ON THE REAL NATURE OF THE SO-CALLED ARTIFICIAL GLOBULIN.

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A number of observations have been made during the last few years which tend to show that albumins can be converted into globulins. Starke studied the action of heat on diluted egg white and concluded that all the albumin could thereby be transformed into ovoglobulin. A similar change occurred, according to Moll, when the serum of the rabbit or of the horse was incubated at 55°C., the transformation being dependent on the presence of free hydroxyl ions. The various proteins were identified by their solubilities in water and salt solutions, their elementary composition, and particularly by their sulphur content. Moll found that the percentage of sulphur in the body formed artificially from albumin was much less than that in serum albumin, whilst it approximated roughly to that in natural serum globulin.

A conversion of albumin into globulin has been recorded by Sikes as occurring in albuminous urine on standing, and the alteration in the composition of blood serum following the administration of antipyrine has also been ascribed by Cervello and by Breinl to a similar cause. Morawitz has invoked the aid of the same hypothesis to account for the primary appearance of serum albumin during the re-formation of the blood proteins after hæmorrhage, whilst the observations of Wiener on the composition of arterial and venous blood have led him to suggest that this change from serum albumin to serum globulin is effected by the agency of the tissue cells.

Quite recently, Kämmerer and Aubry have confirmed Moll's original observations. They state that on warming serum for half an hour, the precipitation zones of the proteins are altered, *i.e.* the albumin is converted into globulin.

These views are opposed to those of Abderhalden and Hammarsten who contend that the proteins of the body have a specific

structure and that no direct change of one into the other takes place. A consideration of the analyses which have been made of serum albumin and serum globulin makes it clear that the transformation of the one into the other can only be brought about by radical changes resulting in, firstly, the appearance of glycocoll and of carbohydrate groups, secondly, a diminution in sulphur-containing radicles, and thirdly, the introduction of phosphorus from some unknown source. Gibson has already shown by the application of Hausmann's process that the distribution of nitrogen in the natural and artificial products does not support the view in favour of their identity, and in the following pages we give some direct evidence on the matter, based on the determination of the content of carbohydrate, phosphorus, and sulphur in the protein substances under consideration.

Preparation and analysis of serum albumin, serum globulin and the artificial globulin.

Serum albumin. Horse blood was procured, which had been prevented from clotting by the addition of 1 % sodium citrate. The plasma was obtained free from corpuscles either by centrifugalising or by allowing it to stand over night until the corpuscles had subsided. The plasma was then siphoned off. Six to seven litres at a time were thus treated. The serum globulin and fibrinogen were removed by adding an equal bulk of saturated ammonium sulphate solution and filtering. From the filtrate the serum albumin was crystallised out by Gürber's method; that is, by adding saturated ammonium sulphate solution till a slight turbidity is produced, re-dissolving this in the least possible amount of water and then adding 10 % sulphuric acid till distinctly turbid and allowing to stand. After 12–24 hours the crystals were filtered off on a Buchner funnel. The crystals were re-dissolved in water and ammonium sulphate again added till a slight turbidity appeared and then acid added as before. The serum albumin thus re-crystallised was dissolved in water, placed in sausage skins of vegetable parchment and dialysed against running tap water for two days, followed by two days against running distilled water. The albumin solution remaining in the dialyser was then filtered, diluted with water and dilute acetic acid added until the re-action was just acid to litmus. It was then coagulated by boiling and the coagulated albumin filtered on a Buchner filter and washed with boiling water until the filtrate did not give the slightest turbidity with barium chloride solution. This washing required at least four days. Boiling alcohol was next poured over it,

and then, after drying and powdering, it was extracted with ether for 24 hours. The product after powdering was then submitted to analysis.

The sulphur content of the serum albumin was determined by Wolf and Österberg's modification of Benedict's method as this admits, at the same time, of testing for phosphorus in the filtrate from barium sulphate. In no case, however, was any phosphorus precipitate detected. The carbohydrate was estimated by Pavy's potash method in which the protein is hydrolysed with 10 % KOH for half an hour and poured into ten volumes of alcohol. The precipitated carbohydrate is dissolved and hydrolysed with 5 % hydrochloric acid for 90 minutes, neutralised, boiled with alumina, made up to a definite volume and filtered through a dry filter. The glucose in the filtrate is determined by Pavy's solution.

1. 1·3612 g. serum albumin gave 0·2048 g. $BaSO_4$.
2. 1·5403 g. ,, ,, 0·2214 g. ,,
3. 1·8921 g. ,, ,, 0·2747 g. ,,
4. 1·6420 g. ,, ,, 0·2516 g. ,,
5. 7·2160 g. ,, ,, 0·0156 g. Dextrose.
6. 8·3200 g. ,, ,, 0·0243 g. ,,

Composition of serum albumin.

	Sulphur %	Carbohydrate %	Phosphorus %
	2·05	0·21	0
	1·96	0·28	0
	1·99	—	—
	2·14	—	—
Mean	2·04	0·25	0

The results obtained by other observers with reference to the sulphur content of serum albumin are as follows:—

Michel 1·90 %, Hammarsten and K. Starke 1·84 %, L. Moll 1·93 % to 2·61 %. It may therefore be concluded that the sulphur content of serum albumin (horse) is about 2 %. The value 2·61 % given by Moll is exceptionally high and unconfirmed by other workers.

The amount of carbohydrate present in serum albumin has been found to average 0·25 %. This amount may be taken as the maximum amount present in serum albumin only twice crystallised, since the results of Abderhalden, Bergell and Dörpinghaus seem to show conclusively that even this small amount is due to impurity. The last mentioned observers have prepared a serum albumin which did not respond to Molisch's very delicate reaction.

Serum globulin. Horse blood was used which had been allowed to clot. After 24 hours the clear serum was taken and ammonium

sulphate added to half saturation. The globulin was filtered off, dissolved in water and re-precipitated with ammonium sulphate. This process was repeated three times. The solution in water was then acidified with dilute acetic acid, heated to boiling, and the coagulated globulin washed with boiling water till free from sulphates. In one case the washing was continued for 14 days; in others for at least five days. After treatment with hot alcohol, powdering and drying, it was extracted with ether for 24 hours, and again dried in a desiccator over sulphuric acid.

The sulphur and carbohydrate in the product were estimated in the same manner as has been described in the case of serum albumin.

The presence of phosphorus in serum globulin was first noticed by Hardy. This has been confirmed by fusing serum globulin with fusion mixture, dissolving, and testing filtered solution with ammonium molybdate. A slight yellow precipitate showed the presence of phosphorus.

1.	1·3132 g.	serum globulin	gave	0·1094 g. $BaSO_4$.
2.	1·4490 g.	,,	,,	0·1024 g. ,,
3.	2·0132 g.	,,	,,	0·1708 g. ,,
4.	5·200 g.	,,	,,	0·1926 g. Dextrose.
5.	3·720 g.	,,	,,	0·1118 g. ,,
6.	2·487 g.	,,	,,	0·0599 g. ,,

Composition of serum globulin.

	Sulphur %	Carbohydrate %	
1.	1·14	—	
2.	1·20	—	
3.	1·16	—	
4.	—	3·7	
5.	—	3·0	washed 14 days
6.	—	2·99	
Mean	1·17	3·23	

According to Hammarsten the average sulphur content of serum globulin is 1·11 %. Mörner gives 1·02 %. The results detailed above are in accordance with these observers. Moll's results on the other hand vary greatly. He gives the sulphur content of serum globulin as varying from 1·1 to 1·5 %. This has not been confirmed either in these results or the results of other workers.

With regard to the amount of carbohydrate present in serum globulin, Pavy finds 2·8 %, which figure agrees well with the results given above. Langstein finds that there is at least 1 %; his method, depending upon the preparation of the benzoyl ester, cannot, however, be considered as quantitative, but as affording only minimal values.

Artificial globulin. Re-crystallised serum albumin was prepared as detailed before, dialysed till free from salts and the solution filtered. The percentage nitrogen was determined in 5 c.c. of this solution by Kjeldahl's method and then the main bulk of the serum albumin solution was diluted with distilled water till it contained between 2 and 3 % of protein. An equal bulk of N/66 sodium carbonate solution was added, the mixture warmed up to 60°C. and kept at that temperature for one hour. At the end of this time the solution was cooled, an equal bulk of saturated ammonium sulphate added and the precipitate filtered off on a Buchner filter. The residue was mixed with water, in which it partially dissolved giving an opalescent solution. To this was added an equal bulk of saturated ammonium sulphate and the precipitated globulin again collected and well washed with 50 % ammonium sulphate solution. The product dissolved in water to some extent, but never gave a clear solution. It was heated up to boiling and then a few drops of 2 % acid added, when the whole was thrown down in a coagulated form, which was washed on a Buchner filter with boiling water for four or five days till the washings were free from sulphate as shown by the barium chloride test. The washed product was treated with boiling alcohol, dried, powdered and extracted with ether for 24 hours and after drying at 100°C. the substance was analysed.

The sulphur and carbohydrate were estimated as before, and the filtrates from the barium sulphate precipitates examined for phosphorus, without however, obtaining any indication, in any case, of its presence.

1.	2·3162 g.	artificial	globulin gave	0·2552 g. $BaSO_4$.
2.	1·7840 g.	,,	,,	0·2006 g. ,,
3.	1·6418 g.	,,	,,	0·2060 g. ,,
4.	0·7438 g.	,,	,,	0·0910 g. ,,
5.	5·3400 g.	,,	,,	0·0108 g. Dextrose.
6.	6·3180 g.	,,	,,	0·0153 g. ,,

Composition of artificial globulin.

	Sulphur %	Carbohydrate %	
1.	1·51	0·20	5
2.	1·54	0·24	6
3.	1·72	—	
4.	1·68	—	
Mean	1·61	0·22	

The product was also tested for phosphorus by fusing up with fusion mixture, extracting with water and treating with ammonium

nitrate, nitric acid and ammonium molybdate. No indication of the presence of phosphorus was, however, obtained.

Collecting the mean values of what it will be noted are closely concordant figures for the percentage of sulphur and carbohydrate in each of the three products under consideration,

	Sulphur	Carbohydrate	Phosphorus
Serum albumin	2·04	0·25	0
Serum globulin	1·17	3·23	+
Artificial globulin	1·61	0·21	0

it will be at once apparent that the artificial globulin differs very essentially from serum globulin, and, in fact, has a composition still approximating to that of serum albumin.

In concluding from these figures that serum albumin is *not* converted into serum globulin, it is nevertheless evident that some change does occur in the albumin as evidenced more especially by the increased precipitability by ammonium sulphate. Since the so-called transformation occurs only in the presence of alkaline salts, the obvious inference was that the increase in the protein precipitable on half saturation with ammonium sulphate was due to a partial formation of alkali albumin.

The following series of experiments with egg albumin was arranged with the object of ascertaining whether under the conditions of Moll's experiments alkali albumin was formed, and, if formed, whether produced in quantity sufficient to account for the supposed increase in the globulin fraction.

Experiments with egg white (carried out in duplicate).

An egg white solution was prepared by taking the whites of three eggs, squeezing through linen three times to separate the membranes then diluting with water and filtering. Of this solution a series of 25 c.c. portions were taken and treated as follows:—

(1) 25 c.c. were pipetted off, an equal bulk of saturated ammonium sulphate solution added, the globulin filtered off and well washed with 50% ammonium sulphate. The filtrates and washings were acidified and coagulated and the coagulum brought on to a filter paper and washed till free from soluble nitrogenous substances. The nitrogen in the precipitate was then estimated by Kjeldahl's method. The globulin was dissolved in water, coagulated, filtered, well washed and the nitrogen estimated as before.

(2) 25 c.c. of the solution were pipetted off, an equal bulk of N/66 sodium carbonate solution added and the mixture heated to 60°C. for

one hour. After this time, it was cooled, half saturated with ammonium sulphate, filtered, the albumin in the filtrate coagulated, washed, and its nitrogen estimated as before. The fraction precipitated by 50% ammonium sulphate was similarly dissolved in water, coagulated, washed and its nitrogen estimated by Kjeldahl's method.

(3) 25 c.c. of the solution were pipetted off, diluted with an equal bulk of sodium carbonate solution and heated as before for one hour at 60° C. After cooling, the mixture was neutralised with 2% acetic acid to throw down the alkali albumin produced, the precipitate was filtered off, washed with distilled water and its nitrogen estimated. The filtrate and washings were then half saturated with ammonium sulphate and the globulin filtered off. The nitrogen of the globulin and also of the albumin in the filtrate were then determined after coagulation in the manner already described.

From the figures obtained, the protein represented was in each case determined by multiplying by 6·25.

		Weight of protein in grams		
		No. 1	No. 2	Mean
1. 25 c.c. separated by $(NH_4)_2SO_4$ into globulin and albumin	globulin	0·13	0·16	0·15
	albumin	0·51	0·50	0·51
2. 25 c.c. heated with N/66 Na_2CO_3 and then separated as described	globulin	0·40	0·37	0·39
	albumin	0·26	0·27	0·27
3. 25 c.c. heated with N/66 Na_2CO_3 and neutralised before separating into globulin and albumin	alkali albumin	0·22	0·22	0·22
	globulin	0·07	0·08	0·08
	albumin	0·30	0·30	0·30

From the figures it is apparent that after heating with the dilute sodium carbonate the increase in the globulin fraction ($0·39 - 0·15 = 0·24$ g.) which is equal to the diminution in the albumin fraction ($0·51 - 0·27 = 0·24$ g.) is almost exactly accounted for by the amount of alkali metaprotein produced (0·22 g.).

On repeating these experiments using serum albumin instead of ovalbumin, it was found that on the addition of dilute acid to the solution previously heated with the dilute sodium carbonate, a turbidity was in every case produced, pointing to the presence of metaprotein. Unfortunately, owing doubtless to the other proteins present, the precipitated metaprotein was in too finely divided a state to permit of a sufficient amount being obtained for analysis; so, instead, a quantity of the material was prepared in the following manner.

Alkali metaprotein.

A dialysed solution of re-crystallised serum albumin was warmed to 70 or 80°C. with a little caustic potash and then neutralised by sulphuric acid after the addition of a little ammonium sulphate. The precipitated alkali metaprotein was mixed with water, boiled and coagulated, and then as usual washed with boiling water till free from sulphates. After treatment with boiling alcohol, the product was dried, powdered and extracted with ether for 24 hours.

The sulphur and carbohydrate content were estimated in the manner already described.

1.	1·2490 g.	alkali	metaprotein	gave	0·1208 g. $BaSO_4$.
2.	0·8792 g.	,,	,,	,,	0·0844 g. ,,
3.	0·8040 g.	,,	,,	,,	0·0780 g. ,,
4.	6·512 g.	,,	,,	,,	0·0144 g. Dextrose.
5.	7·386 g.	,,	,,	,,	0·0118 g. ,,

Composition of alkali metaprotein.

	Sulphur	Carbohydrate
1.	1·43	—
2.	1·32	—
3.	1·32	—
4.	—	0·22
5.	—	0·16
Mean	1·36	0·19

It will be observed that the action of the alkali in the preparation of the metaprotein from serum albumin has been confined chiefly to the sulphur-containing part of the molecule.

No indication of the presence of phosphorus in the alkali metaprotein could be obtained, either by means of fusion with fusion mixture or in the filtrates from barium sulphate.

Discussion.

In surveying all the evidence for the establishment of the change of an albumin into a globulin, two main facts will have been noticed. In the first place, the change has not been observed to occur in albumin solutions which do not contain either alkaline salts or substances of basic reaction. Even the experiments of Cervello and Breinl involving the use of antipyrine fall under this category since this substance is known to have marked basic properties. Secondly, the main evidence for the identification of the artificial product with the

natural globulin rests on the fact that both substances have similar zones of precipitation with saturated ammonium sulphate. But identity of this zone of precipitation with ammonium sulphate cannot alone be taken as the criterion of a globulin, since, as will be remembered, besides globulins, there are derivatives of albumin—acid and alkali metaproteins, which are also precipitated by half saturation with ammonium sulphate. Since these metaproteins are formed by the action of dilute acids and alkalis on all animal albumins, it is surely not permissible to claim that a body formed from serum or egg albumin by dilute alkali, is a natural globulin because it is precipitated by half saturation with ammonium sulphate.

The experiments with egg white that have been described, in which the egg white, after heating with alkali, was neutralised with acetic acid before separating into albumin and globulin, show that a certain amount of alkali metaprotein has been formed (since its globulin is soluble in neutral salts, it would not be precipitated on neutralising). Moreover this amount of metaprotein corresponds with the increase in the globulin fraction of similarly heated but not neutralised egg white controls.

Much the same effect was noticed in the case of the parallel experiments with serum, but as has already been remarked the precipitated alkali metaprotein was not obtained in sufficiently large colloidal aggregates to admit of filtering and weighing. These experiments, however, combined with the fact that Moll and others who have supported his conclusions did not neutralise their solutions before separating the globulin and albumin, renders it most probable that the so-called artificial globulin is in reality alkali metaprotein.

A comparison of the chemical properties of these protein substances fully confirms this view. Natural serum globulin has been shown in the foregoing results to contain at least 3% of carbohydrate, some phosphorus, and not more than 1·2% of sulphur. The artificial globulin has only a minute amount of carbohydrate (0·21%), no phosphorus, and not less than 1·5% of sulphur. With such radical differences in their composition, no doubt can be entertained of the essential dissimilarity of these two substances. On the other hand, the specimens of alkali metaprotein which have been prepared and analysed bear a close resemblance to the so-called artificial globulin. Like their common parent—serum albumin—they contain no phosphorus and the amount of carbohydrate is practically unaltered. The sulphur figures too are just what might be expected. Owing to the

greater action of the stronger base (potash) used in the preparation, employed at a higher temperature, a greater cleavage of the sulphur has occurred. Whereas, taking mean figures, serum albumin contained 2·0 % sulphur, and the artificial globulin 1·5 %, the alkali metaprotein contains only 1·36 %. All these proteins are however still richer in sulphur than serum globulin, which has about 1·17 % sulphur. Since then the artificial globulin stands intermediate between serum albumin and ordinary metaprotein, it must be regarded as a variety of alkali albumin and not in any sense as a globulin.

SUMMARY.

1. The so-called artificial globulin, serum albumin and serum globulin have been prepared and examined with reference to their sulphur, phosphorus and carbohydrate content.

2. The analytical figures obtained prove that the artificial product is not identical with natural globulin.

3. Experiments with egg white and blood serum have shown that the artificial globulin is in reality alkali metaprotein.

4. Analyses of alkali albumin prepared from serum albumin have confirmed the view that a transformation of albumin into globulin does not take place, only alkali metaprotein being produced.

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