CLXXXVII. THE DETERMINATION OF BLOOD- PROTEINS BY ACID-ACETONE.

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DURING the course of experimental work with various methods for the determination of total protein in blood-plasma and serum, an endeavour was made to weigh the protein resulting from precipitation with trichloroacetic acid followed by washing with alcohol and ether. The results, compared with the direct gravimetric method of Knipping and Kowitz [1924] as modified by Starlinger and Hartl [1925], and the total nitrogen as determined by the Kjeldahl process, were invariably low, the following being a typical example.

	Total protein-nitrogen %
Kjeldahl direct, less N.P.N.	1.075
Precipitation with 95 % alcohol followed by Kjeldahl	1.11
Precipitation with 95 % alcohol; washed with ether; Kjeldahl	1.07
Gravimetric. Starlinger and Hartl. Factor 6.45	1.11
Precipitation with trichloroacetic acid and washing with alcohol and ether	0.66

On investigating the source of the loss it was found to be due entirely to the alcohol used for washing the precipitated protein.

Later it was found that 75–85 % acetone could be substituted for the alcohol and the conditions were worked out for obtaining the maximum yield of the acetone-soluble portion. The method ultimately adopted was as follows. 1 cc. of plasma or serum was diluted to 8.5 cc. in a 15 cc. centrifuge-tube and the total protein precipitated by the addition of 1.5 cc. of 20 % trichloroacetic acid drop by drop with constant shaking followed by inversion of the tube closed by the thumb. This is necessary to obtain a finely divided precipitate. The tube was centrifuged at a high speed for 10 min. and the supernatant liquid poured off; 0.25–0.30 cc. of 20 % CCl_3COOH was added; the volume made up to 2 cc. with water and then to 10 cc. with acetone. After stirring with a glass rod the precipitate and solvent were violently shaken and then centrifuged for 10 min. This usually resulted in a residue of insoluble protein occupying about 1 cc. from which the supernatant liquid could be poured off without loss of the deposit; the solvent was poured into a Kjeldahl flask and the precipitate again extracted with 80 % acetone containing 2.5 cc. of 20 % CCl_3COOH per 100 cc. The combined acetone washings were evaporated to dryness in a beaker of hot water and the residue in the tube used for the determination of total insoluble protein by the Folin-Kjeldahl technique.

The supernatant liquid from the first precipitation with trichloroacetic acid can, if desired, be used for the estimation of the N.P.N.

Whilst this work was in progress the proteins from egg-white were prepared by precipitation with ammonium sulphate followed by dialysis, but it was found that when these were examined by the above process, afterwards referred to as the S.P. (standard process) the globulin fractions were totally insoluble and that only a small fraction of the albumin was soluble.

These results opposed the idea, tentatively formed, that the acetone was merely separating the albumin and globulin, but when a number of routine hospital samples taken for the determination of the suspension stability had been examined, it was found that the acetone-soluble fraction showed some degree of correlation with the suspension stability and with the tryptophan content, both of which are related to the albumin/globulin ratio.

When pure albumin and globulin were prepared from human plasma, the albumin was found to be entirely soluble and the globulin totally insoluble in 80 % acetone containing trichloroacetic acid. Sheep- and ox-albumin were also found to be entirely soluble and in all subsequent work on pure proteins animal preparations were used.

The results so far obtained suggested that the process might be used as a clinical method for the determination of albumin and globulin, and various mixtures were accordingly prepared and analysed by the standard process with the following results.

Albumin (%)		
Theory	Found	Difference
6.4	5.4	- 1.0
10.4	8.4	- 2.0
16.0	13.6	- 2.4
29.6	25.3	- 4.3
46.6	43.3	- 3.3
57.3	53.5	- 3.8
79.6	77.4	- 2.2

These results are fairly satisfactory but with very small amounts of globulin the precipitation of the globulin is somewhat delayed unless the concentration of the acetone is increased to 90 %. With 5 % of globulin (38 mg. of albumin and 2 mg. of globulin) and 80 % acetone the percentage error was 40-50 % but the separation of even 1 mg. of globulin from such a mixture cannot be regarded as entirely unsatisfactory.

A series of oxalated plasmata was analysed concurrently by the S.P. and by Howe's method [1921] in which sodium sulphate is used as the precipitant, with the following results, which are expressed as cc. of *N*/10 acid required to neutralise the ammonia formed from 1 cc. by the Kjeldahl process.

In the plasmata containing the higher amounts of albumin the differences were usually small and varied in direction but in the pathological samples containing an excess of globulin the Howe process invariably gave higher results.

Globulin	Albumin		Difference H. - A.
	Acetone-soluble	Howe	
1.50	5.10	5.15	+0.05
2.00	4.85	4.50	-0.35
3.20	4.55	4.80	+0.25
2.85	4.40	4.25	-0.15
2.75	4.40	4.17	-0.13
3.40	4.15	4.40	+0.25
3.70	4.00	4.35	+0.35
3.25	3.97	4.03	+0.06
3.90	3.95	4.50	+0.55
2.80	3.90	4.25	+0.35
3.75	3.50	3.85	+0.35
4.10	3.35	3.55	+0.20
4.65	3.00	4.05	+1.05
5.40	2.75	3.13	+0.38

According to Merklen, LeBreton and Adnot [1931] complex lipoids hinder the precipitation of globulin from serum either by CO_2 or by neutral salts. On removing the lipoids by extraction with 80 % acetone, followed by dissolution of the precipitate in water and reprecipitation with acetone, they obtained the following results.

	Precipitant	Globulin. g. per litre	
		Ordinary serum	Serum after acetone treatment
Pig blood	$\text{MgSO}_4(\text{NH}_4)_2\text{SO}_4$	44.7	52.7
		41.6	48.0
Chicken blood	$\text{MgSO}_4(\text{NH}_4)_2\text{SO}_4$	28.8	32.1
		30.4	38.8

Two points are worthy of note in connection with these results (1) the increase due to acetone extraction and (2) the variable results given by magnesium and ammonium sulphates both before and after acetone extraction. The latter point is also a feature of Howe's results and neither MgSO_4 nor $(\text{NH}_4)_2\text{SO}_4$ gave figures in accordance with the 21.3 % Na_2SO_4 recommended by him. Howe [1921, p. 101] records the following figures.

Precipitant	Albumin in filtrate. g. N per 100 cc.		
	Pig	Goat	Horse
Na_2SO_4 21.3 %	0.393	0.399	0.443
$(\text{NH}_4)_2\text{SO}_4$ half saturated	0.526	0.443	0.477
MgSO_4 saturated	—	0.323	0.385

The percentage differences, calculated on the Na_2SO_4 results are

$(\text{NH}_4)_2\text{SO}_4$	+34	+11	+8
MgSO_4	—	-19	-13

In a second paper, Howe [1921, p. 113] gives further figures for these types of blood which show variations of similar magnitude and direction, $(\text{NH}_4)_2\text{SO}_4$ always giving larger, and MgSO_4 smaller, amounts of albumin than Na_2SO_4 .

It is probably generally appreciated that there are, in the usual precipitation methods, small inherent errors such as the dilution factor and the volume contraction (1-2 % in the case of adding half saturated $(\text{NH}_4)_2\text{SO}_4$ to an equal volume of serum) but the variations in the above figures are of a

very different order and strongly suggest that when applied indiscriminately to normal and pathological serum and plasma, such methods are somewhat empirical.

The effect of acetone extraction carried out according to the method of Merklen *et al.* [1931] was determined on 3 samples of human plasma, using Howe's process and the acetone-acid method previously described. The results, expressed as nitrogen in terms of N/10 acid, were:

Sample	T.P.N.	Method	Albumin-nitrogen	
			Before acetone treatment	After acetone treatment
I	7.33	Na ₂ SO ₄	4.20	3.62
		Acetone, S.P.	3.90	4.00
II	7.78	Na ₂ SO ₄	4.20	3.46
		Acetone, S.P.	3.75	4.02
III	6.42	Na ₂ SO ₄	2.43	1.40
		Acetone, S.P.	1.20	0.95

The acetone treatment was carried out as recommended by Merklen, *viz.* 1 cc. of plasma was diluted with 1 cc. of water, 8 cc. of acetone added and centrifuged. The precipitate was dissolved in 2 cc. of water and made up to 10 cc. with acetone. After centrifuging it was deemed desirable to remove the acetone mixed with the precipitate as this might affect the subsequent precipitation with Na₂SO₄. This was effected in a vacuum desiccator, care being taken not to remove all the water. The precipitate was then dissolved in water at 37°; the volume made up to about 11 cc.; 3.05 g. of anhydrous Na₂SO₄ added; and the volume made up to 15 cc. after the sulphate had completely dissolved. After incubation at 37° for 4 hours the precipitate was filtered off and the N determined in the filtrate.

It might be suggested that the acetone treatment would produce some irreversible coagulation of the albumin, but this is not supported by the results obtained by the standard process nor by the fact that a mixture of pure albumin and globulin gave practically identical results with Na₂SO₄ before and after treatment with acetone.

The above results definitely indicate that acetone removes some substance which materially interferes with the precipitation of globulin by Na₂SO₄ and that this substance is present in greater amount in plasma showing a low albumin/globulin ratio. This substance also affects the results obtained with the acid-acetone process but to a smaller degree.

Attempts to produce this effect synthetically have so far proved unsuccessful. The acetone treatment would more or less completely remove the lipoids together with some lecithin but it was found that the addition of a lecithin emulsion to a mixture of pure albumin and globulin had no effect; the same results were obtained by the Na₂SO₄ and acid-acetone processes both before and after acetone treatment.

A further attempt was made by adding the substances extracted by 80 % acetone. 100 cc. of ox-serum were precipitated by adding 400 cc. of acetone,

the proteins filtered off, and the acetone removed from the filtrate at room temperature. The resulting emulsion had also no effect on the precipitation of globulin from a mixture of this substance with pure albumin.

Albert Fischer [1932] has recently stated that on the addition of anti-prothrombin (heparin, Howell) to albumin it forms a complex which is identical with genuine globulins when considered in relation to solubility, precipitation with weak acids, denaturation, salting out, solubility in excess of heparin, and isoelectric point.

The claims regarding the effect on denaturation and salting out with Na_2SO_4 could not be confirmed in the author's laboratory with the only heparin preparation available (Parke Davis and Co.).

These failures to affect the precipitability of globulin are merely negative evidence and do not disprove the hypothesis that the increased amount of globulin, as determined by the usual salting out methods after acetone treatment, is due to the removal of lipoids and allied substances. These substances are not present in plasma and serum in the free condition, nor is it probable that the various proteins known as euglobulin, pseudoglobulin and albumin exist in serum as such; they are probably present as one or more large complexes which break down in a manner directed by the relatively small amount of substances associated with the proteins. As to the nature of this process practically nothing is known, though the recent work of Schmitz and Wulkow [1932], who employed spectroscopic methods, suggests that in certain pathological sera not only is the micellar system changed but the structure of the individual components is also altered.

If the adsorption of phosphorus-containing substances is capable of altering materially the solubility of globulin, as is indicated by the work of Chick [1914], it is quite possible that there may also be substances which render the whole group of globulins less amenable to the precipitating action of salts.

There is also some collateral evidence that the usual methods for the determination of albumin and globulin in serum and plasma are not satisfactory. Lang [1930], on estimating the serum-protein-sulphur, found that the results differed from those calculated from the albumin and globulin content: in 100 cases, 46 agreed with the theoretical within 10 % but in the remaining 54, variations up to 50 % were obtained. In the latter either the sulphur content of the individual fractions varied or the estimations of the albumin and globulin were inaccurate. Probably both factors were involved, for whilst the above results throw suspicion on the latter one, some unpublished work of the author on the tryptophan content of pathological plasma suggests that one or more of the proteins has changed "qualitatively."

The above data indicate that whilst the usual salting out methods for the estimation of albumin and globulin may yield accurate results with mixtures of pure proteins, they are unreliable when applied to pathological sera and plasmata and that the results vary with the nature of the salt employed.

The suggested acid-acetone process, while giving slightly low results for albumin in mixtures of pure proteins, is probably just as accurate as salt methods with normal sera and plasmata and gives much smaller errors with pathological samples.

Various factors affecting the standard process.

(1) *Effect of salts.* This was determined by adding varying amounts of salts to 1 cc. of a well dialysed albumin solution and determining, by the standard process, the amount rendered insoluble.

Added salt	% of albumin insoluble
1 cc. 0.9 % NaCl	10
Ringer's solution, 6 cc.	42
" " 3 cc.	32
" " 1 cc.	1
CaCl ₂ (7 mg. per 100 cc.), 1 cc.	Nil
Na ₂ SO ₄ , 100 mg.	10
" 85 "	9
" 65 "	2
" 50 "	1
" 25 "	0.5
" 10 "	0.5

Although the addition of 1 cc. of isotonic NaCl rendered a very appreciable amount of the albumin insoluble in 80 % acid-acetone, the results obtained with Ringer's solution suggested that the salts normally present in plasma and serum would not interfere with the separation of albumin and globulin in such solutions. This was confirmed by the analysis of four samples of plasma before and after dialysis and also by removal of the salts by ultra-filtration.

(2) *Effect of added acid.* This is shown by the following results obtained with oxalated human plasma.

Added acid cc. 20 % CCl ₃ .COOH	p_H centrifuged solution	cc. N/10 acid equal to nitrogen as	
		Globulin	Albumin
Nil	2.0	1.45	—
0.08	1.8	1.95	—
0.17	1.6	2.15	—
0.25	1.5	2.10	4.7
0.30	1.45	2.10	—
Howe's process	—	2.35	4.45

The p_H in this, and in all subsequent work, was estimated colorimetrically and no allowance was made for the protein error. This, however, is very small as the maximum concentration was under 0.4 %.

When the added acid was less than 0.17 cc. (p_H 1.6) the globulin was incompletely removed from solution and the acetone was slightly turbid after centrifuging for 10 mins. 0.25–0.30 cc. always gave bright clear solutions and was the amount adopted in all later work.

(3) *Other acids.* The trichloroacetic acid used in this process is not specific since dichloroacetic acid was found to give equally good results; sulphosalicylic acid, another protein precipitant, gave very poor results.

The comparative results on 1 cc. of human plasma were:

	Protein-N as cc. N/10 acid		
	Trichloroacetic	Dichloroacetic	Sulphosalicylic
Acetone-soluble	3.25	3.40	1.40
Acetone-insoluble	4.55	4.45	6.10
	7.80	7.85	7.50
N.P.N.	0.25	0.25	0.55

It was found that whilst 1.5 cc. of 20 % $\text{CCl}_3\text{.COOH}$ was quite sufficient for the complete precipitation of the protein, about 3 cc. of $\text{CHCl}_2\text{.COOH}$ were required. Sulphosalicylic acid did not precipitate the protein completely and also failed to bring all the albumin into solution. With a solution of pure albumin sulphosalicylic acid acted similarly, only about 60 % of the original albumin being found in the acetone solution.

In the later stage, dichloroacetic acid, but not the more weakly dissociated acetic acid, may be substituted for trichloroacetic acid. It was found that when 1 cc. of a 4 % solution of albumin was mixed with an equal volume of water and 8 cc. of acetone added, complete solution of the precipitate could not be obtained by the addition of acetic acid. When the acetone concentration was reduced the precipitate dissolved completely at a higher p_{H} .

On the addition of a more strongly dissociated acid, HCl , to a solution of albumin and making the acetone content up to 80 % by volume, the protein remained in solution until the p_{H} was reduced below 1.5 and was completely precipitated at about p_{H} 0.7 (*vide* Fig. 1). Przyłęcki [1931], in experiments on the heat-denaturation of serum-albumin in the presence of HCl followed by the addition of alcohol (85 % in final solution), found coagulation at p_{H} 4.5, opalescence at p_{H} 4.2–3.9, clear solutions at p_{H} 3.2–1.4, turbidity when the p_{H} was <1 , and acid coagulation at 0.5–0.7 M .

When trichloroacetic acid was used with 80 % acetone, serum-albumin gave perfectly clear solutions even at p_{H} 1.25 and 0.8 and these remained clear until 14 days later when they became faintly opalescent; after 16 days a gelatinous precipitate formed and slowly settled down.

Solutions of serum-albumin when treated by the standard process also gave at p_{H} 1.6–1.05 clear solutions which were stable for days.

The results indicate that HCl may only be substituted for $\text{CCl}_3\text{.COOH}$ in the second stage of the process if the p_{H} is rigidly controlled within narrow limits. Below p_{H} 1.5 the albumin becomes insoluble and above p_{H} 1.7 globulin becomes soluble. This is well illustrated by the following figures obtained with human plasma.

Added acid		p_{H}	% of total protein insoluble in acetone
$\text{CCl}_3\text{.COOH}$.	20 % 0.3 cc.	1.5–1.6	63
HCl	<i>N</i> 0.37 cc.	1.2–1.3	97
HCl	<i>N</i> 0.25 cc.	1.5	77
HCl	<i>N</i> 0.12 cc.	1.6	69
HCl	<i>N</i> 0.07 cc.	1.7	61
Nil		1.8	55

Paraglobulin (pseudoglobulin) when treated with HCl and 80 % acetone, *i.e.* without preliminary precipitation with CCl_3COOH (afterwards referred to as Process II) is completely precipitated (*vide* Fig. 2). With this concentration of acetone the usual isoelectric point is completely obliterated and clear solutions can only be obtained by the addition of alkali. On reducing the acetone concentration the isoelectric zone appears first as a broad band which narrows to a point on further reduction of the acetone concentration.

Fig. 1. Albumin and 80 % acetone.

Fig. 2. Pseudoglobulin and 80 % acetone. Process II.

When CCl_3COOH is used in Process II with paraglobulin, precipitation commences immediately at about p_H 2.0–2.1 and is complete at about p_H 1.2.

When treated by the standard process, paraglobulin is almost entirely (about 90 %) irreversibly coagulated by the preliminary precipitation and on adding sufficient CCl_3COOH to bring the p_H to 1.6–1.5, the precipitate becomes quite insoluble in 80 % acetone.

The essential facts emerging from these experiments so far as they relate to the standard process are that after precipitation with CCl_3COOH , serum-albumin is completely soluble in 80 % acetone even when the p_H is reduced to <1 by adding CCl_3COOH and that serum-paraglobulin becomes entirely insoluble when the p_H is reduced to 1.6–1.5. These results obtained with pure proteins accord with those obtained with plasma and recorded above. The amount of CCl_3COOH required to ensure the correct p_H is 0.25–0.30 cc. of a 20 % solution per cc. of plasma.

The agreement between the results obtained with pure albumin and paraglobulin on the one hand and with plasma on the other suggest that fibrinogen and euglobulin are also completely precipitated under the conditions obtaining in the standard process.

(4) *Acetone concentration.* At an early stage in these experiments the effect of varying the acetone concentration in the standard process was determined on solutions of serum-albumin and paraglobulin and the results are shown in Figs. 3–6.

In these diagrams the curves marked immediate were drawn from the results obtained by centrifuging the solutions immediately after the acetone had been added and thoroughly mixed with the precipitated protein. The other curves represent the results similarly obtained after the time interval stated.

Fig. 3. Albumin and acetone standard process.

Fig. 4.

Fig. 5.

Fig. 6.

It will be seen (Fig. 6) that whilst globulin is entirely insoluble in all acetone concentrations from 10 to 90 %, the albumin curves (Fig. 3) show a distinctly anomalous feature at the lower concentrations. Between 25 and 30 % acetone the incompletely purified albumin became quite insoluble but only a further 10 % was necessary to produce complete solution.

On the advice of Sir F. G. Hopkins, these experiments were repeated with

the substitution of alcohol for acetone and the results are given in Figs. 4–6, together with similar curves obtained with Process II, *i.e.* without the preliminary precipitation with CCl_3COOH .

It will be seen that all the curves, with the exception of those showing total insolubility at all concentrations, exhibit the same node at about 25–30 % whether the solvent be alcohol or acetone, and the solute albumin or globulin.

The curves suggested a change in the physical properties of the solvent at about 30 % concentration by volume and as surface tension is a factor in precipitation a search was made for relevant data.

Fig. 7.

Morgan and Scarlett [1917] determined the surface tension of acetone-water mixtures by the drop weight method and suggested that the results indicated the formation of a definite compound $(\text{CH}_3)_2\text{CO} \cdot 10\text{H}_2\text{O}$. This was based on the theory of Denison [1912] which, as stated by Morgan and Scarlett, is that “the maximum deviation of the property (surface tension) as observed from that calculated by the mixture law is invariably found to exist at a concentration of the mixture corresponding to a definite simple molecular proportion of the two constituents, and this same concentration continues to exhibit the maximum deviation at all temperatures.” The case of acetone-water mixtures was studied intensively by Morgan and Scarlett and the maximum deviation was found at 24–25 % by weight (30 % by vol.) of acetone and was constant between 0° and 45° .

The surface tension of alcohol-water mixtures has been investigated by Grunmach [1912]. Calculations based on Grunmach’s data give a maximum deviation from the mixture law at 26.5 % of alcohol by weight by the dynamic method and 31 % by weight by the static one. The maximum deviation obtained from the data in the Smithsonian tables, probably derived by a static method, is at 29.5 % by weight.

When the obvious difficulties connected with the protein precipitation method are taken into consideration, the agreement with the surface tension methods is very satisfactory and leaves little doubt as to the cause of the nodes in the various curves.

In the construction of Figs. 3 and 5, two different preparations of albumin were used, one purified by repeated precipitation from half saturated $(\text{NH}_4)_2\text{SO}_4$ followed by prolonged dialysis, and the other prepared in a similar manner but precipitated with cold acetone prior to dialysis. With alcohol as the precipitant (Fig. 5) the two albumins gave very similar results, but with acetone (Figs. 3 and 5) the gelation and precipitation of the purer specimen were very markedly delayed.

The acetone-treated albumin is also less susceptible to precipitation by alcohol in the absence of acids and alkalis (*vide* Fig. 7) and a well purified specimen of serum-albumin requires a high concentration of alcohol for complete precipitation.

In accordance with the experience of Mayer and Terroine [1907] no specimen was obtained which could not be precipitated with alcohol, but it was found that if traces of acid were present even a concentration of 95 % of alcohol produced no precipitate within 5 months.

Various proteins.

An examination of various proteins by the standard acetone process has yielded the following results.

	Acetone-soluble (%)	
	Albumin	Globulin
Human serum	100	Nil
Ox serum	100	"
Sheep serum	100	"
Horse serum	100	"
Egg	Nil	"
Caseinogen		29-30
Globin		65
Haemolysed erythrocytes		43-69

With proteins from mammalian blood the acetone value coincides with the solubility in half saturated $(\text{NH}_4)_2\text{SO}_4$, but egg-albumin behaves anomalously, the acetone solubility being that of a globulin. The reason for this will be discussed later.

Some of the earlier preparations of egg-albumin showed a slight but definite (up to 10 %) solubility by the standard process, but it was later found that by carefully avoiding an excess of acetic acid during the precipitation by the method of Hopkins and Pincus, followed by recrystallisation and a short rapid dialysis through a fairly permeable collodion membrane, the acetone-soluble content could be reduced to less than 1 %.

By adding further acetic acid to the filtrate from the egg-albumin, an acid albumin with 4 % solubility in acid-acetone was obtained and by adding

further ammonium sulphate to the filtrate from this protein a preparation was obtained with an acetone value of 10 %.

These higher values may be due either to the presence of acid albumins with appreciable acetone values or to partial hydrolysis of the egg-albumin during its longer contact with a greater concentration of acid.

Basis of standard process.

The process for the separation of blood-albumin and globulin depends primarily upon the difference in the velocity of the denaturation process and the coagulation of the denatured protein.

When albumin is precipitated with CCl_3COOH under the conditions obtaining in the S.P., the precipitate is entirely soluble in water and does not give a nitroprusside reaction after reduction with cyanide. Serum-globulin, when similarly treated, gives a precipitate which is about 90 % insoluble in water and contains the $-\text{S}-\text{S}-$ group.

These results indicate that whilst globulin is denatured and irreversibly coagulated neither process occurs with albumin within the period of time required to complete the determination.

A slow denaturation of serum-albumin does occur, however, and in one experiment with ox-albumin gelation took place after 54 days and the protein separated out as a precipitate 7 days later. This precipitate gave a marked nitroprusside reaction after reduction with cyanide.

Egg-albumin.

The anomalous behaviour of this albumin when treated by the standard process is apparently due to the rapidity with which it undergoes denaturation and irreversible coagulation during the preliminary treatment with CCl_3COOH . The precipitate so obtained was insoluble in water and gave a marked sulphhydryl reaction.

The complete change of ovalbumin to the coagulated state (denaturation; lyophile-lyophobic) under the influence of urea and urethane in the vicinity of the isoelectric point was found by Hopkins [1931] to proceed much more rapidly than with blood-proteins. With high concentrations of urea he obtained an intense $-\text{S}-\text{S}-$ reaction at once with blood-proteins and suggested that although the chemical changes associated with denaturation proceed at once with blood-proteins the colloids change more slowly than ovalbumin from the lyophile to the lyophobic condition.

The difference in the nitroprusside reactions with blood-proteins may be due to the marked difference in the p_{H} in the experiments, that used by the author being much more acid than was employed by Hopkins and sufficient to convert the proteins into the acid compounds.

If the acid albumin precipitate obtained with CCl_3COOH were dissolved in 80 % alcohol and neutralised, the protein slowly precipitated and gave a definite $-\text{S}-\text{S}-$ reaction.

It is evident that the complete change to the lyophobic state takes place with greater velocity with both electrically neutral and acid ovalbumin than with serum-albumin.

When treated as in Process II, ovalbumin gave very different results with CCl_3COOH ; on diluting 0.5 cc. of a 3 % ovalbumin solution to 2 cc. and adding 8 cc. of acetone followed by 0.25 cc. of 20 % CCl_3COOH , no precipitate was obtained and the liquid remained clear until 48 hours had elapsed. On adding the same amount of CCl_3COOH before the addition of acetone, 39 % of the ovalbumin was precipitated at once.

On adding HCl to the ovalbumin and then acetone, the following results were obtained:

Added acid		p_{H}	% albumin insoluble
HCl	0.3 cc. <i>N</i>	1.25	63
"	0.2 cc. <i>N</i>	1.6	11
"	0.1 cc. <i>N</i>	1.85	Nil
CCl_3COOH	0.1 cc. <i>N</i>	2.1	4

With 0.5 cc. of 3 % ovalbumin and 0.1 cc. of *N* HCl and varying amounts of acetone the results were:

Acetone concentration (%)	% albumin insoluble
80	Nil
85	58
90	100
95	100

Lepeschkin [1922] found that if various acids were tested at the same molar concentration, the denaturation velocity was determined by the H ion concentration and that the nature of the acid had no influence, but the above results with 0.1 *N* HCl and CCl_3COOH indicate that this generalisation does not hold completely and that some additional factor is involved in the case of such an efficient protein precipitant as trichloroacetic acid.

SUMMARY.

1. A process is suggested for the estimation of serum-albumin and globulin by precipitation with trichloroacetic acid and extraction of the precipitate with acidified acetone.
2. Treated by this process, serum-albumin is entirely soluble and serum-globulin is entirely insoluble.
3. The process gives comparable results with those obtained by Howe's method (Na_2SO_4 precipitation) for normal sera and plasmata, but gives lower figures for globulin in pathological specimens.
4. Experiments with acetone extraction indicate that Howe's process gives low globulin results with pathological specimens and that more accurate ones are obtained by the acid-acetone method.
5. In experiments with pure proteins and varying concentrations of acetone (or alcohol) a node was found in the curves at 25-30 % of acetone

(or alcohol) corresponding to the concentration at which the surface tension shows the maximum deviation from the mixture law.

6. All the blood-albumins tested (human, sheep, ox and horse) were entirely soluble in acid-acetone, but egg-albumin was insoluble.

7. The solubility of the various proteins is determined by the rate of denaturation and coagulation in acid-acetone.

REFERENCES.

- Chick (1914). *Biochem. J.* **8**, 404.
Denison (1912). *Trans. Faraday Soc.* **8**, 20.
Fischer (1932). *Biochem. Z.* **244**, 464.
Grunmach (1912). *Ann. Phys.* **38**, F 3, 1018.
Hopkins (1931). *Nature*, **126**, 328, 383.
Howe (1921). *J. Biol. Chem.* **49**, 93.
Knipping and Kowitz (1924). *Z. physiol. Chem.* **135**, 84.
Lang (1930). *Arch. exp. Path. Pharm.* **148**, 221.
Lepeschkin (1922). *Biochem. J.* **16**, 678.
Mayer and Terroine (1907). *Compt. Rend. Soc. Biol.* **62**, 317.
Merklen, LeBreton and Adnot (1931). *Compt. Rend. Acad. Sci.* **192**, 1053.
Morgan and Scarlett (1917). *J. Amer. Chem. Soc.* **39**, 2275.
Przyłęcki (1931). *Biochem. J.* **25**, 713.
Schmitz and Wulkow (1932). *Biochem. Z.* **245**, 408.
Starlinger and Hartl (1925). *Biochem. Z.* **160**, 113.