XLV. THE APPARENT FORMATION OF EU-GLOBULIN FROM PSEUDO-GLOBULIN AND A SUGGESTION AS TO THE RELATIONSHIP BETWEEN THESE TWO PROTEINS IN SERUM.

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(*Received July 15th*, 1914.)

Introduction.

A great difficulty in completely separating water-insoluble protein has been the common experience of all who have attempted to prepare pure salt-free samples of pseudo-globulin. In the course of fractionating horse-serum for viscosity measurements [Chick 1914], I found that, however carefully the euglobulin of the serum had been previously separated, the final product invariably contained more or less water-insoluble protein. Haslam [1913] devoted much attention to this question and came to the conclusion that the presence of the latter was due to incomplete removal of the euglobulin in the original serum. This is to some extent true, but my experience has led me to believe that the bulk of the water-insoluble material cannot be accounted for in this manner, for a protein, having the characteristics of euglobulin, was found to increase progressively in amount as the purification of the pseudo-globulin proceeded. A small amount of water-insoluble protein can usually be detected immediately after the first separation of the pseudo-globulin by salt precipitation and this does appear to consist of euglobulin originally present in the serum. The comparatively large quantity (in one case, Exp. IV, Table III, nearly one-fifth of the total protein) present at the end of a lengthy dialysis appears, on the other hand, to be derived, at least in part, from a transformation of the pseudo-globulin itself. The experiments about to be described show that, under certain circumstances, pseudo-

globulin may undergo change into a protein possessing the properties of euglobulin and suggest that such may very likely be the origin of the euglobulin present in serum.

Origin of the water-insoluble protein found present in pseudo-globulin.

In one set of experiments (Table I) pseudo-globulin was prepared from diluted horse-serum by the following method, rapidly executed. Ammonium sulphate was first added to one-third[1] saturation, the precipitate separated and the filtrate brought to half saturation of ammonium sulphate, this second precipitate being pressed free from mother liquor and afterwards dissolved in a small amount of water. The presence of a small amount of insoluble protein was always apparent on dilution, especially if the solution were adjusted to a definite, slightly acid, reaction.

This insoluble protein we may regard as euglobulin not thrown out from serum by one precipitation in one-third saturated ammonium sulphate for if, in addition to this process, as much as possible of the euglobulin is previously removed by careful acidification of the diluted serum, the water-insoluble protein which can be detected in the precipitated pseudo-globulin is considerably reduced (see Table II).

When pseudo-globulin (horse), after undergoing one or more re-precipitations, is subjected to a lengthy dialysis to remove ammonium sulphate, it is found to contain a comparatively large amount of water-insoluble protein. As a general rule from one-tenth to one-fifth of the total protein is found to be in this condition. This insoluble material exhibits a striking likeness to euglobulin. It is dispersed by small quantities of dilute acid or alkali and when first separated is dissolved by neutral salts, which latter property, as is the case with euglobulin, becomes impaired with increasing age of the preparation.

It has not been possible to determine with certainty the origin of the whole of this "euglobulin." One view is that it is derived from the pseudo-globulin by some process of "denaturation"[2] or degradation taking place

[1] "One-third" and "one-half" saturation indicate the concentrations of ammonium sulphate obtained when a saturated solution of ammonium sulphate is added to twice its volume or an equal volume of water.

[2] "Denaturation" of proteins cannot at present be identified with any chemical changes in the material. The term "denaturated" (German, *denaturiert*) is applied to proteins possessing a certain group of solubility characteristics, including a total insolubility in water at the iso-electric point, in absence of electrolytes. Solution (dispersion) in water occurs in the presence of acid, alkali or electrolytes, and is associated with the acquisition of an electric charge by the protein particles, while the system displays a high degree of viscosity. The best-known example is that

after separation from the serum and the results of Exps. I–IV set forth in Tables I–III may, on the whole, be said to support this view. On the other hand, it may be urged that the results of these experiments do not necessarily prove that dialysis of salt-precipitated pseudo-globulin is accompanied by its "denaturation," but that removal of all salt may be essential to permit complete precipitation of any "denaturated" protein (euglobulin) present from the beginning. If this is the case, the concentration of euglobulin in serum must be far higher (two or three times) than is generally believed and the usual method of separation, by dilution and acidification, very inadequate.

In order to throw light upon this point, some experiments were made in order to ascertain with what degree of accuracy water-insoluble protein can be estimated in presence of salts and other soluble protein. In one experiment euglobulin was mixed with a sample of thoroughly dialysed pseudo-globulin (from which all insoluble protein had been separated) and ammonium sulphate added; the mixture contained 0·462 % euglobulin, 2·02 % pseudo-globulin and 1·2 % ammonium sulphate. The concentration of insoluble protein was then estimated by the dilution and acidification method described in detail below. The value obtained was 0·337 %, or 73 % of that added. The concentrations of protein and salt in the final acidified, and diluted, solution were 0·048 % and 0·024 % respectively.

A second experiment was made with a thoroughly dialysed pseudo-globulin solution containing 4·77 % total protein, of which about one-seventh was in the "denaturated" condition and present as a precipitate. Estimations of the insoluble protein present in 10 cc. of the above solution were made both before and after addition of 5 cc. of strong ammonium sulphate (10·3 %). In the control, dilution 50-fold, the proportion of insoluble protein was found to be 0·70 %, in the presence of ammonium sulphate the estimated concentration was only 0·23 % and 0·26 % when diluted 50-fold and 100-fold respectively. In this experiment the final concentration of protein and ammonium sulphate was 0·10 % and 0·093 % respectively in the 50-fold dilution and half these amounts in the 100-fold dilution. The proportion of the total insoluble protein which could be precipitated under these conditions was 33 % and 37 % in the two cases.

of proteins which have been acted upon by hot water [see Chick and Martin 1910, 1911 and 1912]. The vegetable globulins and the euglobulin naturally occurring in serum may be regarded as "denaturated" proteins [see Michaelis and Rona 1910, 2]; their property of forming electrically-neutral solutions with certain salts is, however, a special characteristic not shared by heat-"denaturated" proteins.

When whole horse-serum is diluted ten times, as is usual when euglobulin is separated from serum, the total concentration of protein is from 0·6 % to 0·8 % and that of sodium chloride from about 0·08 % to 0·07 %. Under these circumstances, it is quite possible for some euglobulin to escape precipitation when the diluted serum is adjusted to the iso-electric point of euglobulin by the addition of acetic acid. On the other hand, it must be remembered that a chloride is a less efficient solvent for euglobulin than a sulphate [see Mellanby 1905]. Further, there is, as a rule, no suggestion of incompleteness about the process. The euglobulin usually settles with comparative ease, leaving a clear liquid on top.

The results of Exp. III, Table II also throw some light upon this question. In this case a solution of pseudo-globulin made immediately after its precipitation from the horse-serum contained 5·2 % ammonium sulphate, 12·86 % total protein, and only a trace of water-insoluble protein, when subjected to analysis by the method described below. Part of the material was kept in the laboratory as a control and part was dialysed, toluene being present in both cases to prevent decomposition. At the end of two months the water-insoluble material in the control was equal to 8·6 % of the total protein. In the portion which had been dialysed for 51 days the proportion estimated was 14·1 %. This experiment shows that water-insoluble protein, if present, can be detected in presence of electrolytes and other soluble protein. Therefore, a large proportion, at least 61 % (8·6 parts out of a total of 14·1) of the euglobulin found present at the close of dialysis must be regarded as formed from the pseudo-globulin by some process of denaturation taking place after separation from the serum. The remaining 39 % may, on the other hand, be regarded as euglobulin which was present from the first, although it could not be separated in presence of salt. But, from the fact that immediately after preparation only a trace of insoluble protein could be detected in the salt-containing pseudo-globulin, it seems probable that part of this may also be derived from the pseudo-globulin, showing that the denaturation of this protein is accelerated by dialysis.

Experimental methods.

In the experiments set forth in Tables I to III pseudo-globulin was prepared in various ways by salt-precipitation from horse-serum, the details of themethod employed being given at the top of each table. Estimations of the denaturated protein were made before, after, and, in some cases

(Experiments I and II, Table I), during the dialysis, by determining the amount of protein which was precipitated by dilution and adjustment to the iso-electric point.

The method of estimation was as follows. A small portion of the material was taken for a preliminary trial, and largely diluted, so that the protein-content did not exceed 0·1 or 0·2 %. If electrolytes are present the dilution should be as large as possible. 10 cc. of this diluted solution were then placed in each of a series of test tubes and various small quantities of N/100 acetic acid added. If any denaturated protein were present an opalescence or slight turbidity was usually apparent on mere dilution and, when the solution was adjusted to the iso-electric point of the protein by addition of the required amount of acid, a precipitate was formed which aggregated and settled. The series of trial test tubes was always allowed to stand several hours and the composition of the material in the tube in which the precipitate was largest and agglutination most perfect was taken as the pattern for the estimation.

5, 10, or 15 cc. (according to the protein-content) were taken for analysis, diluted suitably and addition made of dilute (N/10 or N/100) acetic acid in the amount necessary for complete precipitation of the denaturated protein, as calculated from the results of the preliminary trial. The whole was allowed to stand 24 hours and then centrifuged, the deposit being transferred to a small, weighed, centrifuge tube, spun down again, washed with distilled water slightly acidified and centrifuged a third time. The final deposit was dried in the weighed tube at 105° until constant in weight.

It is not advisable to permit the estimation to extend over more than one day, as, in some cases, the denaturation of the pseudo-globulin seems to be continued at an appreciable rate in the dilute solution and, when several days are spent over the estimation, higher values are frequently obtained.

If the material to be analysed is slightly acid, small quantities of dilute ammonia must be substituted for the dilute acetic acid. In cases where the material has been dialysed for a long time and the reaction is approaching that of the iso-electric point, dilution with distilled water containing about one-thirtieth of its volume of water saturated with carbon dioxide is often found to be a quick and efficient method of precipitating all the denaturated protein.

If electrolytes are present, denaturated protein may be detected by the above method, but precipitation, under certain circumstances, is incomplete (see above p. 405). In any case it is advisable to make the dilution as great as is possible.

The above method has been described in detail, because the estimation of denaturated protein in presence of other, water-soluble, protein is by no means an easy matter even in the absence of electrolytes, the difficulty being to ensure complete precipitation of the former. When this has not been accomplished, faint turbidity can be detected in the supernatant fluid after centrifuging; in a successful estimation the aggregation of the precipitate should be complete and the top liquor clear. Some of the earlier values for concentration of "denaturated" proteins given in Tables I to III were obtained when the method was not perfected and may be a little inaccurate; the later ones were more satisfactory, as may be seen in some cases where the results of duplicate analysis are inserted.

It was possible that this water-insoluble protein, like euglobulin [Freund and Joachim 1902, Gibson 1906, Banzhaf 1911], might be thrown out by addition of sodium chloride to saturation, and this would have proved a convenient method of separation and estimation. It was tried in Exp. II,

Table I, but without success. After diluting the material 10-fold and saturating with sodium chloride the residual protein (calculated on the original, undiluted, material) was 10·78 %, the total being 11·12 %; that is to say only 0·34 % protein had been thrown down. The concentration of "denaturated" protein as separated by the above method was, however, found to be 0·96 %.

Precipitation by saturated sodium chloride is of little value for the accurate separation of proteins, the amount precipitated depending, to a greater degree than is the case with other salts, upon external conditions, such as the total concentration of protein and the reaction of the material. For example, on one occasion, with horse-serum, the precipitate obtained on saturation with sodium chloride was four times greater when the serum was diluted 1 in 10 than when the dilution was only 1 in 2; in the latter case the higher concentration of total protein protected more than three-quarters of the otherwise precipitable protein. Further, if diluted serum is made slightly acid in reaction, the whole of the pseudo-globulin and albumin is precipitated as well as the euglobulin.

Discussion of Results.

The results of the experiments in Tables I–III suggest that the pseudo-globulin undergoes a progressive denaturation after its separation from the serum. For example, in Exp. I, Table I, the pseudo-globulin immediately after precipitation from the serum showed only a small amount of water-insoluble protein capable of separation by dilution and adjustment of the reaction to the iso-electric point. At the end of dialysis for 10 and 18 days, however, 9·1 % and 12·9 % respectively, of the total protein was found to be in the insoluble condition. It is probable that the ammonium sulphate present interfered with a complete separation of any euglobulin present at the beginning of the experiment, but it is unlikely that the difference in amount of the traces of salt present after 10 and 18 days' dialysis respectively could be responsible for the large difference in amount of insoluble protein found on these two occasions.

Experiment IV, Table III is a second instance. Here material dialysed for five days against tap water contained 11·1 % denaturated protein; after a further dialysis against distilled water lasting 29 days this proportion was increased to 18·1 %.

TABLE I.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared by half-saturation of undiluted horse-serum with ammonium sulphate, after separation of euglobulin by one-third saturation with ammonium sulphate.

Exp.	Material	Date of analysis	Length of dialysis, days	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein: in water-soluble fraction	Phosphorus-content, g. P per 100 g. protein: in water-insoluble fraction
I.	Prepared 2. 12. 13; re-precipitated twice; final precipitate dissolved in water. Dialysed in presence of toluene against distilled water, changed daily, from 5. 12. 13 to 23. 12. 13	5. 12. 13			small amount			
		15. 12. 13	10	14·56	1·33	9·11		
		14. 3. 14[1]	18	13·55	1·75	12·9	0·018	0·171
II.	As in Exp. I but not re-precipitated	6. 12. 13	3	11·17	present			
		26. 1. 14[2]	3	11·12	0·964	8·67		
	(*a*) dialysed against dist. water from 13. 2. 14 to 25. 3. 14	3. 4. 14[3]	43	4·77	0·654	13·7		
		9. 7. 14	43	4·77	0·700	14·7		
	(*b*) as above, but dialysed against dist. water saturated with CO_2 from 13. 2. 14 to 25. 3. 14	26. 3. 14	43	5·51	{0·652 / 0·660}[4]	11·9		

[1] Kept in cold room from 23. 12. 13 until 14. 3. 14.
[2] Kept in cold room from 6. 12. 13 until 13. 2. 14; dialysis then recommenced.
[3] Kept in cold room from 25. 3. 14 until 9. 7. 14.
[4] Duplicate analyses.

TABLE II.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared from diluted (1 *in* 20) *horse-serum by half-saturation with ammonium sulphate, after successive removal of euglobulin precipitates obtained* (1) *by acidification of the serum* (1 *in* 10 *dilution*), *and* (2) *by one-third saturation of the serum* (1 *in* 15 *dilution*) *with ammonium sulphate: once re-precipitated and dissolved in a small quantity of water.*

Material	Date of analysis	Length of dialysis, days	Concentration of $(NH_4)_2SO_4$, %	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein: in water-soluble fraction	Phosphorus-content, g. P per 100 g. protein: in water-insoluble fraction
Control, immediately after preparation	4. 2. 14	—	5·23	12·86	trace	—	—	—
(*a*) Control, allowed to remain at laboratory temperature, in presence of toluene	3. 4. 14	—	5·23	2·86	1·107	8·61	—	—
(*b*) Dialysed against distilled water, changed daily, in presence of toluene from 2. 2. 14 to 25. 3. 14	30. 3. 14	51	—	4·86	{0·688 / 0·681}[1]	14·1	0·012	0·262

[1] Duplicate analyses.

TABLE III.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared from horse-serum (diluted 1 in 4) by precipitation with one-half saturated ammonium sulphate, this first precipitate extracted with saturated brine, brine-extract precipitated by addition of acetic acid to a concentration of 0·25 %[1]; this second precipitate drained, pressed and mixed with washing soda to a concentration of 3 % and dialysed for five days against tap water.

Material	Date of analysis	Length of dialysis, days	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein: in water-soluble fraction	Phosphorus-content, g. P per 100 g. protein: in water-insoluble fraction
Control	3. 3. 14	(5)[2]	11·72	1·301	11·10	—	—
(*a*) Dialysed further against dist. water, changed daily, in presence of toluene from 6. 3. 14 to 4. 4. 14	4. 4. 14	29 (+5)	5·87	1·055	17·98	0·0062*	0·156
(*b*) as (*a*) the distilled water being saturated with CO from 6. 3. 14 to 4. 4. 14	4. 4. 14	29 (+5)	6·40	1·161	18·14	0·0066*	0·098

[1] Gibson's [1906] process.
[2] Against tap water.
* Approximate only (less than 1 cc. N/10 NaOH neutralised).

The results of Experiments I to IV also suggest in every case that the degradation of the pseudo-globulin is approaching a limit. For example, in Exp. II, Table I, after three days' dialysis 8·7 % of the protein was found to be water-insoluble. After an interval of more than two months, including a second dialysis lasting 40 days, the proportion had only risen to 13·7 % and, after having been kept in the refrigerator for a further period of over three months, to 14·7 %. A similar result is shown in Exp. I. In many cases, the experiments were continued beyond the date of the last analysis given in the tables, and the denaturation process was found to be practically at an end. The degraded material was separated as completely as possible by a modification of the method described above, and the clear, top liquor adjusted to the iso-electric point of the denaturated protein and kept for a considerable time in the cold room under observation. In the case of Exps. III and IV (*a* and *b*), only a trace of insoluble material was deposited after periods of two months and one month respectively.

Phosphorus-content of this water-insoluble pseudo-globulin.

The resemblance between the denaturated pseudo-globulin obtained in these experiments and euglobulin led me to make estimations of the phosphorus contained respectively in the insoluble and unchanged pseudo-globulin.

Hardy [1905] demonstrated the presence of phosphorus (0·07 to 0·08 %) in euglobulin (ox-serum) and considered it to be one of the characteristics marking off this protein as a chemical entity. The pseudo-globulin (separated from the serum by saturation with magnesium sulphate) he found to contain a trace only (about 0·009 %) of phosphorus. Haslam [1913] also found phosphorus to be a constant constituent of euglobulin (ox-serum), even after repeated purification, and to be absent from the purest samples of pseudo-globulin which he was able to obtain. In consequence, he used the absence of phosphorus as a criterion of the purity of pseudo-globulin.

In the present case phosphorus was determined by Neumann's method, using N/10 alkali and acid for the final titration, the quantity to be estimated being very small. In some cases where the ammonium molybdate precipitate was only just visible and the total amount of standard sodium hydrate neutralised less than 1 cc., the values obtained must be regarded as approximate only; such values are indicated in the tables by an asterisk.

The results of the phosphorus estimations showed the resemblance between

the denaturated pseudo-globulin and euglobulin to be maintained in this respect also. The former was found to remove almost the whole of the small amount of phosphorus contained in the original pseudo-globulin preparation. In Exp. I, Table I, the water-insoluble protein at the close of the experiment contained nearly 10 times, in Exp. III, Table II, more than 20 times, as much phosphorus as the unchanged pseudo-globulin; in both cases that present in the latter was reduced to a mere trace. In Exp. IV the contrast was even greater; the denaturated pseudo-globulin contained 0·16 % phosphorus, while that remaining with the soluble protein was too small in amount to be estimated with any degree of accuracy.

Two different explanations might be advanced to resume these facts. According to the first, the process described in the last paragraph is merely the separation of the last traces of phosphorus-containing euglobulin derived from the original serum and present from the beginning. On this theory the cessation of the gradual precipitation of insoluble protein would naturally coincide with the disappearance of the phosphorus originally present in the solution. On the other hand, we may regard this precipitate containing protein and phosphorus, which gradually makes its appearance, as the product of a gradual "denaturation" of the pseudo-globulin which requires the co-operation of some phosphorus-containing body (probably a serum-lipoid) which is present in small quantity in the preparations employed. When this serum-lipoid is exhausted, the process is at an end.

The second explanation would appear to be the correct one for the following reason. After the removal of phosphorus from the pseudo-globulin solution, by the separation of the insoluble protein precipitate, addition of a weak emulsion of lecithin, in presence of salt, causes additional formation of a protein resembling euglobulin which can be precipitated on subsequent dialysis.

Handovsky and Wagner [1911] showed that, when emulsions of lecithin, and other lipoids extracted from serum, were added to dialysed serum, a precipitation of the protein took place, which was prevented if salts were present. I have been able to confirm these observations. Addition of a dilute lecithin emulsion produces only slight turbidity when added to solutions of pure egg- or serum-albumin, a fact also noticed by Handovsky and Wagner. In the case of a dialysed pseudo-globulin, an immediate precipitation of protein takes place, which is prevented if a small concentration of salt (1 % sodium chloride), acid, or alkali is present.

I have prepared artificial euglobulin from pseudo-globulin in the following

manner. A small amount of a watery emulsion of lecithin [1] was added to the salt-free material obtained at the close of Exps. III and IV, in which the denaturation process had ceased and from which the insoluble protein had been separated. An immediate precipitate occurred, which was prevented by the presence of alkali in minute proportion or of salt (NaCl), to a concentration of about 1 %. On subjecting the solution to dialysis for 14 days precipitation occurred of a protein, containing phosphorus, and with the characteristics of euglobulin.

Suggestions as to the origin of euglobulin in serum.

On consideration of the results of the foregoing experiments it seems not improbable that euglobulin in serum is a complex material, formed from pseudo-globulin by association with some serum-lipoid, to the presence of which it owes its phosphorus-content.

Hardy [1905], on the other hand, has regarded phosphorus as an integral part of the euglobulin molecule. He [1905, p. 331] was unable to remove all the phosphorus from heat-coagulated euglobulin (ox-serum) by treatment with strong acetic acid and subsequent extraction with alcohol and ether. He therefore concluded that it could not be "due to entangled lecithin." Haslam [1913, p. 514] found that extraction of euglobulin (ox-serum) with boiling alcohol removed a yellow, fatty substance which contained phosphorus, the proportion of the latter remaining in the euglobulin being reduced to about one-half the original. It is, however, quite possible that complete extraction with alcohol or ether is rendered very difficult by the state of aggregation of the protein and that if fresh surfaces could in succession be exposed to the action of these solvents a larger proportion of phosphorus could be removed.

In this connection it is worthy of note that the available analyses of euglobulin, some of which are collected in Table IV, show no approach to constancy in the proportion of phosphorus present. Hardy found 0·07 to 0·08 % phosphorus in euglobulin prepared from ox-serum by dilution and acidification; Haslam found 0·108 % and 0·105 % respectively present in two different samples. In three specimens prepared by the same method from three separate samples of horse-serum, I found 0·12 %, 0·032 %, and 0·065 % phosphorus respectively.

[1] For this preparation I am indebted to the kindness of Dr H. Maclean. The lecithin was prepared by his own method [1914] from heart muscle.

TABLE IV.

Phosphorus-content of euglolubin.

No.	Description of material and mode of preparation from serum	Method	G. phosphorus per 100 grms. protein	mean	Authority
1.	Euglobulin (ox-serum) by dilution and acidification	Neumann	0·11 0·11	0·11	Haslam
2.	Euglobulin (ox-serum) by dilution and acidification	Carius	0·07 0·08	0·075	Hardy
3.	Euglobulin (horse-serum *A*) by dilution and acidification once re-precipitated and washed	Neumann	0·122 0·117	0·119	Chick
4.	Euglobulin (horse-serum *A*) after three subsequent re-precipitations	Neumann	0·092 0·096	0·094	Chick
5.	Euglobulin (horse-serum *A*) as (4), but after extraction of the euglobulin suspension with acetone followed by dry ether	Neumann	0·019* 0·020	0·020	Chick
6.	Euglobulin (horse-serum *B*) by dilution and acidification, twice re-precipitated	Neumann	0·033 0·032	0·032	Chick
7.	Euglobulin (horse-serum *C*) by dilution and acidification. four times reprecipitated	Neumann	0·069 0·062	0·065	Chick

* Less than 1 cc. of N/10 NaOH neutralised.

TABLE V.

Proportion of phosphorus contained in a sample of whole horse-serum and in the various proteins after separation by different methods.

No.	Material	G. P. per 100 g. protein	Mean
1.	Horse-serum; whole serum dried in vacuo at 37°	0·098 0·103	0·100
2.	Total proteins of horse-serum, precipitated by dropping into a large volume of absolute alcohol; washed with alcohol and ether; and dried at 105°	0·027 0·018*	0·027
3.	Total proteins coagulated in hot water, coagulum washed with water, alcohol and ether; dried at 105°	0·004* 0·008*	0·006*
4.	Euglobulin prepared from No. 6 by one-third saturation with ammonium sulphate (re-precipitated six times)	0·071	0·071
5.	Euglobulin prepared by dilution and acidification of the original serum (re-precipitated four times and washed twice with distilled water)	0·069 0·062	0·065
6.	Total globulin, prepared by one-half saturation with ammonium sulphate (re-precipitated six times)	0·023	0·023
7.	Serum albumin precipitated in the filtrate from No. 6 by saturation with ammonium sulphate (re-precipitated five times)	0·032	0·032

* Approximate only, less than 1 cc. N/10 NaOH neutralised.

A further set of analyses, see Table V, was made of the total globulin, and euglobulin of horse-serum, as well as the total protein precipitated by various methods. The specimens used were all prepared by Dr P. Hartley with great precautions and carefully purified and I am greatly indebted to him for generously placing this valuable material at my disposal. The results showed that none was free from phosphorus. In some cases the proportion contained showed great variation according to the method employed in preparation (*e.g.* nos. 1, 2, and 3, Table V). The euglobulin contained the greatest amount of phosphorus, and the content was about the same whether precipitated with ammonium sulphate or by dilution and acidification of the serum. This would indicate that the phosphorus contained in euglobulin was in closer association than the traces found present in the purified samples of the other proteins.

Any direct evidence regarding the character of the union of phosphorus in euglobulin is at present scanty, but the following results are worthy of consideration. Haslam [1913] was able to remove about half the total phosphorus from a sample of euglobulin by means of extraction with alcohol and ether. Absolute alcohol must, however, in case of proteins be regarded as a powerful reagent, causing serious changes ; all are rendered permanently insoluble if contact is long enough and the temperature is allowed to rise above a low maximum. If previous to extraction with ether, acetone[1], in place of alcohol, is employed to remove the water, no damage appears to be suffered.

The material can then be extracted with ether, three or four changes being employed to remove completely all acetone, and the euglobulin remaining in contact with ether at room temperature for one or two days. The last ether extract being removed, the euglobulin is shaken up once more with ether, which is evaporated by pouring the euglobulin suspension on to a warmed porcelain basin and allowing a gentle current of warm air to play over it, the whole operation taking place in a hot room (about 36°). The extracted euglobulin is then obtained as a fine, white powder, which is exceedingly hygroscopic. The solvents employed should all be carefully purified and freed from water.

The extracted euglobulin was found to have lost nearly all its phosphorus ; on one occasion it was reduced to less than one-quarter of the original (see Table V, original phosphorus-content 0·094 % ; final 0·02 %). It is possible that the care taken to free all materials from water may account for the

[1] Acetone can be employed to remove water from the proteins of whole serum, withou impairing their solubility in water.

large proportion of the phosphorus removed from the euglobulin in these experiments when compared with those of Haslam.

The ether-extracted euglobulin, like that treated by acetone, was readily soluble in water containing a small amount of acid or alkali. Its solubility in dilute salt solution was, however, always found to be reduced to a greater or less extent. On one occasion about 13 % was found to be soluble in dilute sodium chloride (0·85 %); in most cases the ether-extracted material was totally insoluble in salt solution.

The acetone and ether extracts, when taken to dryness, were found to contain fatty substances, presumably the lipoids contained in the original euglobulin. These fatty materials, which contained a considerable proportion of phosphorus, amounted in one instance to as much as 13·3 % by weight of the protein taken for the experiment. On one occasion, when the extracted euglobulin was rubbed up in a mortar with the fatty residue and salt solution, the power of salt solubility was found to have been restored to some extent, and a similar result was obtained with petrol-extracted euglobulin and the residue from the petrol extract. These two experiments were made with the same sample of euglobulin. With a second sample of euglobulin the experiment was unsuccessful and with a third a trace only was rendered soluble in dilute salt solution by this means. In the case of the first, successful, experiment a large proportion of the lipoid extracted from a comparatively large quantity of euglobulin was devoted to a small fraction of the treated protein and this procedure appears to be necessary to obtain any union and consequent restoration of salt solubility.

Some experiments with ether-extracted euglobulin were also made in which a lecithin emulsion was substituted for the fatty substance extracted from the euglobulin itself. In some instances the lecithin-treated material had distinctly greater solubility in sodium chloride, in others little or no difference from the control could be demonstrated. The question is still under investigation.

The evidence contained in the preceding paragraphs in favour of regarding euglobulin as a mechanical complex consisting of a protein (pseudo-globulin) and a lipoid may briefly be summarised as follows. The phosphorus-content is quite inconstant and shows wide variation in specimens prepared from different samples of serum. By extraction with acetone and ether, under certain circumstances, almost all the phosphorus can be removed together with a small amount of a fatty body. By this procedure the euglobulin, otherwise unchanged, loses its property of salt solubility, which characteristic

has, in some cases, been restored by addition of a lipoid to the ether-extracted material.

As regards the nature of this complex, euglobulin in serum must, in my opinion, be regarded as the product of the interaction of two colloidal systems, viz. the colloidal solution of pseudo-globulin and the emulsion of some lipoid present in the serum. The process is not reversible. The serum-lipoid remains firmly associated with the protein, and cannot be separated by repeated dispersion and re-precipitation, but can be removed by extraction with acetone followed by ether. The freed protein is in the "denaturated" condition, the pseudo-globulin having apparently undergone this irreversible change in the first instance under the influence of the lipoid.

Walpole [1914] has pointed out that, in some respects, euglobulin shows analogies with certain artificial colloidal systems which he has investigated, as, for example, solutions of gelatin to which suspensions of mastic or emulsions of oil have been added. In these mixtures of two colloidal constituents with widely separated iso-electric points, the dual nature of the system is apparent in the want of accurate coincidence between the iso-electric point and that of optimum flocculation. The same phenomenon has been demonstrated in the case of euglobulin [Chick 1913] and may doubtless be similarly explained. Feinschmidt [1912] determined the iso-electric point of six samples of lipoid from various sources to lie at a concentration of hydrogen-ions between about 10^{-2} and $10^{-3\cdot7}$ normal. The iso-electric point of the protein, on the other hand, is at a concentration of hydrogen-ions somewhere between 10^{-5} and 10^{-6} normal. [Michaelis and Rona 1910, 1, Michaelis and Davidsohn 1911, Sörensen and Jürgensen 1911, Chick and Martin 1912.]

The identification of the protein in euglobulin with pseudo-globulin is justified on chemical grounds by some recent work of Hartley[1]. He has shown that on examination of the three proteins (euglobulin, pseudo-globulin and albumin) of serum (horse and ox) by van Slyke's method, the composition of pseudo-globulin and euglobulin is found to be practically the same while a marked difference exists in case of serum albumin.

Summary.

1. Pseudo-globulin, prepared from diluted serum by precipitation with ammonium sulphate, the euglobulin having been previously removed as far as possible by dilution and bringing the solution to the iso-electric point,

[1] Communicated to the Physiological Society, Feb. 14, 1914 (Lister Institute, London) and to the Biochemical Society, July 11, 1914 (Oxford).

was always found, at the end of a long dialysis, to contain large quantities of a water-insoluble protein, resembling euglobulin.

2. Evidence is adduced to show that this insoluble material is not merely euglobulin present in the original serum and precipitated on dialysis, but is largely derived from the pseudo-globulin by a gradual process of " denaturation."

3. The gradual precipitation of the insoluble protein is accompanied by a disappearance from the solution of the small amount of phosphorus originally present in the pseudo-globulin preparation, after which there is no further separation of water-insoluble protein.

4. On addition of a watery emulsion of lecithin to thoroughly dialysed pseudo-globulin, from which insoluble protein and phosphorus had been removed as above, the " denaturation " process could be re-initiated.

5. The presence of minute amounts of acid and alkali or of a small concentration of salt (e.g. 1 % sodium chloride) prevents the precipitation of pseudo-globulin by a watery lecithin emulsion but, on dialysis, an insoluble protein, containing phosphorus and displaying the properties of euglobulin, is separated.

6. This artificial euglobulin appears to be a mechanical complex resulting from the interaction and mutual precipitation of the two colloidal systems : (*a*) the solution of pseudo-globulin and (*b*) the lipoid emulsion.

7. It is suggested that the euglobulin in serum is a protein-lipoid complex of similar origin. In support of this view the following facts are brought forward :

(*a*) The inconstant phosphorus-content.

(*b*) The readiness with which phosphorus can be removed from euglobulin by extraction with acetone and ether, during which process the property of salt solubility is lost. This can, in some cases, be restored by addition of the extracted fatty substance or of lecithin to the extracted euglobulin.

(*c*) The want of accurate coincidence between the iso-electric point of euglobulin and the point of optimum flocculation, as pointed out by Walpole [1914].

(*d*) The similarity in chemical composition between pseudo-globulin and euglobulin, as shown by Hartley.

In conclusion I desire to express my indebtedness to Prof. C. J. Martin, F.R.S. for valuable criticism.

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