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ON THE MEAN LYTIC POINT OF RED BLOOD CORPUSCLES AND THE APPARENT TONICITY OF SHEEP SERUM.

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(*From the Quick Laboratory, Cambridge.*)

(With 5 Charts.)

The mean lytic point of red blood corpuscles of different orders.

IN preparing washed red blood corpuscles of various animals for use in sensitization experiments the differences of corpuscles of different orders in their resistance to lysis by hypotonic salt solutions are striking.

As no published papers on this subject supplied the information required in a sufficiently accurate form I determined the respective resistances of the corpuscles of eight animals whose blood is constantly made use of.

The method employed for obtaining washed corpuscles was the usual one. For each observation a series of test tubes was prepared containing sodium chloride solution in descending percentages, each tube differing from the adjoining ones by 0·017 % NaCl. Then 0·05 c.c. of the washed corpuscles was dropped into each tube from a calibrated pipette. After having been shaken up the tubes were put into an ice-chest and the points of "trace" and "complete" lysis were noted next morning.

Chart No. 1 shows that the corpuscles of the eight species each occupy a definite position on the salt scale. It will be observed that the mean range of lysis is very nearly the same for each species, the average range of the eight being 0·144 % NaCl, and that the position of any particular species on the scale appears to be immaterial as far as

the range of its lytic points is concerned. In other words, the majority of the red corpuscles of any particular species are lysed at or about a particular dilution of salt, and comparatively few are either much more or much less resistant than the majority. The variations in position on the scale may depend merely on the strengths of the envelopes of the corpuscles of the different species, but, as will be

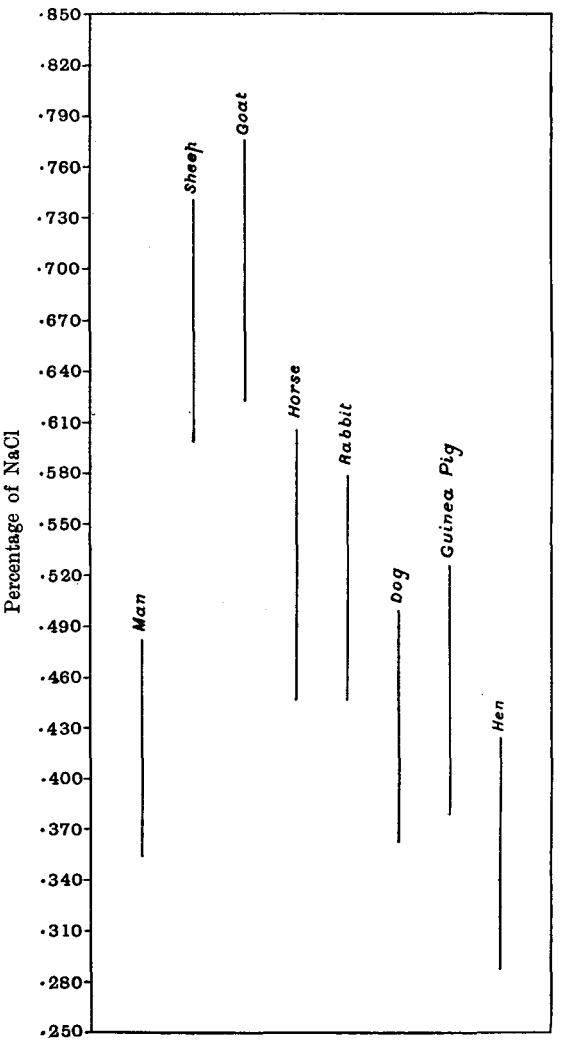

Chart 1. Showing the position on the salt percentage scale of the mean range of lysis of the red blood corpuscles of eight species.

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seen later, other factors must be considered before this point can be decided. The tonicities of the serums did not vary proportionately with the resistances of their respective red corpuscles. The mean of the points of "trace" and "complete" lysis is called the *mean lytic point* of the corpuscles concerned. The values obtained are exhibited in Table I.

TABLE I.

Mean lytic points of the red blood corpuscles calculated from 20 observations on each species.

Goat	...	...	0·706 % NaCl.
Sheep	...	...	0·670 ,,
Horse	...	...	0·527 ,,
Rabbit	...	...	0·514 ,,
Guinea-pig	...	...	0·453 ,,
Dog	...	...	0·432 ,,
Man	...	...	0·418 ,,
Hen	...	...	0·365 ,,

The daily variation in the lytic points of the corpuscles of a healthy goat on 14 successive days was also observed and found to be comparatively small.

Another series of experiments showed that there was no obvious relation between the resistance of the corpuscles of any species to hypotonic lysis and their resistance to lysis by normal serums derived from other orders. Corpuscles derived from goats which have a high mean lytic point are not more susceptible to the lysins normally found in the serums of other animals than corpuscles derived from men or hens which have a low mean lytic point. The data which form the basis of the foregoing remarks are the results of experiments made at the Pasteur Institute of Southern India. The experiments which follow were made in the Quick Biological Laboratory, University of Cambridge. In these sheep red blood corpuscles and sheep serum were used exclusively.

The relation between the apparent tonicity of a sheep serum and the mean lytic point of the red blood corpuscles of the same individual.

It is as well to define at this point the exact meaning which is attached in this paper to the term "*tonicity.*"

The tonicity of a serum or of a fluid is expressed in terms of NaCl per cent., and the expression means that that serum or fluid acts just as

a solution of NaCl of the percentage named would act on washed red blood corpuscles suspended in it as regards preserving them from lysis or causing them to lyse.

The "mean tonicity" is calculated from the observations made by diluting the serum or fluid with distilled water and noting the points of "trace" and "complete" lysis of the red corpuscles used as an indicator. "Actual tonicity" refers to the effect due to the presence of inorganic salts alone, while "apparent tonicity" is used to express the effect of other substances in the fluid either with or without inorganic salts.

Has the mean lytic point of sheep corpuscles in hypotonic solutions of NaCl any relation to the tonicity of the serum of the same animal, or not, and, when the tonicity of the serum is high, is the mean lytic point of the corpuscles lowered and the converse? It might be considered probable that when the tonicity of the serum is high the corpuscles are in proportion more resistant to lysis by hypotonicity and would require a solution of lower tonicity to lyse them than would be required when the tonicity of the serum is lower. If this were so the differences in the mean lytic point of the corpuscles of individuals of the same species might be accounted for. Experiments were made with the corpuscles from 31 different sheep taken from citrated blood and washed three times in 0·85 % NaCl. The same procedure was followed as in the previous experiments except that the adjoining tubes in the series differed by only 0·014 % NaCl, and the mean lytic point for these 31 English sheep worked out at 0·676 % NaCl. In Table I on p. 247 it will be seen that the mean lytic point of the corpuscles of 20 Indian sheep was 0·670 % NaCl.

The tonicity of the serum was judged by putting up a series of tubes containing diminishing quantities of serum and increasing quantities of distilled water and reading off the points of "trace" and "complete" lysis. These, compared with the readings of the like points in salt and water, gave by a simple calculation two values for the tonicity of the serum, and the mean of these values was taken as the tonicity of that serum.

TABLE II.

Highest tonicity observed	...	...	1·164 % NaCl.
Lowest ,, ,,	...	...	0·996 ,,
Mean of 23 observations	...	...	1·072 ,,

A simple way of displaying whether or no high and low tonicities of serum are accompanied by decreases and increases in the mean lytic point is to calculate in each case the percentage deviation from the mean. Chart No. 2 is a graphic representation of the values arrived at from observations on 23 sheep. No definite relation is discovered by these observations between the tonicity of a serum and the mean lytic point of the corpuscles of the same individual.

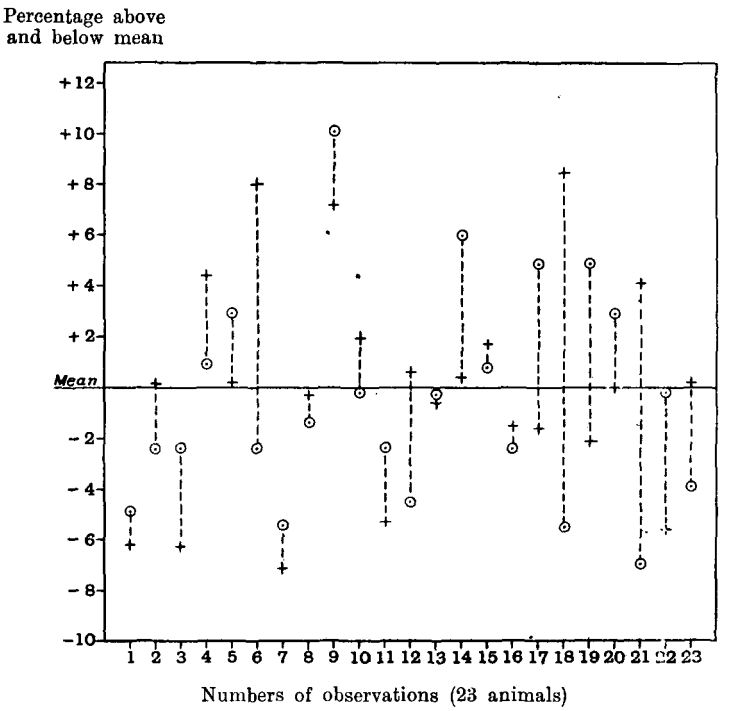

Chart 2. Showing the percentage above or below their means of the mean lytic point of sheep corpuscles ⊙⊙⊙, and of the mean tonicity of the serum of the same animals + + +.

Numbers 6, 18 and 21 show highly tonic serums accompanied by resistant corpuscles; numbers 1, 3, 7 and 11 show serums of low tonicity accompanied by resistant corpuscles; number 9 shows a highly tonic serum accompanied by corpuscles of low resistance; numbers 17 and 19 show serums of low tonicity accompanied by corpuscles of high resistance. Number 12 is a serum of average tonicity with corpuscles of high resistance and number 14 is a serum of average tonicity with corpuscles of low resistance. The remainder are near the mean line. There was

in the first place no reason for supposing that the mean lytic point of corpuscles may be dependent on the tonicity of the serum in which they swim and no relation is apparent in the data now collected. It is, however, evident that the tonicity of serum is only apparent, that is to say it cannot be attributed entirely to the osmotic pressure of the inorganic salts dissolved in it.

In fact, if the apparent tonicity of a serum be calculated in terms of NaCl per cent. and a solution of NaCl in distilled water be prepared of that same strength and a parallel series of dilution experiments put up, it will be found that the serum can be diluted far below its corresponding NaCl solution without lysis of the indicating corpuscles taking place. The average dilution which sheep serum will sustain before the mean lytic point of sheep corpuscles is reached is 1·59 times, but the range of different specimens is considerable (*vide* Table II). As a serum does not act on dilution towards corpuscles in the same manner as a simple solution of NaCl, it is clear that there must be some substance in the serum which protects corpuscles from lysis when the serum is diluted sufficiently to lower the osmotic pressure of the dissolved salts beneath that which keeps the envelopes of the corpuscles intact.

To what is the apparent tonicity of a serum due?

To decide to which constituent of serum the protective influence is due a further series of experiments was undertaken. Chart No. 3 shows the form of the curve obtained by diluting serum. The method employed was the following: the apparent tonicity of the serum was estimated in terms of NaCl in the way described on a previous page. Sundry dilutions of the serum were then prepared, using 0·85 % NaCl as the diluting fluid, and series of tubes were put up as before. The apparent tonicity of each dilution was then estimated separately to give the points for the curve.

In Chart 3 serum *A* is a serum of high tonicity. If its effect on corpuscles suspended in it had been due solely to its contained inorganic salts, on dilution the observations made would have fallen along the straight line *B*.

Serum *C* was a serum of low tonicity initially. If it had followed the course of a salt solution of the same tonicity the curve *C* would have been replaced by the straight line *D*. Clearly, serum contains some substance which has a marked influence in protecting corpuscles from lysis by hypotonic solutions, and the influence of this substance is

easily recognisable even when the concentration of the serum is reduced to only 1 % or 2 %.

Here it is advisable to insert an experiment which shows the extent of error to which the method employed is liable. A solution of NaCl 1·12 % was prepared and from it several dilutions were made with NaCl 0·85 %, precisely as had been done with the serum. The

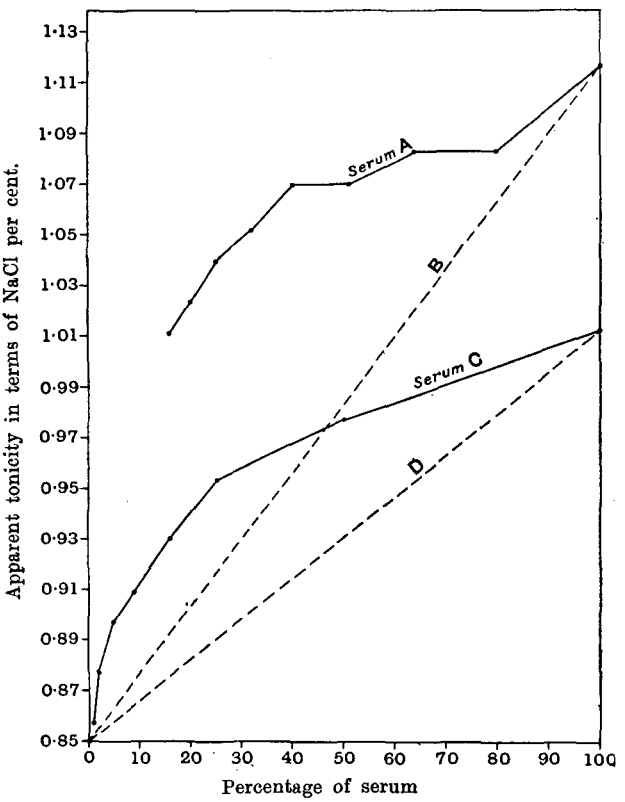

Chart 3. Showing that dilutions of serum do not behave towards corpuscles in the same way as solutions of NaCl isotonic with the undiluted serum would behave.

tonicities of these dilutions were estimated from observations and the resulting points are indicated in circles on Chart 4, in which the straight line marks the actual tonicities for comparison. The difference between the actual and the observed points is not great, and shows that the method is of sufficient accuracy to enable conclusions to be drawn.

The same method was then applied to some of the various constituents of serum in turn.

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Albumins and globulins.

Having failed to obtain specimens of albumin and globulin, that were completely soluble, by the usual method of salting out with ammonium sulphate and dialysing the products, I applied to Mr W. B. Hardy, F.R.S., who was kind enough to suggest the method he has himself employed of precipitating the serum proteins by alcohol and

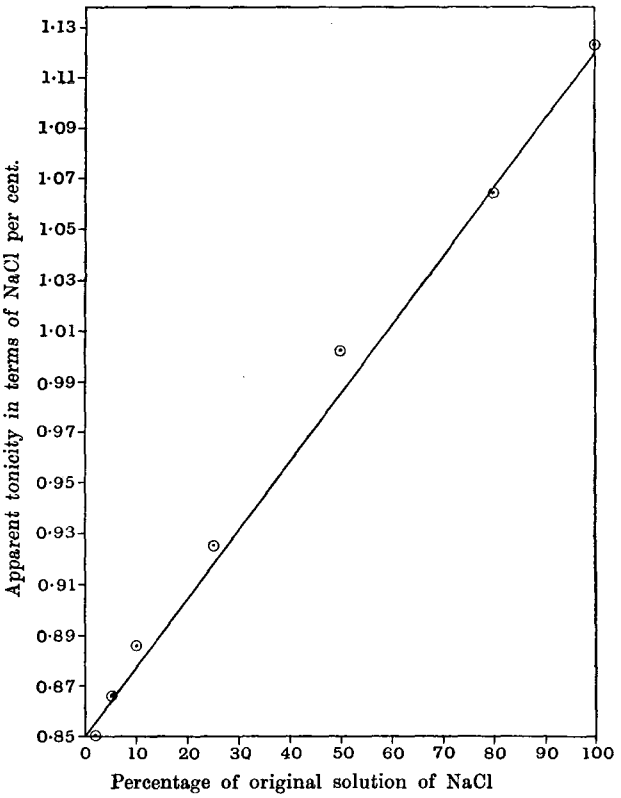

Chart 4. Showing the extent of the errors of observation inseparable from the method employed.

keeping the fluids below freezing point in a freezing mixture during the whole process of precipitation and filtration. The precipitate is subsequently extracted with anhydrous ether to remove the lipoids, dried and dissolved in 0·85 % NaCl equal in volume to the serum originally taken.

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This solution did not protect corpuscles from lysis in the same way as the original serum.

A similarly negative result was given by solutions of albumin and globulin used separately.

The globulins were precipitated by semi-saturation of serum with ammonium sulphate, filtered off and purified by re-solution and re-precipitation. The ammonium sulphate was removed by prolonged dialysis under toluol. The final solution was made up to a volume equal to that of the original serum and NaCl was added to 0·85 %. The filtrate, containing the albumins, was dialysed till all but a trace of ammonium sulphate was removed. The albumins were then precipitated by alcohol in the cold, extracted with anhydrous ether, dried and dissolved in 0·85 % NaCl equal to the original serum in volume.

Neither the globulin solution nor the albumin solution had the protective influence on corpuscles possessed by whole serum.

It is concluded that the protective element in serum is not a protein.

Influence of heat.

The fluid expressed from serum coagulated by heat in a water bath was found to retain the protective element unaltered by exposure to a temperature of 100° C.

This fluid contained alkali-albumin incoagulable by heat, mucoids, lipoids, pigments and salts.

To deal with a protein-free solution it is necessary to precipitate the proteins of serum by alcohol and to filter. The precipitate is well washed with 75 % alcohol and then extracted with ether to remove adherent lipoids. The ether extract is distilled and the residue added to the residue from the alcoholic filtrate after removal of the alcohol by evaporation. These residues contain all the constituents of the serum but protein and are dissolved in distilled water equal in volume to the original serum. The result of these operations is a yellowish fluid which contains the protective element. Curves similar in form to those depicted in Chart 3 could be constructed by using these fluids in various dilutions just as the whole serum was diluted.

Lipoids. After precipitation of the proteins from serum by alcohol the filtrate was evaporated to dryness and the residue was collected and extracted several times with anhydrous ether. To this extract was added the ethereal extract of the protein precipitate. The ether was then distilled off and the residue, a yellow, oily deposit, was taken up

in a volume of 0·85 % NaCl equal to that of the original serum. The solution was white and soapy looking and lathered easily, but it did not separate out on standing. It was unaltered in appearance by being boiled, but on the addition of strong nitric acid red nitric oxide gas was evolved freely. The solution contained the lipoids of the serum and a small quantity of mucoids. It was neutral to litmus, and volumetric estimations showed that it contained no NaCl beyond that which had been added to it. Any effect on corpuscles shown by this solution can, therefore, have been due only to the presence of ether soluble lipoids and mucoids. The effect found is displayed by the curve *L L L* in Chart 5, which is seen to be of the same general form as the whole serum curve in Chart 3. If the lipoids had possessed no protective influence the curve would have been replaced by the dotted straight line.

In an endeavour to distinguish between the lipoids another protein-free residue was dried and extracted with several relays of pure acetone. Acetone dissolves cholesterin but not lecithin.

The remainder was further extracted thoroughly with anhydrous ether which dissolves lecithin. Two extracts were thus obtained, one containing cholesterin and the other lecithin. The solvents were distilled off and the oily residues taken up with 0·85 % NaCl equal in volume to the original serum. Neutral, white, soapy solutions were formed, containing no salt but that which had been added.

Both of these solutions showed the protective effect on corpuscles (*vide* cholesterin curve *E E E* and lecithin curve *A A A* on Chart 5). None of the curves shown in the charts can be compared as regards position but only as regards form, since different serums were used.

It must, therefore, be concluded that both cholesterin and lecithin are concerned in preserving the integrity of the red corpuscles in the blood from lysis by hypotonicity. It is well known that cholesterin is able to protect corpuscles to some extent from lysis by both normal and specific lysins. Hence it seems logical to suppose that reduction of the quantity of lipoid normally present in the blood facilitates lysis of the red corpuscles and that agents which normally would be impotent can then assert themselves, leading to anaemia, haemoglobinaemia, or even pronounced haemoglobinuria.

The total effect of lipoids on corpuscles in a test tube is in the main similar to that of perchloride of mercury, though the mechanism is not the same. The latter salt hardens the envelopes of the corpuscles, so that they can be exposed to hypotonic solutions without rupture.

On the removal of the $HgCl_2$ by sodium thiosulphate lysis by hypotonicity at once occurs.

Corpuscles suspended in hypotonic salt solution and cholesterin remain intact, but when the cholesterin is cautiously neutralised by saponin hypotonic lysis manifests itself.

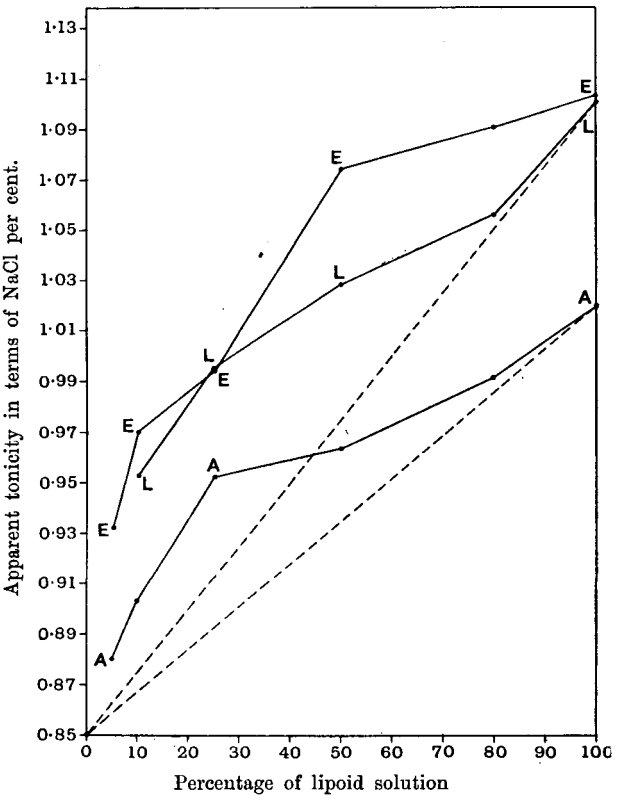

Chart 5. Showing that dilutions of the lipoids of serum do not behave towards corpuscles in the same way as solutions of NaCl isotonic with the undiluted lipoid solutions would behave.

Total lipoid curve *L L L*.
Cholesterin curve *E E E*.
Lecithin curve *A A A*.

Salts. After removal of the proteins by precipitation with alcohol and of the lipoids by extraction with ether little remains but the salts of the serum, some pigment and some mucoid. When taken up in a quantity of distilled water equal to the original volume of serum,

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a faintly yellow, opalescent solution is obtained which is alkaline to litmus.

Volumetric analysis of one such solution showed it to contain 0·677 % NaCl and its alkalinity was equivalent to 0·072 % NaOH. Red corpuscles suspended in solutions of these residues were sometimes lysed immediately, in other specimens lysis occurred slowly. Even when the residue was taken up with 0·85 % NaCl instead of distilled water complete or partial lysis still occurred, proving that the effect was not due merely to hypotonicity, but that some lytic agent was present. This agent is probably the alkaline phosphates and carbonates of the serum. When a series of tubes was prepared containing diminishing quantities of the fluid and increasing quantities of distilled water a point could be reached at which lysis was only partial; the tubes containing the greater concentrations of the fluid showing complete lysis due to alkalinity, and the tubes containing the greater quantities of distilled water showing complete lysis due to hypotonicity. The tubes in the middle of the series contained too little alkali to procure complete lysis by alkalinity and too much NaCl to permit complete lysis by hypotonicity. It is doubtful from the experiments carried out whether sheep serum contains sufficient neutral inorganic salts to maintain the corpuscles intact in the absence of lipoids. The mean lytic point of sheep corpuscles lies between 0·67 and 0·68 % NaCl and this appears to be about the percentage of NaCl ordinarily present in the serum. In order that there may be no lysis at all, even of the weaker corpuscles, the osmotic pressure of the serum must not be less than from 0·75 to 0·85 % NaCl.

The lipoids, therefore, it would seem, have a double duty to perform: they have to protect the corpuscles from lysis in the serum by hypotonicity, and they have to counteract the lytic effect of the alkaline salts.

My sincere thanks are due to Professor G. H. F. Nuttall, F.R.S., in whose laboratory the greater part of the above work was carried out.

Summary.

1. The red blood corpuscles of different species have definite and distinct mean lytic points.

2. The apparent tonicity of sheep serum has no relation to the mean lytic point of the red corpuscles of that individual.

3. The apparent tonicity of sheep serum is not due to its proteins but to its lipoids.

4. The residue of sheep serum, after removal of proteins and lipoids, is sufficiently alkaline to cause lysis.

PROTOCOLS.

CHART 2.

Numbers of animals	Serum: Percentage above or below the mean tonicity	Corpuscles: Percentage above or below the mean lytic point
1	−6·2	−4·9
2	+0·1	−2·4
3	−6·3	−2·4
4	+4·4	+1·9
5	+0·2	+2·9
6	+8·0	−2·4
7	−7·1	−5·5
8	−0·3	−1·4
9	+7·2	+10·1
10	+1·9	−0·2
11	−5·3	−2·4
12	+0·6	−4·5
13	−0·6	−0·3
14	+0·4	+6·0
15	+1·7	+0·8
16	−1·5	−2·4
17	−1·6	+4·9
18	+8·5	−5·5
19	−2·1	+4·9
20	±0·0	+2·9
21	+4·1	−7·0
22	−5·6	−0·2
23	+0·1	−3·9

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CHART 3.

	Serum A			Serum C		
	Observed tonicity	Calculated tonicity	Difference	Observed tonicity	Calculated tonicity	Difference
Whole serum	1·116	...	...	1·012	...	...
80 % ,,	1·083	1·062	+0·021	...	..	...
64 % ,,	1·083	1·020	+0·063	...	...	...
51 % ,,	1·070	0·985	+0·085	...	...	...
50 % ,,	...	...	...	0·977	0·931	+0·046
40 % ,,	1·070	0·956	+0·114	...	...	...
32 % ,,	1·052	0·935	+0·117	...	...	...
25 % ,,	1·040	0·916	+0·124	0·953	0·890	+0·063
20 % ,,	1·023	0·903	+0·120	...	...	...
16 % ,,	1·011	0·892	+0·119	0·930	0·875	+0·055
9 % ,,	...	...	...	0·909	0·864	+0·045
5 % ,,	...	...	...	0·897	0·858	+0·039
2 % ,,	...	...	...	0·877	0·853	+0·024
1 % ,,	...	...	...	0·857	0·851	+0·006

CHART 4.

	Observed tonicity	Calculated tonicity	Difference=experimental error
Undiluted solution	1·123	1·120	+0·003
80 %	1·064	1·066	−0·002
50 %	1·002	0·985	+0·017
25 %	0·925	0·917	+0·008
10 %	0·886	0·877	+0·009
5 %	0·866	0·863	+0·003
2 %	0·850	0·855	−0·005

CHART 5.

	Total lipoids			Acetone extract			Ether extract		
	Observed tonicity	Calcd. tonicity	Difference	Observed tonicity	Calcd. tonicity	Difference	Observed tonicity	Calcd. tonicity	Difference
Whole fluid	1·101	...	...	1·020	...	...	1·103	...	...
80 %	1·056	1·050	+0·006	0·991	0·986	+0·005	1·091	1·052	+0·039
50 %	1·028	0·975	+0·053	0·963	0·935	+0·028	1·074	0·976	+0·098
25 %	0·995	0·912	+0·083	0·952	0·892	+0·060	0·994	0·913	+0·081
10 %	0·953	0·875	+0·078	0·903	0·867	+0·036	0·970	0·875	+0·095
5 %	...	...	...	0·880	0·858	+0·022	0·930	0·862	+0·070

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