any. It is the most nearly homogeneous human albumin preparation which we have obtained and suggests that the method of transport cataphoresis has considerable possibilities in the preparation of pure serum albumins where these are not crystallisable. It should be noted in particular from Table IV that the boundary spreading coefficient of the albumin corresponds well with

the value for recrystallised horse serum albumin in corresponding concentration [*cf.* 1935, 1, Fig. 4]. The curves from the globulin solutions are also satisfactory and show no signs of an A fraction such as contaminates the human globulin preparations of Figs. 2*a* and 2*b*. The discrepancy between measured and calculated total $n_1 - n_0$ is rather high in this case. We conclude from this experiment that the cataphoresis method is on the whole a satisfactory one for fractionating human sera.

The object of this experiment was to obtain human albumin and globulin preparations which could be mixed together again and the mixture examined in the centrifuge. We obtained just sufficient of the above solutions to make a mixture of 3·57 g./100 ml. total protein concentration, and we show in Fig. 4*b* a curve of this mixture obtained from an exposure taken 55 minutes after reaching full speed. From the curves of this experiment we calculate the albumin/globulin ratio to be 2·9. The ratio of the quantities of albumin to globulin added was 1·3. Even if we regard the globulin preparation as only 80 % monodisperse it is clear that on mixing the two together a considerable shift has occurred in the ratio of the fractions. From this experiment it is deduced that the equilibrium between the fractions in human serum is reversible just as we have shown it to be in the case of horse serum fractions. It is, however, not reversible in one respect. It will be noted that the A curve in Fig. 4*b* is quite symmetrical, no signs of an X fraction being detectable. We have noted [1935, 2] the same irreversible change when cow and horse sera are treated with ammonium sulphate. The significance of the phenomenon is obscure.

Exp. 5. Globulin fractions from antidiphtheritic horse serum.

It will have been noted in the examination of the total globulins of normal sera that no distinct molecular types exist in these which might correspond to the pseudoglobulin and euglobulin fractions. If these two fractions represent different proteins we must conclude either that the two have the same molecular weight but different chemical constitutions, or that they are produced by the process of electrodialysis which leads to their separation. To investigate this matter we prepared the total globulin from an immune serum[1] [1935, 3, Exp. 1, Serum 2] by one precipitation with ammonium sulphate and electrodialysed a portion of this to give pseudoglobulin and euglobulin fractions.

Results.

10 ml. of serum 2 gave 16·34 ml. of globulin solution.

Refraction increment of globulin	0·00833
Therefore refraction increment of globulin in original serum ...	0·01361
Total protein refraction increment of original serum	0·01770
Therefore proportion of total protein refraction increment of original serum due to total globulin fraction	77 %

It may be mentioned here incidentally that antidiphtheritic serum 1 [1935, 3] when subjected to the same analytical procedure gave a globulin proportion of 79·5 %.

The total globulin of serum 2 was centrifuged and Fig. 5*a* shows the curve of an exposure taken 50 minutes after reaching full speed. It will be seen from this and from the results in Table V that the preparation is nearly homogeneous

[1] The fractionation of immune sera into eu- and pseudo-globulin fractions is now a process of considerable commercial importance.

which is surprising for a single precipitation. We may assume that the proportion of homogeneous globulin in the original serum is slightly less than the analytical figure—say 75 %—on account of traces of albumin in the globulin preparation. Ultracentrifugal analysis of the undiluted serum [1935, 3] showed that exactly equal amounts of A and G fractions were present. This means that

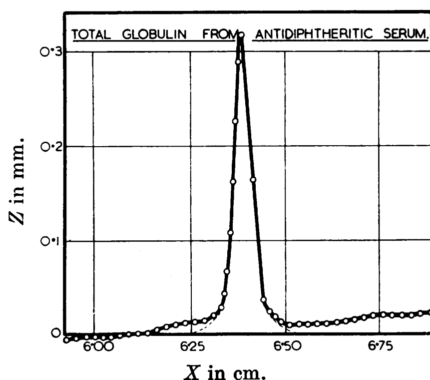


Fig. 5a.

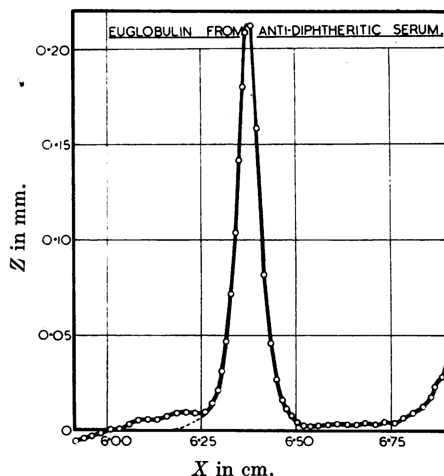


Fig. 5b.

Table V. *Results obtained in the investigation of globulin fractions from an immune serum.*

	Fractions from antidiphtheritic horse serum		
	Total globulin	Pseudo-globulin	Euglobulin
Cell thickness (mm.)	2·00	2·00	2·00
Optical scale distance (cm.)	1·38	1·68	2·28
Cell temperature (first exposure)	34·8°	33·0°	32·7°
Cell temperature (last exposure)	35·2°	35·5°	34·9°
Approximate duration of run (hours)	2	$1\frac{3}{4}$	$1\frac{3}{4}$
Mean centrifugal force used (times gravity)	255,000	255,000	255,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\, m\mu$	0·00833	0·00619	0·00915
Corresponding total protein concentration (g./100 ml.)	4·48	3·33	4·92
Total $n_1 - n_0$, corrected to $\lambda = 366\, m\mu$	0·00899	0·00668	0·00988
Average $S_{W,20} \cdot 10^{13}$	5·81	6·31	6·16
Average $n_1 - n_0$ from curve of sedimentation diagram, $\lambda = 366\, m\mu$	0·00788	0·00603	0·00499
"% diff." $n_1 - n_0$	−12·3 %	−9·7 %	−49·5 %
Spreading coefficient of boundary (cm.²/sec. × 10⁷)	0·15	0·36	1·3

the area of the A curve corresponds to twice the amount of albumin which is recoverable from this serum and the area of the G curve to two-thirds the recoverable globulin. These figures give some idea of the extent to which the albumin/globulin ratio in an untreated immune serum may be altered by the equilibrium between the fractions.

10 ml. of the total globulin of serum 2 were dialysed at 4° against distilled water and then electrodialysed according to the procedure described by Ettisch and Ewig [1928]. As recommended by Ettisch and de Loureiro [1933] we have

used at the cathode a membrane of parchment and at the anode a membrane of glycine-collodion. The electrodialysis was completed inside an hour with no detectable rise of temperature in the cell. The liquid in the centre compartment was stirred up and poured off. The precipitate was separated by centrifuging, drained and stirred up with 1 % sodium chloride in which it redissolved slowly and incompletely to an opalescent solution. After standing overnight in contact with the insoluble globulin the solution of euglobulin was dialysed against 1 % sodium chloride and examined in the ultracentrifuge. The pseudoglobulin solution was dialysed against 1 % sodium chloride and similarly examined. The results are shown in Table V, and Fig. 5*b* shows the curve of an exposure taken 43 minutes after reaching full speed in the experiment on the euglobulin fraction. The pseudoglobulin fraction differs in no significant details from the original total globulin preparation. The euglobulin preparation, on the other hand, is largely polydisperse, as is shown by the shape of the curves, the large "% diff." values, and the abnormally large boundary spreading coefficient. Svedberg and Sjögren [1930] and Mutzenbecher [1931] found both the pseudoglobulin and euglobulin of normal horse sera to be polydisperse, the former fraction much less so than the latter. Svedberg and Sjögren did not use differently charged membranes and their electrodialysis lasted for 36 hours. Mutzenbecher used the same technique as that described above but electrodialysed whole serum instead of total globulin. These differences may explain why our pseudoglobulin preparation appears to be more nearly homogeneous than theirs. Alternatively, antidiphtheritic horse serum may be capable of giving a relatively homogeneous pseudoglobulin fraction while normal horse serum is not. We may say at least that the polydisperse nature of euglobulin seems to be fully substantiated. Our experiment also shows that the pseudoglobulin of antidiphtheritic horse serum does not contain any homogeneous fraction of significantly different molecular weight from that of the total globulin.

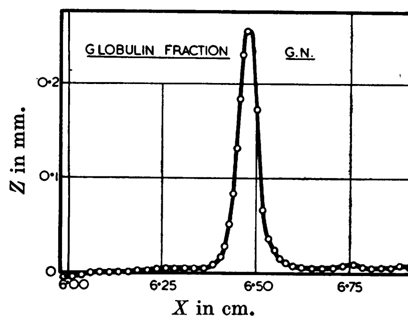
Exp. 6. Arbitrary globulin fractions from the serum of G.N. (septic pneumonia).

It has been shown in Exp. 8 of a previous paper [1935, 3] that in the untreated state the protein of G.N.'s serum is approximately equally distributed between *A*, *G* and *X* fractions. We employed for this serum a purely arbitrary method of fractionation. 4·24 ml. of serum were diluted with 40 ml. of 1 % sodium chloride, and saturated ammonium sulphate was added with shaking until a substantial precipitate had appeared as judged by the turbidity of the solution. This required 23 ml. of the ammonium sulphate solution. The precipitate was centrifuged off, dialysed and examined in the ultracentrifuge (fraction 1). More saturated ammonium sulphate (4 ml.) was added until another substantial precipitate had appeared and this was also separated and dialysed (fraction 2). Sufficient ammonium sulphate (17·24 ml.) was now added to make half saturation and the remainder of the globulin separated off (fraction 3). From the results of the refractive index measurements we obtain the following values for the proportions of the total refraction increment of the original serum which are due to each fraction.

Fraction	1	15 %
,,	2	16 %
,,	3	30·5 %

The results of the ultracentrifugal examination of these three fractions are shown in Table VI, and Fig. 6*a* shows the curves of an exposure taken 56 minutes

after reaching full speed in the experiment on fraction 2. The results for the three globulin fractions indicate that these have approximately the same composition, if we except differences in the "% diff." values which indicate mainly



X in cm.

Fig. 6.

Table VI. *Results obtained in the investigation of certain arbitrary globulin fractions from a pathological human serum (G.N.).*

	Globulin fraction (1)	Globulin fraction (2)	Globulin fraction (3)
Cell thickness (mm.)	2·00	2·00	2·00
Optical scale distance (cm.)	2·14	1·68	1·08
Cell temperature (first exposure)	32·6°	33·9°	29·6°
Cell temperature (last exposure)	35·5°	36·1°	33·0°
Approximate duration of run (hours)	$1\frac{3}{4}$	$1\frac{3}{4}$	$1\frac{3}{4}$
Mean centrifugal force used (times gravity)	250,000	255,000	245,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\,m\mu$	0·00687	0·00728	0·00875
Corresponding total protein concentration (g./100 ml.)	3·14	3·32	4·00
Total $n_1 - n_0$, corrected to $\lambda = 366\,m\mu$	0·00742	0·00786	0·00945
Average $S_{W,20} \cdot 10^{13}$	6·18	6·20	5·65
Average $n_1 - n_0$ from curve of sedimentation diagram, $\lambda = 366\,m\mu$	0·00578	0·00680	0·00665
"% diff." $n_1 - n_0$	−22 %	−13·5 %	−29·5 %
Spreading coefficient of boundary (cm.²/sec. × 10⁷)	0·26	0·27	0·18

differences in the amount of polydisperse protein in each fraction. It is interesting to find also that the curves of all three fractions show very small proportions of *A* fractions to be present—much less than we have found in the globulin fractions of normal human sera. These erratic results all support the view already stated that some factor—probably electrochemical—of which we have no precise knowledge determines the ultimate degree of homogeneity of the globulin precipitates obtained by treating serum with ammonium sulphate.

Exp. 7. Total globulin fractions from the sera of A.J. (malignant disease) and G.L.E. (myeloma).

4 ml. of A.J.'s serum analysed by procedure (*a*) gave 2·0 ml. of a globulin solution.

Refraction increment of globulin	0·0106
Therefore refraction increment of globulin in original serum ...	0·00503
Total protein refraction increment of original serum	0·0128
Therefore proportion of total protein refraction increment of original serum due to globulin fraction	39·3 %

4 ml. of G.L.E.'s serum yielded 8 ml. of globulin solution.

Refraction increment of globulin	0·00796
Therefore refraction increment of globulin in original serum ...	0·01592
Total protein refraction increment of original serum	0·0220
Therefore proportion of total protein refraction increment of original serum due to globulin fraction	72·4 %

The results are shown in Table VII, and Fig. 7*a* shows a curve of A.J.'s globulin (from an exposure taken 44½ minutes after reaching full speed), and Fig. 7*b* a curve of G.L.E.'s globulin (from an exposure taken 65 minutes after reaching full speed).

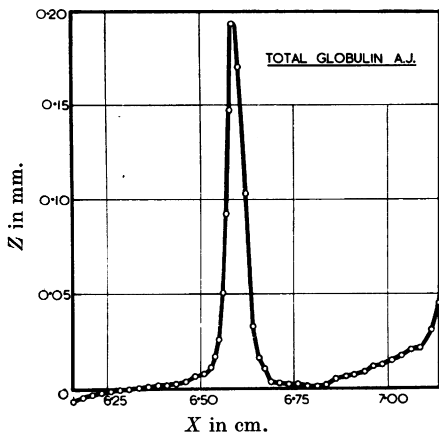


Fig. 7*a*.

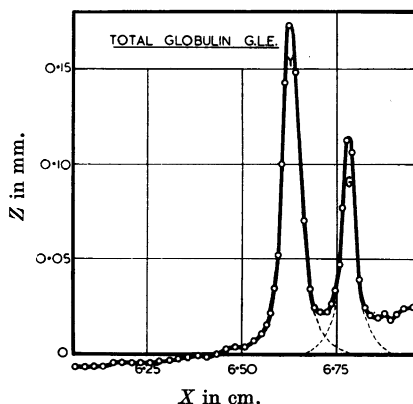


Fig. 7*b*.

Table VII. *Results obtained in the investigation of the total globulins from the sera of A.J. (malignant disease) and G.L.E. (myeloma).*

	Total globulin from serum of A.J.	Total globulin from serum of G.L.E.
Cell thickness (mm.)	2·00	2·00
Optical scale distance (cm.)	0·78	1·38
Cell temperature (first exposure)	33·2°	31·1°
Cell temperature (last exposure)	35·7°	33·9°
Approximate duration of run (hours)	1¾	2
Mean centrifugal force used (times gravity)	255,000	255,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\, m\mu$	0·0106	0·00796
Corresponding total protein concentration (g./100 ml.)	4·84	3·64
Total $n_1 - n_0$, corrected to $\lambda = 366\, m\mu$	0·0115	0·00860
Average $S_{W,20} \cdot 10^{13}$	5·01	5·56 (*Y*) 6·77 (*G*)
Average $n_1 - n_0$ from curve of sedimentation diagram, $\lambda = 366\, m\mu$	0·00930	0·00443 (*Y*) 0·00247 (*G*)
Total $n_1 - n_0$ from curve of sedimentation diagram, $\lambda = 366\, m\mu$	0·00930	0·00690
"% diff." total $n_1 - n_0$	−19 %	−20 %

The total globulin from A.J.'s serum appears to be almost completely free from any *A* fraction and except for the discrepancy of −19 % between calculated and measured refraction increments it has the characteristics of a homogeneous protein. It is also noteworthy in this experiment that the proportion of globulin fraction obtained by precipitation analysis, *viz.* 39·3 % is in exact agreement with the proportion obtained by ultracentrifugal analysis of the serum diluted with 4 volumes of 1 % sodium chloride.

Fig. 7*b* and the results in Table VII show that the globulin precipitate from G.L.E.'s serum is composed of two molecular types corresponding approximately in sedimentation constants to the *Y* and *G* fractions of the original serum. Both fractions appear to be homogeneous.

Exp. 8. Total albumin and total globulin fractions from the serum of K.M. (malignant disease).

In the earlier examination of this serum [1935, 3] we found that four molecular fractions were present and the evidence was in favour of the view that the lightest fraction is an abnormal type and the three heavier fractions correspond to the A, X and G fractions of normal sera. The serum was fractionated by the simplest possible procedure. The total globulin precipitate obtained according to procedure (*a*) was redissolved, dialysed and examined in the centrifuge. The mother-liquor from this precipitation was dialysed under pressure whereby all the ammonium sulphate was removed and the protein was obtained in concentrated solution. This solution was then examined in the centrifuge.

Results.

4 ml. of K.M.'s serum yielded 2·4 ml. of globulin solution.

Refraction increment of globulin	0·00662
Therefore refraction increment of globulin in original serum ...	0·00397
Total protein refraction increment of original serum	0·0118
Therefore proportion of total protein refraction increment of original serum due to globulin fraction	33·6 %

The results of the ultracentrifugal experiments are shown in Table VIII. The curve of Fig. 8*a* is from an exposure taken 100 minutes after reaching full speed in the experiment on the total albumin; that of Fig. 8*b* is from an exposure taken 46½ minutes after reaching full speed in the experiment on the total globulin. These curves and the results obtained from them demonstrate clearly what has happened to the new molecular type in the processes of fractionation. The new (A_1) molecule has remained in solution in close association with the A fraction. The ratio of the concentration of A_1 to A molecules in the albumin fraction is however markedly different from their ratio in the original serum. There are several possible explanations of this, but none that can be substantiated in the meantime.

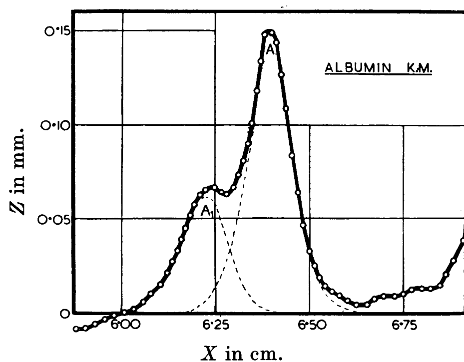


Fig. 8*a*.

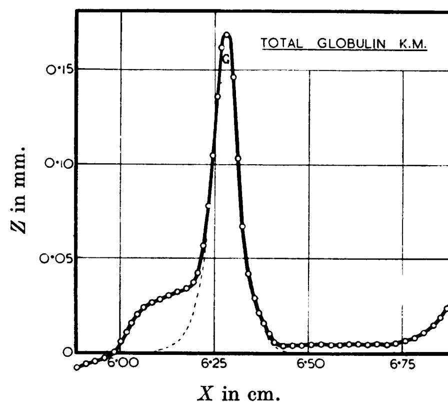


Fig. 8*b*.

The globulin fraction contains slightly more than 20 % of an A fraction. In this respect it differs from the preceding pathological globulin preparations and resembles the preparations from normal human sera (Exp. 2).

Table VIII. *Results obtained in the examination of the albumin and total globulin fractions from the serum of K.M. (malignant disease).*

		Albumin fraction	Total globulin
Cell thickness (mm.)		2·00	2·00
Optical scale distance (cm.)		3·18	2·28
Cell temperature (first exposure)		30·2°	30·9°
Cell temperature (last exposure)		35·8°	34·3°
Approximate duration of run (hours)		3	2
Mean centrifugal force used (times gravity)		250,000	255,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\,m\mu$		0·00659	0·00662
Corresponding total protein concentration (g./100 ml.)		3·30	3·02
Total $n_1 - n_0$, corrected to $\lambda = 366\,m\mu$		0·00712	0·00715
Average $S_{W,20} \cdot 10^{13}$		2·70 (A_1) 3·71 (A)	6·23 (G) —
Average $n_1 - n_0$ from sedimentation diagram, $\lambda = 366\,m\mu$	Fractions	0·00177 (A_1) 0·00458 (A)	0·00410 (G) 0·00109 (A)
	Total	0·00635	0·00519
"% diff." total $n_1 - n_0$		-11 %	-27 %

General discussion.

The view that the albumin and globulin which can be isolated from serum represent stable chemical individuals probably existing as such in the original untreated serum has been widely accepted for many years. The popularity of the view is due to the agreement which has been obtained by many workers studying various physical and chemical properties of these proteins. Serum albumin has become generally regarded as an amphoteric substance of well-defined elementary chemical composition, molecular weight, isoelectric point, electrophoretic mobility, specific refraction increment, specific light absorption and optical rotation. When one considers the possibilities for differences between the results of various observers which are due to differences and errors in the techniques of observation, it is probably fair comment to say that the general measure of agreement in regard to the properties of serum albumin is satisfactory and in support of the view that all observers are examining essentially the same substance. The same cannot be said confidently of serum globulin, because in this case the measure of agreement among different observers has not been good. There has been, however, a strong undercurrent of belief in regard to this substance that the disagreements arise from the presence of quantities of contaminating substances, and that in the preparation of each observer there is present in greater or smaller concentration, a common substance.

Sørensen, to whose earlier work on egg albumin is due probably more than to anything else, the inception of the view that the proteins are stable chemical entities, has recently [1930] given good reasons for doubting the fundamental principles on which this view is based. It is now Sørensen's contention that the uniformity of the results obtained for the serum proteins is not a criterion of the eventual individuality and purity of these substances but only of the reproducibility of the physical and chemical conditions under which the proteins are prepared. He regards both proteins of serum as component or "co-precipitation" systems made up of stable main valency units (probably polypeptides) which are loosely held together by residual valencies. The ultimate size, number and general properties of these loosely constructed units is determined by the physical conditions which hold in the solution at any instant. Since practically all observers have obtained their albumin and globulin fractions from solutions

of closely prescribed ammonium sulphate content it is to be expected that they should obtain fractions of similar composition.

Sørensen's conclusions would be more convincing to us if his carefully conducted experiments had been carried out with fresh untreated serum instead of with serum powder dried by the Hardy-Gardner procedure, and also if his proof had contained more definite physical evidence of the existence of his protein co-precipitation systems in native protein solutions. The facts which we have related in the foregoing series of papers, however, neither support nor disprove his theory because our proof of the existence of albumin and globulin fractions in mixtures over a range of concentrations is purely arbitrary. Our practice has been to find first an empirical relationship between the concentration and sedimentation constant of a protein, and thereafter to use the sedimentation constant to identify the protein in any concentration. The original relationship may depend on the effects of viscosity alone or on a combination of viscosity effects and changes in the molecular dimensions of the protein. The real test of Sørensen's theory is whether or not the observed molecular weights of the serum proteins tend to a constant value in dilute solutions. From a consideration of the data of Svedberg and Sjögren [1930] on the sedimentation equilibrium of serum proteins, and of Adair and Robinson [1930] on the osmotic pressure of the serum proteins we incline to the view that serum albumin at least has a constant molecular weight in high dilutions.

We can say from the results of this work that untreated sera or concentrated mixtures of the serum proteins do not contain a collection of residual valency units which are all of the same particle size. Sørensen does not say whether his co-precipitation systems might be expected to contain under a given set of conditions a small or a large number of different particle sizes. The ultracentrifuge shows that in normal serum there are present three fractions each of which if not completely homogeneous can only be heterogeneous within very narrow limits. We may assume with Sørensen that the serum represents a set of three co-precipitation systems, but we must postulate that specific molecular forces are operative in producing and maintaining only those three sets of loosely bound residual valency units. Sørensen's theory does not explain the nature of these forces.

In one important respect Sørensen's views coincide strikingly with the facts of our experiments. To quote his own words "an interaction must be assumed, for example in serum, between the protein systems mutually as well as between these and other substances present". The equilibrium which we have shown to exist between A and G fractions in serum appears to agree with his prediction in all essentials. It must be remarked, however, that this equilibrium phenomenon does not necessarily require that the A and G fractions separately should be reversibly dissociable systems. The phenomenon is equally well explained by the theory that two stable proteins are able to form loose combinations when present in sufficient concentration. Neither point of view offers any explanation of the really unusual fact about this equilibrium, *viz.* that the protein complex or compound (according to the viewpoint adopted) has lower molecular dimensions than at least one of the disruption systems (or stable proteins) to which it gives rise on dilution.

Whether Sørensen's view that the proteins as a class are reversibly dissociable polypeptide systems is true or not is of great importance in connection with the results of investigations on isolated proteins. On the other hand it has only a small significance for the results of investigations on untreated serum. In the latter case it is frequently only desired to know the nature of the protein

fractions as they exist in serum and it is of minor importance whether these fractions represent proteins which may be isolated from the serum or dissociable systems of no fixed composition. In general, however, the prospect of enlarging our knowledge of the protein constitution of serum will be greatly increased if it is possible to separate the proteins in stable form and examine the pure solutions. For this reason we have conducted the preliminary fractionation investigations reported in this paper.

The outstanding feature of these few experiments is the lack of uniformity in the composition of the globulin precipitates obtained by half saturation with ammonium sulphate. Whereas certain sera, *e.g.* normal human and cow sera, gave rise to globulin precipitates which contained relatively large quantities of albumin, other sera, *e.g.* normal horse and some pathological human sera, gave rise to globulin fractions which contained only small amounts of albumin. The results of the latter experiments suggest that under certain conditions even a single precipitation with ammonium sulphate is capable of giving rise to satisfactorily homogeneous globulin preparations. The results of the experiments as a whole, however, suggest that we have still to find out what constitute ideal conditions for quantitatively fractionating serum by means of ammonium sulphate.

An experiment with the transport cataphoresis method of fractionation has indicated that there are possibilities inherent in this technique, particularly with regard to the fractionation of human sera. On theoretical grounds the method appears to be an ideal one for the preparation of serum proteins and it is hoped to attempt its application later to the isolation of the new molecular types which have been shown to exist in certain pathological sera.

We have examined the ammonium sulphate fractions from two pathological sera in which the evidence for the existence of new molecular types could be regarded as quite definite. The results indicate that in both cases the new molecular types have retained their individuality after the treatment with ammonium sulphate, a behaviour which does not seem to be shared by the X fraction of normal sera. This fact holds out considerable prospect that they represent stable proteins which like albumin and globulin are capable of being isolated in a comparatively pure state. The only satisfactory proof of this is, of course, actually to isolate them and demonstrate their purity. We anticipate, however, that many methods of fractionation will need to be tried and the results tested at every step by ultracentrifugal analysis before a suitable fractionation technique will be found.

SUMMARY.

Fractions obtained from various sera have been investigated in the ultracentrifuge.

1. The total globulin from a normal cow serum, whether precipitated from concentrated or dilute solution with ammonium sulphate, was found to be markedly heterogeneous due to the presence mainly of albumin.

2. The total globulins precipitated from two normal human sera in dilute solution with ammonium sulphate also contained quantities of albumin.

3. Albumin and globulin fractions were isolated from a human serum which had been dried with ether and alcohol. In spite of repeated precipitations with ammonium sulphate these fractions contained large amounts of polydisperse protein.

4. Albumin and globulin fractions obtained by cataphoresis of a normal human serum were found to be satisfactorily homogeneous.

5. The total globulin from an antidiphtheritic horse serum after one precipitation with ammonium sulphate contained only insignificant amounts of albumin. The pseudoglobulin obtained from this preparation by electrodialysis had approximately the same composition as the total globulin but the euglobulin was at least 50 % polydisperse.

6. In the fractionation of two pathological human sera in dilute solution by means of ammonium sulphate, homogeneous globulin fractions were obtained.

7. Two pathological sera in which the presence of new proteins had previously been demonstrated were fractionated by means of ammonium sulphate. In one case the new protein was found in the albumin fraction and in the other in the globulin fraction.

The general conclusion is drawn that a single precipitation with ammonium sulphate is not a satisfactory method of fractionating sera particularly for purposes of quantitative analysis.

It is a pleasure to acknowledge the unfailing interest and valuable advice of Prof. Svedberg in the course of this work and the generous hospitality afforded in his laboratory. To his assistants, particularly to Dr Kai O. Pedersen, I wish to express my great indebtedness. The expenses of this investigation have been defrayed by grants from the Andersson Foundation, the Nobel Foundation and the Rockefeller Foundation.

It will be possible to continue this work at the Lister Institute, London, where an ultracentrifuge now under construction in Upsala will be installed in the near future.

REFERENCES.

Adair and Robinson (1930). *Biochem. J.* **24**, 1864.
Cullen and Van Slyke (1920). *J. Biol. Chem.* **41**, 587.
Ettisch and Ewig (1928). *Biochem. Z.* **195**, 175.
—— and de Loureiro (1933). *Biochem. Z.* **266**, 422.
Howe (1923). *J. Biol. Chem.* **57**, 241.
McFarlane (1935, 1). *Biochem. J.* **29**, 407.
—— (1935, 2). *Biochem. J.* **29**, 660.
—— (1935, 3). *Biochem. J.* **29**, 1175.
Mutzenbecher (1931). *Biochem. Z.* **235**, 425.
Roche and Bracco (1934). *Compt. Rend. Acad. Sci.* **199**, 98.
Sørensen (1925). *Compt. Rend. Carlsberg Lab.* **15**, No. 11, 1.
—— (1930). *Compt. Rend. Carlsberg Lab.* **18**, No. 5, 1.
Svedberg (1930). *Kolloid Z.* **51**, 10.
—— and Sjögren (1930). *J. Amer. Chem. Soc.* **52**, 2855.
Theorell (1934). *Biochem. Z.* **275**, 1.
Wu (1922). *J. Biol. Chem.* **51**, 33.