THE PRECIPITATION OF THE PROTEINS OF HORSE SERUM.

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CONTENTS.

Historical. The proteins of serum have been investigated mainly by experiments involving their precipitation.

Panum[1] drew attention to the fact that the proteins of serum could be differentiated into two groups by dilution with water and neutralisation with acetic acid. The precipitate obtained by this procedure—globulin—is insoluble in water but soluble in dilute neutral salt solution, the protein remaining in solution—albumin—cannot be precipitated by dilution nor by the addition of acid. These properties are quite definite and serve as an adequate means of differentiating the two classes of proteins.

But the power of neutral salts in large concentrations to precipitate proteins from solution served, according to Hammarsten[2], to further differentiate these two classes of proteins. He stated that the saturation of serum with magnesium sulphate precipitated globulin completely and globulin only.

Hofmeister stated that the same separation could be effected by half saturation with ammonium sulphate, and Kauder[3] using this salt came to the conclusion that the precipitate produced from serum by

magnesium sulphate was a single substance, since fractional precipitation yielded no evidence of a double nature. The extension of these salt precipitation limits to the definitions of globulin and albumin determined to a large extent the nature of the investigations which were subsequently made on the proteins of serum.

Burkhardt[4] stated that although Hammarsten had conclusively shown that saturation of a globulin solution with magnesium sulphate precipitated that protein completely, yet he had not proved that the addition of this salt in a like quantity to serum precipitated globulin only and no albumin. He precipitated serum by saturation with magnesium sulphate, dissolved the precipitate in water and dialysed the salt away. No complete precipitation took place even on further dilution and addition of acetic acid. Therefore, he said, some serum albumin was precipitated with the globulin.

Hammarsten[5] estimated the total globulin obtained by dilution and acidification as 10 % of the total protein of serum, whilst in using magnesium sulphate the quantity increased to 43 %. To explain this discrepancy and the results obtained by Burkhardt he assumed that there were present in the serum substances which influenced the solubility and precipitability of globulin. He based this assumption on experiments in which he treated serum with sodium chloride. Afterwards on dialysis the quantity of precipitate produced was increased. The sodium chloride was supposed to have altered the substances which kept the globulin in solution. However, it was evident that the magnesium sulphate precipitate did consist of two parts. But instead of reverting to the old definition of globulin as that protein of serum which was insoluble in water but soluble in dilute salt solution the extended definition of precipitation by magnesium sulphate was retained.

There thus came to be recognised two globulins.

(*a*) A globulin soluble in water (Pseudo). (*b*) A globulin insoluble in water (Eu).

E. Marcus[6] stated that these two globulins had the same coagulation temperature, composition and rotatory power and differed only in their solubilities.

Fuld and Spiro[7]—when working on the effect of blood on the coagulation of milk—found that the globulin fraction of serum (obtained by half saturation with ammonium sulphate), could be differentiated into three parts: (i) precipitated by 21·5 % ammonium sulphate, (ii) precipitated by 28 %—33 % ammonium sulphate, (iii) precipitated

by 34 %—46 % ammonium sulphate. (i) had no effect on the rennet coagulation of milk, (ii) increased the effect, (iii) diminished the effect.

In 1902, Freund and Joachim[8] redetermined the properties of serum globulin (defined as that protein of serum which is precipitated by half saturation with ammonium sulphate). As a result of their analysis they stated that the precipitate so produced consisted of four fractions (exclusive of fibrin globulin). Precipitated by $\frac{1}{3}$ saturated with Am_2SO_4: (*a*) insol. in H_2O Paraeuglobulin, (*b*) sol. in H_2O Euglobulin. Precipitated by $\frac{1}{2}$ saturated with Am_2SO_4: (*a*) insol. in H_2O Parapseudoglobulin, (*b*) sol. in H_2O Pseudoglobulin. It may be noted that this involved analysis depends for its existence on two statements.

(*a*) That half saturation with ammonium sulphate precipitates globulin and globulin only from serum.

(*b*) That one-third saturation of serum with ammonium sulphate precipitates a protein which assists rennet in the coagulation of milk and that that produced from the filtrate by the addition of this salt to one-half saturation hinders this action.

As far as the first statement is concerned it is accepted that half saturation of serum by ammonium sulphate precipitates two proteins which differ according to their solubilities in water. The question is how far the precipitated protein soluble in water differs from the soluble protein left in the serum. There has been no adequate proof adduced that any such differences exist—as far as the properties of this soluble protein are concerned they are identical with those ascribed to albumin. The name globulin applied to it rests solely on the assumption that half saturation with ammonium sulphate or full saturation with magnesium sulphate precipitates globulin only.

The existence of two substances in the different ammonium sulphate fractions augmenting and inhibiting the action of rennet cannot be taken as a proof that the addition of salts in these quantities differentiates the main mass of precipitated proteins. The addition of the majority of proteins to milk inhibits its coagulation by rennet to some extent—even finely divided coagulated egg white is capable of producing this effect. As far as the rennet augmentor effect is concerned the presence of a very minute quantity of a proteolytic ferment attached to the precipitated protein would produce this result and does not necessarily indicate a definite property of the whole of the precipitated protein. The accuracy of this statement is evident from a consideration of the properties of diphtheria antitoxic serum.

The impossibility of separating vegetable globulins and albumins when their solutions are mixed by means of their precipitation limits with ammonium sulphate has been proved by Osborne and Harris[9]. The question arises as to whether ammonium sulphate and magnesium sulphate should be retained as salts capable of differentiating definite classes of proteins when added to solution of proteins in stated amounts. From the foregoing pages it is clear that they are incapable of separating serum globulin from serum albumin as originally defined. In the succeeding pages the curves for the precipitation of the proteins of serum by these salts are given, and it is evident from these curves that the differentiation of proteins by means of them does not rest on a well defined basis. In the precipitation curves there exist no definite breaks such as are necessary for the assumption that the addition of a certain quantity of salt precipitates one protein only and the subsequent addition of more salt initiates the precipitation of a second protein.

(A) Precipitation by water.

When serum is added to ten volumes of water a precipitate of protein is produced. If a little acetic acid be added to the diluted serum to neutralise its alkalinity the amount of protein precipitated is increased. The protein precipitated by this procedure is the serum globulin of Panum. Kuhne considered that the precipitate obtained by simple dilution differed from that obtained subsequently by the addition of acetic acid. The first precipitate he considered to be paraglobulin and the second sodium albuminate. But Heynsius[10] showed that there was no distinction between paraglobulin and alkali albuminate. To prove this point more conclusively the solubility curves of the two globulin fractions in sodium chloride were determined. The two curves coincided, showing that so far as the property of solution in neutral salts is concerned, the two fractions are identical.

From a general consideration of the properties of globulin there is no indication that the two fractions are different. Globulin may be dissolved either by neutral salts or by acids or alkalies. When dissolved in neutral salts simple dilution is required to effect precipitation; when dissolved in acids or alkalies neutralisation of the solvent is required. In serum both types of solvent are present. But when globulin is dissolved in acids or alkalies the presence of small quantities of neutral salts initiates precipitation. Now salts are present in serum in a

quantity about equal to ·8 % sodium chloride. And if globulin exists in solution combined with the alkali present the problem arises as to why this globulin is not precipitated by the salts in solution. Evidently the ultimate solution of globulin in serum must be of a different type from that which obtains *in vitro*.

A. Schmidt considered that serum globulin did not exist in blood but was formed from some precursor during the process of coagulation. The differences in the properties of globulin obtained from plasma and from serum indicate the accuracy of this conclusion. Hammarsten estimated the amount of globulin precipitated from serum by dilution and the addition of acetic acid at about 6 % of the total protein. The amount present in horse serum is variable, a maximum quantity being about 4 % of the total protein.

The influence of the dilution of serum on the quantity of electrolytes combined with the proteins has been dealt with in a previous paper [11], and it was stated that although there is evidence of the decomposition of the protein salt complexes on dilution yet there is no sign of the precipitation of protein. It is evident therefore that in horse serum about 96 % of the total protein is soluble in water.

(B) Precipitation by neutral salts.

(1) *Ammonium sulphate.* The quantitative precipitation of serum by ammonium sulphate was determined in the following way. 10 c.c. of serum were placed into each of a series of test tubes. To these known weights of ammonium sulphate were added and dissolved. The tubes were allowed to stand for twelve hours and were then filtered. A measured amount of each filtrate was taken, coagulated, filtered on a hardened paper and the coagulum washed free from salt. The coagulum was then transferred to a weighed crucible and dried in an air oven at a temperature not exceeding 120° C. After drying and cooling the crucible was again weighed and the gain in weight gave the weight of the coagulum in it. From this weight and the quantity of protein in the serum the percentage of protein precipitated by a given quantity of ammonium sulphate was readily determined. The results obtained are given below.

A saturated solution of Am_2SO_4 contains about 52 % of the pure salt. This, in terms of the Eq. Wt. = 100, is 786. Half saturation or 26 % equals 393.

Am_2SO_4 in terms of Eq. Wt. = 100	Percentage of protein precipitated
227	18
266	42
303	57·5
341	70
378	73
417	81·7
453	85·3
492	91·8
530	98·1

The above results are depicted on Fig. 1. It may be noted that the curve is of a regular form. Half saturation with ammonium sulphate corresponds to the point (*X*) on the curve. At this point there is no indication of a change in the curve such as would warrant the statement that previous to this degree of saturation globulin only, and after it albumin only, was precipitated.

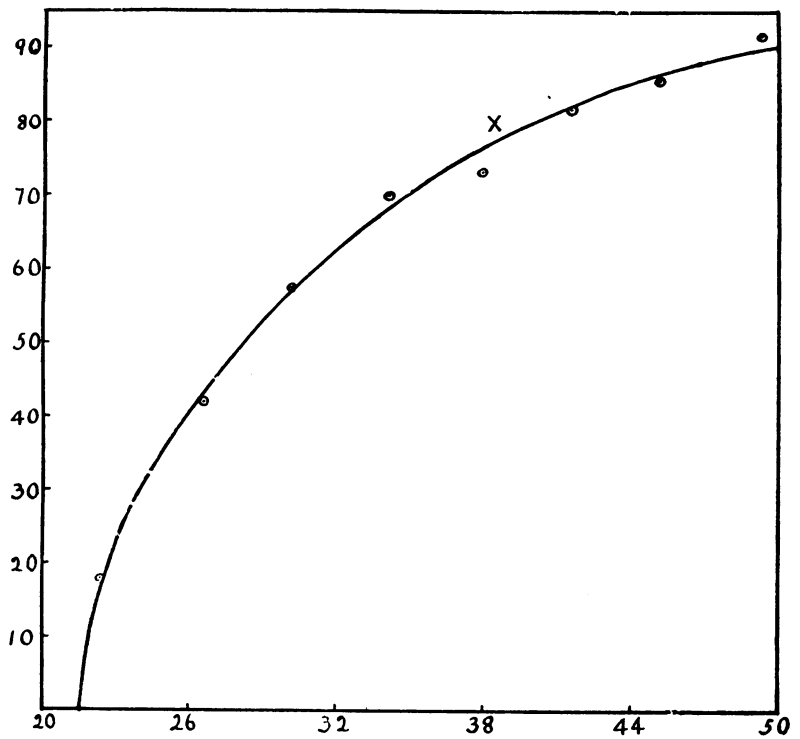

Fig. 1. Ordinates represent percentages of protein precipitated. Abscissæ represent amounts of ammonium sulphate present. 10 = Eq. Wt. of Am_2SO_4.

The influence of dilution. Serum was diluted so that solutions containing one volume of serum in three, five and ten volumes were obtained. The quantitative precipitations of these solutions by ammonium sulphate were determined by the method described above. The results obtained are given below.

Am_2SO_4 in terms of Eq. Wt.=100	Percentage of protein precipitated		
	Vol. 3	Vol. 5	Vol. 10
245	14	—	—
263	—	9	6·5
286	—	—	19
300	45·8	—	—
312	—	43·5	36
327	57·5	—	—
355	66	—	60
408	78·7	76·7	74
436	83·4	—	—
456	—	83·5	83·5
463	91·5	—	—
490	—	89·3	89·1

These results are shown on Fig. 2. It may be noticed that as the serum is diluted the efficiency of any given quantity of ammonium sulphate as a precipitant diminishes.

The influence of temperature. Temperature has very little effect on the precipitation of the proteins of serum by ammonium sulphate. At 0° C. the salt is a little more effective as a precipitant than at 40° C. but the difference is so slight that the temperature coefficient was not determined.

(2) *Magnesium sulphate.* The quantitative precipitation of the proteins of serum by magnesium sulphate was determined. Three series of experiments were done, using serum of different dilutions. In the first series 10 c.c. of serum were placed into a series of test tubes, and magnesium sulphate solution and water were added in varying quantities so that the total volume was 20 c.c. in every case. In the second and third series 7·5 c.c. and 5 c.c. of serum were taken, the total volume in these series after the addition of water and magnesium sulphate being 20 c.c.

The precipitations were done at room temperature and after the addition of the magnesium sulphate and water the mixtures were allowed to stand for twelve hours. The contents of each tube were then filtered and a known quantity of each filtrate heated to 100° C. for ten minutes. The coagula were adequately washed and then transferred to weighed crucibles and dried at 120° C. From the weights

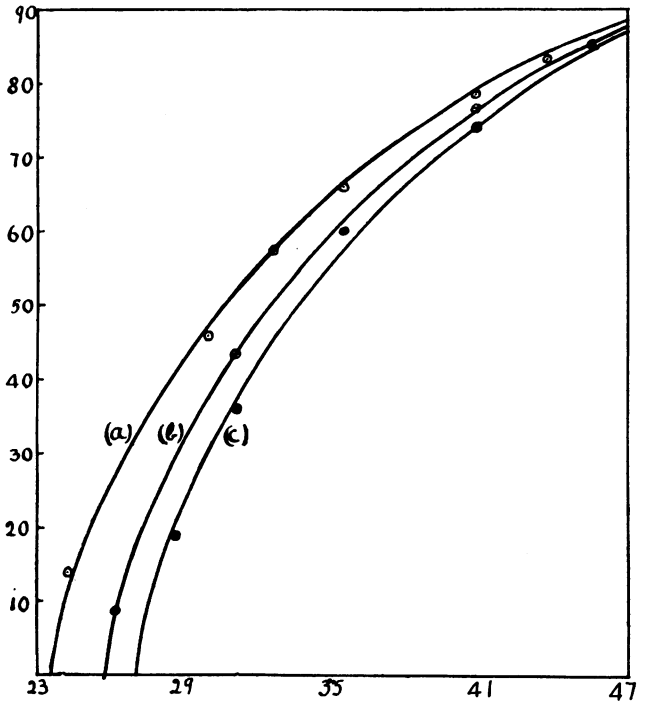

Fig. 2. Ordinates represent percentages of protein precipitated. Abscissæ represent amounts of ammonium sulphate present. 10=Eq. Wt. of Am_2SO_4. (*a*) Serum diluted three times, (*b*) serum diluted five times, (*c*) serum diluted ten times.

of these coagula and the amount of protein in the original serum the amount of protein precipitated was calculated. The following results were obtained:

$MgSO_4$ in terms of Eq. Wt.=100	Percentage of protein precipitated		
	10 c.c. serum	7·5 c.c. serum	5 c.c serum
277	7·56	3·5	3·35
316	18·5	15·5	14·3
358	—	29·0	30·2
396	40·6	35·2	36·6
430	45	—	—
476	—	49·2	46·0
495	52·2	—	—
540	58	—	—

From these figures it may be seen that the precipitation of the proteins of serum by magnesium sulphate is of the same general type as by ammonium sulphate. In the case of this salt also the dilution of the serum diminishes the precipitating efficiency of a given quantity

of salt. But with magnesium sulphate the precipitation is never complete.

(3) *A comparison of ammonium sulphate and magnesium sulphate as precipitants.* Since Hofmeister's statement that half saturation with ammonium sulphate could be substituted for full saturation with magnesium sulphate in the precipitation of globulin these two salts have been assumed to possess similar precipitating properties when used in corresponding concentrations. A quantitative comparison of the precipitating efficiencies of the two salts is, therefore, of importance.

Serum was diluted four times and the precipitations of the proteins of it by ammonium sulphate and magnesium sulphate were determined. The following results were obtained:

Ammonium Sulphate.		*Magnesium Sulphate.*	
Am_2SO_4 in terms of Eq. Wt. = 100	Percentage of protein precipitated	$MgSO_4$ in terms of Eq. Wt. = 100	Percentage of protein precipitated
265	11·6	277	3·3
272	27·5	316	14·3
280	33·2	358	30·2
287	34·7	396	36·6
296	39·2	476	46·0
378	66·0		
455	77·5		
530	85·0		
605	91·0		

These results are plotted on Fig. 3 (*B*, *C*).

It may be noted that although both salts start to precipitate the proteins of serum at about the same concentration yet ammonium sulphate is a much more effective precipitant than magnesium sulphate. From the two curves we can readily see how half saturation with ammonium sulphate and full saturation with magnesium sulphate correspond. Half saturation with ammonium sulphate corresponds to about 26 % of that salt. This, in terms of the equivalent weight as 100, equals 395. At this point on the ammonium sulphate curve 71 % of the total protein is precipitated. Full saturation with magnesium sulphate is obtained when about 30 % of the anhydrous salt is present. This, in terms of the equivalent weight as 100, equals 500. At this degree of concentration 47·5 % of the total protein of the serum is precipitated. The difference between the two quantities of precipitated protein is too great to permit these quantities of the two salts to be interchanged in any series of precipitations.

(4) *Comparison of the precipitations of serum and a globulin solution by ammonium sulphate.* By the phrase "a globulin solution" is meant a solution of that protein of serum which is obtained by dilution with water and neutralisation with acetic acid.

The figures above give the quantitative precipitation of the proteins of serum by ammonium sulphate.

A suspension of globulin was obtained by the method described in a previous paper [12]. This suspension was dissolved in a little ammonium sulphate and the quantitative precipitation of the solution by larger quantities of salt determined.

Am_2SO_4 in terms of Eq. Wt. = 100	Percentage of globulin precipitated
252	31·5
265	44·7
278	53·3
290	69·5
303	78·7
314	85·8
330	100·0

These results are shown graphically on Fig. 3 (*A*).

From the globulin results the way in which half saturation with ammonium sulphate came to be stated as a precipitant for the globulin fraction of serum can be seen. Pure globulin is entirely precipitated from solution only when about 42 % of saturated ammonium sulphate is present. When ammonium sulphate is added to serum to this extent about 50 % of the total protein is precipitated. Hence arose the statement that half the proteins of serum consist of globulin. But in the two cases the characters of the precipitated protein are entirely different. Pure globulin is quite insoluble in water; of the 50 % of protein precipitated from serum only about 2 % is insoluble in water.

The comparative curves are of value in showing how much more effective ammonium sulphate is as a precipitant for a protein which is insoluble in water than for a protein which is completely soluble in water. With the former class of protein complete precipitation occurs with about half the quantity of salt required to fully precipitate the latter class.

(C) Precipitation by neutral salts in the presence of acids.

The neutral salts which precipitate serum are limited to ammonium sulphate, magnesium sulphate and sodium sulphate. The last salt is difficult to work with since the precipitations must be done at a

temperature greater than 34° C., *i.e.*, at a temperature at which the salt exists in solution as Na_2SO_4 and not as the hydrated salt.

But when acid is added to serum not only do smaller quantities of the above salts precipitate the proteins but the number of precipitating salts is greatly increased. The precipitation of serum by neutral salts in the presence of acids is, however, limited. Small quantities of acids left in contact with serum for some time, especially at a temperature above 25°C., tend to produce a jelly-like condition, and the serum in this state is

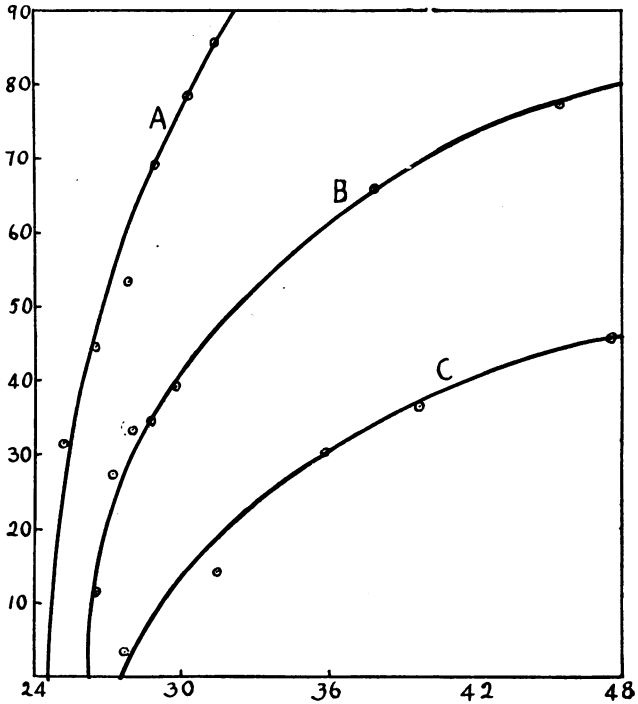

Fig. 3. Ordinates represent percentages of original protein precipitated. Abscissæ represent salt present. 10=Eq. Wt. of Am_2SO_4 or $MgSO_4$. (A) Precipitation curve for globulin by Am_2SO_4. (B) Precipitation curve for serum by Am_2SO_4. (C) Precipitation curve for serum by $MgSO_4$.

unworkable. The acid produces a chemical change in the serum as is evidenced by this jelly-like condition and the bleaching of the pigment. How far the effects on salt precipitation are due to a chemical change in the state of the protein and how far to altered physical conditions has not been accurately determined.

The precipitation of serum by sodium chloride in the presence of

varying quantities of acetic acid, hydrochloric acid and sulphuric acid was determined. Acid was added to serum to the required amount. 10 c.c. of this acidified serum was placed into each of a series of test tubes, and sodium chloride was added to the required amount. The mixtures were allowed to stand three hours and were then filtered. A known quantity of the filtrate was dried down in every case, and from the weight the quantity of protein precipitated was determined. The following results were obtained:

$\frac{N}{12}$ HA. NaCl in terms of Eq. Wt. = 100	Percentage of protein precipitated	$\frac{N}{6}$ HA. NaCl in terms of Eq. Wt. = 100	Percentage of protein precipitated
376	24·4	256	9·7
408	38·8	298	15·5
438	45·5	342	42·1
472	53·3	385	57·7
503	69	427	72·0

$\frac{5N}{12}$ HA. NaCl in terms of Eq. Wt. = 100	Percentage of protein precipitated
136	33·3
171	45·5
205	59·0
240	71·4

The above results are depicted graphically on Fig. 4. The precipitating effect of sodium chloride may be seen to markedly increase with the increase in acidity. Since the precipitating curves for various strengths of acids are straight lines the effectiveness of any acid can be readily determined.

The precipitation of serum by sodium chloride in the presence of hydrochloric and sulphuric acids was determined.

$\frac{N}{36·5}$ HCl. NaCl in terms of Eq. Wt. = 100	Percentage of protein precipitated	$\frac{2·5N}{36·5}$ HCl. NaCl in terms of Eq. Wt. = 100	Percentage of protein precipitated
478	52·2	60	52·2
513	56·4	73	59·3
547	71·1	128	68·2
582	82·0	171	76·8

$\frac{N}{48}H_2SO_4$. NaCl in terms of Eq. Wt. = 100	Percentage of protein precipitated
512	47·8
531	61
547	65·5
581	80

These results are shown graphically on Fig. 5. From the above we see that HCl and H_2SO_4 are practically equal to one another as regards their influence on the precipitation of serum by sodium chloride.

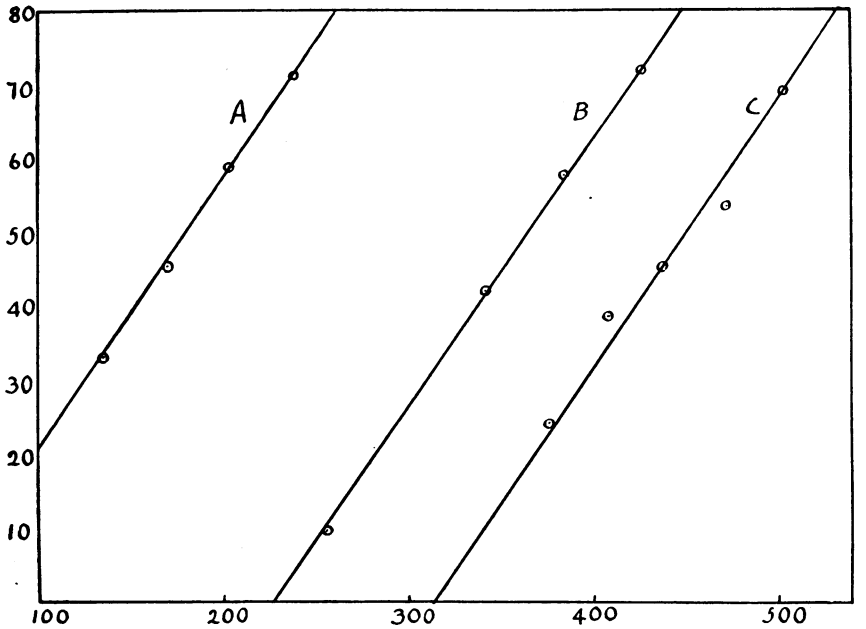

Fig. 4. Ordinates represent percentages of protein precipitated. Abscissæ represent amounts of sodium chloride present. 100 = Eq. Wt. NaCl.

(A) $\frac{5N}{12}H\bar{A}$; (B) $\frac{2N}{12}HA$; (C) $\frac{N}{12}H\bar{A}$.

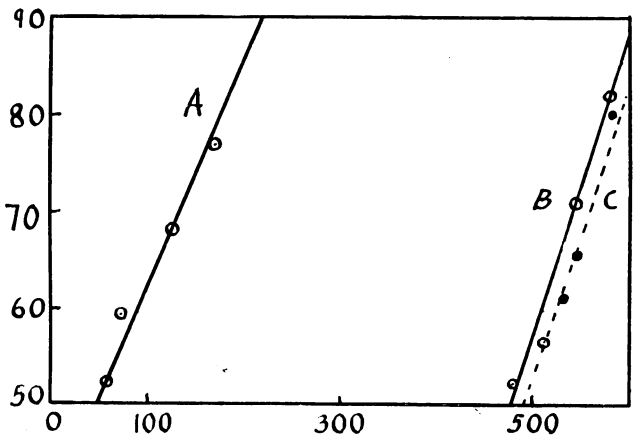

Fig. 5. Ordinates represent percentages of protein precipitated. Abscissæ represent amounts of sodium chloride present. 100 = Eq. Wt. NaCl.

(A) $\frac{2{\cdot}5}{36{\cdot}5}$ N HCl; (B) $\frac{N}{36{\cdot}5}$ HCl; (C) $\frac{N}{48}H_2SO_4$.

From the acetic acid and hydrochloric acid curves we may compare the effects of various quantities of acids. Since the curves are all straight lines we may do this by taking any particular percentage of protein precipitated and comparing the quantities of sodium chloride required to effect this in every case. Suppose we take the amounts of salt required to precipitate 50 % of the protein of the serum. We see that this requires

$$\left.\begin{aligned} &\tfrac{60}{100}\,\text{NaCl (N) with } \tfrac{\text{N}}{36{\cdot}5}\,\text{HCl} \\ &\tfrac{475}{100}\,\text{NaCl (N) with } \tfrac{2{\cdot}5\,\text{N}}{36{\cdot}5}\,\text{HCl} \end{aligned}\right\}$$

$$\left.\begin{aligned} &\tfrac{180}{100}\,\text{NaCl (N) with } \tfrac{5\,\text{N}}{12}\,\text{HA} \\ &\tfrac{366}{100}\,\text{NaCl (N) with } \tfrac{2\,\text{N}}{12}\,\text{HA} \\ &\tfrac{430}{100}\,\text{NaCl (N) with } \tfrac{\text{N}}{12}\,\text{HA} \end{aligned}\right\}$$

If we plot the quantities of acetic acid required as ordinates and the corresponding quantities of sodium chloride as abscissæ we see that the effects of varying quantities of acids are directly proportional to one another, *i.e.*, the curve is a straight line. If we do the same for hydrochloric acid and assume that in this case also the relation is represented by a straight line we can determine the relative effects of acetic acid and hydrochloric acid. The relation works out as follows:—

acetic acid : hydrochloric acid : : 8 : 100.

We have seen that sulphuric acid has about the same effect as hydrochloric acid. These values correspond roughly with the avidities of these acids, and if we are permitted to argue from these few results we may state that the effect of acids on the precipitation of the proteins of serum by neutral salts is proportional to the chemical avidities of these acids.

The quantitative results for the precipitation of serum by various salts composed of ions of different valencies have not been worked out. But there is one point of interest which may be noticed. The effect of the addition of acid is to make all the precipitation curves straight lines, a fact which points to the parabolic form of the neutral salt precipitation curves being due to the forces involved in precipitation and not to any fundamental physical difference in the proteins of the serum.

To determine the fate of an acid when added to serum various conductivity experiments were made.

The conductivity of serum when hydrochloric acid is added to it in varying amounts. To 10 c.c. of serum, hydrochloric acid and water were added, so that the total volume was 20 c.c. and the required acidity was produced. The conductivities of the resulting solutions were determined.

	Conductivity $\times 10^{-3}$	Increase in conductivity due to HCl $\times 10^{-3}$	Conductivity of HCl $\times 10^{-3}$
Original serum	·158	—	—
Original serum $+\frac{N}{100}$ HCl	·166	·008	·0806
Original serum $+\frac{N}{50}$ HCl	·173	·015	·166
Original serum $+\frac{N}{20}$ HCl	·196	·038	·477
Original serum $+\frac{N}{10}$ HCl	·460	·302	·894

From these figures it is evident that up to $\frac{N}{20}$ HCl the hydrochloric acid does not exist free in the serum. Addition of acid to this strength causes a small proportionate increase in the conductivity of the serum, but this increase in conductivity is only about one-tenth of that of the original hydrochloric acid.

The effect of adding hydrochloric acid to fractions of the same serum containing different percentages of solids was next determined. Serum was frozen and then allowed to melt at room temperature. When resolution was complete various layers were syphoned off. The effect of low temperatures on serum has been considered in a previous paper [13]. To 10 c.c. of each of these serum fractions, water and hydrochloric acid were added, so that the total volume was 20 c.c. and the acidity $\frac{N}{20}$ HCl in every case.

The conductivities and other constants of these solutions were then determined.

Serum fraction	Total solid in 100 c.c.	Conductivity $\times 10^{-3}$	Conductivity $+$HCl$\times 10^{-3}$	Difference due to HCl$\times 10^{-3}$
1	·93	·053	·385	·332
2	2·14	·092	·297	·205
3	4·05	·137	·216	·079
4	5·32	·168	·241	·073
5	6·19	·182	·262	·080
6	7·0	·203	·276	·073
7	7·7	·212	·288	·076
$\frac{N}{20}$ HCl	—	·455	—	—

From the above figures it may be seen that the increase in the

conductivity of the serum fraction on the addition of $\frac{N}{20}$ HCl is large until about four per cent. of total solid is present. After this the increase in conductivity after adding $\frac{N}{20}$ HCl is small and is practically constant over a large increase in the total solid present. The results of the above experiments indicate that the addition of a definite quantity of hydrochloric acid to serum results in its neutralisation. The maximum amount of acid which may be neutralised depends upon the quantity of total solids in the original serum. There are two constituents of serum which may be mainly responsible for the neutralisation of the added acid, (1) the alkaline salts, (2) the proteins. From the results of the first experiment it may be seen that the addition of $\frac{N}{20}$ HCl results in the conductivity of the serum being increased by ·000038. In the second experiment the addition of the same quantity of hydrochloric acid causes an increase in the conductivity of ·00007. If the hydrochloric acid were neutralised so that sodium chloride only was formed the increase in conductivity would have been ·00012, a larger quantity than was obtained in either of the above experiments.

It has been previously shown that the various constituents of serum do not exist as separate entities but that they form complex systems. If we assume that this is true, then when hydrochloric acid is added to serum it probably enters into combination with these systems. That all the constituents of serum are affected by the acid is evident from the effects observed on the addition of it. The influence of the acid on the precipitation of the proteins of serum by neutral salts has been considered above. The influence of the acid on the alkaline salts and pigment is evident from the observation that if a sufficient quantity of acid be added the reaction of the fluid becomes acidic and the yellow colour becomes progressively less evident until the fluid becomes colourless.

The fact that acids affect the precipitation of the proteins of serum by neutral salts in degrees proportional to their avidities indicates that an acid combines with a protein salt complex rather than divides itself among the various separate constituents. If then the acid enters into combination with the protein salt complexes the problem arises as to what the small increase in conductivity is due. In the first experiment above it may be seen that the addition to serum of $\frac{N}{100}$ HCl increases

the conductivity by $\cdot 08 \times 10^{-4}$: $\frac{N}{50}$ HCl increases the conductivity by $\cdot 15 \times 10^{-4}$: $\frac{N}{20}$ HCl increases the conductivity by $\cdot 38 \times 10^{-4}$.

These numbers are approximately proportional to one another and they may indicate that the union of a protein salt complex with the hydrochloric acid results in a definite proportion of electrolytic salt being turned out of the system. But they probably indicate that the entrance of the acid into the protein salt complexes definitely increases their conductivity. The effect of electric potentials on serum has been considered in a former paper [14]. The migration of the protein systems to the anode under the influence of a constant current is probably due to the charge which these protein systems carry by virtue of the electrolytes which are contained in them. If hydrochloric acid enters these systems it can be readily appreciated that if the above assumption is correct then this combination would increase the conductivity of the solution containing them. An effect similar to this has been shown by Hardy [15] in the case of globulin. He found that when acetic acid is added to a globulin suspension the conductivity was always decreased by 10 %. He explained his results by assuming that the combination of the globulin with the acid caused it to acquire a definite conductivity.

The evidence in favour of the hydrochloric acid entering into combination with the protein salt complexes is strengthened by a consideration of the effect of adding sodium hydroxide to serum. The addition of alkalies to serum materially influences the precipitation of the proteins by neutral salts. Many neutral salts which do not precipitate normal serum do so when a strong alkali as sodium hydroxide is added to it. The effect of alkalies on the neutral salt precipitation of the proteins of serum was not determined owing to the great liability of the serum when made alkaline to form gelatinous and unfilterable solutions. But the effect of sodium hydroxide on the conductivity of serum was determined under a variety of conditions. Various serum fractions were obtained by freezing and thawing a quantity of serum. 10 c.c. of each of these fractions were taken and sodium hydroxide and water were added until the volume was 20 c.c. and $\frac{N}{20}$ NaOH present. The conductivity of the serum fractions before and after the addition of the sodium hydroxide was determined.

Fraction	Total solid in 100 c.c.	Conductivity $\times 10^{-3}$	Conductivity after adding $\frac{N}{20}$ NaOH $\times 10^{-3}$	Difference $\times 10^{-3}$
1	·93	·053	·237	·184
2	2·14	·092	·241	·149
3	4·05	·137	·233	·096
4	5·32	·168	·252	·094
5	6·19	·182	·273	·091
6	7·0	·203	·290	·097
7	7·7	·212	·310	·098
$\frac{N}{20}$ NaOH	—	·241	—	—

From these figures it is evident that the addition of sodium hydroxide to serum has an effect on the conductivity similar to that produced by hydrochloric acid. But in the case of sodium hydroxide there are no acidic salts to neutralise. We must therefore assume that in the case of alkalies a direct combination between the protein complexes and alkali takes place.

It is interesting to note that in the case of $\frac{N}{20}$ HCl and $\frac{N}{20}$ NaOH the added acid or alkali has a large effect on the conductivity until about four per cent. of solid matter is present in the serum. This indicates that both the acid and alkali combine with a definite quantity of protein, these quantities being probably the same in the two cases. Suppose we assume that the sodium hydroxide combines with a protein salt complex containing a definite quantity of protein. In the above experiment all the alkali is combined when 4·05 % of solid matter is present in the serum. In this serum fraction there were 3·38 % of coagulable protein. Therefore on the above assumption $\frac{N}{20}$ NaOH = 3·38 grams of protein or a gram molecule of sodium hydroxide combines with 676 grams of protein.

The increase in the conductivity of ·00009 (about) on adding $\frac{N}{20}$ NaOH to serum containing an excess of protein is probably due to the same cause which has been supposed to explain the hydrochloric acid results—namely that the entry of the sodium hydroxide into the protein system increases its conductivity by a proportionate amount.

(D) PRECIPITATION BY SALTS OF THE HEAVY METALS.

It is characteristic of the proteins of serum that they are precipitated by very small quantities of the salts of heavy metals. The protein precipitates so produced are insoluble in water and after thorough washing retain the colour of the precipitating salt. Thus the precipitate produced by copper sulphate has a characteristic blue tint. Similarly for the precipitates produced by other salts. Although the protein precipitates produced are insoluble in water yet they may be made to go into solution by any method which splits off the precipitating salt. The precipitate produced by zinc sulphate may be readily dissolved by dilute sodium hydroxide; that produced by copper sulphate by the prolonged passage of sulphuretted hydrogen. The precipitation of the proteins of serum in an insoluble form by salts of the heavy metals does not necessarily involve a chemical change in the protein, a point which will be referred to in the consideration of the properties of diphtheria antitoxin.

The precipitation of serum by salts of the heavy metals possesses some theoretical interest and was worked out quantitatively in the cases of copper sulphate and zinc sulphate.

The precipitation of serum by copper sulphate. 5 c.c. of serum were placed into each of a series of test tubes, and 3·2 % copper sulphate solution and water were added until the required percentage of copper sulphate was obtained and the total volume in each tube was 20 c.c. The amount of precipitate produced in each case was determined by the method previously described.

The following results were obtained:

$CuSO_4$ in grams	Protein precipitated in grams	Percentage of original protein precipitated
·04	·425	22·6
·048	·553	29·5
·056	·701	37·2
·064	·900	47·8
·072	1·07	57
·08	1·45	77
·12	1·6	85
·16	1·63	87
·24	1·67	88·6

These results are plotted on Fig. 6. It is evident from the results above that serum contains two definite types of protein. The first protein is readily precipitated by copper sulphate and forms about 85 % of the total protein present; the other protein is precipitated only by

relatively large amounts of copper sulphate. Also it is clear that when once precipitation has been started the amount of the first protein which is precipitated by copper sulphate is roughly proportional to the amount of salt added. But that there is at present in serum some substance which inhibits the precipitation of the protein by copper sulphate is evident from the observation that at the beginning of precipitation ·04 grams of salt precipitate only ·435 grams of protein, whilst the addition of a further ·04 grams of salt increases the precipitate to 1·45 grams. If we assume that the precipitation of the main

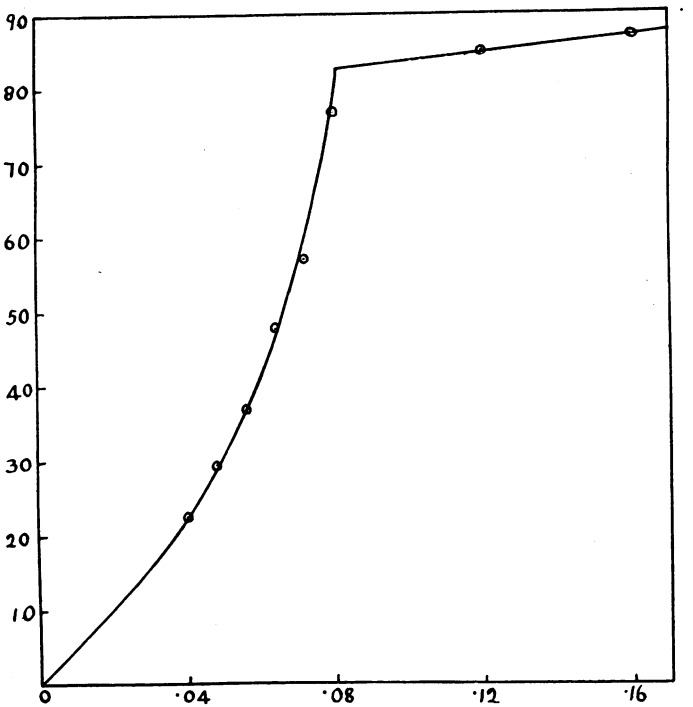

Fig. 6. Ordinates represent percentages of protein precipitated. Abscissæ represent amounts of copper sulphate in grams present.

protein is proportional to the amount of copper sulphate added after the initiation of precipitation we find that ·04 grams of copper sulphate precipitate 1·025 grams of protein or 160 grams of copper sulphate precipitate 4100 grams of protein, or a gram molecular weight of copper sulphate combines with 4100 grams of protein. The inhibition of the precipitation of serum by copper sulphate is evident also from a

comparison with the above results of the precipitation of serum when diluted in the proportions of 7·5 c.c. and 10 c.c. to 20 c.c.

$CuSO_4$ in grams	Protein precipitated in grams		Percentage of protein precipitated	
	(7·5 to 20)	(10 to 20)	(7·5 to 20)	(10 to 20)
·04	·295	—	10·5	—
·08	·945	·62	33·5	16·5
·096	1·05	—	37·3	—
·112	1·37	—	48·6	—
·128	1·81	1·14	64	30·5
·144	2·12	—	75	—
·16	2·34	2·44	83	65
·20	2·44	2·93	87	78
·24	—	3·17	—	84

The differentiation of the two types of protein is evident from these precipitation results also.

It has been previously stated that protein probably exists in solution as a complex aggregate consisting of protein, pigment, inorganic salts, etc. There is strong evidence in favour of the assumption that it is the protein complex which is precipitated by the salt of the heavy metal rather than the protein molecule itself. The pigment of the serum is always associated with the precipitate, as may be readily seen by re-dissolving it. And in the description of the influence of electric potentials on serum it was found that when serum was electrolysed through zinc sulphate electrodes the accumulation of insoluble protein at the anode was not accompanied by any increase in the electrolytes present in that region. This result would not have been obtained if the zinc sulphate had combined with the protein only and in so doing had liberated the inorganic salt ions from the protein complex. The above calculation, therefore, of the amount of protein precipitated by a gram molecule of copper sulphate should have been in terms of a protein complex.

The precipitation of the proteins of serum by zinc sulphate is of the same order of magnitude as by copper sulphate, as may be seen from the following figures.

Serum diluted 1:4.

$ZnSO_4$ in grams	Protein precipitated in grams	Percentage of protein precipitated
·05	·41	19
·075	·87	40·5
·1	1·16	54
·2	1·72	80
·4	1·91	89

The points which have been noted in the case of the precipitation of serum by copper sulphate are evident in the case of this salt also. Apparently zinc sulphate is a less efficient precipitant than copper sulphate, although the results obtained are comparable in order of magnitude in the two cases.

The existence in serum of a substance inhibiting the precipitation of its protein complexes by the salts of heavy metals renders the calculation of the amounts of protein precipitated by definite quantities of the added salts of little value.

(E) Precipitation by alcohol.

Alcohol has long been recognised as an efficient precipitant and coagulant for the proteins of serum. But its action has not been quantitatively determined. In the following pages these quantitative results are given.

(i) *The precipitation of serum by alcohol, the volume being kept constant with water.* 10 c.c. of serum were put into each of a series of flasks. To this serum alcohol and water were added in varying quantities so that the total volume in each was 50 c.c. The flasks were kept at constant temperature (18° C.) for three hours and the contents were then filtered. 10 c.c. of each filtrate were dried down in a weighed crucible and from the additional weight the amount of solid in the filtrate was determined. This weight subtracted from the total weight of solid in the serum gave the amount of protein precipitated. From the total protein in the serum the percentage amount precipitated could be calculated. The following figures give a typical result:

Percentage of alcohol	Percentage of protein precipitated
30	5·1
35	7·9
40	42·0
45	64·8
50	78·5
55	95·6
65	98·0
75	100·0

These results are shown on Fig. 7. It may be seen that the curve indicates that there are three proteins in serum which possess different precipitation limits with alcohol. The quantities of these proteins and their alcohol precipitation limits are as follows:

(*a*) This protein forms about 5 % of the total protein present in this serum. It is precipitated by 30 % alcohol.

(*b*) This protein forms about 85 % of the total protein. It is precipitated between 35 % and 55 % alcohol.

(*c*) This protein forms about 10 % of the total protein. It is precipitated between 55 % and 75 % alcohol. A characteristic of this protein is that it is slowly precipitated by alcohol and that it is rendered insoluble only after being in contact with alcohol for a considerable time.

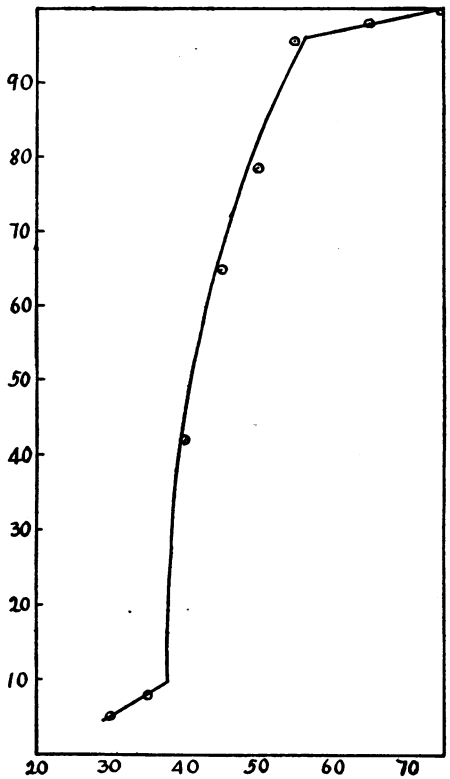

Fig. 7. Ordinates represent percentages of protein precipitated. Abscissæ represent percentages of alcohol present.

The first portion of the protein precipitate is formed by the globulin present in the serum. It may be seen that the quantity given by this method is about the same as that obtained by diluting the serum with water and neutralising with acetic acid. The third portion of the protein precipitate is probably formed by that protein of serum which can be crystallised. But the curve shows no characteristic differentiation of the main bulk of the proteins of serum.

(ii) *The precipitation by alcohol of the serum of different horses.* A determination of the quantitative precipitation of the proteins from the sera of a number of horses is of interest. This determination shows the value of alcohol as a precipitant and the constancy of the physical properties of the sera of a number of animals.

From the following data it may be seen that alcohol has the same general precipitation type in all the sera. But the precipitation curves do not coincide, showing that there are individual differences. These differences are small compared with the differences in the amount of

solid matter contained in the sera used. Thus serum (A) contains 8·35 % of solids, whilst serum (C) contains 12·17 % of solids.

Serum	Total solid in 100 c.c.	Amount of protein in 100 c.c.
A	8·35	7·4
B	8·65	7·6
C	12·17	11·3
D	9·35	8·5
E	9·9	9·0

Percentage of alcohol	Percentage of protein precipitated				
	A	B	C	D	E
25	—	—	—	—	4·5
30	5·9	11·3	5·1	5·7	9·0
35	—	18·9	7·9	15·9	26·8
38	13	23·7	—	—	—
40	—	—	42	19·5	48·2
42	30	36·8	—	—	—
45	46·8	50	64·8	48·4	67
50	77·3	64·4	78·5	72	—
55	—	—	95·6	90	90
50	95	93	—	—	—
65	—	—	98	96	96
70	97	98	—	—	

From the comparative coincidence of the precipitation curves for sera A and C we might deduce that the precipitation of the proteins of serum by alcohol was not influenced by the total quantity of protein present. From the figures giving the total solids and the quantity of protein present in the various sera it may be seen that although there are large variations in the amount of protein present, yet the quantities of other substances present are fairly constant throughout the series—about ·9 %.

(iii) *The influence of time on the precipitation by alcohol.* In the experiments described in the previous pages comparative experiments were done under similar conditions. In the following experiment the influence of time in alcohol precipitation is detailed.

Serum was precipitated with varying quantities of alcohol and at different times the amount of protein precipitated was determined.

35 % alcohol. 15 % of protein precipitated in one hour, 23 % of protein precipitated in four hours, 41·5 % of protein precipitated in eleven hours.

40 % alcohol. 42 % of protein precipitated in one hour, 46·5 % of protein precipitated in four hours, 56 % of protein precipitated in eleven hours.

45 % alcohol. 68 % of protein precipitated in one hour, 73 % of protein precipitated in three hours, 77 % of protein precipitated in twenty-three hours.

50 % alcohol. 85 % of protein precipitated in thirty minutes, 89 % of protein precipitated in three hours, 89 % of protein precipitated in ten hours.

It may be seen that with lower concentrations of alcohol time is a very important factor in any consideration of the amount of precipitate produced. With higher concentrations of alcohol equilibrium is soon produced. It is probable that the action of alcohol is first to produce some chemical change in the proteins of the serum and these changed proteins are afterwards precipitated.

(iv) *The rate of coagulation of the proteins by alcohol.* This was determined in order to see whether the property gave any evidence of protein differentiation.

Alcohol was added to serum to the required percentage. After different times 10 c.c. of the mixture were taken and added to 40 c.c. of water. The uncoagulated portion of the protein precipitate dissolved whilst the insoluble portion settled to the bottom of the flask. By a series of weighings the quantity of protein precipitated from the serum by a particular percentage of alcohol and the amounts of insoluble and soluble protein, after various times were determined. The following results were obtained from the precipitates produced by 60 % and 50 % alcohol:

	Time in minutes	Insoluble portion of precipitate	Soluble portion of precipitate
60 % alcohol.			
	7	·17 grams	·91 grams
	12	·28	·80
	17	·39	·69
	27	·41	·67
	37	·46	·62
	47	·49	·59
	57	·52	·56
	67	·53	·55
	117	·58	·50
	162	·62	·46
	1167	·87	·21
50 % alcohol.			
	11	·09	·91
	20	·14	·86
	68	·23	·81
	128	·27	·77
	188	·31	·74
	254	·37	·68
	1370	·43	·62

The relations between the times and the amounts of insoluble protein are plotted on Fig. 8. From either of the curves it appears that there are at least two proteins in the precipitate differing in the rates of coagulation by alcohol. Thus in the 60 % alcohol curve about 50 % of the precipitate is coagulated in one hour, whilst the remaining portion

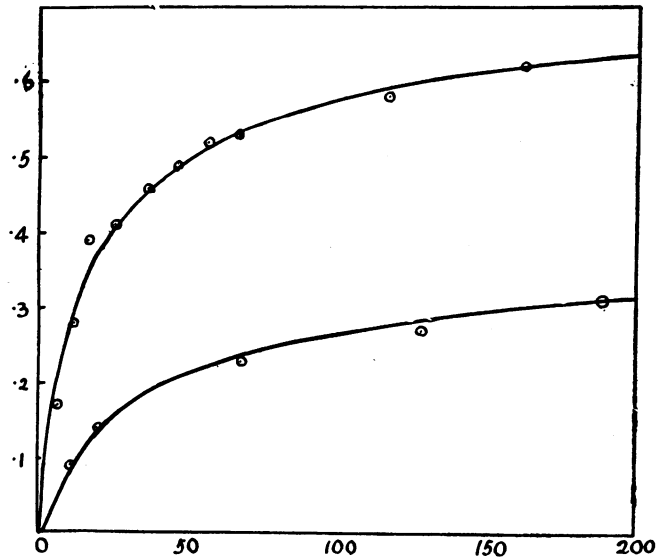

Fig. 8. Ordinates represent grams of insoluble protein. Abscissæ represent time in minutes.

takes days. But if we compare the two curves it is evident that the particular form of them does not depend upon any protein differentiation, but upon the mechanical factors which influence coagulation. With both 50 % and 60 % alcohol practically the same amount of precipitate is produced from 10 c.c. of serum—about one gram. From the 60 % alcohol time curve half of this protein appears to be readily coagulable; from the 50 % alcohol curve only about one-quarter of it. The only variable in the two cases is the alcohol. We must assume, therefore, that the type of curve depends upon the action of the alcohol and not on any protein differentiation. Probably the slowing of the rate of coagulation after the first hour is due to the formation of a film of coagulated protein on the precipitated particles. The effectiveness of any film of a definite thickness is determined by the amount of alcohol present.

(v) *The temperature coefficient.* In the above experiments the temperature has not been considered beyond the fact that comparative

experiments were done at the same temperature. A study of the temperature coefficient for the precipitation of serum by alcohol gives interesting results. From a series of rough experiments it may be seen that alcohol as a precipitant of the proteins of serum has a critical temperature. This temperature varies slightly for different sera but is usually about 14° C. If we increase or decrease the temperature of the alcohol serum mixture above or below this critical point the precipitating efficiency of the alcohol increases. But there is a striking difference between the type of precipitation above and below the critical point. Above this temperature the precipitate shows varying degrees of insolubility; below it the protein precipitate is completely soluble. This critical temperature, therefore, indicates a double point.

(*a*) A change from it in either direction increases the efficiency of alcohol as a precipitant of the proteins of serum.

(*b*) If the temperature be increased above the critical point the protein precipitated is partially insoluble; if the temperature be decreased the precipitate is completely soluble.

A quantitative determination of the temperature coefficient of the precipitation of serum by alcohol is given below.

Serum and alcohol were cooled to the required temperature. 10 c.c. of this cooled serum were then placed into each of a series of test tubes and alcohol was added to the required percentage. The mixtures were kept at constant temperature for three hours and were then filtered. The percentage of solid matter in the filtrate was determined by drying and weighing a known volume.

0° C. Percentage of alcohol	Percentage of protein precipitated
17·5	25·4
20	38·2
27	57·5
30	66·0
35	78·3

4° C. Percentage of alcohol	Percentage of protein precipitated
20·0	17·5
22·5	28·3
25·0	37·7
27·0	43·4
30·0	53·7
32·0	59·0
35·0	65·6
40·0	80·0

10° C. Percentage of alcohol	Percentage of protein precipitated
27	17
30	26·5
32	37·3
35	47
37	53
40	64·3
45	83·3
50	95

16° C. Percentage of alcohol	Percentage of protein precipitated
33	18·6
35	30·6
38	46·2
41	62
44	73·5
47	88
50	96
60	98

40° C.	Percentage of alcohol	Percentage of protein precipitated
	20	12·8
	22·5	32·0
	25	40·5
	27	51·3

Weight of solid in 100 c.c. of original serum = 11·6 grams.
Weight of protein in 100 c.c. of original serum = 10·6 grams.

The results obtained are plotted on Fig. 9. The precipitation at 16° C. was slightly above the critical temperature. The precipitate produced at this temperature was not completely soluble.

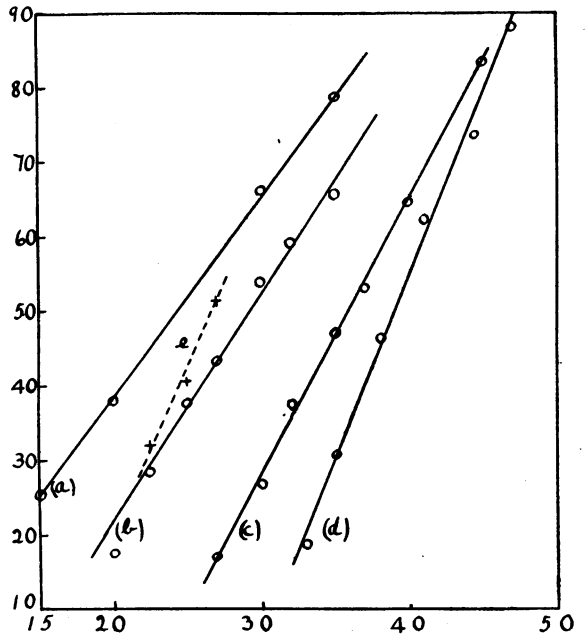

Fig. 9. Ordinates represent percentages of protein precipitated. Abscissæ represent percentages of alcohol present. (*a*) temperature 0° C.; (*b*) temperature 4° C.; (*c*) temperature 10° C.; (*d*) temperature 16° C.; (*e*) temperature 40° C.

It may be seen that the curves for 0° C., 4° C. and 10° C. keep the same proportionate distance from one another throughout their course. but that the actual distance diminishes the greater the amount of protein precipitated. This diminishing temperature coefficient with increase in the protein precipitated may be readily appreciated by determining the actual coefficient at each percentage of protein

precipitated. Thus the coefficients have the following values at 25 %, 50 % and 75 % of protein precipitated.

$$25\,\% : 50\,\% : 75\,\% :: 26 : 23 : 18.$$

The five precipitation curves on Fig. 9 allow an accurate determination to be made of the critical temperature of that particular serum. Suppose we take a point at which 32 % of the protein is precipitated. We find that this quantity of protein is precipitated by

18·5 % of alcohol at 0° C., 23·5 % of alcohol at 4° C., 31·5 % of alcohol at 10° C., 35·5 % of alcohol at 16° C., 22·5 % of alcohol at 40° C.

These values are plotted on Fig. 10. The crest of the curve is at 14° C. This temperature, therefore, is the critical temperature for the alcohol precipitation of the proteins of that serum. If we take the

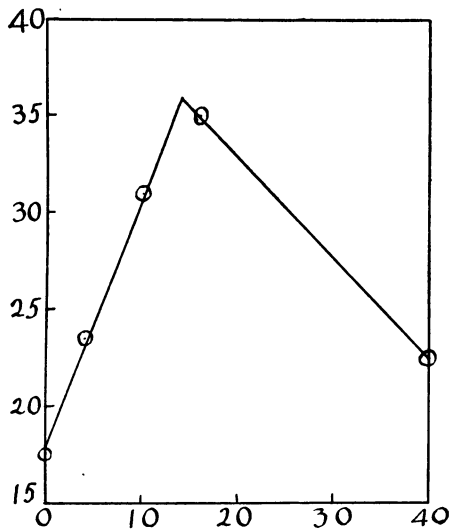

Fig. 10. Ordinates represent percentages of alcohol. Abscissæ represent temperatures in degrees.

values of the temperature coefficient above and below the critical temperature, we find that the ratio of these coefficients is as eleven is to twenty-four. There is thus a third difference in the precipitation of the proteins of serum by alcohol above and below the critical point—the temperature coefficient below this point is about twice as large as above it.

(vi) *The influence of the concentration of serum on alcohol precipitation.* Serum was frozen and allowed to melt gradually. The resulting

liquid was syphoned off in a series of layers. The serum fractions obtained contained the following weights of solid in each 100 c.c.:

(*a*) 2·075 grams. (*b*) 4·37 grams. (*c*) 7·89 grams.
(*d*) 11·06 grams. (*e*) 15·6 grams. (*f*) 19·6 grams.

The quantitative precipitations of these serum fractions by alcohol at 0° C. were determined. The results are given below.

Percentage of alcohol	Percentage of total solid not precipitated					
	a	*b*	*c*	*d*	*e*	*f*
17·5	77·5	—	—	91	93·5	94·2
20	—	70	75	80·4	86	87
22·5	67	—	—	74	77	80·6
25	64	63	64	66	—	73·5
30	—	57	58	—	64·3	—
35	58	53	52·8	52	55	53·6
40	—	46	44·8	42·5	43·5	40
45	51	33·5	33	26	—	23
50	30	28·3	—	14	14	11

It may be seen that the percentages of protein precipitated by a given percentage of alcohol vary with the different fractions. But these variations are not of the same order as the differences in concentration of the original serum fractions. Thus we see that the amount of solid in the serum fractions range from 2·075 grams to 19·6 grams in each 100 c.c., whilst in these two cases the percentage of protein precipitated by a given quantity of alcohol does not vary more than 20 %.

If we carefully examine the above figures we find that with serum fractions containing a low percentage of solid a given percentage of alcohol has at first a higher precipitating efficiency and later a lower precipitating efficiency than with fractions containing a large percentage of solid. The following figures make this point clear: 17·5 % of alcohol precipitates 22·5 % of solid from serum (*a*), 17·5 % of alcohol precipitates 5·8 % of solid from serum (*f*); 50 % of alcohol precipitates 70 % of solid from serum (*a*), 50 % of alcohol precipitates 89 % of solid from serum (*f*).

An hypothesis based upon this result is that alcohol has a slightly increased precipitating efficiency with higher concentrations of proteins, but that this precipitation is inhibited by ionised salts. In a previous paper it has been shown that the ratios of the amounts of the various constituents of the serum fraction are constant throughout the series and are the same as in the original serum. Therefore, in the fractions containing a greater amount of solid the percentage of salts is increased

as well as the percentage of protein. There are thus two antagonistic factors—the increased salts diminish the precipitation effect of the alcohol and the increased protein augments it. At the beginning of the precipitation of serum fractions containing a large percentage of solids, the inhibitory effects of the salts predominate; later, the effects of the increased protein become evident. In the above tables it may be seen that these two factors balance one another at about 35 % alcohol.

(vii) *The influence of sodium chloride on the precipitation by alcohol.* To test the value of the hypothesis that ionised salts inhibit the precipitation of the proteins of serum by alcohol a definite series of experiments were made.

Sodium chloride was added to serum in varying quantities and the amount of protein precipitated by a definite percentage of alcohol at 0° C. was determined. The following results were obtained:

30 % alcohol as precipitant.	Normal serum	53 % of protein precipitated.
	Serum + $\frac{N}{27}$ NaCl	39·5 % ,, ,,
	Serum + $\frac{2N}{27}$ NaCl	34 % ,, ,,
	Serum + $\frac{4N}{27}$ NaCl	28 % ,, ,,

From the above results which are typical of a number obtained it is evident that neutral salts inhibit the precipitation of the proteins of serum by alcohol.

(viii) *The influence of acids.* But although neutral salts diminish the precipitating effect of alcohol free acids increase it. This increase is evident both above and below the critical temperature for the precipitation of serum by alcohol.

Hydrochloric acid was added to serum in varying quantities and the precipitations for definite quantities of alcohol at 0° C. determined. The following results were obtained:

Percentage of alcohol	Percentage of protein precipitated			
	a	*b*	*c*	*d*
11·0	2	16	22·2	—
12·6	8·5	—	—	38·6
16·2	21·6	32	—	—
19·8	37·5	49	59	—

(*a*) (*b*) (*c*) and (*d*) are sera containing ·05 %, ·1 %, ·15 %, ·2 % of HCl respectively. It may be seen from the above figures that the addition of acid produces a large increase in the quantity of protein precipitated.

A series of experiments to determine the influence of hydrochloric acid on the precipitation of serum by alcohol at 16° C. were done. The following is the type of result obtained.

Serum was precipitated with 34 % alcohol. At 16° C. only a very slight precipitate occurred. 10 c.c. of the filtrate were placed into each of a series of test tubes and a constant volume of varying strengths of hydrochloric acid added. The amount of protein precipitated out was determined in each case.

34 % alcohol filtrate. Temp. 16° C.	Percentage of hydrochloric acid	Weight of protein precipitated
	·0095	·007
	·024	·014
	·0475	·048
	·07	·105
	·095	·215
	·19	·189
	·285	·160

It may be seen that after the addition of about ·1 % hydrochloric acid the amount of precipitate produced decreases. This transition point is independent of the percentage of alcohol contained in the filtrate being the same with 35 %, 38 %, 41 %, 44 %, 48 %, and 50 % alcohol. It probably depends upon the types of protein in the serum. The diminution of the protein precipitate with higher concentrations of acid does not go on until all the protein is redissolved. It reaches a limit when about 50 % of the protein has again gone into solution. This resolution with higher concentrations of acid is evidence of some chemical differentiation of the proteins of serum.

(ix) *The influence of alcohol on the electric conductivity of serum.* In many of the experiments recorded in the previous pages there has been an apparent influence of electrolytic salts on the precipitation of the proteins of serum by alcohol. To determine the precise effect of alcohol on the free ions contained in serum a number of conductivity experiments were done. The addition of alcohol to serum markedly diminishes its conductivity. This is evident from the following figures:

	Conductivity $\times 10^{-3}$
Serum	·447
Serum + 9·09 % alcohol	·362
,, + 20 % ,, ...	·272
,, + 33·3 % ,, ...	·208

But these results do not settle the problem whether a definite ionic change is associated with the advent of precipitation.

To solve this problem the following experiment was done. 3·3 c.c. of alcohol were added to 10 c.c. of serum. The conductivity of the resulting solution was determined at a series of temperatures.

Temperature	Conductivity $\times 10^{-3}$
2° C.	·1075
5	·127
9	·154
13·5	·187
18	·222
23·5	·258
30	·303
38	·365
47	·435

These results are plotted on Fig. 11. The relation between the temperatures and conductivities is represented by a straight line; at no point is there any indication of an abrupt change in conductivity. But the serum alcohol mixture existed in three distinct states during these changes of temperature. At 2° C. there was a large amount of protein precipitated from solution. As the temperature rose from 2° C. to 13° C. this quantity of precipitated protein gradually diminished until at 13° C. the solution was quite clear. From 13° C. to 23° C. the mixture remained clear, no precipitate being present. From 23° C. onwards there was a gradually increasing protein precipitate present. The first and second protein precipitates differed in their solubilities in the manner previously described. From this experiment it is evident that whatever mechanism may be involved in the alcohol precipitation of serum, when that precipitation is initiated no abrupt change in the ionisation of the serum takes place.

The effect of adding alcohol to serum on the conductivity temperature coefficient may be seen by comparing these results with those given in a previous paper [16]. With pure serum the conductivity temperature coefficient is $\frac{\cdot 00062}{50} = \cdot 000012$ or the increase in conductivity per degree is ·000012. With serum containing 25 % of alcohol the increase in conductivity per degree is $\frac{\cdot 00028}{40} = \cdot 00007$, that is about one-half of that of serum free from alcohol. It may be seen from Fig. 11 that when the serum began to be precipitated by 25 % alcohol at 13° C. its conductivity was ·00018; when the second precipitation occurred at 23° C. the conductivity of the mixture was ·000255. Therefore, if there is any connection between the ions present in serum and the precipitation

of its proteins by alcohol, two different mechanisms must be involved when precipitations take place above and below the critical temperature.

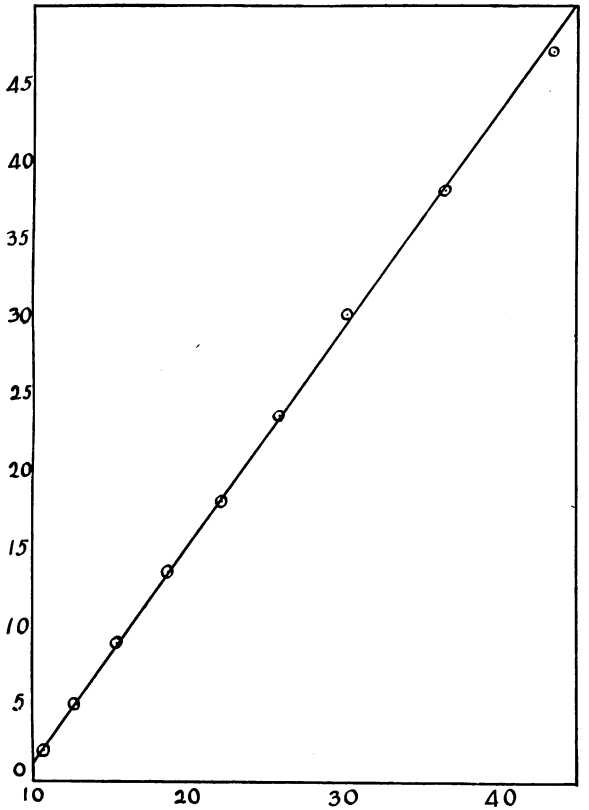

Fig. 11. Ordinates represent temperatures in degrees. Abscissæ represent conductivities. 10 = ·00010.

Remarks and Conclusions.

To explain any series of phenomena in which two opposite effects such as solution and precipitation occur it is natural to look for two factors which have some property alike in kind but opposite in sign. In the case of the precipitation of serum by salts the obvious opposing factors in the active agent are the kation and anion.

Pauli[17] in a series of papers has described the results of his investigations on the precipitation of egg white by salts of the alkalies, of the alkaline earths and of the heavy metals. His experimental results

are quantitative to the extent that he added definite quantities of salts and noted whether immediately or after twenty-four hours the solutions were clear, opalescent, or precipitated to some extent. From the analysis of his results he states that the active precipitating agent of a salt is the kation whilst the anion hinders this action. When precipitation is produced by salts of the alkalies the inhibitory action of the anion is so considerable than some salts when added to saturation do not precipitate. To compare these salts with those which do precipitate, he either uses them in super-saturated solution or adds a second salt to produce a precipitating effect. The precipitating effect of several electrolytes he assumes to be equal to the sum of the precipitating effects of their constituent ions. To explain the precipitating effects observed with the salts of the earth alkalies he assumes that the protein enters into strong combination with the kation and this prevents the anion from having any great inhibitory effect. In the case of precipitation by salts of the heavy metals the inhibitory action of the anion is supposed to be negligible.

A number of results are not adequately explained by Pauli's theory, but probably the most cogent objection to it is that the precipitating effect of an ion is not related to any known property beyond the sign of its charge. The valencies and velocities of the constituent ions of a salt are not related to its precipitating efficiency and the relative efficiencies of various salts of the same type are not correlated to their degrees of dissociation. He has drawn up a table giving the order of potency of various anions and kations to produce solution and precipitation [18]. The order observed in this table cannot be correlated to any known property of the ions.

The precipitation of colloids has been investigated by Matthews [19] also. From a series of experiments on egg white he deduces that ions of opposite sign precipitate whilst those of the same sign dissolve, and their action is inversely proportional to their solution tension coefficients. The valency of a precipitating ion may be important because a polyvalent ion links several colloidal particles together.

Recently the precipitation of serum has been investigated by Iscovesco [20]. He used serum diluted ten times and observed the precipitation produced in it by colloidal ferric hydroxide and colloidal arsenious sulphide. He concluded that serum contains two proteins, one negatively charged, the other positively charged, the former being present in much larger quantities than the latter. It may be noted that in the case of the precipitation of his diluted serum with colloidal

iron about 1 c.c. of a 1 % colloidal ferric hydroxide was required to precipitate 1 c.c. of his diluted serum. The diluted serum contained about 1 % of protein, and consequently if the precipitation were due to the union of the positively charged colloidal iron with the negatively charged protein then the active mass of the iron must have been approximately of the same size as the protein complex. But the quantitative results are very similar to those described by Pauli for the precipitation of protein by salts of the heavy metals, and in the dilutions he used an impurity of ·01 % of ferric chloride would produce the precipitation effects observed. He confirmed the presence of negatively and positively charged protein in serum by submitting it to the influence of an electric field. But the experiment described is invalid since he put his platinum electrodes directly into the diluted serum and the chemical effects generated at the electrodes would explain the results observed.

Galeotti[21] has also described a series of experiments on the precipitation by salts of the heavy metals. He concluded that

(*a*) There is no constant relation between the metal and protein in the sense of valency, but that the precipitate varies with the composition of the fluid.

(*b*) The precipitation is reversible, being soluble in excess of either of the constituents.

(*c*) The relation of the precipitate and solution obey the laws of chemical equilibrium.

An analysis of the experimental results described in the previous pages affords some insight into the nature of the proteins of serum and the processes involved in their precipitation.

(1) *Neutral salts.* If we examine Fig. 1 which gives the quantitative precipitation of serum by ammonium sulphate, we see that the curve has a regular form. A determination of the angles made by the tangents to this curve at various points gives the following results.

Percentage of protein precipitated	Angle of tangent at this point
30	$\frac{44}{40}$
40	$\frac{39}{40}$
50	$\frac{32}{40}$
60	$\frac{25}{40}$
70	$\frac{18}{40}$

The relation is given approximately by a straight line. If we express this result by a formula, we have:

$$\frac{d \text{ salt}}{d \text{ protein}} = K \text{ protein} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{(i)}$$

where protein refers to the percentage of protein precipitated, salt to the required salt in terms of Eq. Wt. = 100, and K is a constant. The solution of equation (i) is:

$$\text{salt} = Ka\,(\text{protein})^2 + b.$$

The equation is that of a parabola, and this therefore is the form of the curve showing the precipitation of serum by ammonium sulphate. The value (b) in the above equation expresses the amount of salt required to initiate precipitation. This may be seen by putting protein = 0, when from the equation, salt = b. The value of (b) is constant only for the particular serum precipitated. For instance in the curves expressing the influence of dilution on the precipitation of serum by ammonium sulphate, we see that in serum diluted 3 times precipitation starts with 232 Am_2SO_4; serum diluted 5 times precipitation starts with 254 Am_2SO_4; serum diluted 10 times precipitation starts with 270 Am_2SO_4.

These values are plotted on Fig. 12. The relation is again given by a straight line, so that the equation for the value of (b) is:

$$b = A + \frac{D}{K},$$

where A is the amount of salt required to initiate precipitation with serum of the dilution D, and K is the tangent of the angle formed between the straight line and the abscissa in Fig. 12. The value of (b) is interesting, for in the limit when the serum is infinitely diluted b has the value 288. From this we see that when the percentage of protein is infinitely small precipitation with ammonium sulphate starts when salt has been added to the extent of 2·86 times its equivalent weight, and is completed only when about 4·6 equivalent weights have been added.

From the magnesium sulphate precipitation curve it may be seen that the parabolic relation exists between the amounts of salt and precipitated protein with this neutral salt also. The relative efficiencies of ammonium sulphate and magnesium sulphate after precipitation has started are given approximately by the value of the tangent of the angle to the curves at a particular percentage of protein precipitated.

If we compare curves (*B*) and (*C*) in Fig. 3, we see that the tangents of the angles at 30 % protein precipitated are in the proportion of 17 to 8. Therefore after precipitation has started ammonium sulphate is more than twice as effective a precipitant as magnesium sulphate. This preponderance is still more marked if we compare the total amount of salt added, since in the ammonium sulphate curve precipitation was initiated with 2·4 equivalents of salt whilst 2·72 equivalents of magnesium sulphate were required to do this. With magnesium sulphate, also, the advent of precipitation is delayed by dilution of the serum.

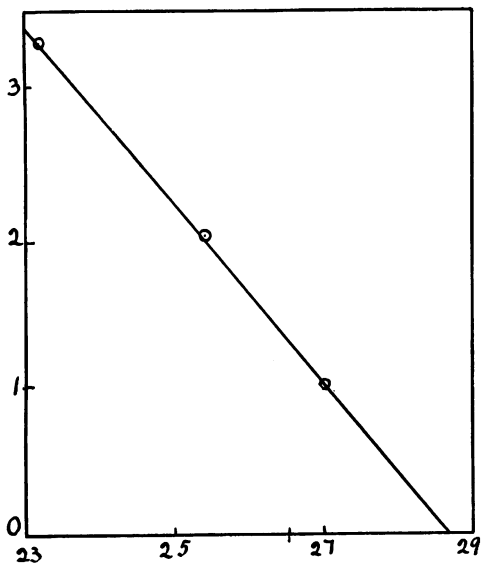

Fig. 12. Ordinates represent reciprocals of dilution. Abscissæ represent amounts of ammonium sulphate present. 10=Eq. Wt. Am_2SO_4.

In any theory dealing with the nature of the precipitation of the proteins of serum by neutral salts the following observations must be adequately explained:

(1) At any stage of the precipitation the amount of salt added is proportional to the square of the percentage of protein precipitated.

(2) With extreme dilution of the serum, precipitation is extended over a definite range of salt concentrations.

(3) The effect of temperature is very small; at 0° C. only a slightly greater quantity of precipitate is produced than at 40° C.

(4) Ammonium sulphate is a more efficient precipitant than mag-

nesium sulphate—an approximate calculation of their efficiencies is as about two is to one.

(5) Precipitating salts of the alkali metals are practically limited to ammonium sulphate, magnesium sulphate and sodium sulphate.

None of the facts stated above are capable of being readily explained by the assumption that precipitation is due to the action of the constituent ions of a salt. An examination of the curve for the precipitation of serum by ammonium sulphate shows that precipitation does not start until 2·32 equivalents of the salt are present and is complete (as far as the main protein is concerned) when 4·6 equivalents have been added. If we apply Pauli's theory to this we must assume that the solvent and precipitating effects of the anion and kation are exactly balanced until 2·32 equivalents of the salt are present, but that after this concentration the superior influence of the kation becomes sufficiently great to precipitate the protein on adding a further 2·3 equivalents of the salt. On Matthew's theory we must assume that at this concentration the solutions tensions of the two ions suddenly change to produce a corresponding effect. There is no known physical property of ammonium sulphate to warrant such assumptions.

In a paper[22] on the precipitation of globulin by ammonium sulphate and magnesium sulphate the author came to the conclusion that the quantitative results obtained could be most readily understood if it was assumed that the precipitate produced by the neutral salt depended on the formation of a molecular complex between the protein and salt, this complex being stable only in the presence of an excess of the salt. The explanation of the precipitation of globulin on an ionic basis was difficult, since it had been previously shown that the solution of this protein by neutral salts depended upon the combined action of both ions,—ions of the same valency whether positive or negative being equally efficient in producing solution and the efficiency of ions of different valencies being proportional to the square of these valencies. If these laws for the solution of globulin are correct a peculiar redistribution of ionic energy in more concentrated solutions of salts would be required to explain the subsequent precipitation of this protein as due to a preponderating precipitant action of the kation over the solvent action of the anion. There is no obvious quantitative difference between the effects observed during the precipitation of globulin by neutral salts and those seen with albumin. The logical conclusion therefore is to apply the theory which was most adequate to explain the precipitation of globulin from solution by neutral salts to the phenomena

observed when albumin solutions are so precipitated. The assumption that albumin is precipitated from solution by neutral salts owing to the formation of a molecular salt complex, stable only in excess of the precipitating salt, is adequate to explain most of the facts stated above. The breaking up of the protein salt complex is readily effected by water, since on dilution resolution readily takes place. The amount of salt required therefore to neutralise the hydrolytic effect of the water is represented by that quantity which is required to initiate precipitation. On this theory the very small temperature coefficient and the direction in which it changes—a neutral salt is a slightly more efficient precipitant at 0° C. than at 40° C.—are readily appreciated. The hydrolytic effect of water at 0° C. is slightly less than at 40° C. and therefore less excess of salt is required to render the protein salt complex stable. This temperature effect and the effect observed on dilution would be difficult to explain on any ionic hypothesis. The results observed on dilution of the serum, *i.e.* that the more dilute the serum the more salt is required to initiate precipitation are readily understood when the active mass of the protein concerned is considered.

But the actual relation between the amount of salt required to precipitate and the amount of protein precipitated are not capable of a simple explanation. The first point to be noticed is that although globulin and albumin start to be precipitated with about the same amount of ammonium sulphate present yet the rate of precipitation of globulin is twice as rapid as that with albumin. This rate must be determined by the type of protein to be precipitated. But that the rate is not determined purely by the physical properties of the protein is evident from the observations of Osborne and Harris[23] that some vegetable globulins were more slowly precipitated by ammonium sulphate than typical vegetable albumins.

There is the further point that the precipitation of the proteins of serum is initiated by practically equivalent amounts of ammonium sulphate and magnesium sulphate, but that the subsequent rate of precipitation by ammonium sulphate is about twice as great as that by magnesium sulphate. In this case we possibly have an example of the different types of molecular complexes formed between the precipitating salt and the protein—the complex formed with ammonium sulphate being much more stable than that formed with magnesium sulphate.

From the above it will be seen that the hypothesis that the precipitation of proteins by neutral salts is due to the formation of a molecular complex between the protein and salt is capable of

explaining some of the phenomena observed. The same explanation is applied to the phenomena observed in the precipitation of serum by salts by the heavy metals—the only difference in this case being that the molecular complexes so formed are relatively stable in the presence of water. In a previous section the effect of acids on proteins has been dealt with and in this it has been shown that these influences are so marked that the analysis of the phenomena observed when salts of the heavy metals (which are often markedly acidic in solution) are added to proteins must be carefully analysed before an adequate explanation of the forces involved can be set forward.

In Fig. 2 it may be seen that the curves expressing the precipitating relations with various dilutions of serum meet at about 85 % of the total protein precipitated. It may be assumed therefore that at this point all the protein for which the observations above mentioned hold true has been precipitated and that there remains only that protein in solution to which frequent reference has been made in a previous paper. A detailed consideration of the precipitation of this protein has not been made, but from the experiments described in the previous pages the following statements may be made about it.

(*a*) The amount of neutral salt required to precipitate it is not materially influenced by the dilution of the serum.

(*b*) It is precipitated by alcohol at temperatures above the critical point only very slowly and may be left in contact with alcohol for a long time without being coagulated.

(*c*) It is not readily precipitated by salts of the heavy metals.

(*d*) It is not associated with the inorganic salts of the serum.

It is probably that protein which can be crystallised from horse serum by means of ammonium sulphate and sulphuric acid.

(2) *Neutral salts in the presence of acids.* The precipitation of serum by neutral salts in the presence of acids has been considered in the previous pages. It was found that the efficiencies of various acids were proportional to their avidities, a result indicating that the acids entered into direct combination with the protein complexes. This hypothesis was supported by the experiments showing the effect on the conductivity of adding acid to serum. The forces involved in the precipitation of this acidified serum by neutral salts have not been fully worked out so far as the neutral salts are concerned. In the case of sodium chloride the precipitation curves are approximately straight lines, indicating that the major portion of the protein of serum possesses identical physical properties when treated in this way. The direct

proportion which holds between the amount of salt added and the quantity of precipitate produced points to the formation of a chemical compound between the two substances.

(3) *Alcohol.* The existence of a critical temperature in the alcohol precipitation of serum is of interest. Below this temperature the proteins of serum are precipitated in a perfectly soluble form; above it the precipitate produced shows varying degrees of insolubility. At the critical temperature the efficiency of any percentage of alcohol as a protein precipitant reaches a minimum. A change of temperature in either direction increases its precipitating effect. Below the critical temperature the alcohol precipitation curve is a straight line showing that the amount of precipitate produced is directly proportional to the quantity of alcohol added. In this respect the precipitation is of the same kind as that observed in the case of acidified serum by neutral salts.

The straight line relation shows no indication of any differentiation of the proteins of serum. But above the critical temperature the alcohol precipitation curve shows well defined variations, as may be seen in Fig. 7. In this precipitation curve we may see evidences of the existence of three distinct proteins in serum. From this difference between the precipitation curves of serum by alcohol above and below the critical point we may conclude that in the former case the protein is changed by the action of the alcohol and in the latter case no such chemical change is produced. The action of alcohol on the protein of serum at temperatures above the critical point is of two kinds: (*a*) It acts on the protein when in solution, as may be seen from the influence of time on the precipitation of serum by alcohol. (*b*) It coagulates the protein when precipitated. From a series of experiments it was found that protein which was precipitated by one percentage of alcohol was rendered insoluble by the addition of more alcohol. Also that the rate of coagulation by alcohol was proportional to some extent to the strength of alcohol in contact with the precipitate.

The differences between the alcohol temperature coefficients above and below the critical point indicate that two different mechanisms are involved in these precipitations. If a chemical change is involved in the precipitation of the protein of serum by alcohol at temperatures above the critical point, the question arises as to what forces are involved in this precipitation below the critical point. In this precipitation the following observations have been made: (*a*) The precipitation curves are straight lines; (*b*) the lowering of the temperature greatly increases

the precipitating efficiency of any given quantity of alcohol; (*c*) the addition of neutral salts diminishes the precipitation produced by any given quantity of alcohol; (*d*) the addition of acid increases the effect; (*e*) the amount of precipitate produced by a given quantity of alcohol is proportional to the percentage of protein in the serum; there is an indication that with large quantities of protein in solution the percentage of protein precipitated by a given quantity of alcohol may be increased; (*f*) there is no break in the electrical conductivity curve at temperatures at which precipitation starts either above or below the critical point.

The reverse effects produced by the addition of acids and neutral salts to serum on the efficiency of alcohol as a precipitant suggest that two different mechanisms are involved by these two classes of compounds. Acids, as we have previously seen, enter into combination with the protein complexes and probably produce their effects by altering the constitution of these complexes. Neutral salts on the other hand exist almost entirely free in solution. Probably therefore the inhibitory effects produced by them are due to the influences exerted by their ions.

A confirmation of this hypothesis is derived from a comparison of the temperature coefficients for the precipitation of serum by 25 % alcohol and the conductivity of serum to which this percentage of alcohol has been added. From the curve given on Fig. 11 we see that the conductivity of serum to which 25 % alcohol has been added is ·000180 at 13° C. and ·000090 at 0° C. That is by lowering the temperature from 13° C. to 0° C. the conductivity has been lowered 50 %. From the curves given on Fig. 9 we see that the efficiency of 25 % alcohol as a precipitant for the proteins of serum has the following values:

Temp.	0° C.	51 % of protein precipitated
„	4° C.	36 % „ „ „
„	10° C.	11 % „ „ „

These values are approximately proportional to one another and from them we see that precipitation with 25 % alcohol starts at 12·5° C. Therefore by diminishing the temperature from 12·5° C. to 0° C. the amount of protein precipitated increases by about 50 %. In the same temperature range the conductivity of serum to which 25 % alcohol has been added also diminishes by 50 %. The equality of the two values obtained suggests that the increasing efficiency of alcohol as a precipitant of the proteins of serum with diminishing temperature is

due to a diminution in the ionic energy produced by the lower temperatures. This deduction together with the observation that the amount of protein precipitated by a given percentage of alcohol is proportional to the percentage amount of protein in solution suggests the following hypothesis for the mechanism involved in alcohol precipitation of serum at temperatures below the critical point.—The alcohol enters into combination with the protein complexes in quantities directly proportional to the active masses of these substances; the compounds thus formed are unstable and are readily broken down by the ions existing in solution. Precipitation ensues only when the quantity of alcohol present in excess is sufficient to diminish the energy of these ions below a minimal value. From the precipitation curves given on Fig. 9 we see that before precipitation starts about 22 % of alcohol must be present in the serum alcohol mixture at a temperature of 10° C. On the above hypothesis this quantity of alcohol is the quantity necessary to depress the energy of the elecrolytic ions to the required amount. The hypothesis that alcohol enters into combination with the protein complexes derives additional support from the observation of the action of alcohol on solutions of electrolytes. To explain the results obtained it is necessary to assume that the alcohol causes the production of complex ions even in moderate dilutions. The observation that acids increase the precipitating efficiency of alcohol at temperatures below the critical point indicates that the acid protein complex forms a system with the added alcohol which is more stable in the presence of ionised salts.

The above hypothesis affords a ready explanation of the alcohol critical temperature for serum. To explain this phenomenon we must assume that at temperatures above 14° C. the alcohol, instead of merely uniting with the protein complex, acts directly upon it and changes its composition. This changed protein is precipitated more readily than the original protein, since with increased temperature and consequently increased ionic energy the amount of protein precipitated is augmented. The chemical action of alcohol on protein at temperatures above the critical point is evident from the time factor on the amount of protein precipitated by a given percentage of alcohol, and the observations on the rate of coagulation of serum protein by alcohol.

Alcohol must act on the different proteins at different rates, since the precipitation curves at temperatures above the critical point show a differentiation of the protein into three groups.

(i) Globulin—comprising about 3 % of the total protein;

(ii) Albumin (α)—comprising about 85 % of the total protein;

(iii) Albumin (β)—comprising about 12 % of the total protein.

(4) *Salts of the heavy metals.* The precipitation of the proteins of serum by salts of the heavy metals has been considered in detail in the previous pages. The precipitate is probably produced by the molecular combination of the salt with the protein complex. The presence of a substance in serum inhibiting the formation of these combinations prevents an accurate determination of the amount of protein combined with the salt from being made. The readiness with which the proteins of serum enter into combination with the added salt indicates that there are two proteins in serum:

(*a*) Forms about 85 % of the total protein and is precipitated by salts of the heavy metals;

(*b*) Forms about 15 % of the total protein and is not readily precipitated by these salts.

The observation that the protein precipitated by the salts of heavy metals is probably in the form of a protein complex indicates that it is these complexes rather than the pure protein which are precipitated in all the methods previously described. The constant association of the pigment with the precipitated protein lends support to this view.

SUMMARY.

1. There is no indication from the precipitation curves of the proteins of serum by neutral salts that half saturation by ammonium sulphate or full saturation with magnesium sulphate differentiates two classes of proteins.

2. The precipitation of the proteins of serum by neutral salts is suggested to be due to the entry of the salt molecules into the protein complexes, the resulting phase being readily broken up by water and stable only in the presence of an excess of salt molecules.

3. Acids increase the precipitatory efficiency of a neutral salt at a rate proportional to their avidities.

4. The precipitation of the proteins of serum by salts of the heavy metals is explained on the same basis as for neutral salts, the only difference being that the resulting complexes are stable in the presence of water.

5. Alcohol shows a definite critical temperature for the precipitation of the proteins of serum.

The precipitate produced below the critical temperature is suggested to be due to the formation of alcohol protein complexes, these complexes being unstable in the presence of electrolytic ions. Above the critical temperature the alcohol exerts a chemical action on the protein, the degree of action depending on the percentage of alcohol present, the temperature, and the time.

6. The phenomena observed in the precipitation of serum indicate that there are three main proteins present in serum.

(*a*) Globulin—about 3 %;
(*b*) Albumin (*a*)—about 85 %;
(*c*) Albumin (*β*)—about 12 %.

The evidence points to the conclusion that albumin (*β*) is that protein which can be crystallised.

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