CXXXVI. THE ELECTROPHORETIC ANALYSIS OF NORMAL HUMAN SERUM

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It has been shown by Stenhagen [1938], using the Tiselius [1937, 1] electrophoresis technique, that normal human serum contains four electrophoretically distinct protein components, viz. albumin, and α, β and γ globulins. In this respect it is similar to the normal serum of other animals, e.g. horse, rabbit [Tiselius, 1937, 2].

The optical method used by Stenhagen does not allow of the accurate estimation of the relative quantities of the protein components of normal human serum. Such information is important, particularly for the proper interpretation of the changes which can be observed in sera of pathological human conditions.

This paper is an account of the investigation of several normal human sera, in which the electrophoretic behaviour has been observed by means of the Lamm [1928; 1929; 1937] scale method. It has thus been possible to make accurate estimates of the relative amounts of the protein components present.

Experimental

(1) *Treatment of sera*

The sera, obtained from normal fasting adults, were dialysed at constant volume in cellophane tubes against suitable buffers of ionic strength $\mu = 0{\cdot}1$, in a bath at 2°. The volume of buffer for dialysis was 5–10 times that of the serum sample. The dialysate was changed twice a day until ionic equilibrium was attained. Refractive index measurements ($\lambda = 546$ mμ) were made with the dipping refractometer on fresh buffer, and on buffer which had been in contact with the serum for 12 hr. When no difference in refractive index could be observed between these samples, it was assumed that equilibrium had been attained. The process usually took 3–4 days.

The dialysed serum was centrifuged to remove the slight precipitate which invariably formed, and the refractive index measured. For electrophoretic examination the serum was then diluted with buffer until the difference in refractive index between the protein solution and the buffer, $(n_1 - n_0)$, was 0·00300, where n_1 = refractive index of protein solution and n_0 = refractive index of buffer. This involved a fourfold dilution approximately.

The electrophoresis was carried out in a bath at 0° the potential gradient applied usually being about 5 V./cm.

(2) *Optical methods*

The electrophoretic migration was observed both by the Toepler "Schlieren" method [Tiselius *et al.* 1937] and by the Lamm scale method. A sodium vapour lamp was used as a light source and photographs taken on Ilford Half Tone Panchromatic Plates.

In the scale method a double equal distance scale was photographed through the U-tube, one scale through each limb. The scale was situated approximately 4·0 cm. from the centre of the U-tube. Immediately after the formation of the boundary in any experiment, the boundary being at that time hidden by the centre plate of the U-tube, an exposure was made which was used as a reference scale. The boundary between buffer and diluted serum was then brought into view by the compensator, the current switched on, and exposures taken subsequently at suitable intervals, usually every 30 min. "Schlieren" photographs, taken immediately before each scale photograph, were used for the selection of the most suitable scale photographs for measurement and plotting.

The electrophoresis curves were obtained by plotting the deviations of the scale lines, Z, against the deviated scale line position, X, in the cell, in accordance with the accepted method.

(3) *Estimation of amounts of components*

The type of curve obtained from the scale photographs shows some variation, depending on whether the electrophoretic migration takes place in the direction of the buffer or of the diluted serum. Greater spreading of the individual boundaries occurs in that limb of the U-tube in which the direction of migration is towards the diluted serum, and diagrams from this limb are therefore less suitable for the estimation of the amounts of all the components present.

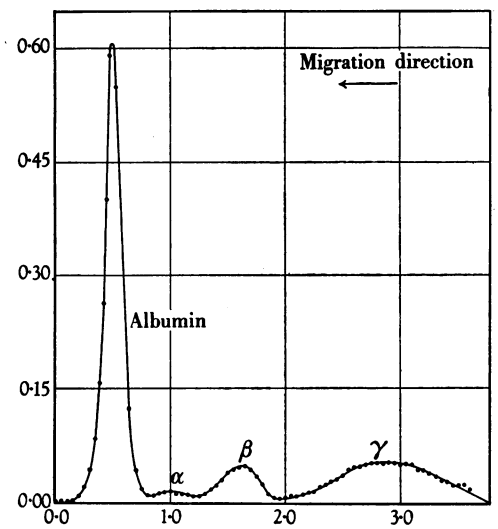

Fig. 1. Normal human serum. 3. Electrophoretic analysis. Phosphate buffer *p*H 8 $\mu=0{\cdot}1$ 4·9 V./cm. Exposure 152 min. after starting current. *Anode limb.* Abscissa: distance in U-tube in cm. Ordinate: scale line displacement in mm.

In Fig. 1 is shown a typical scale diagram of a normal human serum, from that limb of the U-tube in which migration was taking place in the direction of the buffer layer. It is obvious that by slight extrapolations to the base line it is possible to measure the area enclosed by each peak, and this is proportional to the refractive increment due to each component [McFarlane, 1935, 1].

It can be seen from Fig. 2, a scale diagram for the opposite limb (migration towards diluted serum), that in this case the extrapolations are more uncertain. Such curves have been used for the estimation of the relative amounts of albumin and total globulin, but not for the estimation of the individual globulins.

The amounts of the components are given as percentages of the total refractive increment due to the protein present. It is not possible to express these values in terms of g. protein per 100 ml. serum as the specific refractive increments of the components probably differ, and reliable values for human serum proteins are not yet available.

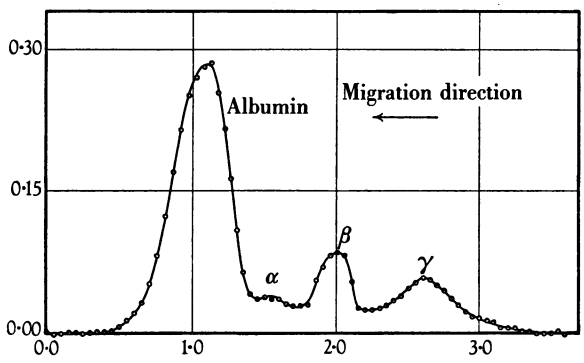

Fig. 2.

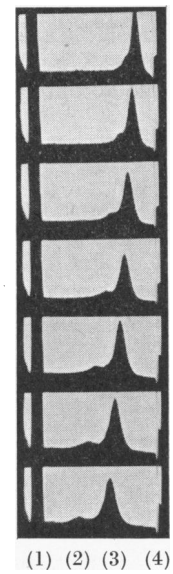

Fig. 3.

Fig. 2. Normal human serum. 3. Electrophoretic analysis. Phosphate buffer *p*H 8, $\mu = 0{\cdot}1$, 4·9 V./cm. Exposure 124 min. after starting current. *Cathode limb.* Abscissa: distance in U-tube in cm. Ordinate: scale line displacement in mm.

Fig. 3. Normal human serum. 5. Ultracentrifugal analysis. Phosphate buffer *p*H 8, $\mu = 0{\cdot}1$. $n_1 - n_0 = 0{\cdot}00300$. Interval between exposures 10 min. Field strength 270,000 × gravity. (1) Cell index, (2) globulin, (3) albumin, (4) meniscus.

(4) *Ultracentrifuge technique*

Samples of the solutions used for the electrophoresis experiments were examined in the ultracentrifuge in a field strength of 270,000 times gravity. The "diagonal schlieren" method [Philpot, 1938] was used for the observation of the sedimentation, a high pressure mercury arc being used as a light source. Photographs were taken on Ilford Half Tone Panchromatic Plates, monochromatic light $\lambda = 546$ mμ, being isolated by a suitable filter.

Fig. 3 is a series of photographs obtained by this method, showing the sedimentation behaviour characteristic of normal human serum. For the estimation of the amounts of the components, the centrifuge photographs were projected, with a magnification of six diameters, on to transparent graph paper. The contours of the enlarged image were traced freehand on the graph paper and the curves so obtained analysed geometrically. An arbitrary straight base line was drawn connecting those portions of the actual base line visible at the

extremities of the diffusion boundary. A symmetrical curve was constructed for the albumin peak and the remaining area assigned to globulin.

That the use of such an arbitrary base line was justified under the experimental conditions used, was shown by experiment. Following a normal serum sedimentation run, buffer alone was put into the cell and exposures taken at times corresponding to those in the serum run, with identical optical settings. By this means a true base line for each exposure was obtained. An analysis for albumin and globulin using the true and arbitrary base line gave the following figures: true base line, albumin 79 %, globulin 21 %; arbitrary base line, albumin 81 %, globulin 19 %.

Using either the arbitrary or true base line, the curve for the globulin component obtained by the geometrical analysis showed slight deviations from a perfectly symmetrical appearance. These deviations, representing about 2 % of the total curve area may have been due to the inclusion of some of the *X* fraction [McFarlane, 1935, 2] with the globulin. The quality of the curves, however, did not seem to justify a finer analysis.

Results

The area enclosed by curves obtained by the Lamm scale method at a diffusion boundary such as is under consideration is given by the equation

$$A = Gab\,(n_1 - n_0),$$

where A = area.

G = photographic enlargement factor.
a = thickness of fluid column.
b = optical distance from the scale to the middle of the cell.
n_1 = refractive index of the protein solution.
n_0 = refractive index of the buffer in which the protein is dissolved.

Substituting the values $G = 0{\cdot}75$, $a = 2{\cdot}5$ cm., $b = 3{\cdot}0$ cm. (corrected for the refractive index of water etc.), and allowing for a plotting magnification of 5×10^3, a value of 84 cm.^{2} is obtained for A. Comparison with the data of Table I shows that the areas found experimentally are in fair agreement with this value. The fact that the anode curve areas are generally slightly greater than those for the cathode will be referred to in the discussion.

Table I. *Comparison of anode and cathode curve areas*

Run no.	pH	Exposure no.	Curve areas	
			Anode	Cathode
12	8·0	7	74·8	71·3
13	8·0	6	79·4	76·7
14	8·0	6	79·9	77·5
15	8·0	7	80·7	78·9
16	7·0	6	75·4	78·3
17	4·0	6	71·9	71·2

The curve areas are in cm.^{2}

In Table II, the relative quantities of albumin and total globulin, calculated from corresponding anode and cathode limb exposures are presented for a number of sera. The amount of albumin calculated from the cathode limb curve is in each case somewhat higher than that from the anode limb, and may be related to the area discrepancy noted above.

Table II. *Percentages of albumin and total globulin calculated from corresponding anode and cathode limb exposures*

Serum no.	*p*H	Exp. no.	Anode limb		Cathode limb	
			Albumin	Globulin	Albumin	Globulin
3	8·0	6	60·0	40·1	66·1	33·9
4	8·0	6	60·7	39·3	65·8	34·2
5	8·0	6	56·6	43·4	62·0	38·0
6	8·0	7	54·6	45·4	57·9	42·1
6	7·0	6	58·5	41·4	60·3	39·9
		Mean	58·0	42·0	62·5	37·5

Mean values given to 0·5%.
Values are percentages of total refractive increment.

It has been pointed out by Tiselius [1937, 1] that it is usually most convenient to separate the protein components of sera at *p*H 8. This *p*H has also been found most useful for the present experiments. There remains the possibility, however, that the amounts of the components might vary with *p*H, and also, in view of the results of McFarlane [1935, 1, 2] with the ultracentrifuge, the amounts of the components might be expected to vary with the total protein concentration. These points have been investigated and the relevant data are given in Table III. The amounts of the components represent the mean values obtained from two successive exposures taken 30 min. apart with the current running continuously. The values refer to the limb in which migration was taking place towards the buffer; in most cases this was the anode limb. These conditions apply also to Table IV.

Table III. *Percentages of components at various* p*H and concentrations*

*p*H	$n_1 - n_0$	Albumin	Globulins		
			α	β	γ
8·0	0·00300	57·1	3·0	13·5	26·5
8·0	0·00150	57·2	4·7	13·0	25·3
7·0	0·00300	58·9	3·7	13·1	24·5
4·0	0·00300		79·4		20·6

Data obtained from normal human serum 6.
Values are percentages of total refractive increment.

Table IV. *Percentages of components in various normal human sera.* p*H 8*

Serum no.	Albumin	Globulins		
		α	β	γ
1	58·9	4·4	11·0	25·7
3	59·4	2·8	10·0	27·9
4	62·4	5·2	9·9	22·6
5	57·8	6·6	11·7	24·0
6	57·1	3·0	13·5	26·5
Mean	59·0	4·5	11·0	25·5

Analysis at *p*H 8. $n_1 - n_0 = 0{\cdot}00300$.
Mean values given to 0·5%.
Values are percentages of total refractive increment.

Samples of a single normal human serum were dialysed as described against buffers of ionic strength $\mu = 0{\cdot}1$ of *p*H 4, 7 and 8 and an electrophoretic analysis

made at the standard protein concentration. In addition the sample at *p*H 8 was investigated at half the standard concentration, viz.:

$$n_1 - n_0 = 0{\cdot}00150.$$

It will be noticed that the values for the amounts of the components determined at *p*H 7 and 8 are in excellent agreement. At *p*H 4, even after prolonged electrophoresis, it was possible only to estimate the amount of γ globulin, as there was insufficient separation between the albumin, α and β globulin curves to allow any further analysis. The value obtained for the amount of γ globulin is in reasonable agreement with the values at *p*H 7 and 8.

Under the experimental conditions used there was no change in the amounts of the components with protein concentration. Owing to the design of the U-tube it was not possible to carry the investigations above a total protein concentration corresponding to $n_1 - n_0 = 0{\cdot}00300$, as the scale deviations became so large as to be immeasurable, because of the out of focus effect.

In Table IV the results from the analysis at *p*H 8 of five normal human sera are assembled. It will be noticed that the relative quantities of the various components show a satisfactory constancy throughout the series. No attempt has been made to calculate standard deviations for the mean values given at the bottom of the table, owing to the rather small number of sera investigated.

Table V. *Comparison of electrophoretic and ultracentrifugal analysis of same sera*

			Electrophoretic		Ultracentrifugal	
Serum	*p*H	$n_1 - n_0$	Albumin	Globulin (total)	Albumin	Globulin
1	8·0	0·00300	58·9	41·1	80·0	20·0
3	8·0	0·00300	59·4	40·7	81·5	18·5
4	8·0	0·00300	62·4	37·7	80·0	20·0
5	8·0	0·00300	57·8	40·3	75·0	25·0
6	8·0	0·00300	57·1	43·0	76·0	24·0
6	8·0	0·00150	57·2	43·0	74·0	26·0
6	7·0	0·00300	58·9	41·3	78·5	21·5
6	4·0	0·00300	—	—	73·0	27·0

Values are percentages of total refractive increment.

No figures are included relating to the mobilities of the components in the various sera, as these would appear to be of doubtful significance when determined in the presence of other proteins, because the magnitude of the viscosity effects is unknown.

In Table V the ultracentrifugal analysis for each serum is compared with the corresponding electrophoretic analysis. The ultracentrifuge data represent the mean values from two exposures in each case; the electrophoretic data are the mean values from two anode limb curves.

The discrepancy between these two sets of results is marked and will be referred to in the discussion.

Discussion

Referring to Table I, the question of the difference in the areas enclosed by the anode and cathode curves calls for some comment. The total area of the curves is very dependent on the accuracy with which the position of the base

line can be determined, and discrepancies of the same order as those shown in Table I frequently occur between areas from exposures on one side of the U-tube alone. The consistency with which the effect occurs, however, suggests that it may be significant, and this is supported to some extent by the data of Table II, for reasons that appear below.

Under the experimental conditions used in the work reported in this paper the only indication of a δ boundary [Tiselius, 1937, 2] has been a slight inflexion in the curve for γ globulin from a few of the exposures. Stenhagen [1938], with experimental conditions closely similar to those used here, reports the absence of a δ boundary. It has been suggested by Longsworth & MacInnes [1939] that the δ boundary is due to differences in buffer salt concentration between the protein solution and buffer, occurring on account of the Donnan equilibrium set up in the initial dialysis. It can be shown that dialysis at high protein concentration, followed by dilution with buffer, the technique used in these experiments, tends to minimize such differences. There has been no indication whatsoever of any ϵ boundary [Longsworth & MacInnes, 1939], from the cathode limb curves.

A δ boundary effect would tend to give a greater total area for the anode limb curves than for the corresponding cathode curves, and also tend to increase the relative amount of globulin as calculated from the anode limb, over that from the cathode limb curves.

The data of Tables I and II are consistent with this argument, though the effects are rather small. Further, the δ boundary effect would in these experiments be included in the γ globulin peak, resulting in too high a proportion for this component.

Tiselius & Kabat [1939], having excluded the δ boundary from their anode curves, found larger total areas for the cathode than for the anode curves, and report that "the occurrence of a δ boundary does not alter the relative concentration of the other components".

In view of the reservations of the previous paragraphs, the data of Table IV show satisfactory agreement, the amounts of the components being independent of *p*H and of concentration in the region examined (Table III). The significance of the marked discrepancy between the electrophoretic and ultracentrifugal analyses (Table V) is difficult to assess. The ultracentrifugal data are in general agreement with the results of McFarlane [1935, 1] and the protein concentration is such that any "mixture effect" is not detectable by further dilution. If the value for the amount of γ globulin is too high, as discussed above, this would be an aggravating factor, but it would seem that further analysis of the situation depends on a study of artificial mixtures of the electrophoretically separated components of normal human serum.

Summary

1. The quantities of the protein components present in normal human serum have been determined by electrophoretic analysis.

2. Discrepancies between the electrophoretic and ultracentrifugal analyses are reported and discussed.

3. Electrophoretic analysis gives mean values of 59% albumin and 41% total globulin, the latter consisting of 4·5, 11·0 and 25·5% of α, β and γ globulin respectively.

By ultracentrifugal analysis the mean quantities of albumin and globulin found are 78 and 22% respectively.

4. The probable modifications in the electrophoretic analytical values consequent on a slight δ boundary effect, have been discussed.

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