LXXIX. THE ULTRACENTRIFUGAL PROTEIN SEDIMENTATION DIAGRAM OF NORMAL HUMAN, COW AND HORSE SERUM.

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It has been shown in a previous paper [McFarlane, 1935] that the diagrams which are obtained in the refractive index method of following protein sedimentation in the ultracentrifuge are capable of yielding a great deal of valuable qualitative and quantitative information relating to the composition of the protein solution in the centrifuge cell. In particular, they are capable of giving the concentration of each molecular fraction which separates in the centrifugal field and an estimate of the relative degree of dimensional homogeneity of the constituent molecules of each fraction. In the work to be reported here we have obtained the sedimentation diagrams for various sera and have attempted to establish the following general points: (1) the extent to which the ultracentrifugal sedimentation diagram is a strictly reproducible characteristic of a serum; (2) the nature and extent of any changes in the diagram caused by treating the serum in various ways; (3) whether the serum from a series of members of the same species gives the same sedimentation diagram; and (4) to what extent the serum diagram of normal members of different species is the same.

It is to be emphasised at the outset that the work which will be reported is essentially of the nature of a general survey of this new field of investigation, the object being more to discover the possibilities of the field as a whole than to establish any particular points in great detail.

Since each complete ultracentrifugal experiment takes some considerable time it has been necessary throughout to minimise the number of experiments. The evidence in regard to (1) is, therefore, largely obtained incidentally in the experiments designed to investigate (2) and (3). We have used cow serum in investigating the effect of treatment on the same sample of a serum because a large quantity was readily available to cover all the experiments. It was more convenient, and also of greater interest, to use human serum in investigating (3), and for the species comparison in (4) we have used human, cow and horse sera.

It is necessary to refer the reader to the previous paper [1935] for a full description of the theory and technique of an ultracentrifuge experiment and for the details of the methods of calculating the results. We shall require in the present communication to refer frequently to that paper.

SECTION 1. THE SEDIMENTATION DIAGRAM OF NORMAL COW SERUM.

In this section we shall describe a series of experiments all performed on the same sample of cow serum and designed to investigate the effect of treatment of various kinds on the sedimentation diagram of the serum.

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A large sample of blood was obtained from a cow which had no signs or history of disease.¹ The blood was allowed to clot spontaneously and the serum was separated after 12 hours. Portions of this serum were used in the following experiments. During the period of these experiments (10 weeks) the serum was kept at 4° under sterile conditions, and no signs of decomposition could be detected.

A portion of the serum was placed in a collodion dialysis bag arranged as for an osmotic pressure measurement. The bag was placed in 10 times its volume of 1 % sodium chloride solution and the level of serum in the capillary kept constant by externally applied pressure from a water manometer. When equilibrium had been reached, as indicated by constant manometer pressure, the apparatus was dismantled and the refractive index of solution and dialysate measured by means of the Pulfrich refractometer and using the sodium D line. The dialysate gave a negative protein reaction with salicylsulphonic acid. By subtracting the two values we obtained the total protein refraction increment of the serum, subject to the assumptions regarding the effect of the diffusible ion concentrations discussed in the previous paper.

In calculating protein concentrations from our refractometric data we have used the following specific refraction increment values throughout this work:

Protein	Specific refraction increment	Author
Horse serum albumin	0.00183	Adair and Robinson [1930, 1]
Horse serum globulin	0.00186	Adair and Robinson [1930, 1]
Total horse serum	0.00184	—
Human serum albumin	0.00200	Schretter [1926]
Human serum globulin	0.00219	Schretter [1926]
Total human serum	0.00202	Schretter [1926]
Cow serum albumin	0.00185	—
Cow serum globulin		
Total cow serum		

The value for total horse serum protein is a mean of Adair and Robinson's values for horse serum albumin and globulin, and the cow serum values are assumed to be the same as the horse and sheep serum values which Adair and Robinson have given. The use of these values can only give approximate concentrations because of these assumptions, and because there is a good deal of difference of opinion in the literature as to the exact values of the specific refraction increment of the serum proteins, and particularly of human serum protein [cf. Linder *et al.*, 1924; Starlinger and Hartl, 1925; Schretter, 1926]. In this work we are primarily concerned to measure and compare differences in refractive index ($n_1 - n_0$), concentration measurements being of secondary importance. We have, therefore, not made any more accurate determinations of concentrations than are given by the refractometric data, and we have avoided basing any important conclusions on the concentration values given.

In order to be able to compare the measured refractivity of the protein with the value calculated from the sedimentation diagram we have applied to the measured values the wave-length correction factor which Pedersen and Andersson, as described in our previous paper [1935], have obtained for horse serum proteins, *viz.* 1.08.

Exp. 1. Untreated cow serum. A portion of the serum was examined in a centrifuge cell 2.0 mm. thick. The centrifuge reached 58,000 r.p.m. (equivalent to a mean centrifugal force of 240,000 times gravity) after 40 mins. and was

¹ I am indebted to Med. Kand. O. Mellander for the supply of this serum.

kept at this speed to within ± 300 r.p.m. throughout the run which lasted a further 3 hours and 3 mins. up to the time of the last exposure.

It was found on viewing the scale from the camera when regular sedimentation had been established that an optical scale distance of 1.54 cm. gave satisfactory results, *i.e.* a large line displacement with good definition of the displaced lines, and this was used in all exposures. The temperature of the cell at the time of the first exposure (which we will call T_f) was 33.4° , and at the time of the last exposure (T_l) was 35.2° . The photographs were taken on Cramer plates using the mercury line at $366 m\mu$, which was isolated by means of a Corning nickel oxide glass filter. The exposure time was 25 secs., and the photographic enlargement factor 0.996.

Fig. 1.

The curve shown in Fig. 1 was obtained from a photograph taken 108 mins. after reaching full speed. The position of the meniscus (not shown in the figure) is 5.64 cm. from the centre of rotation. It will be seen that three molecular fractions are present. $S_{W,20}$ for the lightest fraction is 2.67 and for the heaviest fraction is 4.60. These $S_{W,20}$ values are corrected for the density and viscosity of the electrolyte medium in which the protein is dissolved (*cf.* equation (5) of previous paper) but take no account of the concentration of the protein. The value of $S_{W,20}$ at infinite dilution (or the true sedimentation constant) of the *A* fraction may be obtained by extrapolating to infinite dilution a series of values of $S_{W,20}$ at various concentrations. It is not so easy to obtain the true sedimentation constant of the *X* or *G* fractions because it is necessary to take into account the effect of the concentration of the lighter fraction or fractions, as the case may be. In this work we are mainly concerned to identify a particular fraction, and for this purpose it is not necessary to obtain the extrapolated sedimentation constant. It is to be emphasised, however, that the $S_{W,20}$ values in this paper may not be compared with the true sedimentation constants published for other proteins, since these latter are all extrapolated and represent natural constants characteristic of the proteins and independent of the concentration. $S_{W,20}$ for the intermediate fraction cannot be obtained with any degree of accuracy because a clear separation of this curve from that of the lightest fraction was not obtained. We shall in future label the three fractions, in order of increasing sedimentation velocity, as *A* fraction (presuming a relationship

with serum albumin); *X* fraction (of unknown nature); and *G* fraction (presuming a relationship with serum globulin).

It is impossible to produce here more than one curve from each experiment. This is a disadvantage because to obtain a complete picture of the sedimentation process it is necessary to consider all the curves.

We may discuss here in some detail a point of importance which arises in connection with the *A* and *X* fractions. We find in the case of cow serum the phenomenon of two boundaries which, in the process of sedimentation, reach the bottom of the cell without completely separating. The question arises whether under these conditions of incomplete separation we may attempt to resolve the composite curve graphically on the assumption that it consists of two symmetrical component curves. The problem is more comprehensible if we consider the boundaries in terms of protein concentration instead of refraction gradient. We have represented in Fig. 2A the refraction diagram of a hypo-

Fig. 2. Illustrating the type of curve-distortion caused by the overlapping boundaries in undiluted serum.

thetical composite *A* + *X* boundary with the corresponding protein concentration diagram shown in association with it (Fig. 2B). In both diagrams the component curves are represented by thin lines and the composite curve by a heavy line. Above the level "*a*" in the cell (*i.e.* to the left of the line "*a*" in the diagram) only *A* molecules are present, and we may therefore assume that the shape of this part of the curve will correspond to that of the sedimenting boundary curve of a pure solution of *A* molecules. As we have discussed in the previous paper, the concentration distribution of these molecules is determined by the forces of natural diffusion and an opposing force due to the variation of sedimentation velocity with concentration. Below the level "*c*" the shape of the curve should correspond to that of the boundary curve of a solution of *X* molecules sedimenting in a homogeneous medium of *A* molecules. Between

the levels "a" and "b" both molecular types are present in continuously varying concentration and it is difficult to visualise clearly the forces which will determine the ultimate shape of the composite concentration curve. It seems at least definite that in this region the X boundary gradient will be increased by the effect on the sedimentation velocity of the X molecules of the increasing concentration of A molecules as we proceed from "a" to "b". If this were the only factor present we should expect a composite curve of the type shown in Fig. 2c. Actually, however, we obtain curves of the general asymmetrical shape shown in Fig. 2d. The degree of distortion is not marked in the case of cow and horse sera but is characteristically pronounced in the case of untreated normal human sera. To explain the shape of this curve it is necessary to assume that in addition to the increase of the X concentration gradient there has also occurred an apparent "sharpening" of that part of the A boundary which lies between the levels "a" and "b", so that the limb of the curve nearer to the bottom of the cell comes to lie more vertically. It seems unlikely that this sharpening can be attributed to any effect of the concentration of X molecules on the sedimentation velocity of A molecules because the X molecules are sedimenting faster than the A molecules. It is possible, however, that the diffusion of A molecules in this region will be influenced by the changing viscous effect of the solution of X molecules. The question of the influence of the charge on the molecules also arises, but a significant charge effect is regarded as unlikely in the presence of approximately 1 % neutral electrolyte. The possibility may not be excluded, however. It is not possible to obtain a more exact idea of the phenomenon until we have more data regarding the behaviour of the individual components of a mixture under the conditions of a simple diffusion experiment. We may note, however, that since the asymmetry shown in Fig. 2d is probably due to an external mechanical influence it should not affect the total area enclosed by the curve and the base-line.

When such an asymmetrical curve is obtained it may be questioned whether we know that the boundary is really composite and that the asymmetry is not due to some obscure mechanical factor acting on a homogeneous boundary. The evidence against this is (1) that the base of the curve is much too broad for a homogeneous boundary of the same sedimentation constant; (2) that in several experiments with human serum we have found an asymmetrical $A + X$ curve in the early exposures which separated later into two symmetrical curves; (3) in experiments with certain concentrated globulin solutions the depression of the boundary diffusion is much greater than in the case of undiluted serum and no apparent asymmetry of the curve is present.

The only method we know of attempting to resolve graphically such a composite curve as that shown in Fig. 2c is to locate the points "a" and "c", delete the portion of curve between these points and try to reconstruct the ideal component curves by consideration of symmetry. The procedure is only possible when the portion of curve to the left of "a" is substantially more than half the whole A curve, and even then the accuracy must be very limited. In the case of undiluted cow serum we have usually found that it is possible to reconstruct the A curve in an apparently reproducible way, but the X curve cannot be satisfactorily drawn. We have, therefore, obtained a very approximate value for the area of the X component curve by subtracting the area of the symmetrical A curve from the total boundary area.

The general results of the first experiment are collected in Table I.

As in the previous paper we have obtained a comparative value for the discrepancy between measured and calculated protein concentration (entitled

Table I. *Results obtained with undiluted cow serum.*

	Average ($n_1 - n_0$) calculated* $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w,20} \cdot 10^{13}$
<i>A</i> fraction	0.00952	4.76	2.67
<i>X</i> fraction	0.00253	1.27	3.2 ± 0.3
<i>G</i> fraction	0.00249	1.25	4.60
Total protein	0.01454	7.28	—
Total ($n_1 - n_0$), by refractometric measurement on the serum and its dialysate, and correction of the result to $\lambda = 366 m\mu$ 0.0153			
Total protein concentration, measured... .. 7.64 g./100 ml.			

* These values are obtained from the area of the component curves on the sedimentation diagram by means of equation (3) of the previous paper [1935]. They represent the refraction increments of the individual fractions. The value for the total protein is the sum of the calculated individual values and represents, therefore, the calculated refractive equivalent of the total area of the sedimentation curve.

“‘% diff.’ total protein”) by subtracting the value for the total protein refraction increment calculated from the area of the sedimentation diagram from that obtained by measurement on the serum and its dialysate, and expressing the difference as a percentage of the latter.

“% diff.” in total protein values -5.2% .

The individual values for the *A* and *X* fractions are only to be regarded as approximate for the reasons already discussed, but the combined value is exact to the limits of the experimental accuracy of the method. The calculated proportion of the total protein which is represented by the *G* fraction is 17.1% . The *A* boundary sediments to the bottom of the cell with a steady, but very slight, diminution in the breadth of the boundary, thus giving rise to a negative spreading coefficient.¹ We regard this as evidence in favour of the dimensional homogeneity of the molecules of the *A* fraction. The *G* boundary spreading coefficient can only be determined with a very low degree of accuracy and since, in any case, the values are bound to vary with the concentration of *A* molecules, and, therefore, have little significance, we shall ignore them.

Exp. 2. Undiluted cow serum heated to 56° for $2\frac{1}{2}$ hours. The object of this experiment was to find if any change in the molecular dimensions of the serum proteins accompanies the destruction of the complementary substance in immunological reactions.

A different 2.0 mm. cell from that used in Exp. 1 was employed in this case, and an optical scale distance of 1.38 cm. was found suitable. Mean centrifugal

Table II. *Results for heat-inactivated cow serum.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w,20} \cdot 10^{13}$
<i>A</i> fraction	0.00968	4.85	2.68
<i>X</i> fraction	0.00198	0.99	3.2 ± 0.3
<i>G</i> fraction	0.00284	1.42	4.94
Total protein	0.01450	7.26	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$ 0.0153			
Total protein concentration, measured 7.64 g./100 ml.			
“% diff.” in total protein values -5.5%			
Calculated proportion of <i>G</i> fraction 19.6%			
Spreading coefficient of <i>A</i> boundary Negative			

¹ The spreading coefficient is defined in the previous paper and is calculated according to equation (8) of that paper.

force = 255,000 times gravity. $T_f = 33.5^\circ$. $T_i = 35.6^\circ$. The photographic arrangements were the same as in Exp. 1. The curves are all so closely similar in shape to those in Exp. 1 that we have not reproduced a specimen here. The results are shown in Table II.

The only change which might be a possible accompaniment of the heat-inactivation process is an increase in the concentration of the *G* fraction with a compensatory decrease in the concentration of the *X* fraction. The change, however, is not great enough to enable this conclusion to be definitely drawn. In all other respects the data for the normal and heat-inactivated sera are identical.

Exp. 3. The effect of simple dilution on the sedimentation diagram of cow serum. In the first experiment 1 volume of cow serum was diluted with 4 volumes of 1 % sodium chloride and the solution was examined in a 2.0 mm. cell. Mean centrifugal force = 235,000 times gravity. Optical scale distance = 10.14 cm. $T_f = 32.2^\circ$. $T_i = 33.6^\circ$. Photographic enlargement factor = 0.993. The diagram shown in Fig. 3 was obtained from an exposure taken 84 mins. after reaching full speed, and Table III gives the results.

Fig. 3.

Table III. Results obtained for cow serum diluted with 4 volumes of 1 % sodium chloride.

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w, 20} \cdot 10^{13}$
<i>A</i> fraction	0.0018	0.92	4.23
<i>G</i> fraction	0.0007	0.37	6.63
Total protein	0.0025	1.29	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$	...	...	0.00306
Total protein concentration, measured	...	...	1.53 g./100 ml.
"% diff." in total protein values	...	...	-18.0 %
Calculated proportion of <i>G</i> fraction	...	...	28.0 %
Spreading coefficient of <i>A</i> boundary	...	...	6.1×10^{-7}

Owing to the increased spreading of the boundaries it is impossible to say whether the *A* fraction is homogeneous or consists of *A* + *X* fractions. It will be noted that there has been a substantial relative increase in the concentration of the *G* fraction and that the percentage discrepancy between measured and calculated ($n_1 - n_0$) has increased considerably as compared with the previous two experiments.

In a second experiment the serum was examined after dilution with 7 volumes of 1 % sodium chloride. A 4.0 mm. cell was used. Optical scale distance = 8.14 cm. Mean centrifugal force = 235,000 times gravity. $T_f = 31.7^\circ$. $T_i = 34.5^\circ$. Photographic enlargement factor = 0.996.

Table IV. *Showing the results obtained for cow serum diluted with 7 volumes of 1 % sodium chloride.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w, 20} \cdot 10^{13}$
A fraction	0.00117	0.59	4.43
G fraction	0.00043	0.22	6.70
Total protein	0.00160	0.81	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$	...	...	0.00201
Total protein concentration, measured	...	...	1.01 g./100 ml.
"% diff." in total protein values	...	...	-20.4 %
Calculated proportion of G fraction	...	...	27.2 %
Spreading coefficient of A boundary	...	...	7.7×10^{-7}

The relative concentration of the G fraction has not been increased by this further dilution and it appears probable, therefore, that the maximum "globulin" content of the serum obtainable by ultracentrifugal analysis is approximately 28 %. This will be referred to in a later paper where we shall have occasion to discuss the albumin and globulin concentrations in this serum obtained by precipitation methods. It is also of interest that the percentage discrepancy between measured and calculated ($n_1 - n_0$) should again be so large in comparison with the case of undiluted serum. It suggests either that simple dilution of cow serum leads to the production of polydisperse protein or that in some way our technique is not exactly the same in the examination of concentrated and dilute solutions.

Exp. 4. Cow serum dialysed against 1 % sodium chloride. A sample of the cow serum was dialysed against several changes of 1 % sodium chloride until ionic equilibrium was obtained. During the process a slight dilution of the serum occurred. Cell thickness = 2.0 mm. Optical scale distance = 1.69 cm. Mean centrifugal force = 255,000 times gravity. $T_f = 32.7^\circ$. $T_i = 36.9^\circ$.

Table V. *Results obtained for cow serum dialysed against 1 % sodium chloride.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w, 20} \cdot 10^{13}$
A fraction	0.00852	4.26	3.09
X fraction	0.00135	0.67	—
G fraction	0.00225	1.13	5.07
Total protein	0.01212	6.06	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$	...	...	0.0133
Total protein concentration, measured	...	...	6.66 g./100 ml.
"% diff." in total protein values	...	...	-8.9 %
Calculated proportion of G fraction	...	...	18.5 %
The spreading coefficient of the A boundary in this case is significantly zero.			

We may note here the slightly increased sedimentation constants and boundary spreading coefficients as compared with the untreated serum in Exp. 1. These increases may be due to the dilution which occurred inadvertently during the dialysis.

It is also possible that the rather low value of $(n_1 - n_0)$ for the X fraction is due to the slightly increased spreading at the A and X boundaries which makes the graphical separation of the two curves more uncertain than usual. In general, however, we conclude from this experiment that dialysis against 1 % sodium chloride does not affect significantly the protein sedimentation diagram of undiluted cow serum.

Exp. 5. Cow serum dialysed against 0.1 % sodium chloride. The dialysed serum was examined in a 2.0 mm. cell with an optical scale distance of 2.28 cm. The fact that we were able to use this greater scale distance indicated early in the experiment that a significant change had occurred in the sedimentation diagram. Mean centrifugal force = 255,000 times gravity. $T_f = 32.9^\circ$. $T_i = 25.2^\circ$.

Table VI. *Results for cow serum dialysed against 0.1 % sodium chloride.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W, 20} \cdot 10^{13}$
A fraction	0.00786	3.93	2.76
X fraction	0.00167	0.84	—
G fraction	0.00304	1.52	5.45
Total protein	0.01257	6.29	—
Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$	...	...	0.0134
Total protein concentration, measured	...	...	6.70 g./100 ml.
"% diff." in total protein values	...	...	-6.0 %
Calculated proportion of G fraction	...	...	24.2 %
Spreading coefficient of A boundary	...	...	0.6×10^{-7}

Fig. 4.

Fig. 5.

We reproduce in Fig. 4 the curve of an exposure taken 56½ mins. after reaching full speed. It will be seen on comparison with the curve for untreated serum in Fig. 1 that the two curves have the same general shape, but as the calculations show, the G curve in Exp. 4 has a definitely greater area than the G curve in Exp. 1, and the A curve in Exp. 4 has a smaller area than the A curve in Exp. 1. The sedimentation velocity of the G fraction appears to be abnormally high. The increase in the boundary spreading coefficient of the A fraction was expected since charged proteins tend to diffuse more rapidly in the presence of lower concentrations of diffusible electrolytes. In order to find if further reduction in the concentration of diffusible electrolytes would give rise

to a further increase in the *G* fraction we examined next electrolyte-free cow serum.

Exp. 6. Electrolyte-free cow serum. The cow serum was dialysed for several days in a collodion bag against distilled water during which procedure a rather scanty flocculum of euglobulin collected at the bottom of the bag. The supernatant solution was then centrifuged in a 2.0 mm. cell. Mean centrifugal force = 255,000 times gravity. Optical scale distance = 3.78 cm. $T_f = 33.8^\circ$. $T_i = 35.8^\circ$.

Total ($n_1 - n_0$), measured and corrected to $\lambda = 366m\mu$...	0.011
Total protein concentration, measured ...	5.51 g./100 ml.

Fig. 5 shows the curve of an exposure taken $52\frac{1}{2}$ mins. after reaching full speed. The base-line is sloping probably because of the high potential gradient set up in the solution by the movement of charged protein in an electrolyte-free medium. It is, therefore, not possible to make calculations of ($n_1 - n_0$) from this curve which will have any precise significance, but we may obtain approximate values for the sedimentation constants of the *A* and *G* fractions. The values are:

$S_{W,20}$ for the <i>A</i> fraction ...	$3.4 \cdot 10^{-13}$
„ „ <i>G</i> „ ...	$7.4 \cdot 10^{-13}$

It is impossible to say whether any *X* fraction is present or not.

The high value of $S_{W,20}$ for the *G* fraction conforms with the high value of the previous experiment. It may be due to charge effects or to an increase in the size of the *G* molecules. We may also notice in Fig. 5 the marked increase in the degree of spreading at both boundaries. In connection with certain arguments discussed in the previous paper concerning the charge on the proteins in untreated serum, this phenomenon certainly suggests that both fractions in electrolyte-free serum are charged. It may not be inferred, however, that the same is true of untreated serum until we have more evidence that the constitution of the soluble protein in serum is not affected by the removal of electrolytes.

Exp. 7. Cow serum dialysed against M/20 acetate buffer. p_H 4.6. Cell thickness = 2.0 mm. Mean centrifugal force = 250,000 times gravity. Optical scale distance = 1.24 cm. $T_f = 33.4^\circ$. $T_i = 35.9^\circ$. The results are shown in Table VII.

Table VII. Results with cow serum dialysed against M/20 acetate buffer.

	Average ($n_1 - n_0$) calculated $\lambda = 366m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
<i>A</i> fraction }	0.00983	4.92	3.29
<i>X</i> fraction }			
<i>G</i> fraction	0.00236	1.18	5.10
Total protein	0.01219	6.10	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366m\mu$...	...	...	0.0125
Total protein concentration, measured ...	...	...	6.26 g./100 ml.
"% diff." in total protein values ...	...	...	-2.4 %
Calculated proportion of <i>G</i> fraction ...	...	...	19.4 %
Spreading coefficient of <i>A</i> boundary ...	...	...	0.3×10^{-7}

Owing to a certain amount of leakage from the cell at the beginning of this run the sedimentation path available was shorter than usual, with the result that the presumably merged *A* and *X* boundaries reached the bottom of the cell before any detectable separation of the two had time to occur. It was therefore only possible to calculate combined values for ($n_1 - n_0$) and $S_{W,20}$.

for this boundary. In all other respects, and taking into account the slight dilution during dialysis, we regard the curve as showing no significant points of difference from the curve of untreated cow serum.

Exp. 8. Cow serum dried and redissolved. A portion of the serum was rapidly dried under reduced pressure in a desiccator containing phosphorus pentoxide and then mixed with the same volume of water as there was serum originally. In spite of stirring the mixture gently at intervals for 24 hours a rather bulky greyish-brown residue remained. The clear solution after centrifuging off the insoluble material was then dialysed under pressure against 1 % sodium chloride until equilibrium was reached and the total protein concentration was approximately the same as that of the original serum. The solution was examined in a 2.0 mm. cell. Mean centrifugal force = 240,000 times gravity. Optical scale distance = 1.54 cm. $T_f = 33.3^\circ$. $T_i = 34.4^\circ$.

Fig. 6 shows the curve of an exposure taken 66 mins. after reaching full speed, and Table VIII shows the general results of the run.

Table VIII. *Results for cow serum which had been dried and redissolved.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w, 20} \cdot 10^{13}$
A fraction	0.00940	4.73	2.37
X fraction	0.00183	0.92	—
G fraction	0.00235	1.18	4.37
Total protein	0.01358	6.83	—
Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$	...	...	0.0161
Total protein concentration, measured	...	...	8.06 g./100 ml.
"% diff." in total protein values	...	...	-15.5 %
Calculated proportion of G fraction	...	...	17.3 %
The spreading coefficient of the A boundary has a negative value.			

It will be seen from Table VIII and from Fig. 6 that the results generally are in rather close agreement with those for untreated cow serum, except that there is a greater discrepancy in the total protein values. The values of the sedimentation constant and spreading coefficient are in keeping with the slightly higher total protein concentration in this experiment than in Exp. 1, but the calculated value for $(n_1 - n_0)$ is definitely less. It appears, therefore, that, compared with the untreated serum, some 8–10 % more of the total protein in the redissolved serum is completely heterodisperse in the centrifugal field and does not show on the apparent base-line. It is of interest that, if we disregard this polydisperse protein and the insoluble protein left on redissolving the dried serum, the sedimentation characteristics of the remaining soluble protein after concentration and the concentration ratio of its constituent fractions should be so closely similar to those of the untreated parent serum.

Exp. 9. Cow serum, dried, ether-extracted and redissolved. In order to investigate whether the serum lipoids have any influence on the character of the sedi-

Fig. 6.

mentation diagram we have subjected a portion of the serum powder (prepared as in Exp. 8) to the action of warm ether in a Soxhlet extractor for 3 days. The residue in the extraction thimble was exposed to the laboratory atmosphere for a day to get rid of all traces of ether and then redissolved and dialysed against 1 % sodium chloride. As in the previous experiment there was a bulky residue of insoluble protein. The protein solution after dialysis was examined in a 2.0 mm. cell, using a mean centrifugal force of 255,000 times gravity. Optical scale distance = 2.88 cm. $T_f = 33.7^\circ$. $T_i = 35.7^\circ$.

Table IX. *Results for cow serum which had been dried, ether-extracted and redissolved.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w, 20} \cdot 10^{13}$
A fraction	0.00592	2.96	3.38
X fraction	0.00117	0.59	—
G fraction	0.00185	0.93	5.40
Total protein	0.00894	4.48	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$	...	...	0.0119
Total protein concentration, measured	...	...	5.96 g./100 ml.
"% diff." in total protein values	...	...	-24.8 %
Calculated proportion of G fraction	...	...	20.6 %
Spreading coefficient of A boundary	...	...	1.0×10^{-7}

The most notable feature is the fact that almost one-quarter of the total protein present has failed to appear on the diagram. A discrepancy of the same order was found in Exp. 3 using diluted cow serum and there we could not exclude the possibility that an error might arise owing to the different conditions (cell thickness, scale distance, time of experiment *etc.*) under which a dilute and a concentrated solution are examined. In this case we feel justified in drawing the definite conclusion that a considerable amount of polydisperse protein is present because the conditions of the experiment are practically identical with those in Exps. 1, 2, 4, 5, and 7, in which an average loss of only 5.5 % is recorded. It is also interesting to find in this experiment that the refraction equivalent of the total area of the curve of the first exposure, *viz.* 0.0107, is much higher than in the later exposures where it has a constant value of 0.00894. The spreading coefficient of the A boundary also appears to be high, but this may be due to the rather low concentration of the homogeneous protein which is present.

It is again interesting to find that in spite of the protein which was lost in the form of the insoluble residue, and of the polydisperse protein which fails to show its presence on the sedimentation diagram, the remaining protein appears to be normal from the sedimentation point of view. We should have expected that the protein loss would fall more heavily on one or other of the fractions. We must conclude from this that the denaturation process affects each fraction in proportion to the concentration in which it is present, or else that the three fractions are in equilibrium and removal of part of one fraction is followed by an internal molecular rearrangement which restores the fractions to their normal relative concentrations. This latter possibility does not seem to us unlikely in view of the equilibrium which exists in mixtures of serum albumin and globulin. We also conclude from this experiment that the serum lipoids which are extractable with ether do not exert an essential influence on the character of the sedimentation diagram.

Exp. 10. Cow serum in the presence of ammonium sulphate. In a preliminary test on a sample of cow serum it was found that globulin precipitation began when 3.9 ml. saturated ammonium sulphate had been added to 10 ml. of serum. We then added to a 1 ml. sample of the serum 0.3 ml. saturated ammonium sulphate and examined the clear solution in the centrifuge. The object of the experiment was to find whether any marked change is induced in the sedimentation diagram by ammonium sulphate in amount insufficient to cause precipitation. Cell thickness = 2.0 mm. Optical scale distance = 3.78 cm. Mean centrifugal force = 255,000 times gravity. $T_f = 33.3^\circ$. $T_i = 35.4^\circ$.

Fig. 7.

The curve shown (Fig. 7) was obtained from a photograph taken $88\frac{1}{2}$ mins. after reaching full speed. This and other exposures show base-line irregularities which are due at least in part to sedimentation of the ammonium sulphate ions. Two fractions are present, and owing to the density and viscosity effects of the ammonium sulphate these sediment very slowly, the complete run requiring 4 hours. More blurring of the boundaries thus has time to occur with the result that no signs of a separation of the lighter fraction into *A* and *X* components can be seen. Also, owing to the abnormal conditions of sedimentation, we cannot say from the spreading coefficient of this boundary whether it is homogeneous or not.

Table X. *Results for cow serum in the presence of ammonium sulphate.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W, 20} \cdot 10^{13}$
<i>A</i> fraction	0.00618	3.09	3.07
<i>G</i> fraction	0.00053	0.27	5.36
Total protein	0.00671	3.36	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$	...	...	0.00109
Total protein concentration, measured	...	...	5.46 g./100 ml.
"% diff." in total protein values	...	...	-38.5 %
Calculated proportion of <i>G</i> fraction	...	...	7.9 %

The individual values of ($n_1 - n_0$) for the *A* fraction are interesting, viz. 6.64 after $88\frac{1}{2}$ mins., 5.64 after 155 mins. and 5.77 after 204 mins. The spreading coefficients are also uneven—e.g. calculated between the exposures at $88\frac{1}{2}$ and 155 mins. = $2.46 \cdot 10^{-7}$, and between the exposures at 155 and 204 mins. = $0.86 \cdot 10^{-7}$. As already pointed out, owing to the slow sedimentation we cannot compare these values with the spreading coefficients which we have determined in other

experiments. It is, however, interesting to find a large degree of boundary spreading with falling ($n_1 - n_0$) in the upper part of the cell and a much smaller degree of spreading with constant ($n_1 - n_0$) in the lower part of the cell. The whole picture, including the marked discrepancy between measured and calculated total ($n_1 - n_0$) values, leaves little doubt that a large part of the protein in the solution is polydisperse. Mutzenbecher [1933] has also found that ammonium sulphate causes a dissociation of the serum protein molecules prior to precipitation.

It is also to be noted that the percentage of the total protein refraction increment due to the G fraction is very low. It would appear in this case (as distinct from the state of affairs when serum is dried and redissolved) that the loss of protein has fallen more heavily on the G fraction. This we would expect since it is only necessary to increase the concentration of ammonium sulphate to the point of precipitation and the globulin fraction practically disappears altogether from the diagram.

Exp. 11. Cow serum treated with ammonium sulphate. In this experiment a collodion dialysis bag filled with cow serum and closed was immersed in a large volume of saturated ammonium sulphate. In a few minutes the serum protein precipitated completely and was left so for 24 hours. The bag was then removed from the ammonium sulphate solution and placed in 1 % sodium chloride solution which was changed frequently until the dialysate was free from sulphate ions. It was verified that no protein leakage had occurred through the bag, the change in the total protein refraction increment of the serum—from 0.00141 to 0.00132—being due to incidental dilution during the dialysis process. The serum was then examined in the same cell as was used in the previous experiment. Mean centrifugal force = 255,000 times gravity. Optical scale distance = 2.28 cm. $T_f = 32.3^\circ$. $T_i = 33.7^\circ$.

Table XI. *Results for cow serum treated with ammonium sulphate.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w, 20} \cdot 10^{13}$
$A + X$ fraction	0.00975	4.88	3.11
G fraction	0.00174	0.87	4.71
Total protein	0.01149	5.75	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$	...	...	0.0143
Total protein concentration, measured	...	...	7.13 g./100 ml.
"% diff." in total protein values	...	...	-20 %
Calculated proportion of G fraction	...	...	15 %

The curve shown in Fig. 8 was obtained from a photograph taken 116½ mins. after reaching full speed. At the apex of the A curve there is definite evidence that this fraction is not homogeneous but consists of two molecular species present in approximately equal concentrations. The later exposures from this experiment show the same signs of an early process of resolution of the A curve into two curves of approximately equal areas—a process which is, however, still incomplete when the boundary reaches the bottom of the cell. The spreading coefficient of this boundary is $0.25 \cdot 10^{-7}$, which is in keeping with a heterogeneous fraction in view of the negative values obtained with the untreated serum.

In Fig. 9 we have shown the curve of a late exposure in Exp. 1 (untreated cow serum), and we have also superimposed on it the curve of the corresponding exposure from this experiment. Owing to the steeper boundary gradient in

Exp. 1 the two experiments were carried out with different scale distances. In order to make the two curves strictly comparable, therefore, we have recalculated each scale line position for the exposure in Exp. 11 to the same scale distance as used in Exp. 1, and these recalculated values are plotted in Fig. 9.

Fig. 8.

Fig. 9.

There still remains a difference in the total areas of the two curves owing to the slightly lower total protein area in Exp. 11. The difference is too small, however, to affect significantly the shape of the curves. A comparison of the two demonstrates the difference in the relative concentrations of the A and X molecules in the two cases much more strikingly than the figures which we have been able to give for the spreading coefficient of the A boundary in the two experiments.

We have generally preferred to base our conclusions with regard to the homogeneity of a sedimenting fraction on measurements of the actual boundary spreading which occurs between two levels in the cell rather than on measurements of the dimensions of one boundary curve after a given time. We regard this as more reliable because the dimensions of any particular boundary curve are dependent, among other things, on the time required to accelerate the centrifuge to full speed, and, possibly, also on the degree of convectional disturbances at the meniscus during this period. Also, of course, the dimensions are directly dependent on the scale distance, thickness of cell and photographic enlargement factor, which differ between experiments. On the other hand the effect of these latter variables may be eliminated by recalculating the scale line positions for one experiment to the conditions of the other as we have done here. In all our experiments also we have employed, as far as possible, a standard acceleration period for the centrifuge and approximately the same maximum speeds, and it has been our experience that boundary curves with significantly aberrant dimensions do not occur.

With due consideration of the many difficulties in the way of an exact comparison of the dimensions of curves from different experiments, we feel justified in including the evidence in Fig. 9 of gross changes in the dimensions of the curve together with that of the shape of the curve at the apex and the value of the boundary spreading coefficient as support for the view that ammonium sulphate causes an irreversible change in cow serum. The nature of the change may be defined as such that the concentration of the X molecular fraction is

increased to a value comparable with that of the *A* fraction, and that the increase is at the expense of the *A* fraction. It will be shown in the next section that the same appears to be true for horse serum.

SECTION 2. THE SEDIMENTATION DIAGRAM OF NORMAL HORSE SERUM.

In the previous paper [McFarlane, 1935] we have published certain data and a curve from an experiment with fresh untreated horse serum. We pointed out there that the curve was not symmetrical, but to avoid confusing the special issue of that work, which it did not affect in essential principle, we passed over the question of this asymmetry and discussed the serum as a two-component mixture. In the light of the experiments which will now be reported it is necessary to regard horse serum with cow serum as having a heterogeneous lighter molecular fraction consisting of two component fractions which are only imperfectly separable in the centrifugal field. In this section we shall report the results of some experiments on horse serum which were undertaken largely in order to enable a comparison of the results with those obtained for cow serum.

Exp. 12. Untreated horse serum. We are indebted to Prof. Robin Fåhræus for the supply of this serum from a horse which had not been immunised and had no signs or history of disease. The results of this experiment are taken from the previous paper and given here for purposes of comparison with later experiments. On Fig. 3 (*a*) of the paper referred to there will be found the curve of an exposure taken 117 mins. after reaching full speed. The experimental conditions were: Cell thickness = 2.0 mm. Optical scale distance = 2.14 cm. Mean centrifugal force = 230,000 times gravity. $T_f = 32.3^\circ$. $T_i = 34.0^\circ$.

Table XII. *Results for untreated horse serum.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w,20} \cdot 10^{13}$
<i>A</i> fraction	0.00854	4.32	2.94
<i>X</i> fraction	0.00206	1.04	—
<i>G</i> fraction	0.00269	1.34	4.35
Total protein	0.01329	6.70	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$	...	...	0.0144
Total protein concentration, measured	...	...	7.25 g./100 ml.
"% diff." in total protein values	...	...	- 8.9 %
Calculated proportion of <i>G</i> fraction	...	...	20.2 %
Spreading coefficient of <i>A</i> boundary	...	...	0.8×10^{-7}

Exp. 13. Undiluted horse plasma. A sample of blood from the same horse and taken at the same time as the serum in Exp. 12 was collected in a vessel containing sufficient dry sodium oxalate to make an approximately 0.5 % solution in the plasma. The blood corpuscles were centrifuged off 2 hours later and the clear plasma examined in the same 2.0 mm. cell as was used in the previous experiment. Optical scale distance = 2.14 cm. Mean centrifugal force = 240,000 times gravity. $T_f = 32.9^\circ$. $T_i = 34.1^\circ$.

Fig. 10 shows the curve of an exposure taken 75 mins. after reaching full speed, and the results are shown in Table XIII.

A comparison of the results in Exps. 12 and 13 and of the curve of Fig. 10 with that of Fig. 3 (*a*) of the previous paper reveals a very close similarity in the characteristics of the serum and plasma of the same animal. The additional protein in the diagram of the plasma, corresponding to the fibrinogen which is removed in the clot, is represented apparently by slight increases in all

Table XIII. *Results for undiluted horse plasma.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W, 20} \cdot 10^{13}$
A fraction	0.00868	4.39	2.86
X fraction	0.00240	1.21	—
G fraction	0.00345	1.72	4.44
Total protein	0.01453	7.32	—

Since it was considered inadvisable to dialyse the plasma no measured value for ($n_1 - n_0$) was obtained.

Calculated proportion of G fraction 24 %
Spreading coefficient of A boundary 0.8×10^{-7}

Fig. 10.

Fig. 11.

the fractions, but the most definite increase is clearly associated with the G molecule. We know from previous work that if some globulin were added to the serum used in Exp. 12 it would cause an increase both in the G curve and in the composite A + X curve owing to the equilibrium which exists between those fractions. It appears possible then that the fibrinogen of oxalate plasma is present with a molecular weight approximately the same as that of the globulin fraction and that it takes part in the general protein equilibrium reaction of the serum. This would explain the slight increases in the A and X fractions. Without some such explanation as this we must assume that fibrinogen is not a single substance, but consists of two or three fractions of the same sedimentation constants as the corresponding serum fractions. As a result of this experiment we can say definitely that no new fibrinogen molecule appears in the diagram of the plasma. It is interesting to note in this connection that Verney [1926] found no significant difference in the osmotic pressure per g. of total protein in the case of serum and plasma from the same human subject, indicating that fibrinogen has approximately the same mean molecular weight as the mean molecular weight of the serum proteins.

Exp. 14. Defibrinated horse plasma. We have defibrinated a sample of oxalate plasma from another horse by a usual procedure, *viz.* by adding slightly more solid calcium chloride than is required to precipitate all the oxalate and then

stirring vigorously with a glass rod. The shreds of fibrin were centrifuged off and the clear defibrinated plasma examined in the centrifuge in a 2.0 mm. cell. Optical scale distance = 1.54 cm. Mean centrifugal force = 235,000 times gravity. $T_f = 33.4^\circ$. $T_l = 34.5^\circ$.

Table XIV. *Results for defibrinated horse plasma.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w, 20} \cdot 10^{13}$
A fraction	0.01100	5.56	2.68
X fraction	0.00231	1.17	—
G fraction	0.00349	1.74	3.66
Total protein	0.01680	8.47	—
The preparation again was not dialysed before the experiment so that no measurements of total protein refractivity were made.			
Calculated proportion of G fraction	...	...	20.8 %
Spreading coefficient of A boundary	...	...	0.3×10^{-7}

The curve shown in Fig. 11 is from an exposure taken $120\frac{1}{2}$ mins. after reaching full speed.

It is interesting to find that, in spite of the fact that this serum contains considerably more protein than that of Exp. 12, the sedimentation diagram in the two cases is closely similar. The diminished sedimentation constants and spreading coefficient of the A boundary in the latter experiment are in keeping with the greater protein concentration.

Exp. 15. Horse serum treated with ammonium sulphate. We have undertaken this experiment in view of the change which had been noticed in the sedimentation diagram of cow serum as a result of treatment with ammonium sulphate. The experimental details were exactly as in Exp. 11 with cow serum. A specimen of the horse serum used in Exp. 12 was dialysed against a saturated solution of ammonium sulphate for 24 hours in a closed collodion bag. Thereafter, the serum was dialysed against changes of 1 % sodium chloride until all sulphate ions had disappeared from the dialysate. Cell thickness = 2.0 mm. Mean centrifugal force = 250,000 times gravity. $T_f = 33.6^\circ$. $T_l = 36.5^\circ$.

Table XV. *Results for horse serum treated with ammonium sulphate.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w, 20} \cdot 10^{13}$
A fraction	0.00836	4.23	3.43
G fraction	0.00290	1.44	5.01
Total protein	0.01126	5.67	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$	...	...	0.00130
Total protein concentration, measured	...	...	6.54 g./100 ml.
"% diff." in total protein values	...	...	-13 %
Calculated proportion of G fraction	...	...	25 %
Spreading coefficient of A boundary	...	...	2.2×10^{-7}

The curve shown in Fig. 12 was obtained from a scale photograph taken $95\frac{1}{2}$ mins. after reaching full speed. It will be seen that this A curve shows no signs of dividing into two as was the case with cow serum. It will have been noted, however, in the horse serum experiments that we have never found any

negative values for the spreading coefficient of the *A* boundary such as occurred frequently in the cow serum experiments. In general, the degree of *A* boundary spreading is greater in the horse serum experiments and this may be due to the lower total protein concentration in the horse serum and the slightly greater proportion of *G* fraction. We believe this increased boundary spreading to be the reason for the apparent monodispersity of the *A* boundary in this experiment.

Fig. 12.

On the other hand, the *A* curve in all exposures is perfectly symmetrical, the boundary spreading coefficient appears to be abnormally high, and if the curve in Fig. 12 is replotted to the same scale distance as the curve for the original untreated serum it is seen that there has been a marked change in the general dimensions of the boundary curve similar to the change shown for cow serum in Fig. 9.

In the previous paper we have described an experiment in which albumin and globulin were prepared from this serum by fractionation with ammonium sulphate and then mixed together to form a so-called "synthetic" horse serum. A curve from this experiment is shown in Fig. 3 (b) of that paper, and in it, as in all the curves of that experiment, the *A* boundary curve is perfectly symmetrical.

For these reasons we believe that in the treatment of horse serum as well as cow serum with strong solutions of ammonium sulphate a definite change, which the ultracentrifuge is just able to detect and little more, occurs in the composition of the lighter molecular fraction.

We may not attach any particular significance to the high proportion of *G* fraction in this experiment because it will be noted that slight dilution of the serum has occurred during the dialysis. The data of the previous paper, supported by further evidence derived from the next experiment, show that the concentration relationship of the fractions in horse serum is exceedingly sensitive to changes in the total protein concentration—much more so than in the case of cow serum.

Exp. 16. Horse serum diluted with 3 volumes of 1 % sodium chloride. A sample of the serum used in Exp. 12 was diluted with 3 volumes of a 1 % solution of sodium chloride and examined in a 2.0 mm. cell using an optical scale distance of 8.78 cm. Mean centrifugal force = 250,000 times gravity. $T_f = 32.2^\circ$. $T_i = 33.2^\circ$. Photographic enlargement factor = 0.996.

The curve in Fig. 13 was obtained from an exposure taken 48 mins. after reaching full speed. In the resolution of this curve into *A* and *G* components

Table XVI. *Results with horse serum diluted with 3 volumes of 1 % sodium chloride.*

	Average ($n_1 - n_0$) calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w, 20} \cdot 10^{13}$
<i>A</i> fraction	0.00171	0.87	4.22
<i>X</i> fraction	0.00013	0.07	—
<i>G</i> fraction	0.00105	0.52	6.51
Total protein	0.00289	1.46	—
Total ($n_1 - n_0$), measured and corrected to $\lambda = 366 m\mu$	...	...	0.00353
Total protein concentration, measured	...	...	1.78 g./100 ml.
"% diff." in total protein values	...	...	-18 %
Calculated proportion of <i>G</i> fraction	...	...	36 %
Spreading coefficient of <i>A</i> boundary	...	...	6.6×10^{-7}

it appears that a small part of the total area cannot be included in the area of the symmetrical component curves. This small area lying in the region between the two curves we have called the *X* fraction in Table XVI, but if, like the *X* fraction in the undiluted serum, it has an average sedimentation constant very close to that of the *A* fraction it is probable that what we have classed as "*A*" fraction is not homogeneous but contains significant quantities of an *X* fraction. With the markedly increased boundary spreading in dilute protein solutions it is not possible to attempt a resolution of the lighter component into *A* and *X* fractions with any degree of accuracy. The value which we give in Table XVI for the *X* fraction is, therefore, merely an estimate of the refractive equivalent of the asymmetrical area not included in the *A* and *G* curves and has a doubtful connection with the *X* fraction of undiluted serum.

Fig. 13.

A comparison of the results of this experiment with those of Exp. 3 will show that the increase in the proportion of the *G* fraction on diluting cow serum is much less than the increase on diluting horse serum to the same extent. No doubt this is related to the higher albumin : globulin ratio in cow serum obtained by precipitation methods.

SECTION 3. THE SEDIMENTATION DIAGRAM OF NORMAL HUMAN SERUM.

Our first experiment using fresh undiluted human serum in a 2.0 mm. cell gave rise to a curve of the same general three-component type as the curves of undiluted cow and horse serum, but with two points of difference. The concentration of the *G* fraction was considerably smaller than in those sera, and the composite *A* + *X* curve showed marked distortion of the type illustrated in Fig. 2D. In attempts to reduce this distortion we decided to try diluting the serum with small amounts of 1 % sodium chloride such as would not be expected to give rise to any significant change in the relative concentrations of the fractions.

Exp. 17. Normal human serum diluted with one-third its volume of 1 % sodium chloride. A 2.0 mm. cell was used. Optical scale distance = 1.54 cm. Mean

centrifugal force = 240,000 times gravity. $T_f = 33.2^\circ$. $T_l = 35.1^\circ$. The results are shown opposite the reference initials J.S.P. in Table XVII and the curve in Fig. 14 is from a photograph taken 92 mins. after reaching full speed. It will be seen that the concentration of the G fraction is small, and that a certain amount of distortion is still present in the $A + X$ curve. In the exposure taken 150 mins. after reaching full speed, however, the A and X curves had become sufficiently separated for each boundary to be free of the distorting influence of the other and we find two symmetrical curves. We are able, therefore, to estimate with considerable accuracy the sedimentation constant and concentration of the X fraction in this serum.

Exp. 18. The same normal human serum (J.S.P.) diluted with an equal volume of 1% sodium chloride. The same cell was used as in Exp. 17. Optical scale distance = 2.74 cm. Mean centrifugal force = 235,000 times gravity. $T_f = 30.3^\circ$. $T_l = 32.4^\circ$. The results are also shown in Table XVII.

The curve in Fig. 15 was obtained from a photograph taken 83 mins. after reaching full speed, and shows that practically no change has occurred in the

Fig. 14.

Fig. 15.

concentration of the G fraction, but owing to the increased boundary spreading it is now impossible to obtain an accurate graphical separation of the A and X curves.

Since small additions of 1 % sodium chloride do not appear to affect significantly the concentrations of the fractions in human serum it appears to be permissible to adopt the method of slight dilution in order to obtain a more satisfactory separation of the A and X curves. It is probable that the addition to a normal serum of more than half its own volume of 1 % sodium chloride will defeat the purpose for which it is added and give rise to a curve of the type shown in Fig. 15 which cannot be used to obtain accurate concentration values

for the *A* and *X* fractions. Somewhere between this dilution and a state of no dilution there must be an optimum total protein concentration for symmetrical separation of the *A* and *X* curves. It does not appear that we can predict this dilution beforehand in any particular case because in addition to the total protein concentration, which we can control, there is the concentration ratio of the *A* and *X* fractions, which we cannot alter. We believe from our experiences to date that under the present conditions of an ultracentrifuge experiment the concentration ratio of the *A* and *X* fractions may influence the degree of distortion of the curves. We suspect, indeed, that it is the higher average proportion of *X* fraction in normal human serum which gives rise to the more pronounced distortion in this curve than in the curves of normal cow or horse serum. Our evidence for this, however, is mainly presumptive at present and it is possible that the true reason lies in slight differences in the sedimentation constants of the *A* and *X* proteins in the serum of different species—differences which are too small to be measurable by present methods. It is also possible that even slight changes in the total protein concentration of a serum may cause small changes in the concentration ratio of the *A* and *X* fractions, which, although not great enough to be measurable in the diagrams so far obtained, may nevertheless influence the degree of distortion of the curve.

Altogether, the variable factors are so numerous and the data available at present are so scanty that we have no choice but to resort to a method of trial and error in order to find the best dilution at which to examine a human serum. A factor which we have not mentioned, and which from several points of view must play an important part in the separation of the *A* and *X* curves, is the centrifugal force used. Here again, however, it is not possible to be sure of the nature of the effect, but it seems probable that with higher centrifugal forces the degree of boundary spreading will be reduced and the rate of separation of the two boundaries increased so that we may hope to obtain a satisfactory separation of the *A* and *X* fractions even using undiluted serum. Fortunately, also, much higher centrifugal forces have recently been obtained and a new rotor for serum work is under construction, which Prof. Svedberg hopes will enable us to take advantage of these forces and even permit at the same time of a slight increase in the height of fluid column in the cell. When this rotor is available we hope that it may be possible to investigate as a separate problem the effect of a wide range of centrifugal forces on the diagram of a normal human serum. In the following experiments we have had to employ a standardised centrifugal force in order to make the results more strictly comparable. We have, however, employed various dilutions of human serum so that the curves in some cases are rather more symmetrical than in others.

*Exp. 19. The same normal human serum (J.S.P.) diluted with 7 volumes of 1 % sodium chloride. A 4.0 mm. cell was used and an optical scale distance of 8.0 cm. Mean centrifugal force = 235,000 times gravity. $T_f = 30.3^\circ$. $T_i = 32.4^\circ$. Fig. 16 shows the curve of a scale photograph taken 51 mins. after reaching full speed, and the results are recorded in Table XVII. The concentration of the *G* fraction is still remarkably small, especially in comparison with diluted cow and horse serum. We have in another experiment examined the same serum diluted with 31 volumes of 1 % sodium chloride. The curves obtained all show irregularities which may be due to a disintegration of the protein molecules, or, on the other hand, may arise from convection effects in the cell. We do not, therefore, publish the results but we may state, from the general appearance of the curve, that even at this dilution no dramatic appearance of globulin molecules takes place.*

Fig. 16.

Table XVII. *Results obtained in the investigation of a range of normal human sera.*

Serum	Dilution in vols. serum: vols. 1 % NaCl	Cell thickness mm.	Optical scale dist. cm.	Average $S_{w, 20} \cdot 10^{13}$		
				A mol.	G mol.	X mol.
J.S.P.	3:1	2.00	1.54	2.63	4.47	3.14
J.S.P.	1:1	2.00	2.74	3.30	5.15	—
J.S.P.	1:7	4.00	7.64	4.48	6.64	—
W.L.	5:2	2.00	1.98	2.90	5.14	3.40
C.A.	5:2	2.00	1.38	2.69	5.24	3.20
O.D.B.	3:1	2.00	1.24	2.60	5.05	3.02
R.N.	5:2	2.00	1.69	3.01	5.33	3.31
A.G.P.	5:2	2.00	1.98	3.03	4.86	3.20
M.S.	3:1	2.00	1.84	3.30	5.95	—
I.S.	Undil.	2.00	2.14	2.88	5.39	—
A.McF.	1:1.25	2.00	3.64	3.92	5.67	—
O.Q.	1:1.25	2.00	3.64	3.54	5.47	—

Serum	Average calc. $(n_1 - n_0) \cdot 10^3$ $\lambda = 366 m\mu$				Conc. G mol. % total protein	Calc. total $(n_1 - n_0) \cdot 10^3$ for undil. serum	Total protein conc.* of undil. serum g./100 ml.	Spreading coeff. of A boundary cm. ² /sec. 10^7
	A mol.	G mol.	X mol.	Total				
J.S.P.	8.97	1.10	2.70	12.77	8.6	17.03	8.43	0.4
J.S.P.	5.47	0.625	2.30	8.40	7.5	16.80	8.32	1.9
J.S.P.	1.65	0.30	—	1.95	15.4	15.60	7.76	5.2
W.L.	6.74	0.84	2.24	9.82	8.5	13.75	6.81	1.4
C.A.	7.02	1.47	2.90	11.39	12.9	15.95	7.90	0.13
O.D.B.	8.09	0.67	2.14	10.90	6.1	14.53	7.19	—
R.N.	6.38	0.54	2.40	9.32	5.8	13.05	6.46	0.14
A.G.P.	6.30	1.46	3.29	11.05	13.2	15.47	7.66	0.02
M.S.	6.65	1.12	1.25	9.02	12.4	12.63	6.25	—
I.S.	6.56	0.90	2.81	10.27	8.8	10.27	5.09	—
A.McF.	4.41	0.66	0.65	5.72	11.5	12.87	6.37	1.9
O.Q.	4.32	0.51	0.18	5.01	10.2	11.27	5.58	3.3

* The protein concentration values are obtained from the refraction increment values by means of the value 0.00202 [Schretter, 1926] for the specific refraction increment of total human serum protein.

Exp. 20. A series of normal human sera. We have next examined in turn the serum from a number of apparently healthy normal persons of both sexes. The results are collected in Table XVII. In all cases a 2.0 mm. cell was used and the general experimental conditions were very similar to those in the previously recorded experiments. The general shape of the corresponding curves in all these experiments coincides remarkably, and the curve in Fig. 14 may be taken as characteristic of the curves obtained at about the middle of each run. As we shall show in a subsequent paper the similarity of these curves of normal serum among themselves is quite sufficient to distinguish any of them at a glance from the curves which are obtained from many pathological sera. We regard a curve of the general shape shown in Fig. 14, with due consideration of the conditions of dilution, centrifugal force and time at which it was obtained, as a valuable physico-chemical criterion of the state of the proteins in a normal human serum. In the table of results it will be seen that while the calculated total protein concentration of the untreated serum has varied within wide limits (5.09 g./100 ml. (I.S.) to 8.43 g./100 ml. (J.S.P.)) the proportion of *G* fraction in all cases can be represented by the formula $(10.5 \pm 5) \%$ of the total protein. The concentration of *X* fraction is $(25 \pm 5) \%$ of the total protein, if we exclude the solutions diluted with more than an equal volume of 1 % sodium chloride. In the case of the latter solutions, owing to the increased boundary spreading, the *X* concentration values are very inaccurate, and indeed, must be regarded as little more than an empirical allowance for the small area between the *A* and *G* curves which cannot be included with graphical symmetry in either of these curves. It is probable that the true *X* values are greater than the values given.

The spreading coefficient and sedimentation constant values in Table XVII call for little comment as they are in the main what one would expect to find under the conditions of the various experiments. In certain cases no value is given for the sedimentation constant of the *X* molecules because the separation of the *X* curve was not sufficient to enable a sufficiently accurate location of the position of the "ideal" sedimentation boundary. Within the limits of comparability it appears from these results that the sedimentation constants of the fractions in human serum are the same as those of the corresponding fractions in cow and horse serum.

We have repeatedly obtained an irregular appearance of the *G* curve which will be seen on close consideration of the curve in Fig. 14. It is not clear to what extent this is due to the accentuation of the effect of base-line irregularities in relation to the small *G* curve, but we are of opinion on the whole that the *G* fraction in normal human serum is not homogeneous.

In only two cases in this series have we dialysed the serum beforehand and measured the total protein refraction increment, because we desired to examine the sera in as fresh and untreated a state as possible. The sera subjected to preliminary dialysis were those of C.A. and W.L., and the calculated values of the protein refraction increments were found to be respectively 7.5 and 14 % less than the measured values.

Another feature which we have noted is the occasional presence in human serum of quantities up to 5 % of the total protein of fractions which sediment between two and three times as fast as globulin and can usually only be seen in the first photograph. The molecular weight of these substances must be at least half a million. We have ignored these fractions in this work because they could only be satisfactorily studied with lower centrifugal forces than we wished to use. We mention their presence, however, because they must contribute in

some degree to the discrepancies found between calculated and measured protein refraction increment.

The considerable disagreement among the three calculated values for the total protein refraction increment in the case of J.S.P. shows a phenomenon which we have found generally throughout this work, *viz.* much greater discrepancies between measured and calculated protein refraction increment values in the case of dilute solutions than of concentrated solutions. The explanation of this is not clear.

Exp. 21. The serum of mother and child. It is well known that the serum of a newborn infant contains smaller amounts of protein than the mother's serum. It occurred to us that the small amount of globulin present in normal adult serum might be associated with the development of immunity to various diseases acquired in the early years of life. We have therefore examined in the centrifuge serum from umbilical blood in the newborn infant, and serum from the mother's blood taken at the time of parturition. Owing to the lower concentration of total protein in the former serum we examined this without dilution. The same cell was used in both experiments and the other experimental conditions were comparable in the two cases. The results are shown in Table XVII opposite the reference letters I.S. (infant's serum), and M.S. (mother's serum). It is clear that our original idea is disproved since the infant's serum contains 8.8 % of a *G* fraction. The mother's serum has an abnormally low concentration of *X* fraction, the proportion being approximately half that in the infant's serum.

Exp. 22. A test of the reproducibility of a particular sedimentation diagram. The experiment which we mention here was actually carried out at the beginning of this work. After we had examined the writer's serum (*cf.* Table XVII), diluted 1:1.25 with 1 % sodium chloride, and discovered the surprisingly low concentration of globulin in it, our first concern was to repeat the finding. Another portion of the same diluted serum was, therefore, examined again a few days later using the same cell and approximately the same centrifugal force. The photographs were taken as far as possible at the same time in relation to the start of the experiment. We do not reproduce the results because these do not show any appreciable difference from the results of the earlier experiment shown in Table XVII. We found that if the curves of corresponding photographs were superimposed they coincided in practically every detail. Even some irregularities on the base-line, which we had previously ignored, were faithfully reproduced in the later experiment.

Exp. 23. Normal human serum dried by the Hardy-Gardner method. Serum dried by simple evaporation does not usually redissolve well, a residue of insoluble protein remaining behind on redissolving the powder. Hardy and Gardner [1910] have used a method of drying with alcohol in the cold which yields a white lipid-free powder which redissolves completely. Sørensen [1930] has subjected the redissolved powder to a variety of tests and comes to the conclusion that the procedure "hardly involves any real chemical splitting, but only a purification of the proteins from the contained substances soluble in ether and alcohol". Also "reconstituted plasma can still be made to clot and albumins may be recrystallised" [Hewitt, 1927].

In view of our previous findings with serum dried by simple evaporation we have thought it of value to submit redissolved serum proteins obtained by the Hardy-Gardner method to the rather searching analysis of the ultracentrifuge. We divided a normal human serum into two portions one of which was dialysed (during which process some dilution took place) and examined in the centrifuge after the refractive indices of protein solution and dialysate had been measured.

The other portion was subjected to all the steps in the Hardy-Gardner drying procedure as described and modified by Hewitt [1927]. In particular, great care was taken to keep the temperature low and by the use of a refrigerator, itself contained in a cold room at -4° , the whole procedure was carried out at -16° . The serum-alcohol mixture was allowed to stand at -16° for 2 hours, and the subsequent filtration and washing of the protein precipitate occupied 6 hours at the same temperature. As recommended by Hewitt we have further minimised the time of contact between the proteins and the alcohol by omitting to dry the powder *in vacuo* before extracting with ether.

The final powder was pure white and redissolved completely in water. The solution was then dialysed against 1 % sodium chloride and the total protein concentration was arranged to be approximately the same as that in which the original serum was examined. The same cell, scale distance and centrifugal force

Fig. 17.

were used in the two experiments. The results are shown in Table XVIII and we reproduce in Fig. 17 two curves—one from the original serum obtained 40 mins. after reaching full speed, and one from the dried serum obtained 25 mins. after reaching full speed.

Table XVIII. *Results obtained with a diluted normal human serum and the same serum after drying with ether and alcohol.*

Cell thickness = 3.0 mm. Optical scale distance = 3.64 cm.

Mean centrifugal force = 255,000 times gravity.

	Average $S_{w, 20}$		Average $(n_1 - n_0) \cdot 10^3$		Total	Measured total $(n_1 - n_0) \cdot 10^3$ corr. to $\lambda = 366 m\mu$	“% diff.” total $(n_1 - n_0)$	Calculated proportion of G mol.
	A + X mol.	G mol.	A + X mol.	G mol.				
Original serum	3.9	5.70	5.42	0.56	5.98	7.31	-18.2	15.2
Dried serum	3.8	—	—	—	5.95	8.14	-26.9	—

It is clear that a marked change has taken place on drying. The solution of dried protein appears to contain considerable quantities of polydisperse protein, as shown by the irregularity of the curve and the discrepancy of 27 % between measured and calculated total protein refraction increment. We cannot say

whether any unchanged native protein is still present or not. Signs of a *G* fraction can be discerned. It will be noted from Table XVIII that the average sedimentation constant of a boundary (taken as the position of the apex of the curve) in the case of the dried protein is in good agreement with the value for the same boundary in the case of untreated serum. It is possible that each fraction in the original serum has given rise to a group of polydisperse molecules with the same mean molecular weight as the parent protein fractions. If any of the original serum protein is still present in significant quantity we must conclude that the drying procedure has in some way given this native protein an abnormally high power of diffusion. We can imagine changes in the shape and charge of the protein molecules which might cause small alterations in the diffusion constant, but we cannot imagine a change which, without affecting the weight or homogeneity of the molecules in a fraction, could cause such a gross alteration in the degree of boundary spreading as we are concerned with here. The same phenomenon has been found in the case of a preparation of serum globulin dried in the same way.

SECTION 4. THE OSMOTIC PRESSURE OF UNDILUTED SERUM.

It has been noted by numerous observers [*e.g.* Mayrs, 1926; Verney, 1926; Krogh and Nakazawa, 1927] that when serum is diluted the colloid osmotic pressure falls more rapidly than corresponds to the fall in protein concentration. Various workers have attempted to correlate the osmotic pressure of a serum with the partial osmotic pressures of the albumin and globulin fractions in it [Govaerts, 1925; 1927; Marrack and Hewitt, 1927; Farkas, 1927; Adair and Robinson, 1930, 2; Elias and Goldstein, 1932], and it has been clearly established that a considerable proportion of the colloid osmotic pressure of a serum must be attributed to the uneven distribution of diffusible electrolytes across the membrane. The problem of determining accurately the proportion of the total pressure due to the diffusible electrolytes is one of great difficulty because we are concerned with a non-ideal solution. Adair and Robinson [1930, 2] in a careful consideration of the problem have come to the conclusion that Donnan's formulae, which refer to infinitely dilute solutions, are not applicable to serum as suggested by Hecht [1925] and Marrack and Hewitt [1927]. They put forward a new treatment of the problem which is essentially based on the calculation of the partial pressure of the diffusible ions from the membrane potential by means of the equation

$$p_i = 15.4 J E_m^2,$$

where p_i = the partial osmotic pressure due to the excess of diffusible ions inside the membrane;

J = the ionic strength, or the sum of the concentrations of the ions in the dialysate multiplied by the squares of their valencies;

E_m = the membrane potential in millivolts.

Adair and Robinson have obtained good agreement between the observed osmotic pressure of dilute unfractionated serum proteins and the partial osmotic pressures of the albumin and globulin. They, therefore, suggest that the state of aggregation of these purified proteins is the same as in the diluted parent serum, a view with which the ultracentrifuge results are in agreement. They have also found that, if due allowance is made for p_i in the case of concentrated ox serum proteins, the relationship between the partial protein osmotic pressure and the total protein concentration of the solution approximates to a straight

line. This fact is also not in essential disagreement with our results. Adair and Robinson conclude from the two sets of experiments, however, that the "state of aggregation of the proteins in untreated serum appears to be the same as their state of aggregation in the purified proteins", and with this conclusion we cannot agree.

Adair and Robinson have not attempted to demonstrate quantitative agreement between the observed osmotic pressure of undiluted serum and the partial osmotic pressures of the constituent albumin and globulin. We are informed in a private communication that this omission was intentional because of lack of confidence in the existing methods of estimating the albumin and globulin fractions in serum (which, incidentally, we regard as well justified), and because the osmotic coefficient g_p (a factor representing the effects of the attractive and repulsive forces between the molecules and presumed to be constant), may not be constant in concentrated solutions.

We have endeavoured to apply Adair and Robinson's theory to the results of osmotic pressure measurements which were carried out on the cow and horse serum used in the experiments of Sections 1 and 2, and on a specimen of human serum from Section 3 (C.A., Table XVII) of this work. The serum in each case was dialysed until ionic equilibrium was obtained against approximately ten times its volume of 1 % sodium chloride, and the height of fluid column in the manometer measured. The osmotic pressures are corrected to mm. mercury at 0°. We have also subjected each serum to a standard method of analysis involving precipitation with ammonium sulphate and thus obtained values for the albumin and globulin concentrations in the serum. We have no confidence that these values represent exact albumin and globulin concentrations, but they are as good as any that can be obtained to our knowledge and sufficient for our present purpose.

Osmotic pressure of undiluted cow serum.

Observed osmotic pressure of undiluted cow serum	= 22.3 mm. Hg
Total protein concentration (C)	= 7.64 g./100 ml. solution
Corrected protein concentration (C_v)	= 8.27 g./100 ml. solvent

By precipitation analysis the serum was found to contain 58 % albumin and 42 % globulin. Therefore

$$\begin{aligned} C_{v \text{ albumin}} &= 4.80 \text{ g./100 ml. solvent} \\ \text{and} \quad C_{v \text{ globulin}} &= 3.47 \text{ g./100 ml. solvent} \end{aligned}$$

By reference to Adair and Robinson's curve [1930, 2, Fig. 5, p. 1885] showing the variation of total osmotic pressure ($p_i + p_p$) of crystalline serum albumin with protein concentration we obtain the value 15.5 mm. for an albumin concentration of 4.80 g./100 ml. solvent. This value is made up of 10.5 mm. due to the protein and 5 mm. due to p_i . Similarly, from their curve in Fig. 6 (p. 1886) we obtain the value 3.3 mm. for the total osmotic pressure of a solution containing 3.47 g. globulin/100 ml. solvent. This is made up of 3.0 mm. due to the protein and 0.3 mm. due to p_i . We may not add the total values together because it may not be assumed that the total p_i of a mixture is equal to the sum of the p_i values for the individual components in separate solution. It is more probable that the membrane potentials are strictly additive, especially in view of the rather constant E_m/C_v values which Adair and Robinson obtain for ox serum protein over a range of concentrations. That is

$$E_{m \text{ mixture}} = E_{m \text{ albumin}} + E_{m \text{ globulin}}.$$

Squaring both sides and substituting from the equation given above

$$p_{i \text{ mixture}} = p_{i \text{ albumin}} + p_{i \text{ globulin}} + 2\sqrt{p_{i \text{ albumin}} \times p_{i \text{ globulin}}},$$

p_i will, therefore, be greater in the mixture than the sum of the p_i values due to each protein in separate solution, and by an amount which will be significant when the two components contribute approximately equally to the total p_i of the mixture, and negligible when one contributes much more than the other.

In the present case, the osmotic pressure of the serum

$$= 15.5 + 3.3 + 2\sqrt{5 \times 0.3} = 21.3 \text{ mm.}$$

Alternatively, we may use the albumin and globulin concentration values obtained from the sedimentation diagrams, *viz.* 83 % albumin and 17 % globulin (*cf.* Exp. 1). These are found to correspond respectively to partial osmotic pressures of 24.5 mm. and 1.3 mm. In this case $p_{i \text{ globulin}}$ is negligibly small, and, therefore,

$$\text{Osmotic pressure of cow serum} = 24.5 + 1.3 = 25.8 \text{ mm.}$$

Osmotic pressure of undiluted horse serum.

Observed osmotic pressure of undiluted horse serum = 20.5 mm.

Total protein concentration = 7.25 g./100 ml. solution

$C_{v \text{ total protein}}$ = 7.82 g./100 ml. solvent

By precipitation analysis the serum was found to contain 51 % albumin and 49 % globulin. Therefore

$C_{v \text{ albumin}}$ = 3.99 g./100 ml. solvent

and $C_{v \text{ globulin}}$ = 3.83 g./100 ml. solvent

Again from Adair and Robinson's curves we obtain the corresponding osmotic pressures, *viz.* (4 + 8.2) mm. and (3.6 + 0.1) mm., giving a total osmotic pressure for the serum of $15.9 + 2\sqrt{0.82} = 17.7$ mm.

By ultracentrifugal analysis the same serum was found to contain 80 % albumin and 20 % globulin (*cf.* Exp. 12). In this case, therefore,

$C_{v \text{ albumin}}$ = 6.26 g./100 ml. solvent

$C_{v \text{ globulin}}$ = 1.56 g./100 ml. solvent

and the corresponding osmotic pressures are

Albumin = 21.5 mm.

Globulin = 1.5 mm.

and Total serum protein = 23.0 mm.

Osmotic pressure of undiluted human serum (C.A.).

Observed osmotic pressure of undiluted human serum = 30.8 mm.

Total protein concentration = 7.91 g./100 ml. solution

$C_{v \text{ total protein}}$ = 8.59 g./100 ml. solvent

Precipitation analysis gave 74.5 % albumin and 25.5 % globulin. Therefore

$C_{v \text{ albumin}}$ = 6.40 g./100 ml. solvent

and $C_{v \text{ globulin}}$ = 2.19 g./100 ml. solvent

Corresponding osmotic pressure of albumin = 22.0 mm.

Corresponding osmotic pressure of globulin = 2.2 mm.

and Corresponding osmotic pressure of serum protein = $24.2 + 0.3 = 24.5$ mm.

Ultracentrifugal analysis gave 87 % albumin and 13 % globulin. Therefore

$$C_{v \text{ albumin}} = 7.47 \text{ g./100 ml. solvent}$$

and

$$C_{v \text{ globulin}} = 1.12 \text{ g./100 ml. solvent}$$

$$\text{Corresponding osmotic pressure of albumin} = 2.8 \text{ mm.}$$

$$\text{Corresponding osmotic pressure of globulin} = 1.1 \text{ mm.}$$

$$\text{and Corresponding osmotic pressure of serum protein} = 29.1 \text{ mm.}$$

The results are collected in Table XIX.

Table XIX. *Comparing the observed osmotic pressure of undiluted serum with the theoretical value based on the albumin/globulin ratio obtained by ultracentrifugal and precipitation analysis.*

Undiluted serum	Observed osmotic pressure mm. Hg	Theoretical osmotic pressure	
		Based on precipitation <i>A/G</i> ratio	Based on ultracentrifugal <i>A/G</i> ratio
Cow	22.3	21.3	25.8
Horse	20.5	17.7	23.0
Man	30.8	24.5	29.1

In the derivation of the ultracentrifugal figures we have classed the combined *A* and *X* fractions as albumin, since we are unable to introduce allowances for the molecular weight of the *X* fraction. If we had sufficient data to do so, the serum osmotic pressure values which are based on the ultracentrifugal albumin/globulin ratio would be somewhat lower.

Strictly, we should only utilise Adair and Robinson's curves to obtain values for the osmotic pressure of albumin and globulin in equilibrium with a buffer solution of p_H 7.4 and ionic strength (*J*) equal to 0.3466. We have measured our serum osmotic pressures at approximately the p_H of the untreated serum and against an approximately 1 % solution of sodium chloride. These two factors, however, can only introduce small errors in our results since Mayrs [1926] and Marrack and Hewitt [1927] find the osmotic pressure of serum proteins to be only slightly influenced by changes of p_H in the neighbourhood of neutrality, and the ionic strength of our dialysate is approximately 0.34.

As already mentioned it is not yet proved that Adair and Robinson's theory may be applied in its present form to undiluted serum. We have ventured to apply it here for two reasons. Firstly, we wish to show that if the theory is applicable to undiluted serum it appears to agree with the ultracentrifugal albumin/globulin ratio at least as well as with the ratio obtained by precipitation analysis; and, secondly, we wish to put forward the above figures in order to give some idea of the magnitude of the difference in the theoretical osmotic pressures of undiluted serum based on the two conflicting analytical values for the albumin/globulin ratio.

GENERAL DISCUSSION.

We hesitate to attempt to generalise on the basis of the experimental results which have been given in this paper, because it will be clear that little more than a start has been made in the investigation of a very wide field. We have endeavoured to place on record an exact account of these experiments on normal sera in the hope that they may form a basis for the accumulation in the future of the considerably greater amount of experimental material which is clearly necessary before we may try to establish a comprehensive definition of the molecular dimensional state of the proteins in a normal serum. The necessity

of having a much greater amount of experimental material than is at present available arises not merely because the exact definition of the normal serum of a species can only be obtained by averaging the results of a large number of normal members of the group, but also because we are attempting an exact physico-chemical definition of a non-ideal protein solution, and practically no general principles, such as we are familiar with in the case of dilute ideal solutions, can be applied. It will have been noticed in the foregoing description of our experimental results that the majority of our deductions have had to be based on comparisons between experiments. Our values for the sedimentation constant, for instance, have only a relative significance because of the high viscosity effects in strong protein solutions. Similarly, the molecular homogeneity of the serum fractions can only be estimated in a relative way by comparison of boundary spreading coefficients. We have no theoretical knowledge of the value of the boundary spreading coefficient of a pure protein in the same circumstances. Finally, the absolute concentration values of the serum fractions are subject to inaccuracies because of the effect of the total protein concentration which varies from one serum to another. All these facts raise difficulties in the way of generalising from the results obtained. They do not mean that established generalisations in this field can never be obtained. We believe that all that is required is a great many more results, preferably obtained with rather more standardised conditions of the centrifuge experiment than we have been able to adopt in this work. We believe that in the near future many new facts will be added to those quoted in this paper and we shall, therefore, attempt to draw the results together in the form of a few general deductions in the hope that these may form a framework of theory in relation to which present facts may be more easily viewed as a whole, and future facts may be tested.

In general, we have found that the normal serum of the cow, horse and man is constituted in a similar manner of three molecular species which can be identified by ultracentrifugal analysis. The corresponding fractions in the three types of serum appear to have the same sedimentation constants.

The heaviest of the three fractions separates easily from the other two sediments with a speed approximately the same as that of horse serum globulin in corresponding concentration and is present in variable amount up to 20 % of the total protein. The concentration of this fraction in a range of normal human sera was found to be rather constant, *viz.* (10 ± 5) % of the total protein concentration, which varied widely. The homogeneity of this fraction is doubtful in the case of human serum, which is also distinguished from cow and horse sera by a tendency to contain small amounts of substances sedimenting 2-3 times as quickly as globulin. These substances have not been specially examined.

The two lighter fractions differ so little in average molecular weight that it is difficult under the present conditions of a centrifuge experiment to obtain a satisfactory separation of the boundaries. We have, however, obtained a good separation in the cases of a few human sera by diluting slightly, and in these cases the average concentration of the more rapidly sedimenting fraction is 24 % of the total protein. We have no knowledge of the chemical nature of this substance, and the sedimentation constant tells us little more than that the molecular weight is slightly greater than that of serum albumin and considerably less than that of serum globulin. The sedimentation constant of the lightest fraction corresponds approximately to that of recrystallised horse serum albumin in the same circumstances, and consideration of the degree of boundary spreading suggests that the fraction is homogeneous. The separation of these

two fractions is not so satisfactory in the case of cow and horse serum, and we can only give approximate values for the relative concentrations of the fractions and the sedimentation constant of the heavier of the two. The concentration of the latter is 14 % of the total protein in the cow serum and 16 % of the total protein in the horse serum which we have examined.

All three types of serum on dilution with 1 % sodium chloride show an increased concentration of the globulin fraction with correspondingly diminished concentration of the lighter fractions. In these diluted sera it is impossible to discern a separation of the lighter fraction into two owing to the increased boundary spreading. Whereas the dilution effect is very marked with horse serum it is much less marked with cow and human sera. It is only necessary to dilute horse serum with 3 volumes of 1 % sodium chloride in order to raise the globulin proportion from 20 to 36 %. In the cow serum, on the other hand, 28 % appears to be the maximum globulin obtainable by dilution. Dilution of a human serum with an equal volume of 1 % sodium chloride had no significant effect on the globulin concentration, and dilution with 7 volumes of 1 % sodium chloride could only raise the proportion from 8.6 to 15 %.

It appears that in certain circumstances a solution of the serum proteins may contain a considerable amount of polydisperse protein molecules. We have found that drying cow serum by simple evaporation over P_2O_5 , or drying and then ether-extracting the powder, or treating the serum with ammonium sulphate, all gave rise in varying degree to this polydisperse protein. We have also found that the discrepancy between measured and calculated protein values is always greater when we are examining dilute solutions, and this may also indicate the presence of polydisperse protein in these solutions. These facts suggest a considerable sensitivity on the part of the proteins to environmental changes. Svedberg and collaborators have shown in numerous investigations that changes of hydrogen ion concentration outside certain limits give rise to pronounced associative or dissociative changes, and these facts have frequently been confirmed by other investigators using different methods. Altogether, it would appear that in the examination of protein solutions only a few very restricted methods may be used and most careful standardisation of technique must be observed. We have found in this work that it is at least possible to dialyse serum against 1 % sodium chloride or acetate buffer at p_H 4.6 without affecting the molecular dimensional state of the proteins.

In addition to the production of some polydisperse protein, treatment with ammonium sulphate appears to cause an irreversible alteration in the concentrations of the two lighter fractions in serum. This fact, which will require further investigation, raises difficulties in the way of applying to untreated serum facts derived from a study of the properties of the fractions obtained by ammonium sulphate precipitation.

We have found that treating serum with alcohol at low temperatures, which is an integral step in a method of obtaining dry lipid-free serum protein, gives rise to a marked change in the sedimentation diagram. It is probable that the major part of the protein exists in a polydisperse state, and the mean molecular weight of the fractions of this polydisperse protein which separate in the centrifugal field appears to be the same as that of the fractions in the parent serum. This latter fact explains why the redissolved powder in the hands of various investigators has shown physico-chemical properties very similar to those of the original serum. We find the phenomenon of interest not only because it indicates a serious defect in this technique of drying, but also because we visualise the change which takes place to be an early stage in the complex phenomenon

of denaturation, and there would appear to be placed in our hands a convenient method of studying the latter, *viz.* by examining various proteins after treatment with alcohol at low temperatures.

In the investigation of the various problems dealt with in this paper we have, as already stated, obtained only the barest experimental data in many cases. We hope, however, that in the presentation of these data we have, at least, adequately demonstrated an important aspect of the ultracentrifuge, *viz.* as a unique analytical instrument capable of separating the components of a mixture in respect of their molecular dimensions and estimating with considerable accuracy the concentration of each.

SUMMARY.

1. The ultracentrifugal sedimentation diagram appears to describe a strictly reproducible physical property of a protein solution.

2. The sedimentation diagrams obtained for the serum of the normal cow, horse and man all show the presence of three molecular types characterised by their sedimentation constants. The sedimentation constants of corresponding molecular types in serum from different species are the same. The diagram appears to be a common qualitative characteristic of normal serum generally, such differences as exist between sera being of the nature of quantitative differences in the concentrations of the individual fractions and of the total protein.

3. Heat inactivation of cow serum at 56° has little or no effect on the sedimentation diagram.

4. The sedimentation diagrams in the sera examined are changed fundamentally by simple dilution, the change consisting always in an absolute increase in the concentration of the globulin fraction and a corresponding decrease in the concentrations of the two lighter fractions. In dilute solutions it is not possible to separate the two lighter fractions in the centrifugal field.

The dilution effect is marked in the case of horse serum and much less so in the case of cow and human serum. Slight dilution had no significant effect on the sedimentation diagram of human serum, and dilution with 7 volumes of 1 % sodium chloride only increased the globulin concentration from 8.6 to 15.4 % of the total protein.

5. Dialysis of cow serum against 1 % sodium chloride or acetate buffer does not affect the sedimentation diagram. Complete removal of electrolytes induces certain changes which may be due to charge effects.

6. If cow serum is dried by simple evaporation at reduced pressures, with or without ether-extraction of the dry powder, the redissolved protein gives rise to a solution which differs significantly from the original serum in containing considerable quantities of polydisperse protein. A similar irreversible formation of polydisperse protein is caused by treating the serum with ammonium sulphate.

7. There is evidence that, in addition, ammonium sulphate causes an irreversible increase in the concentration of the intermediate fraction in cow and horse serum at the expense of the lightest (or "albumin") fraction.

8. A study of the behaviour of the serum and plasma from the same horse shows that no characteristic fibrinogen molecule is present in the plasma, but the two show certain differences in the concentrations of the three characteristic protein fractions.

9. In the study of a range of normal human sera there has been found a very close similarity in the general shape of all the sedimentation curves, indicating an approximately constant concentration for the constituent fractions.

The concentration of the heaviest or globulin fractions throughout the series is (10 ± 5) % of the total protein concentration, which latter value varied from 5.09 g./100 ml. to 8.43 g./100 ml.

10. The theory that the small amount of the globulin fraction in normal serum may owe its presence to the acquired immunities of childhood is proved to be wrong by the finding that in the serum of a baby at the time of birth 8 % of this fraction was present. There are some points of difference between this diagram and that of the mother's serum taken at the same time.

11. Drying a normal human serum by a recognised procedure using ether and alcohol at -16° causes a fundamental change in the physical state of the proteins. The interpretation of this change is discussed.

12. Osmotic pressure measurements have been performed on certain of the sera used in this work, and the values obtained are considered in the light of a recent theory of the osmotic pressure of untreated serum.

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