XXXII. THE FACTORS CONCERNED IN THE SOLUTION AND PRECIPITATION OF EUGLOBULIN.

BY HARRIETTE CHICK.

From the Lister Institute.

(Received April 15th, 1913.)

CONTENTS.

	PAGE
I. Introduction	318
II. "Solution" of euglobulin by acids and alkalies	319
III. "Solution" of euglobulin by neutral salts	322
IV. Effect of neutral salts upon acid and alkaline dispersions of euglobulin	332
V. Summary	338

I. INTRODUCTION.

There is no reason for regarding euglobulin, the material precipitated from serum by dialysis or by dilution and acidification, as a chemical entity. Michaelis and Rona [1910, 2] are of opinion that it consists of that portion of the total proteins which owes its solution to the dispersing power of the electric charge upon its particles. It is true that the procedure adopted for separating euglobulin is one which renders it iso-electric with the solution; but the conditions determining its dispersion in serum are not so simple as they suggest. Euglobulin, as shown by Hardy [1905], is dissolved by neutral salts, e.g., sodium chloride, in a concentration of 1/10th normal, to form a colloidal solution in which the particles are electrically neutral, a result which I have been able to confirm (see p. 329, below). Serum contains enough salt to produce this effect and unless it is at the same time diluted, the euglobulin it contains cannot be completely precipitated by the addition of the amount of acid necessary to render it iso-electric. The dispersion of euglobulin in serum is due to two entirely distinct causes: (1) Electric charge on the particles owing to the alkalinity of the fluid; (2) Formation of a "soluble" compound with the salt present,

concerning the mechanism of which there are a variety of theories, which are discussed in detail below.

By the process of "heat-denaturation" proteins acquire many of the properties peculiar to euglobulin, as regards the factors conditioning their solution (dispersion) and agglutination (see Hardy [1900], Michaelis and Mostynski [1910] and Chick and Martin [1912]). The results of the last-named, however, afford no support for the opinion expressed by Starke [1900] and Moll [1904] that "albumin" is converted into "globulin" by heating. Abderhalden [1903, 1904] has found evidence of difference in chemical composition between the two sets of proteins which is not obliterated after the former has been heated. Further, the analogy mentioned above is by no means complete; the union of euglobulin with neutral salts to form a dispersion that is without drift in an electric field has no parallel in the case of denaturated serum-proteins.

II. SOLUTION OF EUGLOBULIN BY ACIDS AND ALKALIES, AND THE ISO-ELECTRIC POINT.

Solution of euglobulin by means of acid or alkali was shown by Hardy [1905] to be associated with the possession by the dispersed particles of an electric charge which was respectively positive or negative in sign.

Michaelis and Rona (1910, 2) found the iso-electric point of euglobulin to be at a concentration of hydrogen-ions equal to 36×10^{-7} normal and to coincide with the point of optimum flocculation for this protein.

The iso-electric point has been re-determined in the present instance and the result of Michaelis and Rona has been confirmed.

The euglobulin was prepared as follows: horse serum was diluted ten times with distilled water and the globulin was precipitated by acidifying with acetic acid (about 3-4 cc. N acetic acid per litre according to the original reaction of the serum). The precipitate was allowed to settle, was centrifuged off, and purified by dissolving in a minimal amount of standard sodium hydroxide solution (according to the amount of the precipitate) and reprecipitating with hydrochloric acid, the precipitate again being separated by centrifuging. This operation was repeated once or twice and the precipitate finally washed with distilled water. A fairly concentrated suspension was made in distilled water from which, by dilution, the material was prepared which was used for the various experiments¹. The particles of

¹ In some experiments, for example those in Tables IV and V, the euglobulin was prepared from horse-plasma (oxalate) by the method described by Mellanby [p. 339, 1905]. The purification in this case was just as above and no differences were detected between samples prepared by the two methods.

suspensions prepared as above invariably had a slight negative charge and the addition of a little acid was necessary in order to render the particles iso-electric with the solution.

In Table I is shown the degree of dispersion of a sample of euglobulin (0.032 % solution) corresponding with various concentrations of acid and alkali. The charge carried by the particles was at the same time determined

TABLE I.

Influence of reaction (hydrogen-ion concentration) upon the dispersion of euglobulin, and the electric charge carried by the protein particles; influence of sodium sulphate.

Concentration of protein = 0.032 %.

Exp. No.	Cc. N/100 HCl (orequi-valent) added in total volume of 10 cc.	Cc. N/100 NaOH (orequi-valent) added in total volume of 10 cc.	Salt added	Concen-tration of salt, in terms of normality	Concen-tration of hydrogen-ions, in terms of normality	Sign of electric charge on the particles	Degree of agglutination
1	—	0.2	—	—	0.36×10^{-7}	—	Faintly opalescent soln.
2	—	0.1	—	—	0.97	—	Opalescent solution.
3	—	0.0	—	—	32	—	Agglutinated.
4	.1	—	—	—	748	—	Agglutinated later.
5	.15	—	—	—	1140	—	Opalescent solution.
6	.2	—	—	—	1390	+	Faintly opalescent soln.
7	.5	—	—	—	5080	—	Clear solution.
8	.2	—	Na ₂ SO ₄	0.03	0.97	—	Opalescent solution ¹ .
9	.5	—	„	0.05	1590	—	Opalescent solution ¹ .

¹ Agglutinated on standing.

by observing their behaviour in an electrical field, using the microscopic method previously employed by Martin and the author [1912, p. 285] in investigating the electrical properties of denaturated proteins. In the 5th column is given the concentration of hydrogen-ions in the various solutions, and the point of optimum agglutination is seen to be at a concentration equal to 32×10^{-7} normal. This figure is in good agreement with that found by Michaelis and Rona.

Confirmation of these values was incidentally obtained in the course of experiments made to elucidate other points. For example, the range of agglutination of another euglobulin suspension, containing 0.6 % protein was determined, after first dissolving in a minimum amount of dilute sodium hydroxide solution, by adding dilute hydrochloric acid to a series of tubes until precipitation occurred and finally dispersion was again obtained. Dispersion corresponded with a concentration of hydrogen-ions equal to 2.2×10^{-7}

normal on the alkaline side and 62.1×10^{-7} normal on the acid side. The solutions, of course, all contained a trace of salt.

In case of a third sample of euglobulin, see Table II (containing 0.016 % protein) the limits of agglutination were found to lie between concentrations of hydrogen-ions equal to 5×10^{-7} and 213×10^{-7} normal and precipitation to be rapid at a concentration of 18×10^{-7} normal.

TABLE II.

Influence of reaction (hydrogen-ion concentration) upon the dispersion of euglobulin and upon the electric charge carried by the protein particles; influence of sodium sulphate.

Concentration of protein = 0.016 %.							
Exp. No.	Salt added	Concentration of salt added, in terms of normality	Cc. N/100 HCl	Cc. N/100 NaOH	Concentration of hydrogen-ions, in terms of normality	Sign of electric charge on particles	Degree of agglutination
			(or equivalent) added in total volume of 10 cc.	(or equivalent) added in total volume of 10 cc.			
1	—	—	0.3	—	—	+	Clear solution.
2	—	—	0.2	—	1280×10^{-7}	+	" "
3	—	—	0.1	—	213 "	+	Agglutination partial.
4	—	—	0.00	—	18.6 "	—	Agglutination complete.
5	—	—	—	0.1	5.41×10^{-7}	—	Agglutination partial.
6	—	—	—	0.2	—	—	Dispersed.
7	Na ₂ SO ₄	.02	0.5	—	1010×10^{-7}	—	Agglutination partial, less good than No. 8.
8	"	.03	"	—	137 "	—	Agglutination best, but not quite complete.
9	"	.04	"	—	65.9×10^{-7}	—	Agglutination less good than No. 8.
10	"	.05	"	—	—	0	Dispersed.
11	"	.07	"	—	—	0	Dispersed.

The experiments as to the amount and sign of the charge carried by the euglobulin particles, detailed in Tables I and II, are not calculated to define the iso-electric point with as great accuracy as those of Michaelis and Rona. As a rule the particles, while agglutinating rapidly, retained the negative charge which they originally held and only showed a positive charge when dispersion by acid was already well begun. Speaking generally, however, the point where the charge on the particles changed its sign coincided with the agglutination-zone. On the whole, there is a tendency to retain the negative charge rather than the positive. A distinctly positive charge was first detected on the globulin particles at a concentration of hydrogen-ions

equal to about 200×10^{-7} normal (see Exp. 3, Table II, and Exp. 2, Table VII).

The agglutination and dispersion of euglobulin by acids and alkalies, in confirmation of the results of Michaelis and Rona, is seen to be primarily dependent upon hydrogen-ion concentration, and in this respect a close analogy is presented with heat-denaturated proteins [Michaelis and Rona, 1910, 1; Sørensen and Jürgensen, 1911; Chick and Martin, 1912]. In both cases flocculation takes place when the protein particles are iso-electric with the solution, and, in cases where the reaction of the solution is more acid or alkaline than the iso-electric point, dispersion of the protein is due to the possession of a positive and negative electric charge respectively.

III. SOLUTION OF EUGLOBULIN BY NEUTRAL SALTS.

There has been some difference of opinion as to the mechanism involved in the solution of euglobulin by electrolytes. Hardy [1905] came to the conclusion that salt-solutions of globulin are without drift in an electric field and the particles of the system must be regarded as electrically neutral. He considered [1905, p. 325] "salt-globulin" to be the result of a molecular union of globulin and salt¹ by a process analogous to the formation of amino-acid-salt compounds², thus:

A similar theory had already been put forward by Pauli [1899].

Mellanby [1905], on the other hand, has expressed the opinion that the solution of globulin by electrolytes is the result of activity of the constituent ions. He found that the efficiency of a salt in "dissolving" euglobulin depended on the valency of its ions, and, moreover, that the amount of globulin dissolved by a given concentration of any salt was proportional to the initial concentration of the protein. This curious fact was also noticed by Hardy [1905, p. 310].

A third view is that of Schryver [1910]. He considers (1) that, when precipitated, euglobulin is in a dehydrated condition, the amphoteric molecules having united by twos in salt formation with loss of the elements of

¹ Osborne and Harris [1905] also interpreted the solution of edestin by certain electrolytes as due to the formation of such compounds.

² These appear to have been prepared in the crystalline form by Pfeiffer and Modelski [1912].

water and (2) that, when dissolved by electrolytes, the molecules remain separate in their hydrated condition, the aggregation in pairs being prevented by adsorption of the electrolytes on the surface of the molecules, thus

The insolubility of the dehydrated euglobulin Schryver attributes to the increased size of the molecule¹. There seems to be, however, better experimental evidence in support of the more usual conception that the euglobulin is precipitated in such circumstances as will allow of the aggregation of the protein particles under the influence of surface tension, that is to say, in the absence of other causes tending to keep them dispersed. Such causes are the presence of acid or alkali, in which case the euglobulin particles have been shown to be charged electrically, or the presence of electrolytes in whatever way the latter may act.

Schryver's view is in accord with that of Hardy in so far as the solution of euglobulin by electrolytes is attributed by both to the agency of the salt as a whole rather than to the activity of the constituent ions. His adsorption hypothesis, however, affords no explanation of the comparatively high concentration necessary for solution in case of many salts.

An additional theory, for which some experimental support is here given, is that dispersion of euglobulin by salts may be due to a specific adsorption by the protein particles of one—the more potent—ion of the electrolyte employed, and the acquisition of an electric charge by this means. Such an hypothesis would explain the influence of valency, demonstrated by Mellanby, and be consistent with what is known of the action of neutral salts in dispersing denaturated serum proteins [Chick and Martin, 1912, p. 281], with which euglobulin is in many ways analogous.

In the experiments detailed below, the "solution" of euglobulin by neutral salts was studied, using a series of electrolytes of more varied character, as regards the valency of the constituent ions, than those employed by the above observers. By this means some facts have been discovered which go to show that while, in some instances, solution of euglobulin by

¹ The conclusion of Dabrowski [1912] that in solutions of crystalline egg-albumin containing ammonium sulphate, the colloidal particles are of smaller size than in the case of the dialysed protein, is of interest in this connection.

neutral salts appears to be due to some action of the whole salt, in other cases it is undoubtedly associated with an acquirement of electric charge by the protein particles, the sign of which is determined by the more potent ion of the electrolyte employed.

1. *Experiments to determine whether salt-globulin is electrically charged.*

A series of experiments with a dilute euglobulin suspension (0.016% protein) and a variety of salts is set forth in Table III. The object was to compare quantitatively the solvent and dispersing power of different ions and to decide whether solution was accompanied by the deposition of an electric charge upon the protein particles. The latter, in this instance, was determined by the microscopic method, using a dark ground and side illumination. The fluids were placed in a specially constructed cell and the behaviour of the particles in an electric field was observed. In using this method with fairly concentrated salt solutions (0.01 normal and upwards) there is some risk of disturbance by gas-bubbles given off at the electrodes, as the result of electrolysis. This has to be very carefully watched. As a rule the difficulty is minimised if the electrodes are previously platinised and the circuit closed only for the short time necessary to make the observation. The electromotive force employed was about 9 volts.

The results of this series of experiments show the relative solvent power of six different salts, viz. the chloride, sulphate and citrate of sodium, the chlorides of barium and calcium and lanthanum nitrate.

It is very clear that the influence of valency in preventing agglutination of euglobulin is far from negligible, multivalent ions being much the more powerful in order of increasing valency. For example, the concentration of lanthanum nitrate and sodium citrate required for the purpose is respectively 1/30th and 1/60th of the necessary concentration of sodium chloride (5th column, Table III).

The electrical condition of the dispersed particles is set out in column 4, and it will be seen that, in four cases, a charge was demonstrated of similar sign to that carried by the more potent ion of the electrolyte employed. In the case of calcium¹ and barium salts, on the other hand, no charge could be detected on the protein particles which remained visible.

The *character* of the dispersion obtained was also different in the case of different salts, and could be grouped according to two types, which I have tentatively called the "electrical" and the "molecular" type of solution.

¹ This was also found to be the case when denaturated serum-proteins were dispersed by calcium salts [Chick and Martin, 1912, Table XIV].

TABLE III.

Solution of euglobulin by electrolytes.

Concentration of protein = 0.016 %.

- × × × Complete agglutination, filtrate protein-free.
 × × Less complete agglutination, filtrate contained trace protein.
 × Partial agglutination.
 × - Almost complete dispersion.
 - Complete dispersion, clear solution.

Salt added	Concentration of salt, in terms of normality	Degree of agglutination	Sign of the electric charge upon the particles	Concentration of salt, in terms of normality, necessary	
				(a) to prevent agglutination	(b) to complete dispersion
NaCl	0.01	× × ×		0.03	—
	0.02	× × ×			
	0.03	× ×			
	0.04	× ×			
	0.045	× ×	— *		
	0.05	×	— *		
Na ₂ SO ₄	0.0005	× × ×		0.02	0.05
	0.001	× × ×			
	0.01	× × ×			
	0.02	× ×	— *		
	0.03	×			
	0.04	× -	— *		
Na ₃ Cit	0.0005	×	—	0.0005	0.003
	0.001	×			
	0.002	× -			
	0.003	—			
CaCl ₂	0.01	× × ×		0.02	—
	0.02	× ×			
	0.03	× ×			
	0.04	× ×			
	0.05	×	0		
BaCl ₂	0.001	× × ×		0.005	0.05
	0.005	× ×			
	0.008	× ×			
	0.01	× ×	0		
	0.02	×	0		
	0.03	× -			
La(NO ₃) ₃	0.05	—	0		
	0.001	× ×	+	0.001	0.02
	0.003	× ×			
	0.005	× ×			
	0.006	× -	+		
	0.008	× -			
	0.01	× -			
	0.02	—			

* Agglutinated particles.

That with a lanthanum salt or a citrate was exactly analogous to dispersion by acids and alkalies, the globulin suspension, with progressive increase in concentration of the salt, passed through various gradings of opalescent solutions until a clear transparent fluid was finally obtained. At the same time, under the microscope, using a dark ground illumination, innumerable particles could be seen which became smaller and smaller, acquiring at the same time an unmistakably positive or negative charge. Finally the whole field became a uniform grey in colour, until at length no particles could be distinguished.

In the case of the other salts used, there was usually no grading of opalescence, but, as the concentration of salt was increased, a decreasing amount of the original suspension remained undissolved in an otherwise clear fluid. Under the microscope there was a black and white effect, fewer and fewer agglutinated masses remaining visible on the black ground. This type of solution obtained with sodium sulphate and the chlorides of sodium, calcium and barium. In the case of the two latter salts, no charge was discovered on the visible particles; with sodium chloride and sulphate a negative charge was observed on the agglutinated particles which remained visible. It must however be remembered that the sign of the charge in both cases was negative, i.e. similar to that carried by the original globulin suspension.

The microscopic method is clearly unsuitable for studying the electrical condition of salt-globulin in such cases as the above, where "solution" is associated with a degree of dispersion which renders the dispersed particles immediately invisible. A series of corresponding experiments were accordingly made, in which the "U" tube method was employed, the object being to find out whether actual kataphoresis of the "salt-globulin" did or did not take place in an electric field.

This was the method used by Hardy [1905] whose conclusion that "salt-globulin" was without electric charge has already been referred to. In his experiments, however, as was pointed out by Michaelis [1909] the protein solution may not have been adequately protected from the influence of the acid and alkali produced at the electrodes by electrolysis of the salt present.

In order to avoid all complications due to electrolysis five U tubes were arranged in series in the present instance. The "salt globulin" occupied the centre tube, which was connected by a three-way tap on either side for convenience in filling. The other four tubes, two on either side, contained a solution of the same electrolyte in the same concentration as that used to disperse the euglobulin. All five tubes were fastened by brass clips to

a stand, so constructed that their position could be altered until the height of liquid in all the tubes was accurately adjusted to a standard level. Only after this was accomplished were the taps opened, and the euglobulin solution placed in contact with the other tubes. Control experiments showed that under these circumstances there was no transference of liquid from one tube to another. The electrodes were placed in the further arms of the two end tubes and the globulin solution was thus securely protected from the influence of any acid or alkali produced there by electrolysis. Litmus and phenol-phthalein were added to the tubes containing the positive and negative electrodes respectively and in all cases the experiment was discontinued long before there was any danger of acid or alkali reaching the centre tube. The resistance of this arrangement was very great, and, although the electrodes (small strips of platinum foil) were connected with the lighting circuit (200 volts) the current which passed amounted to only 0.0001 to 0.005 ampères according to the nature and concentration of the electrolyte employed. At the close of the experiment, which usually lasted from 5 to 10 hours, the contents of the nearer arms of the U tubes adjacent to the globulin tube were tested for the presence of protein by addition of Esbach's reagent or otherwise.

The results are set out in Table IV below, and, in general, confirm the results of Table III. In the case of calcium chloride, barium chloride and

TABLE IV.

*Electrical properties of euglobulin, dispersed by various electrolytes.
(U-tube method.)*

Protein content = 0.16 %.

Exp. No.	Salt	Concentration, in terms of normality	Electric field, volts.	Current, ampères	Duration of experiment, hours	Sign of electric charge upon protein	Appearance of solution	Sign of electric charge, observed by microscopic method
1	Na ₂ SO ₄	0.05	200	0.0025	8.5	0	Clear solution containing some particles undissolved.	— (agglutinated particles)
2	CaCl ₂	0.05	„	0.002	5.5	0	Almost clear solution, containing some particles.	0
3	BaCl ₂	0.04	„	0.005	5.0	0	Almost clear solution, faint opalescence.	0
4	La(NO ₃) ₃	0.005	„	0.0001	5.5	+	Opalescent solution	+
5	Na ₃ Cit	0.003	„	0.001	5.0	—	Opalescent solution	
6	„	0.05	„	0.0015	9.75	0	Clear solution containing some agglutinated particles.	

sodium sulphate in concentrations of 0.05, 0.04 and 0.05 normal respectively, no migration of the dissolved protein was demonstrated. With the last salt, however, the negative charge carried by the undissolved particles was again shown by the settling which took place less rapidly in the "positive" than in the "negative" arm of the centre U tube, showing that in the latter case the action of gravity was reinforced by motion of the particles towards the positive pole.

Euglobulin dispersed by a small concentration of lanthanum nitrate (= 0.005 normal) displayed a marked positive charge and in the case of sodium citrate¹ a negative charge was shown under similar conditions (concentration = 0.003 normal). If the concentration of sodium citrate was increased to 0.05 normal, however, the dispersed euglobulin showed no migration at all.

The case of sodium citrate is a very interesting one, as the character of the dispersion obtained by employing these two concentrations was also quite different, conforming respectively to the "electrical" and "molecular" types of solution described above (p. 324). With the dilute salt the dispersion is to an opalescent solution and is accompanied by the acquisition of a negative charge; in the stronger solution of citrate, the protein is uncharged and the liquid shows a small precipitate suspended in a clear fluid.

The change from the one type of solution to the other can be seen if a series of solutions be made up containing equal amounts of euglobulin and a concentration of sodium citrate ranging from 0.001 to 0.05 normal. Dispersion is already well marked at 0.001 normal and continues through various grades of diminishing opalescence to a concentration of about 0.003 normal. At a concentration of 0.005 normal the opalescence is greater and at 0.01 normal, a distinct precipitate can be seen. In higher concentrations a gradual clearance takes place, but the solution is now of a different character, less and less of the precipitate remaining undissolved in an otherwise clear solution. In those experiments the protein content was 0.16%.

Another interesting experiment is the following: The U-tube apparatus was arranged so that in the centre tube was a dispersion of euglobulin in 0.003 N sodium citrate and in the side tubes 0.05 N sodium chloride. As the current passed a precipitation took place in the negative arm of the centre tube, the zone of which continually progressed towards the positive arm. This was followed by a zone of clear solution, also moving in the same

¹ These citrate solutions were carefully prepared to be quite neutral and the hydrogen ion concentration was approximately 10^{-7} normal.

direction. The interface between the advancing chlorine ions and the citrate ions will not be sharply defined, as the former travel more rapidly and tend to over-run and intermix with the latter. A possible explanation is therefore that as both citrate and chlorine ions move towards the positive pole, the latter replace the former in the solution around the protein which is also moving towards the positive electrode, but at a slower rate than either. In low concentration chlorine ions are unable to disperse globulin and precipitation occurs, to be followed again by solution when the concentration of the chlorine ions is sufficiently increased.

The conclusion to be drawn from these experiments is that solution of euglobulin by neutral salts, in case of the more ordinary electrolytes, is due to the formation of some unionised and uncharged compound of salt and globulin—whether by a molecular union (Hardy) or as the result of adsorption (Schryver) there is not, at present, enough experimental evidence to decide. At the same time the phenomenon of dispersion by salts, at any rate in its beginning, is due to an electric charge being deposited on the particles of the euglobulin suspension by the agency of the ions of the electrolyte. The influence of salts upon dispersions of euglobulin by acids and alkalies, dealt with in the next section, is in support of this view.

In the case of the commoner salts, containing only mono- or divalent ions, the electric charges brought into play are not powerful enough to disperse the euglobulin until the concentration is increased to a point where the second ("molecular") type of solution takes place. With such electrolytes the first or "electrical" type of solution as a rule is negligible. It was, however, detected in the case of sodium chloride and sodium sulphate. With sodium citrate, on the other hand, the one type was seen to give place to the other as the concentration of salt was progressively increased.

When euglobulin is denaturated by heat, it loses its characteristic property of forming electrically neutral solutions with electrolytes. For example, after heating a dispersion of euglobulin in 0.05 normal sodium sulphate, in which no kataphoresis of the protein could be demonstrated, the protein particles were found to migrate to the positive pole in an electric field. At the same time an alteration took place in the appearance of the solution—the degree of dispersion was diminished and a thick opalescence was developed. With dispersions in sodium chloride (0.1 normal) and calcium chloride (0.15 normal) almost complete agglutination took place on heating.

2. *Alteration in electrical conductivity during solution of euglobulin by electrolytes.*

It is clear that if a molecular union or an adsorption compound is formed during solution of euglobulin by electrolytes, there must be some diminution in electrical conductivity. Hardy [1905, p. 307] states that a loss of conductivity takes place equal to 1.4% to 2.4% in case of solution by magnesium sulphate and sodium chloride respectively.

The results of a special set of experiments, made for the purpose, confirm the result of Hardy.

The same sample of euglobulin was used as for the previous set of experiments, and the mixtures when prepared contained 0.6% protein. The conductivity was determined:

- (1) of the "salt-globulin" solution,
- (2) of an equal concentration euglobulin suspension in distilled water.

In the case of (1) and (2) the solutions were allowed to settle or were centrifuged and the conductivity measured in the supernatant liquid.

- (3) of an equal concentration salt solution in distilled water.

The conductivity of the distilled water used was found to be negligible in comparison; hence direct comparison was made between (1) and the sum of (2) and (3). All determinations were made at 18°.

The results are set out in Table V and show a loss of conductivity in every case investigated. The salts used were the chlorides of barium and sodium, sodium citrate and lanthanum nitrate. In the case of what I have termed the "electrical" type of dispersion the diminution in conductivity was proportionally greater than with the molecular type; in the former case it occurred with iso-electric euglobulin to the extent of 13.6% and 11.6% in case of dispersion by weak (0.004 normal) lanthanum nitrate and sodium citrate respectively, see Exp. 1. With more concentrated salt, 0.05 normal, the loss varied from 1-4% in the case of the four salts employed.

3. *Relation of the amount dissolved by a salt to the total amount of euglobulin present.*

Notice has already been made of the observation of Mellanby [1905, p. 342] confirmed by Hardy [1905, p. 310] that the amount of euglobulin dissolved by a given concentration of salt is, within certain limits, approximately proportional to the concentration of protein in the original suspension.

In the case of the salt used by Mellanby (sodium chloride in concentration

TABLE V.

Change in electrical conductivity on solution of euglobulin by electrolytes.

Concentration of protein = 0.6 %, temperature 18° C.

Exp. No.	Condition of euglobulin suspension	Salt	Concentration of salt, in terms of normality	Conductivity, in reciprocal ohms				Percentage loss in conductivity	Concentration of euglobulin in solution as "Salt-globulin," %
				Salt solution	Euglobulin suspension	"Salt-globulin" (found)	"Salt-globulin" (calculated)		
1	Approximately iso-electric	NaCl	0.05	0.1389	0.00050	0.1378	0.1394	1.17	—
		BaCl ₂	0.05	0.1368	0.00126	0.1368	0.1381	0.99	—
		Na ₃ Cit	0.004	0.005489	0.00046	0.005331	0.005949	11.6	—
		"	0.05	0.05289	0.00046	0.05236	0.05335	1.90	—
		La(NO ₃) ₃	0.004	0.006785	0.000345	0.006277	0.007130	13.6	—
		"	0.05	0.06708	"	0.06536	0.006742	3.14	—
2	Alkaline	Na ₃ Cit	0.004	0.005562	0.000569	0.005578	0.006131	9.93	0.28
		"	0.05	0.05357	"	0.05337	0.05414	1.45	0.41
		La(NO ₃) ₃	0.004	0.006845	"	0.006482	0.007414	14.4	0.34
		"	0.05	0.06681	"	0.06511	0.06738	3.5	0.51
3	Acid	Na ₃ Cit	0.004	0.005541	0.002145	0.006907	0.007686	11.3	0.07
		"	0.05	0.05359	"	0.05415	0.05573	2.93	0.42
		La(NO ₃) ₃	0.004	0.006850	"	0.008264	0.008995	8.85	0.45
		"	0.05	0.06794	"	0.06717	0.07008	4.33	0.58

from 0.04 to 0.09 normal) solution is of the "molecular" type. On the assumption that this solution is the result of a molecular union between salt and protein (Hardy) it is difficult to explain the existence of the above relationship. On the other hand, if Schryver's view of the salt-solution of euglobulin be accepted in one respect, that is to say if we consider the second or "molecular" type of globulin to be the result of "adsorption" of the salt as a whole, the amount of globulin dispersed by a given concentration of salt might be approximately proportional to the extent of adsorbing surface i.e. to the concentration of euglobulin¹.

It was also possible, however, that solution-rate might be conditioned by the size of the euglobulin particles (of which there would be every variety in such a suspension as that used by Mellanby) and that in the time of experiment, final equilibrium had not been maintained but only the particles of small size and comparatively large surface had been successfully attacked.

¹ The experimental support for this view is based upon the fact that the globulin-dissolving capacity and surface-tension (against air) of series of solutions of similar salts, were found to be inversely related to one another, which is the result that should obtain, in accordance with the Willard Gibbs hypothesis. The application of surface tension measurements of air against solution to the case of the protein against solution is not, however, without risk of error.

Some experiments were made to examine this theory, using equal volumes of a 3% suspension of globulin and 1/10th normal sodium chloride solution. It was found, however, that almost perfect equilibrium was attained within a few minutes after mixing. Experiments were also made, using in the one case finely divided euglobulin and in the other a similar suspension of globulin previously aggregated by freezing; the amount of protein dissolved in the second case by 0.05 normal sodium chloride, under similar conditions, was about 20% less than in the former. A phenomenon of this magnitude is, in itself, inadequate to explain the relationship observed by Mellanby and Hardy; it may, however, be a contributing factor.

The facts would appear to be best met by some such conception as the following: Solution of euglobulin by such electrolytes as sodium chloride or sodium sulphate, is due to the formation of a "soluble" compound of the globulin and the salt, which is electrically neutral, and to prevent the dissociation of which a large excess of the salt is necessary. Under such circumstances the final equilibrium, i.e. the relative proportion of "dissolved" and "undissolved" globulin, will largely depend upon the concentration of the salt employed and show an approximate constancy if the latter is maintained constant. In other words, the amount of dissolved euglobulin will be roughly proportional to the original concentration (Mellanby).

IV. THE EFFECT OF NEUTRAL SALTS UPON ACID AND ALKALINE DISPERSIONS OF EUGLOBULIN.

The influence of salts in causing precipitation of euglobulin previously dissolved in acid and alkali is analogous to the corresponding action with denaturated serum proteins.

Hardy [1905, p. 317] has drawn attention to the fact that the precipitating action¹ of a salt upon "acid or alkali-globulin" is due to one only of its ions, viz.: that which carries a charge opposite in sign to that carried by the protein, and that the higher the valency of this ion, the greater is the power of the salt.

These results have been confirmed by me and I have also been able to show that, if the concentration of salt is further increased, dispersion will again occur, the particles now taking a charge whose sign is determined by that of the more potent ion of the electrolyte employed. Thus the addition of an appropriate electrolyte to an alkaline or acid solution of euglobulin

¹ Mellanby [1905] has dealt with the precipitation of globulin from its solution in electrolytes. This is a different phenomenon and a high concentration of salt is required (salting out).

may, after first precipitating the protein, cause the particles to disperse again with a charge opposite in sign to that originally carried.

For example, an alkaline dispersion of euglobulin containing 0.016% protein, with the particles negatively charged, was precipitated by the addition of lanthanum nitrate to a concentration of 0.001 normal; at a concentration of 0.008 to 0.01 normal, dispersion again took place, the particles now being positively charged. In a similar experiment with sodium chloride the precipitation took place at a concentration of salt equal to 0.02 normal; at 0.05 normal the globulin was again dispersed, this time bearing a negative charge.

These results present a close analogy with what takes place in case of denaturated serum-proteins, where the degree of dispersion in acid and alkaline solution is also greatly influenced by the presence of neutral salts, and in two ways. In the first place the reaction of protein-containing solutions, whether denaturated or not, becomes altered on addition of neutral salts [Chick and Martin, 1911, p. 21; 1912, p. 280], being shifted in the direction of the neutral point. In acid solution, the concentration of hydrogen ions is lowered and the concentration of hydroxyl ions lessened if the solution be alkaline; the effect is related to the valency of the anion and kation respectively of the electrolytes in the two cases. In the second place, the electric charge carried by the protein particles is modified and may be lessened or even changed in sign by the addition of electrolytes if of opposite sense to that carried by the more potent ion of the electrolyte added.

The effect of the above-mentioned salts in modifying the reaction¹ of solutions containing protein has also been demonstrated in the case of euglobulin (see Tables I, VI and VII). It was therefore necessary to determine in how far the precipitating effect of salts was due to this effect. It is evident that solutions either too acid or too alkaline for precipitation of euglobulin might be adjusted to the iso-electric reaction by the addition of an appropriate electrolyte, and indeed this frequently occurred. For example (Experiment 12, Table VI), addition of sodium citrate to a concentration of 0.002 N, caused precipitation of an acid dispersion of euglobulin (0.016% protein) at the same time reducing the concentration of hydrogen ions to a point very near the iso-electric point for this protein. In some cases the change of reaction extended to the other side of the iso-electric point, e.g. Table VI, Experiment 13; in this case the observed change of sign in electric charge taking place simultaneously with dispersion by sodium citrate, could be explained on the ground of change of reaction alone. By comparison of

¹ The phenomenon in absence of protein is perceptible but negligible.

Experiment 7 with 12 and 13, Table VI, this change in reaction is seen to be increased proportionally with the degree of valency possessed by the ions of the salt employed.

TABLE VI.

The effect of sodium sulphate and citrate upon an acid dispersion of euglobulin (0.016 %).

Exp. No.	Salt added	Concentration of salt in terms of normality	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Concentration of hydrogen ions, in terms of normality	Sign of electric charge carried by the particles	Degree of agglutination
1	—	—	0.0		—	Partial agglutination.
2		—	0.075		—	Agglutinated completely.
3		—	0.1		—	" "
4		—	0.2	$10^{-4.10}$ (810×10^{-7})	+	Dispersed, clear solution.
5		—	0.3		+	" " "
6		—	0.7		+	" " "
7	Na ₂ SO ₄	0.03	0.5	$10^{-4.45}$ (357×10^{-7})	—	Agglutinated.
8		0.04	"		—	Partial agglutination.
9		0.05	"		—	Dispersed.
10		0.07	"		0	"
11	Na ₃ Cit	0.001	0.7		+	Dispersed.
12		0.002	"	$10^{-5.41}$ (39×10^{-7})	—	Almost complete agglutination.
13		0.003	"	$10^{-6.04}$ (9.2×10^{-7})	—	Dispersed.
14		0.004	"		—	Dispersed, faintly opalescent solution.

In many cases, however, acid and alkaline solutions of euglobulin, with their positively and negatively charged particles respectively, were first precipitated and afterwards dispersed, the particles bearing an electric charge of changed sign in solutions whose reaction still remained more acid and more alkaline respectively, than the iso-electric point¹. A good example of this is seen in Table I, Experiment 9, where addition of 0.05 N sodium sulphate to an acid dispersion of euglobulin (0.032 % protein) caused the particles to be dispersed and to carry a negative charge in a solution where the hydrogen ion concentration, equal to 1590×10^{-7} normal, was far on the acid side of the iso-electric point. Experiment 7 of Table II is another instance of the same phenomenon¹.

With an alkaline suspension of euglobulin a corresponding series of results was obtained with lanthanum nitrate. The results are set out in

¹ In higher concentration of sodium sulphate, the dispersed globulin appears to be electrically neutral, see Exp. 10, Table VI.

Table VII. A preliminary set of experiments (1 to 8), in absence of electrolytes, showed a positive charge to be acquired by the protein particles at a concentration of hydrogen ions equal to 204×10^{-7} normal. In the alkaline suspension used, the concentration of hydrogen ions was equal to 0.0013×10^{-7} normal. In presence of 0.006 normal lanthanum nitrate the

TABLE VII.

Effect of lanthanum nitrate upon an alkaline dispersion of euglobulin.

Protein = 0.016 %.							
Exp. No.	Salt added	Concentration of salt, in terms of normality	Cc. N/100 HCl (or equivalent) added in a total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in a total volume of 10 cc.	Concentration of hydrogen ions, in terms of normality	Sign of electric charge carried by the particles	Degree of agglutination
1	—	—	0.2	—			Dispersed, clear solution.
2		—	0.1	—	$10^{-4.69}$ (204×10^{-7})	+	Dispersed, faintly opalescent solution.
3		—	0.05	—	$10^{-7.00}$ (1.05×10^{-7})	—	Agglutinated.
4		—	0	—		—	Dispersed, opalescent sol.
5		—	—	0.5			" " "
6		—	—	0.1			Dispersed, clear solution.
7		—	—	0.2			" " "
8		—	—	0.5	$10^{-9.88}$ (0.0013×10^{-7})		" " "
9	La(NO ₃) ₃	0.0005	—	0.5			Dispersed, opalescent sol.
10		0.001	—	"			Agglutinated.
11		0.002	—	"		+	Agglutinated almost completely.
12		0.004	—	"		+	" " "
13		0.005	—	"		+	Agglutination not complete.
14		0.006	—	"	$10^{-7.63}$ (0.24×10^{-7})	+	" " "
15		0.008	—	"			Dispersed partly.
16		0.01	—	"			" " "
17		0.02	—	"			" " "

alkalinity was reduced almost to the neutral point. At the same time the charge on the particles was found to be positive in a solution whose reaction was on the alkaline side of the iso-electric point, and where, in the absence of any electrolyte, the particles would be negatively charged.

In these instances the effect of electrolytes recalls the exactly similar set of phenomena obtaining in the case of denaturated serum-proteins mentioned

above, and also the analogous influence of salts which has been observed in case of inorganic colloidal solutions [Burton, 1909]. In certain of the latter there has been demonstrated a selective adsorption of the ion bearing a charge opposite in sign to that of the charged colloidal particles [Linder and Picton, 1895; Whitney and Ober, 1902; Freundlich, 1910] and this explanation may be extended to the case of proteins.

In this connection, certain of the conductivity determinations in Table V, i.e., those of Experiments 2 and 3, are of special interest. These experiments were made with suspensions of euglobulin which were slightly on the acid and alkaline side, respectively, of the iso-electric point. The effect of adding electrolytes containing trivalent positive ($\text{La}(\text{NO}_3)_3$) and negative (Na_3Cit) ions was carefully studied. The loss of conductivity was found to be greater for sodium citrate if the globulin suspension were originally acid (i.e. charge on the protein particles positive) and greater for lanthanum nitrate if the globulin suspension were alkaline. At the same time, the degree of dispersion was measured by the content of protein in the supernatant fluid after centrifuging (see last column), and was found to be less. In all cases equivalent solutions of the salts were compared and the effects were more marked in the experiments with less concentrated salt (0.004 normal), in which case dispersion has been shown to be of the "electrical" type.

Analogy with euglobulin presented by caseinogen and other proteins.

Caseinogen has been found to show close analogy with euglobulin as regards the effects of electrolytes upon its solutions in either acid or alkali.

Michaelis and Rona [1910, 2] drew attention to the fact that among the naturally occurring proteins whose solution was accompanied by the acquisition of electric properties, in addition to globulin, were caseinogen, gliadin, and edestin, and they determined the iso-electric point in each case. The result was especially interesting in the case of caseinogen, where the particles were found to be iso-electric with the solution, and to be precipitated when the concentration of hydrogen ions was 1.8×10^{-5} normal, a degree of acidity far beyond that determined for euglobulin or for the heat-denatured proteins of serum.

The following experiments, set out in Table VIII, show the analogy with euglobulin to be closely maintained in respect also of the action of electrolytes. For example, in an opalescent solution (0.05 %) in weak hydrochloric acid, in which the protein particles were yet visible under the microscope using a high power and dark ground illumination, the caseinogen was found

to be positively electrified. This solution was readily precipitated by a minute concentration of sodium sulphate (equal to 0.0005 normal) or sodium citrate (0.00005 normal); with increased concentration of either salt (0.015 and 0.001 normal, respectively) the protein particles were again dispersed and found to carry a negative charge in both cases, see Table VIII, Experiment A.

TABLE VIII.

Influence of electrolytes upon agglutination of caseinogen
(Merck's pure casein).

(A) Dispersed with a little HCl.

(B) Dispersed with a little NaOH.

× × × Complete agglutination.

× Partial agglutination.

× × Almost complete agglutination.

× - Almost complete dispersion.

- Complete dispersion.

Exp. No.	Concentration of protein, %	Salt added	Concentration of salt, in terms of normality	Degree of agglutination	Sign of electric charge carried by the particles
(A)	0.05	0	0.00	-	+
	„	Na ₂ SO ₄	0.0005	× × ×	
			0.001	× ×	
			0.005	× ×	
			0.01	× -	
			0.015	-	-
			0.02	-	
	„	Na ₃ Cit	0.00001	× ×	
			0.00005	× × ×	
			0.0001	× ×	
			0.0005	×	
			0.001	-	-
			0.002	-	
(B)	0.03	—	0.00	-	-
	„	CaCl ₂	0.01	-	
			0.02	× -	-
			0.05	×	-
			0.09	×	0
			0.10	×	
			0.15	×	
			0.2	× -	0
			0.5	-	
	„	La(NO ₃) ₃	0.00002	-	
			0.00005	×	-
			0.00008	×	
			0.0001	× ×	
			0.0002	× × ×	
			0.0005	× -	
			0.001	-	+

Experiment (B) with an alkaline dispersion of caseinogen 0.03%, showed an exactly analogous set of phenomena with lanthanum nitrate.

With calcium salts the action appears to be different in character. In the first place no complete precipitation takes place; the size of the particles is increased, but no complete agglutination occurs although the solution becomes turbid. The particles, when dispersed again by increased concentration of the salt (0.2 to 0.5 normal) do not appear to carry any electric charge. In this respect also the analogy with both denaturated serum-proteins and with euglobulin is maintained.

V. SUMMARY.

1. The iso-electric point for euglobulin has been re-determined and found to coincide with the point of most rapid agglutination, viz.: at a hydrogen ion concentration of about 3×10^{-6} normal, a figure which agrees with that obtained by Michaelis and Rona [1910, 2].

2. The solution or dispersion of euglobulin by electrolytes is shown to be much influenced by the nature (especially as regards valency) of the constituent ions and to be of two general types:

(a) "electrical" type of solution in which the euglobulin dispersion is accompanied by the acquisition of an electric charge by the protein particles, the sign of which is similar to that of the more potent ion of the electrolyte employed.

(b) "molecular" type of solution, in which the dissolved euglobulin is electrically neutral.

In type (a) the dispersion is considered to result from a specific adsorption of the ion possessing the higher valency, in (b) from a molecular union with (Hardy) or adsorption of (Schryver) the salt as a whole. Both types of solution are accompanied by loss of electrical conductivity in the liquid.

The "electrical" type of solution is well seen in case of dispersion by such salts as sodium citrate and lanthanum nitrate in low concentration; in case of the more ordinary salts, containing mono- or divalent ions only, the electric forces concerned are not powerful enough to disperse globulin until the concentration is raised to a point where "molecular" solution takes place. In the case of sodium citrate, the "electrical" type of solution was found to change to the "molecular" type as the concentration of the salt was increased.

3. Euglobulin, when denaturated by heat, no longer possesses the property of forming the "molecular" type of solution with electrolytes. On

heating the latter, in some cases the degree of dispersion is merely diminished, and the protein particles acquire an electric charge, whose sign is determined by the more potent ion of the electrolyte employed; in other cases agglutination takes place.

4. The reaction of acid and alkaline solutions of euglobulin is greatly influenced by the addition of electrolytes, the hydrogen and hydroxyl ion concentration being reduced respectively. In case of the former the effect is much increased with rising valency of the anion and in alkaline solution the result is determined by the valency of the kation.

5. The influence of electrolytes in causing precipitation of globulin dissolved in acid and alkali may, in some instances, be adequately explained by the alteration in reaction, described under 4; in this way solutions too acid or too alkaline for agglutination of the globulin may be adjusted to the iso-electric point by the addition of an appropriate electrolyte.

Precipitation by electrolytes may, however, also take place in solutions whose reaction is still far removed from that of the iso-electric point. In these instances it is attributed to neutralisation of the electric charge originally carried by the protein particles by means of a specific adsorption of the oppositely charged ion of the electrolyte; the effect is related to valency.

6. In the properties regarding solution and precipitation detailed under 1, 4 and 5, euglobulin, in common with caseinogen, and the vegetable globulins presents a very interesting analogy with heat-denaturated proteins. Euglobulin differs from heat-denaturated protein in its capacity to form solutions with electrolytes in which the protein particles are electrically neutral.

In conclusion, I wish to express my indebtedness to Prof. C. J. Martin for much helpful advice and criticism.

REFERENCES.

- Abderhalden (1903), *Zeitsch. physiol. Chem.* **37**, 49
— (1904), *Zeitsch. physiol. Chem.* **44**, 17.
Burton (1909), *Phil. Mag.* (6) **17**, 583.
Chick and Martin (1911), *J. Physiol.* **43**, 1.
— — (1912), *J. Physiol.* **45**, 261.
Dabrowski (1912), *Bull. Akad. Sci. Cracow A*, June, 485.
Freundlich (1910), *Zeitsch. physikal. Chem.* **73**, 385.
Hardy (1900), *Proc. Roy. Soc.* **66**, 110.
— (1905), *J. Physiol.* **33**, 251.
Linder and Picton (1895), *J. Chem. Soc.* **67**, 63.

- Mellanby (1905), *J. Physiol.* **43**, 338.
Michaelis (1909), *Biochem. Zeitsch.* **19**, 181.
—— and Mostynski (1910), *Biochem. Zeitsch.* **24**, 79.
—— and Rona (1910, 1), *Biochem. Zeitsch.* **27**, 38.
—— ——— (1910, 2), *Biochem. Zeitsch.* **28**, 193.
Moll (1904), *Beiträge*, **4**, 563.
Osborne and Harris (1905), *Amer. J. Physiol.* **14**, 151.
Pauli (1899), *Pflüger's Archiv.* **78**, 315.
Pfeiffer and Modolski (1912), *Zeitsch. physiol. Chem.* **81**, 329.
Schryver (1910), *Proc. Roy. Soc. B*, **83**, 96.
Sörensen and Jurgensen (1911), *Biochem. Zeitsch.* **31**, 397.
Starke (1900), *Zeitsch. Biol.* **40**, 494.
Whitney and Ober (1902), *Zeitsch. physikal. Chem.* **39**, 630.