STUDIES ON THE EFFECT EXERTED BY *SHAKING ON SERUM.*

By HANS SCHMIDT

(*From the Bacteriological Department, Lister Institute, London.*)

Introduction.

Owing to the difficulty of satisfactorily explaining many facts in serological work, there has been a tendency in the last few years to consider serological phenomena more and more from a physico-chemical point of view.

A serum, physically considered, is a protein hydrosol containing electrolytes. Different proteins, globulins and albumins, are kept in the serum in the relatively stable state of an hydrophile colloidal solution both by their mutual protecting influence as well as by the influence of the salts. Under the most diverse conditions of a purely physical nature, such as heat, storage, freezing, decrease of salt concentration by dilution, or loss of salts by dialysis, increase of the surface by shaking, etc., the stability of the colloidal phases is altered and more or less energetic alterations of the physical state of the serum take place. These may be reversible or irreversible and are followed by changes in the serum as regards their serological properties.

Shaking a serum produces well-marked alterations in it, visible to the naked eye first of all as cloudiness and followed later by precipitation. The production of cloudiness in a serum by shaking has been already observed by Jakoby and Schuetze (1910), but not until recently in the work of P. Schmidt and M. Liebers (1913), H. Schmidt (1913) and L. Hirschfeld and R. Klinger (1914) has this phenomenon been especially taken into account and recognised as the main alteration which a serum undergoes during shaking and of which all other changes observed are more or less a consequence. Seeing the increase of surface to be the main effect of shaking, and that the mechanical agitation by itself plays

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a less important part, probably only in continuously renewing the surface, it may be assumed that the alteration in the serum as the effect of shaking occurs more quickly the greater, *ceteris paribus*, the surface is, to which the serum is exposed. It is therefore evident that the intensity of the shaking influences greatly the time necessary to produce cloudiness in a serum, for the more intensely shaken a serum is, the finer is the froth and the greater is the development of the surface. S. and S. Schmidt-Nielsen (1909), in their experimental work on the inactivation of rennet by shaking, have observed to what extent the difference in the ratio of shaken liquid to bottle volume produces a variation of the results. Ritz (1912) also drew attention to this relation as regards shaking of serum and in a former paper (1913) I fully confirmed this.

In order to obtain comparable results, the ratio of bottle volume and liquid as well as the intensity of the shaking movement must be kept constant.

Factors concerned in the effect of shaking upon serum.

Supposing that these relations are kept throughout under optimal conditions, there are still many other factors which may by their presence either accelerate or inhibit the effect of shaking on serum. Before quoting them it should be mentioned that all the following observations are made on fresh guinea-pig serum. The complementing power of such a serum is a characteristic property of its freshness and will become lost by any factor which may alter the physical conditions of the serum proteins. The influence which shaking especially exerts upon the complementing power of a serum will be dealt with in a further communication. It is proposed here merely to deal with the effect of shaking in producing coagulation and denaturation of the proteins of a serum.

The concentration of the serum.

In the first experiments made to inactivate the complement of a serum by shaking, it was found by Jakoby and Schuetze (1910) that a longer and more intense shaking is required to render a serum inactive when undiluted, and therefore these authors used the usual dilution of 1 : 10 in their experiments. This dilution has been later found by Ritz (1912) to be an optimum, and my results, published in a former paper (1913), partially confirmed this. In the meantime I found, however, that frequently a serum diluted 1 : 20 or even 1 : 40 with 0·85 % saline

showed the same degree of cloudiness and inactivity as regards complement action as when diluted 1 : 10. If the inactivation by shaking is due to change in surface energy, I can see no reason for an optimal dilution. The process is to be considered as progressing with time. The shaking causes aggregation and coagulation of a certain part of the proteins, as will be more fully explained later. This coagulation affects only a part of the total proteins. Now, since the relation between volume of liquid and gas as well as the shaking movement are kept constant, thus producing by the equal intensity of shaking an approximately uniform froth and therefore an approximately similar extent of surface, the relation of the denaturated protein to the total protein-content will be the smallest in the undiluted serum, which therefore needs a greater time to become altered to the same extent as in the diluted serum. The relation of the amount of the unchanged proteins to the denaturated proteins increases with the concentration of the serum, as Schmidt-Nielsen also found in the inactivation of rennet by shaking. If the complementing power of the serum be taken as a measurement of the effect produced by shaking, the decrease of complementing power by shaking will be partially covered by that produced by dilution. In the case of a serum the conditions are more complicated than in the case of ferments as rennet or pepsin, etc., inasmuch as the shaking produces principally a denaturation of the euglobulins, as well as an alteration of the albumins, which latter play an important part in the stability of the globulins. Since dilution of the serum decreases the absolute content of albumins, this factor must be taken into account, when considering the more rapid inactivation of diluted sera effected by shaking.

Concentration of salts in the serum.

If instead of 0·85 % saline, dist. water is taken as dilution medium, or if NaCl crystals are added to the native serum, the concentration of salts will be altered and, apart from the effect of shaking, this alone will account for a change in the stability of the colloidal proteins.

a. Decrease of salt concentration.

Sachs and Teruuchi (1907) found that dilution 1 : 10 with aqu. dist. and standing $1\frac{1}{4}$ hours at 37° C. will affect a complement-containing serum in such a manner as to render it inactive, if it is again made isotonic. They found that this phenomenon took place more rapidly

at higher temperatures and that a dilution of 1 : 10 was an optimum. Such a serum could be reactivated by addition of the albumin fraction obtained by dilution and acidification with CO_2, while the euglobulin fraction was rarely able to do so, and only then to a lesser degree [Sachs and Bolkowska, 1910]. Marks (1912) found that a serum after dilution to 1 : 10 with aqu. dist. and standing $1\frac{1}{4}$ hours at 37° C. was not completely inactive. The fractions of a serum so treated obtained by CO_2 gave full action, when combined with the corresponding fractions of a normal serum. Bessemans (1913) extended these observations and found that the fractions were inactive when combined with themselves. If the fractions of a normal serum were diluted 1 : 10 with dist. water and then kept for $1\frac{1}{4}$ hours at 37° C., he found, generally speaking, no loss of power in their action. Sometimes a slight reduction could be observed in the action of the globulin fraction, as also observed by Guggenheimer (1911), but no alteration of the albumin fraction ever occurred. Diluted with aqu. dist. produces decrease of the salt concentration. This alters the conditions controlling the solubility of the euglobulin, part of which is therefore thrown out of solution in the form of a suspension, which renders the serum opalescent and cloudy. Addition of salt brings the suspended euglobulin promptly back in solution. After standing a long time however the suspended and precipitated euglobulins undergo alterations, becoming insoluble. The solubility of the euglobulins is favoured by the presence of serum albumins. A solution of euglobulin obtained only by means of salt is always more or less opalescent, indicating that there is no true solution. According to H. Chick (1913) electrically neutral compounds of the salts with the euglobulin are formed, which Schryver (1910) supposes to be due to adsorption. In the serum the albumins, owing to their lower surface tension value, exert a protecting influence on the euglobulin in solution, causing a greater dispersity and thus a true solution of the latter. A more detailed reference to these relations will be given later.

In view of the importance of the relationship between the effect of dilution and standing and the effect produced by shaking, I repeated the experiments of Sachs and Teruuchi and give below the results obtained.

Technique :

Haemolysis was due to the complementing action of the serum in combination with sensitized sheep red corpuscles, of which a 2·5 % emulsion in 0·85 % saline was used. Sensitization was effected with three times the single lysing dose of an inactivated rabbit immune serum.

The following schema has been adopted to illustrate different degrees of haemolysis :

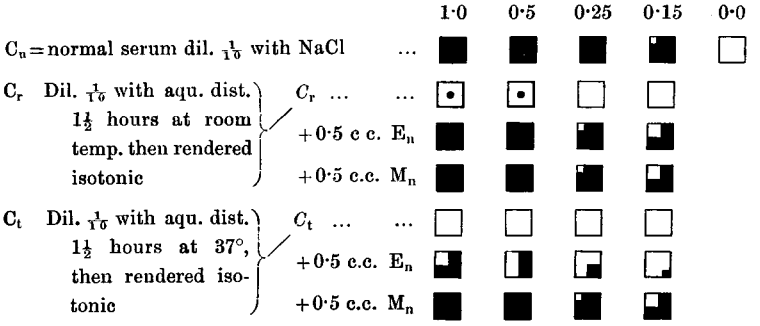
- No haemolysis.
- Faint trace of haemolysis.
- Very slight haemolysis.
- Well-marked haemolysis.
- Half haemolysed.
- Strong haemolysis.
- Very strong haemolysis.
- Nearly complete haemolysis [a slight trace of unlaked cells at the bottom].
- Complete haemolysis.

Experiment showing the influence of dilution with dist. water upon a complement-containing guinea-pig serum.

Fresh guinea-pig serum was diluted 1 : 10 with aqu. dist. and kept for 1½ hours at room temperature and at 37° C. Then the serum, rendered isotonic, was tested for its haemolytic power with 0·5 c.c. sensit. red cell emulsion (AB dose = 3 times the single lysing dose).

Haemolysis after 1 hour at 37° and 15 hours at room temperature.

Total volume in each tube 2·0 c.c.



The thus treated sera, before being rendered isotonic, were treated by CO_2 according to Liefmann's method. The globulin fraction, = M-piece, was not taken up in solution by 0·85 % NaCl until immediately before use. The albumin fraction, = E-piece, was filtered through hardened paper and then rendered isotonic.

The fractions thus obtained from C_n, C_r, C_t are respectively E_n, E_r,

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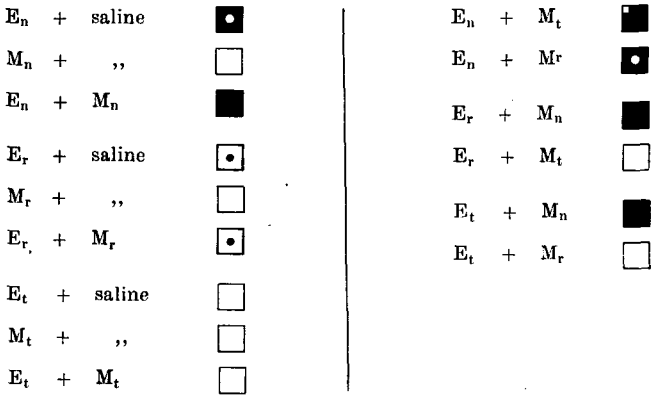
E_t, M_n, M_r^1, M_t. 1 c.c. of each gave with 1 c.c. of each other and 0·5 c.c. of the sensit. red cell emulsion, the following reactions :



The experiment shows that inactivation has taken place, but more completely in the case of the serum which has stood at 37° C. But contrary to the statement of Teruuchi, I found that a fresh globulin-fraction of a normal serum exerted a better restitution effect than the albumin fraction.

As regards the CO_2-fraction of the serum, treated by dilution with aqu. dist. and standing, it follows from the above experiment that the alteration concerns only the globulin fraction, the albumin fraction of a serum thus treated proving to be active when combined with a normal globulin fraction.

If the serum however was not fresh, I found that dilution with dist. water and standing had a far less destructive effect upon the complementing power of a serum. Inactivation will not be complete, even after many hours standing. Cf. Exp. 6 in my paper on the effect of Berkefeld filtration upon Complement (this *Journal*, 1914). What process has taken place during dilution and standing so as to render the complementing power of a serum inactive is difficult to say, for the suspended globulins seem to be in no way altered, the serum becoming quite clear if rendered isotonic. Probably some alteration in the H·-concentration takes place, which may inhibit complementing action, but no definite evidence can yet be given in favour of this conception.

Now, if such a serum, the euglobulins of which are in a state of lability owing to lack of sufficient salt concentration, be shaken, it is evident that these euglobulins will aggregate and coagulate more readily.

In fact, a serum diluted 1 : 10 with aqu. dist. is very rapidly altered by shaking, so that the cloudiness, which in the unshaken control serum will promptly vanish by addition of salt, will not disappear if the serum be rendered isotonic, but will do so on the addition of alkali, thus indicating that the protein particles have undergone denaturation.

Increase of salt concentration.

An increase in the concentration of salt has a somewhat opposite effect upon the influence of shaking. Since Friedberger (1908) drew attention to the use of concentrated salt solution for the preservation of the complementing power of a serum, this method has been frequently and successfully used [Hecht, 1910]. As already mentioned, a certain concentration of salt is necessary for the stability of the euglobulin in the serum [H. Chick (1913)]. This protection however is only a relative one, as the euglobulin is also precipitated in the course of time in a native and otherwise untreated serum. Higher salt concentration prolongs the time during which a serum can be kept clear, by maintaining the dispersity of the protein-phases and thus preserving the complementing power.

If a serum is shaken in a hypertonic condition the time necessary to cause a denaturation and coagulation of proteins will be found to be prolonged, but even as high a concentration as 20 % will not absolutely protect the serum, as well as the isolated protein solutions, from becoming denaturated, as will be seen later. The protecting influence of salts upon the effect of shaking is therefore relative, which fact has already been recognised by Hirschfeld and Klinger (1914) to be the case in human serum.

The influence of temperature upon the effect of shaking.

The accelerating influence of a temperature of 37° C. upon the effect of shaking has been observed by all authors who have worked on the shaking inactivation of complement and ferments. The denaturation and precipitation of a part of the serum proteins occur also at low temperatures. Seeing that it is principally a surface phenomenon, this would be expected to be the case. The acceleration observed at 37° C. is a summation of the effects produced by the temperature and that produced by the shaking. An explanation of this very complicated process will be attempted later. Here, only the fact may be mentioned.

If, however, on the other hand, a serum has been previously heated, the time required to produce cloudiness and coagulation by shaking will be found to be prolonged, the more so the greater the temperature to which the serum was heated. This observation has only been made by Hirschfeld and Klinger (1914) in the case of human serum. They found that exposure to a temperature of 58° C. for 1 hour prevented the serum from becoming cloudy on shaking, as well as by dilution with aqu. dist. They postulate that thermoinactivation as well as a hypertonic condition, the latter in a lesser degree, renders the serum euglobulins more stable; an explanation of this fact however was not given. I think a probable explanation can be given by the results of observations made by H. Chick and C. J. Martin (1913) and H. Chick (1913) in the following way.

The denaturation process of proteins as the result of high temperatures has an abnormally high coefficient of temperature, which apparently effects the denaturation of proteins and their coagulation in the serum to be due to a certain temperature. In reality the process of denaturation takes place already at lower temperatures, but proceeds very slowly. It can however be observed to take place at 37° C. under favourable conditions. The euglobulins form salt compounds in the serum, which remain electrically neutral, when enough salt is present to prevent their dissociation. If the euglobulin has been denaturated by heat, and there is evidence that an exposure to 56° C. for half an hour is sufficient to produce heat-denaturation, the euglobulins lose their characteristic property of forming electrically neutral solutions with salts. The euglobulin particles become electrically charged and any change in dispersity is prevented by their mutual repulsion. The alkalinity of the serum is increased. A decrease of $H^{\cdot}$-concentration takes place [S. T. Sörensen and Jürgensen (1911)], and the dispersed protein particles are negatively charged. As long as in such a serum the $H^{\cdot}$-concentration is kept constant, the stability of the euglobulins remains increased.

This may probably be the principal reason why sera previously heated remain clear much longer on standing and require a considerably longer time to become visibly affected by shaking.

I have often observed in the case of guinea-pig serum that half an hour's heating at 56° C. does not prolong very much the time necessary for coagulation by shaking, but a longer exposure to 56° C. prolongs considerably this time, and I was unable to bring about a visible coagulation by shaking in the opalescent serum diluted 1 : 10 with saline and exposed to the temperature of boiling water. It may be possible that

the resistance against the shaking effect, which can be observed in heated sera, may be partly due to the influence of capillary-active substances, which are formed in the serum during heating and which lower the surface tension against air. Further reference to this will be made later.

Influence of the nature of the gas.

That the coagulation of proteins by shaking is not dependent on the nature of the gas, which is in contact with the serum surface, in so far as it is chemically inactive, has already been shown in the early experiments of Melsens (1851), Harting (1851) and Smee (1863, 1864). Metcalf (1905) stated that the formation of membranes of the surface of colloidal solutions was independent of the oxygen and Ramsden (1894) found that all solutions, the particles of which coagulate under the influence of shaking, show this phenomenon also, if oxygen is excluded. Similar results were obtained by A. O. Shaklee and Meltzer (1909), who found that the inactivation of pepsin by shaking took place equally well whether the experiment was done in O, H, or in CO_2. Contrary to this, Courmont and Dufourt (1912) concluded from their experiments that the inactivation of the complement in a guinea-pig serum by shaking was due to oxidation. This could not be confirmed by P. Schmidt and M. Liebers (1913) nor by myself (1913) working independently. No evidence was found for oxidation being responsible for the shaking effect. However, instead of shaking the serum in oxygen, as I formerly did, in order to determine what rôle, if any, oxidation plays in shaking inactivation, I exposed the complement-containing serum to the influence of oxygen in nascent state obtained by the action of the serum katalase on hydrogen peroxide. I found that if H_2O_2 was added to the serum in such quantities as to produce an 0·5 % H_2O_2-solution, no evidence of loss of complement could be observed in spite of the large amount of oxygen thus developed in the serum, enough being present to render the serum colourless in a few minutes. I am therefore convinced that the effect of shaking on serum is not due to oxidation.

Influence of the age of the serum upon the shaking effect.

Storage of a serum diminishes its complementing power. This loss runs parallel with the formation of a cloudiness and of a precipitate. Apart from the influence of bacterial growth, which according to Hara (1913) accelerates the loss of complementing power, this decrease of complement and the formation of precipitates occur also under sterile

conditions. The precipitates in old sera consist of euglobulin, which has become more and more insoluble. It has been seen that hypertonic condition as well as previous heating increase the stability of the euglobulin to this extent, that sera so treated remain clear for a considerably greater length of time. If a serum which has been kept for a long time be treated by CO_2 according to Liefmann's method, I found that the action of the fractions, thus obtained, varied according to the length of time the serum has been kept. Great individual variations however occur. Generally speaking I found, at the commencement, when the complement activity was not yet completely lost, that both fractions gave a complete action when combined with the corresponding fractions of a fresh serum. Later on the globulin fraction of the old serum loses its power to reactivate a fresh albumin fraction, but not *vice versa*. Finally the albumin fraction of the stored serum loses its action. This however was not found to be of constant occurrence, for sometimes a serum was found, the globulin fraction of which retained its activity with a fresh albumin fraction as long as the albumin fraction did with a fresh globulin fraction. This however must be considered as exceptional. Changes taking place in the euglobulins in the course of storage have been already noted by Liefmann (1911) and Mutermilch (1911). They found that in sera kept for a long time the albumin fraction was the more stable of the two, and this has been confirmed by Bessemans (1913) more recently.

A probable explanation of the process is, that first of all an adsorption of the albumins in the free surface takes place. This size of the surface is of importance, for I could observe that with an increase of the surface against air there is an increase in the rapidity with which the complement of a serum is destroyed. This adsorption of albumins on the surface diminishes the stability of the euglobulins, leading to a gradual precipitation of the latter. At the same time adsorption of salts is possibly taking place, for according to Hecht (1914) the electrical conductibility decreases with the length of time.

The process of inactivation due to storage then closely resembles that produced by shaking. The same alterations occur, but more rapidly, and are therefore followed by a much more rapid loss in the inactivity of the albumin fraction.

It is interesting to notice that the greater stability of the euglobulins produced by heating is paralleled by the observation of Mutermilch (1911), who found that under similar conditions the time required to produce precipitation of the euglobulin by dialysis is considerably

prolonged. The same but to a lesser degree has been found by him (1911) to be the case in an old serum.

This is precisely what occurs with regard to the production of a precipitate in a serum by means of shaking, a well-marked inhibition occurring in the case of a heated serum and a relatively lesser inhibition in an old serum.

The effect of shaking upon the isolated proteins of a serum.

In regard to all these factors, which either accelerate or tend to inhibit the effect of shaking, it is highly probable, that the shaking of a serum results in rendering the euglobulins unstable and thus increasing their tendency to aggregate and precipitate. That the effect of shaking is really of such a nature is rendered certain by a study of the effect of shaking upon the isolated proteins. I undertook first of all experiments showing the effect of shaking upon the isolated fractions, obtained by Liefmann's method [dilution with dist. water and acidification with CO_2]. The precipitate which, re-dissolved in 0·85 % NaCl, represents the globulin fraction, contains the euglobulin and a relatively small part of the pseudoglobulin, which can be demonstrated by the behaviour of the solution, when 1 : 3 or 1 : 2 is saturated with the sulphate of ammonium.

The same method of salting out reveals the fact that the albumin fraction of the serum, *i.e.* the supernatant fluid rendered isotonic, contains all the albumin and the main bulk of the pseudoglobulin.

Now, if the albumin and globulin fractions are shaken under similar conditions the following results are obtained.

The shaking of the globulin fraction produces relatively little and unstable froth, and in a very short time cloudiness is developed and precipitation takes place. If the precipitate is removed by centrifugation, and the shaking continues, no more cloudiness will appear, the liquid remaining clear. Half saturation with the sulphate of ammonium reveals the presence of some pseudoglobulin, euglobulin no longer being present. If the salt concentration of the globulin fraction is increased to 20 %, the time required to coagulate the euglobulins is found to be prolonged but coagulation finally does take place.

The shaking of the albumin fraction however is accompanied by the production of a copious and persistent froth and the liquid is found to be still quite clear at a time when the euglobulins of the globulin fraction are to a large extent precipitated. Prolonged shaking however produces coagulation of some of the proteins in the albumin fraction, also the

smaller the quantity of salts present, the more rapidly does this occur. In any way, the amount of protein in the shaken albumin fraction found to be coagulated is very small compared with the big precipitate in the globulin fraction.

In order to decide, whether this coagulated protein of the albumin fraction is pseudoglobulin or albumin, these serum proteins were prepared and tested separately. I obtained these preparations by third, half and total saturation with the sulphate of ammonium, and the euglobulin as well by dilution of a serum and acidification with acetic acid up to its isoelectric point. The proteins thus obtained and freed from the ammonium sulphate by dialysis, were then shaken under varied conditions as to protein—or salt concentration. Summarising my results I can say:

The production of froth was well marked in the albumin solution, to a slightly lesser extent in the pseudoglobulin solution, but the euglobulin solution gave a froth only as long as shaking was going on and this froth being unstable quickly disappeared. This is interesting in regard to the different influence which these proteins exert upon the surface tension of water. Cloudiness followed by precipitation was first observed to occur in the euglobulin solution, then in the pseudoglobulin solution, and finally in the albumin solution, the latter requiring eight hours' shaking. Higher salt concentration tended to inhibit the precipitation, but this inhibition was less marked in the case of euglobulins.

In view of the great difficulty of getting absolute separation of the different proteins, I am far from considering the proteins, prepared as described, as being pure. I therefore repeated the experiments with preparations of albumin and globulins of horse serum, very pure preparations, which Dr Hartley kindly put at my disposal. I shall refer to these preparations later on in regard to the surface tension. The shaking effect of these proteins was similar to those described above, the albumins requiring the longest time to be precipitated. P. Schmidt (1914) did not evidently shake the albumin long enough, for he did not succeed in getting cloudiness and precipitation.

In spite of the certain restrictions which are to be observed in applying these observations to those observed when shaking the whole serum, I am inclined to consider it highly probable that the euglobulins in the serum are the first proteins which are denaturated and precipitated by shaking. Some alterations in the albumins must also have taken place, because their influence on the dispersity of the euglobulins must

have been lessened. If albumins either in the form of a CO_2-albumin fraction, or in pure state, be added to the serum before shaking takes place, this excess of albumin prolongs the time necessary for the precipitation of the euglobulin, and *vice versa* an excess of globulin is found to shorten this time. Only when a serum is shaken for a very great length of time can it be assumed that part of the pseudoglobulin as well as of the albumin is precipitated in a denaturated state.

In every case, where a precipitate was produced by shaking, the latter has been found to be insoluble except in alkalis, which shows that denaturation has taken place.

Now the question arises what is the real cause of this denaturation of the proteins, which occurs when they are shaken with a gas, and what other processes accompany this shaking-effect.

The effect of shaking explained as a consequence of change in surface energy.

The observation that shaking of a protein solution with a neutral gas produces the separation of a solid body, can be traced back to 1851, when Melsens and Harting first observed this phenomenon, which was later confirmed by Smee (1863) and more closely investigated by Plateau (1873), who considered the formation of solid surface membranes to be of a similar nature. The same observations were made by Naegeli (1880) and Kauder (1886). To Metcalf (1905) we owe a thorough investigation of the nature of the formation of surface membranes, especially those occurring in solutions of peptone. Peptone as well as other substances, the solutions of which tend to form surface membranes, lower the surface tension of water [Freundlich, 1909]. According to Gibbs' thermodynamic principle those substances will be positively adsorbed at the free surface of the liquid, thus producing a relatively higher concentration in the surface. This increase of concentration in the surface may become so great, that gel-formation takes place. The conception of H. Freundlich (1909, p. 79) is probably more correct, namely that the adsorbed substance in the surface is undergoing reversible or irreversible alterations in a less soluble body, especially if the substance is by itself not readily soluble in water as the dispersion medium. This change in solubility has also been observed by Deveaux (1904), who noticed the formation of white insoluble membranes, when egg albumin was dropped on to a clean surface of water. These phenomena are especially well marked in the case of egg albumin and

have been closely investigated by Ramsden (1894). It is evident, that a soluble substance tending to form surface membranes, will show this tendency more so, if by means of shaking the surface is considerably enlarged. Now Ramsden demonstrated this accelerating effect of shaking on the formation of surface membranes, and he succeeded in bringing about almost complete coagulation of the protein content of an egg white solution by means of shaking.

What happens now in the case of a shaken serum? *In vivo* the serum has no free surface against air, but is everywhere surrounded by a wall covered with endothelium. It is probable that the adhesion on this wall is in equilibrium with the cohesion of the serum, thus giving rise to no surface tension of the serum and therefore thus insuring the stability of the serum proteins. If the serum has however a free surface against air, the cohesion will be larger than the adhesion to the air, and a certain positive value of surface tension of the serum against air will result. Now the serum is an electrolyte containing protein hydrosol, and its surface tension against air is less than that of pure water, as J. Traube (1908) first showed. This lower value of surface tension is chiefly due to the presence of proteins. If according to Maraghini (1912) the protein substances are removed by filtration through a collodium membrane, the filtrate will show a surface tension similar to water and even a little higher, this fact being due to the presence of neutral salts, which increase the surface tension. The proteins show therefore a tendency to be positively adsorbed on the free surface against air and the more they lower the surface tension of their dispersion medium, the greater is the tendency for this to occur. Comparative experiments have shown that the loss of surface tension produced by the serum albumins is found to be somewhat higher than that caused by the globulins.

I give below in a table some data, with regard to the surface tension of the different proteins. The figures represent the number of drops given by a stalagmometer of Traube and calculated as mentioned in my former paper (this *Journal*, 1913, 316).

From these data it follows that a serum albumin tends to lower the surface tension of water against air more than the globulins do. This is in accordance with experiments made by F. Bottazzi (1913), but is contrary to the findings of Iscovesco (1910), who found a pure egg albumin to increase the surface tension of water, whereas only the globulins cause a loss of surface tension. Either egg or serum albumin behaves differently as regards its influence upon surface tension, which

			Heated ½ h. at 56°	Boiled
I.	Water	100·00	—	—
	Serum undiluted	111·65	—	—
	Serum diluted 1 : 8 with aqu. dist.	102·51	—	—
	Albumin fraction not rendered isotonic ($\frac{1}{8}$) ...	102·77	107·31	111·02
	,, ,, rendered isotonic ($\frac{1}{10}$) ...	104·95	105·05	112·56
	Globulin fraction in 0·85 % NaCl ($\frac{1}{10}$) ...	102·16	104·95	106·70
	Serum dil. $\frac{1}{20}$ with 0·85 % NaCl	102·67	104·49	109·83
II.	Serum diluted 1 : 4 with aqu. dist.			102·50
	Albumin fraction not isotonic ($\frac{1}{10}$)			104·30
	,, ,, ,, heated ½ hour at 56°			105·81
	,, ,, ,, 2 hours shaken at 37° and filtered ...			103·39
	,, ,, ,, Control 2 hours at 37°			105·06
III.	Serum diluted 1 : 10 with NaCl solution			103·67
	,, ,, ,, ,, heated ½ hour at 56° ...			106·87
	,, ,, ,, ,, 2 hours shaken at 37° ...			103·67
	,, ,, ,, ,, 2 hours control at 37° ...			104·19
	Globulin fraction, *well washed* with aqu. dist. then diluted 1 : 5 with 0·85 % NaCl			101·4
	Globulin fraction, heated ½ hour at 56°			101·75
	,, ,, 1 hour shaken			101·55
	Albumin fraction dil. 1 : 8, not isotonic			103·97
	,, ,, heated ½ hour at 56°			106·06
	,, ,, 2 hours shaken at 37° and filtered			101·22
	,, ,, Control at 37°			103·95

Surface tension of horse serum preparations.

		Aqu. dist.—100
1.	50 c.c. aqu. dist. + 0·5 gr. air dried whole horse serum	103·15
2.	50 c.c. ,, + 0·5 gr. ,, albumin	113·74
3.	50 c.c. ,, + 0·5 gr. ,, globulins (eu- + pseudoglob.) ...	107·08

would be very interesting, or the albumin is not free from the accidental presence of other substances, which lower the surface tension. These may be the serum pigments, which are naturally present in the CO_2-albumin fraction, and also in the albumin preparations obtained by salting out, for a total saturation with the sulphate of ammonium involves the pigments, these latter coming down with the precipitate. This question, viz. how far the observed loss in surface tension in albumin solutions is due to the actual accidental presence of such substances, I must leave open for the present, but for the sake of argument will assume it to be the case, namely that serum albumin produces a considerable loss of surface tension of the water against air.

Now a serum exposing a free surface to air is by that reason alone

rendered unstable, involving as it does the adsorption of albumins. This adsorption decreases the protecting influence which the albumins exert on the dispersity of the euglobulins in virtue of their greater influence on surface tension. This process is favoured by any increase of temperature which produces an increase in the intensity of the Brownian movement. The euglobulin thus becoming more labile tends to aggregation and precipitation, undergoing a slowly but steadily progressing denaturation. This process, which takes place when serum is exposed with a free surface to air, and which is accelerated by an increase of temperature, is accelerated to a considerable extent when, by means of shaking with air, not only is its free surface enlarged but also continuously changed and renewed. This continuous alteration of the surface causes the already aggregated protein particles to disperse again, which process, when continuously repeated, favours the condition of irreversible insolubility of the proteins, as has been already pointed out by Ramsden. The dispersion of already aggregated proteins is the more marked, the higher the protein concentration in the serum is, *i.e.* the more the mutual protecting influence of the different proteins can be exerted. Dilution of the serum favours therefore the occurrence of the condition of coagulation and irreversible insolubility. This irreversible change in solubility affects naturally first the euglobulins, which by themselves show the least tendency to solution. If the serum has been previously heated, which causes a considerable loss of surface tension, as observed by Traube (1908) and confirmed by many other authors, it may be possible to assume that the formation of capillary-active substances by heat, according to L. Berczeller (1913), albumoses and pepton, prevents the proteins from getting adsorbed on the surface, thus allowing them a greater stability against the influence of storage and shaking. This may also be taken into account when considering the influence of the electric charge of the denaturated protein particles upon their state of dispersion.

In a former paper of mine (1913) I was able to show that the surface tension of a serum is increased by shaking. The collected froth shows however lower surface tension than the shaken liquid, which is in accordance with Gibbs' principle.

How far the shaking effect upon a serum is due to alterations of the surface energy is best demonstrated by the fact that the addition to a serum of traces of such a capillary-active substance, as saponin is, completely prevents the proteins from being affected by the shaking, whatever the length of time during which it is shaken.

The possibility that, apart from alterations of the surface energy, other factors may play a more or less important part in the production of the shaking effect, cannot be excluded. It may be assumed, for instance, that the rapid movement of the air bubbles through the liquid gives rise to an electric charge on their surface, the density of which will increase with the curvature of the bubbles, *i.e.* with their smallness, and it may be possible that these electrically charged surfaces play a part in favouring the aggregation and coagulation of the proteins in solution. No definite evidence however is forthcoming to support this view.

Finally I mention the probability that a change in the H·-concentration in the shaken serum has taken place. Using neutral red and rosolic acid as indicators, I could not find however any difference in reaction of a shaken as compared with an unshaken serum. The addition of either traces of alkali or of acid to a serum proved to be without any inhibiting effect upon the effect of shaking, but I found that alkali produced a slight acceleration of the coagulation and precipitation in the shaken serum. In view of the importance of the H·-concentration of a serum as regards its serological properties, the question of any alteration of H·-concentration as a result of shaking deserves a special study.

GENERAL CONCLUSIONS.

Shaking a serum with air produces coagulation and denaturation of the euglobulin, due to alterations in the surface energy of the serum, leading to adsorption of albumin, thus rendering the euglobulins labile, which then coagulate and become insoluble.

This process is not due to oxidation, and it has been shown that conditions which render the euglobulin more stable, such as previous heating and the presence of a hypertonic medium, tend to inhibit the effect of shaking, whereas dilution with water or storage, rendering the euglobulins labile, favours also the effect of shaking.

REFERENCES.

BERCZELLER, L. (1913). *Biochem. Zeitschr.* LIII. 215.
BESSEMANS, A. (1913). *Zeitschr. f. Immunitätsf.* XIX. 380.
BOTTAZZI, F. (1912). *Rend. R. Acc. dei Lincei Roma* [5], XXI. 221, ref. *Kolloidzeitschr.* 1913, XII. 205.
CHICK, H. (1913). *Biochemical Journal*, VII. 318.
CHICK, H., AND MARTIN, C. J. (1913). *Kolloidchem. Beihefte*, V. 49.

COURMONT AND DUFOURT (1912). *Compt. rend. Soc. Biol.* LXII. 1058.
DEVAUX (1904). *Procès verbeaux des Séances Soc. Phys.*, Bordeaux, 7 Jan., cit. of Metcalf.
FREUNDLICH, H. (1909). *Kapillarchemie.*
FRIEDBERGER (1908). *Centralbl. f. Bacteriol.* XLVI. 441.
GUGGENHEIMER (1911). *Zeitschr. f. Immunitätsf.* XI. 393.
HARA, K. (1913). *Zeitschr. f. Immunitätsf.* XVII. 209.
HARTING (1851). *Neederl. Lanc. Sefet*, cit. of Metcalf.
HECHT, H. (1914). *Sitzungsber. d. wissenschaftl. Ges. deutscher Aerzte in Böhmen*, ref. *Münch. med. Wochenschr.* 15. 851.
HIRSCHFELD, L., AND KLINGER, R. (1914). *Zeitschr. f. Immunitätsf.* XXI. 40.
ISCOVESCO, H. (1910). *Compt. rend. Soc. Biol.* LXIX. 622.
JAKOBY AND SCHUETZE (1910). *Zeitschr. f. Immunitätsf.* IV. 730.
KAUDER (1886). *Arch. f. Path. u. Pharm.* XX. 416.
LIEFMANN (1911). Ref. d. Freien Vereinig f. Mikrobiologie. *Centralbl. f. Bacteriol.* L. 151.
MARAGHINI, F. (1912). *Arch. d. Farmac. speriment.* XIII. 53.
MARKS (1912). *Studies from the Rockefeller Institute*, 654.
MELSENS (1851). *Annal. Chim. Phys.* III. 33. 170.
—— *Journal f. prakt. Chem.* LIV. 62. 383.
METCALF, W. V. (1905). *Zeitschr. f. physikal. Chem.* LII. 1–54.
MUTERMILCH (1911). *Compt. rend. Soc. Biol.* LXX. 577; LXXI. Nro. 35.
NAEGELI (1880). *Königl. Bayr. Acad. d. Wissensch. math. phys. Klasse.* XIII. 156.
PLATEAU (1873). *Paris. Statique des liquides*, 2. 261–296.
RAMSDEN, W. (1894). *Arch. f. Anat. u. Physiol. Abt. f. Physiol.* 517.
—— (1903). *Proc. Roy. Soc.* LXXII. 156.
—— (1904). *Zeitschr. f. physikal. Chem.* XLVII. 336.
RITZ (1912). *Zeitschr. f. Immunitätsf.* XV. 145.
SACHS AND BOLKOWSKA (1910). *Zeitschr. f. Immunitätsf.* VII. 778.
SACHS AND TERUUCHI (1907). *Berlin. klin. Wochenschr.* 2139.
SHAKLEE, A. O., AND MELTZER, S. J. (1909). *Americ. Journ. of Physiol.* XXV. 81.
SMEE (1863). *Proc. Roy. Soc.* XII. 399.
—— (1864). *Proc. Roy. Soc.* XIII. 350.
SÖRENSEN, S. P., AND JÜRGENSEN (1911). *Biochem. Zeitschr.* XXXI. 397.
SCHMIDT, H. (1913). *Journal of Hygiene*, XIII. 291, 314.
SCHMIDT, P., AND LIEBERS, M. (1913). *Zeitschr. f. Immunitätsf.* XIX. 373.
SCHMIDT-NIELSEN, S. AND S. (1909). *Zeitschr. f. physiol. Chem.* (LX.) 426.
—— *Zeitschr. f. physikal. Chem.* LXIX. 547.
SCHRYVER (1910). *Proc. Roy. Soc.* B. 83. 96.
TRAUBE, J. (1910). *Biochem. Zeitschr.* X. 380.