The Action of Formaldehyde upon Blood Serum.

By

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I

The living organism is a vast equilibrium system, every component of which is itself an equilibrium system of no less complexity. In studying the physiological or chemical state of the organism one is at liberty to choose as components of the system those constituent parts which are most amenable to the treatment of the subject, provided always that one satisfies the condition that one shall choose the least number of components necessary to form all of its phases. In a study of the nervous mechanism of an organism one might consider the components to be the nervous system, the vascular system and the tissues not included in these two organs. A study of the vascular system could be based upon the selection, as the two components, of the vascular system itself and the extravascular tissues.

But in all cases one must go still further and,

remembering that each of these components is itself heterogeneous, endeavour to understand what composes the nervous or vascular systems or the tissues and to determine the factors governing each of these systems during the normal life of the organism. Eventually one is inevitably led to a chemical view of life-processes and to attempts to formulate these processes in terms of pressure, temperature and the concentration of chemical compounds, - proteins, carbohydrates and their split products, inorganic salts and water.

In accordance with the law of Le Chatelier, every change of one of these factors (pressure, temperature and concentration) governing any of the systems into which the organism is arbitrarily divided, occasions a rearrangement of the system in such a direction that the factor in question experiences a change opposite to the original change. If, for example, one wishes to study the relationship between the vascular system and the extravascular tissues, then one will finally reduce them to systems composed of proteins, carbohydrates, fats, inorganic salts and water. In the normal state of the organism these substances will be present in a definite concentration in each of the two systems. The individual system will

be governed by the factors temperature (the normal body temperature of the organism) concentration of chemical constituents and, probably, pressure. If a rise in temperature occurs, caused, for example, by bacterial invasion, then the system will so rearrange itself, by alteration of concentration and pressure factors, that the normal temperature will again be approached.

Since the blood is relatively easy to analyse chemically and can be obtained without seriously damaging the living organism and since, also, it is quick to show changes in the other component of the body system, the extravascular tissue, the blood has been most frequently used in diagnostic tests. This is especially the case in diagnosis of parasitic diseases, for the efforts of the body to overcome infection are almost always shown by the appearance in the blood stream of new chemical compounds or physical conditions: the chemical or physical interpretation of these changes depending upon one's view point.

The value of a blood test will be decided by its specificity, the degree of accuracy to which it lends itself, the speed with which the blood change tested follows the appearance of a pathological state and the definiteness

of the interpretation which can be placed upon the result. Other things being equal, the most valuable test will be that based upon a definite chemical change, demonstrated by simple chemical methods. Such chemical tests are few in number; so few that any new test suggested should be carefully investigated before being finally rejected as valueless.

It is the object of this thesis to inquire into the mechanism, the applicability and the interpretation of the test known as the Formol-Gel Reaction.

II

The action of formaldehyde upon pathological sera was first noted by Gaté and Papocostas (1920). These workers found that serum drawn from syphilitics, on treatment with a small quantity of commercial formaldehyde solution, set to a clear gel, within twenty-four hours. The reaction was not observed with other sera and it was therefore proposed as a test for diagnosis of syphilis. Later Fox and Mackie (1921) obtained a similar reaction with serum from kala-azar cases and with a few cases of malarial infection. Napier (1921) found that a gel forms in sera infected with kala-azar, malaria, tuberculosis and leprosy.

Admangué and Gonzales (1922) obtained the reaction in dogs infected with taenia and in rabbits suffering from coccidiosis. Combiesco (1922) found the reaction to occur in erysipelas, scarlatina and eruptive diseases. This author also obtained a formol-gel reaction in animals suffering from anaphylactic shock. Panisset and Verge (1922) showed that in some cases the serum of healthy cattle gives the reaction and that it always occurs in the serum of tuberculous cattle. Turkhud (1922) definitely observed the reaction in leprosy and Plantureux (1922) obtained it with the serum of dromedaries suffering from trypanosomiasis. Johnston (1923) has found that the test is frequently given in erysipelas, sometimes in gonorrhoea and in old tuberculosis. He found it to be absent in typhoid and pneumonia and usually absent in dermatosis and cancer.

Obviously these observations prove the test to be useless as a specific method of diagnosis, yet in the opinion of the author, the reaction is sufficiently interesting to be worth careful investigation and may even be found useful as a means of following the progress of the infections to which it is applicable.

III

Gate and Papocostas treated 1 c.c. of clear serum with 2 drops of commercial formalin and shook to make sure of mixing. The test tubes used were covered with cotton and left at room temperature from 24 - 30 hours. Within that time period the serum gelatinised so that the test tube could be reversed without the contents pouring out. The gel was clear, and shook like ordinary jelly. The reaction was obtained with syphilitic serum, whether fresh or not, if uncontaminated. Normal serum gave no action.

Napier discovered the reaction in the case of kala-azar by using formalin as a preservative in place of phenol, which was not available. The serum was obtained from blood drawn from the veins after the injection of potassium antimony tartrate. The lower portion of the serum soon solidified and became opaque. Normal serum, under the same conditions, was quite clear. The reaction was intensified by the addition of a dilute solution of phenol. This worker used the reaction as a routine test. He carried it out by adding 1 drop of commercial formalin (actually 30%) to 1 c.c. of the serum. The mixture was shaken and the test tube placed in a rack at room temperature. The serum became viscid and set in

1 or 2 minutes to a white opaque gel. In 3 to 20 minutes it became solid. It was "like serum coagulated by heat, or the 'white' of a hard-boiled egg". If the serum was originally stained with hemoglobin the coagulum had a pink tinge, which turned chocolate-brown after 24 hours. Napier found that the reaction occurred to a slight extent in leprosy, malaria and tuberculosis. In leprosy and tuberculosis the serum became cloudy during gel formation. In two cases of malaria, in which there were large numbers of parasites in the blood, the solid had a characteristic green tinge. Normal serum remained quite clear and fluid for a more or less indefinite period. In kala-azar cases the tendency to reaction seemed to disappear during treatment with tartar emetic. Napier carefully studied the conditions of the reaction and found that an optimum result was obtained by adding 1 part by weight of formalin to 200 parts of serum. Excess diminished the distinctness of the test and even prevented it in some cases. He obtained the reaction also with acetaldehyde (a 40% solution in distilled water) but gel formation was much slower and less complete. The portion of the serum responsible for the reaction seemed to be the eu-globulins. After 30% saturation with ammonium

sulphate, and removal of the precipitating agent by dialysis, the filtrate gave no action. The precipitate was dissolved in physiological saline and also freed from ammonium sulphate by dialysis. It then gave the formaldehyde reaction. If the serum was dialysed against fifty times its volume of distilled water and, subsequently, most of the globulins precipitated, the speed of the gel formation was decreased. If dialysis was continued in running water and the remaining globulin precipitated thus, the solution did not give the reaction. The precipitated globulins, dissolved in saline, gave the test. It was found also that alteration of the P_H of the serum, in either direction, prevented the production of a gel. A slight increase of P_H in some normal sera caused gel formation with formaldehyde. The author remarked that in kala-azar cases the P_H was slightly increased.

IV

Napier's tracing of the reaction to the eu-globulins suggested that any process which would lead to the increase of these proteins either by increasing the concentration of all the proteins or by increasing the relative quantity of eu-globulins would bring normal serum into a condition

in which gels might be obtained with formaldehyde. Banzhaf (Hiss-Zinsser, Text Book of Bacteriology, New York, 1922, page 272) states that if serum is heated for twelve hours at 56° C., fifty per cent of the pseudo-globulins is converted into eu-globulins. Gibson (1916), Banzhaf (1908) and Heinemann (1916) have used this change in developing methods for the concentration of tetanus, diphtheria and hog-cholera antitoxins. The process was applied to the study of the formol-gel reaction as follows: -

Horse serum, centrifuged and filtered, was heated in a water bath for several hours at a temperature between 56° C. and 60° C. At intervals samples were taken out and after cooling to room temperature were treated with formaldehyde. 2 drops of a commercial solution of formalin, almost neutralised with N/10 caustic soda, (formaldehyde content then 28.9 grams in 100 c.c.) were added to 1 c.c. of the serum and the mixture well shaken. The test tubes were covered with cotton and stood in the ice chest. The course of the reaction was observed at intervals. The results are tabulated below.

Time of heating of serum. Hours	Result
1	No gel formed.
1½	No gel formed.
6¾	Viscid fluid set to gel on long standing.
7¾ (a)	Complete clear, orange gel in about 20 hours.
7¾ (b)	Slight gel, much fluid in 20 hours.
9¼	Clear orange gel in 16 to 20 hours.
10¼	Clear orange gel in 65 minutes.
12	Became viscid in 8 minutes. Clear orange gel in 12 minutes.

All the gels formed were of the same type as those observed in the serum of syphilitics. None showed any tendency to opacity, as in cases of tuberculosis, leprosy or kala-azar.

The work of Banzhaf, Gibson and Heinemann shows that such heating of serum increases the relative amount of eu-globulin in serum. It was thought possible that formation of a gel with serum might in some cases be due to concentration of the whole protein content of the serum, rather than to increase in the proportion of one particular protein. To test this, horse serum was evaporated in vacuo at room temperature (about 18° C.) and samples tested at intervals. In a purely qualitative test it was found that, with increased concentration, the time period between addition of formaldehyde and formation of a gel was progressively decreased. For example, after 3 hours evaporation, a gel formed in 7 days (unevaporated serum gave a gel in 10 days). After evaporation for 13¼ hours the gel formed in 4½ hours (the serum became viscid in 1¼ hours) and after evaporation for 15 hours viscidity set in almost at once and a gel formed in 45 minutes. All these gels were clear and elastic. Later the test was carried out quantitatively. 50 c.c. of serum were evaporated in vacuo until they occupied only 39 c.c. Of these 2 c.c. were taken and treated with formalin. A gel was obtained within 24 hours. The remainder was evaporated until each 1 c.c. of the concentrated serum was equivalent to 2.32 c.c. of the original.

With 2 c.c. of the serum a gel formed in less than 10 minutes. In another experiment 50 c.c. of serum were evaporated to 16 c.c. On addition of formaldehyde, gel formation occurred within 30 seconds. It was found, also, that a serum which would give no gel in the cold, with formaldehyde (at least within a period of weeks) could be converted to a gel by heating below 75° C. The gel formed was transparent and elastic.

While carrying out these experiments I became acquainted with the results of several workers on this subject. Bessemans and Van Boeckel (1922) had also studied the influence of heat on the power of normal serum to give gels with formaldehyde. Their experiments on heating serum were carried out in tubes closed with either cotton or cork. They found that gel formation was least accelerated in the case of serum heated in closed vessels. They found also that the effect was more intense after prolonged heating, especially at a temperature slightly above 56° C. In a later paper, (1923) Bessemans showed that the reactivity of a serum with formaldehyde could be increased by evaporation or addition of salts and that these agents could be placed in the following order of decreasing efficiency: -

Evaporation, Ammonium Sulphate, Sodium Chloride, Magnesium Sulphate.

Evaporation had a far greater effect than addition of sodium chloride sufficient to give, to unevaporated serum, an NaCl content equal to that resulting from evaporation. Bessemans also found that dilution with water, salt solutions, serum, neosalvarsan, arsenobenzol, mercurous cyanide and hemoglobin retarded the formol reaction. My results on evaporation and heating therefore completely confirm those made earlier by Bessemans and his co-worker. But these workers do not, by evaporation, mean evaporation in vacuo. The effects they classify as due to the treatment are really due to the combined effects of heat and concentration.

A more detailed study was made of the influence of the concentration of serum upon the tendency to gel-production with formaldehyde. Into each of eleven test tubes was measured a sample of the serum which had been evaporated so that 50 c.c. of the original serum were condensed into 39 c.c. The quantities of this liquid used varied progressively from 2 c.c. down to 1 c.c. In each test tube the volume was made up to 2 c.c. by adding distilled water where necessary. The test was

made by adding to each tube 2 drops of the neutralised formaldehyde.

Tube	Concentration in terms of original serum as 1.	Result
1	1.437	Viscid in 7½ hours. Gel within 24 hours.
2	1.365	ditto.
3	1.293	ditto.
4	1.222	Viscid in 24 hours. Gel begins to form in 30 hours. Complete within 48 hours.
5	1.15	Viscid in 48 hours. Gel within 72 hours.
6	1.078	Incipient gel in 4 days. Gel in 98 - 100 hours.
7	1.00	Viscid in 9 days. Gel, not quite firm, in 11 days.
8	0.934	Viscid in 11 days. Gel, not firm, in 11½ days.
9	0.863	Gel in 18 days.
10	0.791	Gel in 20 days.
11	0.72	Gel in 28 days.

Under the same conditions the unevaporated serum gave a gel in 10 days. All the gels were clear, and when quite solid, elastic. All eventually became solid.

For the purpose of the study of the formol-gel reaction in disease, the results obtained with serum in tubes 1, 2 and 3 are worth consideration. In a later section I propose to discuss their significance in the light of the changes in the protein content of serum in the course of infection.

The action of such salts as ammonium sulphate, sodium sulphate and magnesium sulphate has obviously no direct bearing upon the changes which blood serum undergoes in the course of disease processes. Yet so large a part of our knowledge of the proteins has been derived from examination of the action of salts upon the physical state and chemical activity of these complex bodies that it is of interest to find how their addition to normal serum will affect the tendency of the serum to react with formaldehyde. A study of such effect may also lead to some explanation of the cause of gel formation in sera.

As mentioned above, Bessemans carried out experiments in this direction. But they were not so complete as to make repetition superfluous. Therefore the action of a series of salts has been studied, the method consisting

of the addition of a measured quantity of a given salt to a known volume of the fresh serum and the subsequent making up of equal volumes (2 c.c.) of the serum in a regular series of salt content. The formaldehyde test was always carried out by adding to each cubic centimeter of the treated serum 1 drop (0.05 c.c.) of a commercial solution of formaldehyde neutralised by N/10 NaOH. This solution contained 28.9 grams of formaldehyde to 100 c.c. of solution. The results are shown in the following tables.

Bessemans found that under certain conditions the gelification of serum with formaldehyde was inhibited or completely absent. These conditions always involved dilution of the serum. There are, however, certain substances which appear to have the power of completely nullifying any gel production by formaldehyde. One of these is urea. A specimen of serum, evacuated in vacuo at room temperature, until it gave a gel in less than 24 hours, was saturated with urea. By addition of further quantities of the serum, samples were obtained which were 1/2 and 1/3 saturated with urea. The formaldehyde test was carried

The Influence of Inorganic Salts upon the Speed of Gelification of Serum by Formaldehyde.

Salt	Time of gelification after addition of HCHO Concentration of salt in gram equivalents per 1000 c.c. of serum			
	2	1	0.75	0.5
Ammonium Sulphate		about 50 hrs.	115 hrs.	150 hrs.
Sodium Sulphate		3 "	4½ "	within 22 hrs.
Magnesium Sulphate		4½ "	6 "	6 - 7 "
Sodium Chloride	over 36 hrs.	2¼ "		4 "
Potassium Chloride		about 15 "		15 - 20 "
Sodium Acetate		40 min.	2½ "	4½ "
Sodium Nitrate	54 hrs.	70 hrs.		

N.B. In all cases, except in that of $NaNO_3$, the gels obtained were white and opaque. Those with $NaNO_3$ were clear, with a very slight opalescence.

out in the usual way, but there was no sign, even of viscidity, much less of gel formation, in any of these samples 11 days after addition of the aldehyde. Since urea is a normal constituent of blood, it is interesting to find at what concentration the compound can inhibit the formol-gel test. A preliminary experiment made on a serum, which after evaporation, in vacuo, at room temperature, gave a gel in 1 to 3 hours, showed that a concentration of 12 milligrams of urea in 100 c.c. of serum had absolutely no inhibiting effect. Hardy found that urea has the power to dissolve serum globulins, when in high concentration. Such action will obviously depend upon the amount of globulin present. In the evaporated serum used, this was abnormally high.

The urea content of normal blood is low being only about 20 milligrams per 100 c.c. In certain pathological conditions it is increased, but the highest concentration reached is only about 375 to 400 milligrams per 100 c.c. In experiments with evaporated serum containing amounts of urea varying between 10 and 78 milligrams per

cubic centimeter no inhibition of gel formation was noticed, though the samples of serum used were gelled by formaldehyde in times varying from 20 minutes to 10 days, (i.e. the sera were at both extremes of gelification time).

Further experiments are being carried out to determine the effect of cholesterol, glucose and bile salts, in concentrations such as appear in blood in the course of disease, upon the speed of gel formation in serum.

V

As already mentioned, the gels produced by the action of formaldehyde upon serum of infected subjects are of two distinct types - a clear, transparent gel, obtained with serum of cases of syphilis and malaria, and a cloudy gel, obtained in cases of leprosy and tuberculosis. The descriptions of these two types of gels as 'like ordinary jelly' and 'like the white of a hard-boiled egg', respectively, suggests that both were elastic.

In my experiments upon the methods of obtaining gels by treatment of ordinary serum, I have obtained the same two distinct types of product. And it is interesting to find that the two types fall into two distinct classes, on the basis of the treatment to which the normal serum

was subjected in their preparation. ALL GELS OBTAINED BY HEATING SERUM, OR CONCENTRATING SERUM BY EVAPORATION IN VACUO, HAVE BEEN CLEAR AND TRANSPARENT. On the other hand, ALL GELS PRODUCED BY ADDITION TO THE NORMAL SERUM EITHER OF SALTS OR OF GLOBULIN HAVE BEEN WHITE AND OPAQUE. The significance of this fact will be discussed in a later section.

The gels do not involve the whole of the serum proteins, indeed they involve but a small portion of the total protein content of the serum. The protein not affected by formaldehyde can always be extracted by shaking with a dilute saline solution. Fractional precipitation of the extract then shows that a small fraction of the eu-globulins is left unattacked, a larger portion of the pseudo-globulins and practically the whole of the serum albumins. The gel itself is not soluble in water or in salt solutions. It is not soluble or but very slowly soluble in acid, but is soluble in alkali (10% NaOH), from which it can be reprecipitated by careful addition of acid (10% acetic acid).

A series of experiments was carried out to determine the least quantity of formaldehyde which would

give the test within 24 hours, in a sample of serum which had been evaporated in vacuo until it gave the reaction within this time period. To each tube, containing 1 c.c. of the serum, was added 1 drop (0.05 c.c.) of a formaldehyde solution of known concentration. The mixture was allowed to stand at room temperature and the time of gel formation noted. The results were -

Tube	Strength H.CHO soln. grams per 100 c.c.	Gel formed in hours
1	28.8	1.884
2	21.6	2.917
3	19.2	5.5
4	14.4	17.33
5	9.6	within 19
6	7.2	" 24
7	5.76	" 70
8	4.8	" 70
9	3.6	
10	2.88	

This serum was concentrated (in vacuo, at room temperature) to about 1.77 times normal strength.

It seems therefore, that a concentration of

formaldehyde as low as that found in tube 6 will give the test within 24 hours. But the actual concentration of the aldehyde here is only 3.43×10^{-3} grams HCHO per cubic centimeter of serum. Yet a concentration much lower than this is sufficient to produce a gel even in a normal serum. The lowest quantity of formaldehyde found to give the gel was 2.99×10^{3} grams per cubic centimeter of serum. This gave a gel in 35 days at room temperature. Now the work of Kossel and Gavrilov (1912) and of Van Slyke and Birchard (1913) has shown that the free amino nitrogen in the unchanged protein molecule corresponds to one half the lysin nitrogen. Since lysin is a diamino acid, this means that for each lysin molecule in the protein chain there is one free amino group. The lysin content of the serum proteins has been estimated as

Albumin	11.08%	Serum Globulin (1)	6.72%
		(2)	6.75%
		Average	6.735%

(Lock and Thomas, quoted from Mathews, Physiological Chemistry, 3rd Edition, p. 553). The sample of serum used in estimating the minimum requirement of formaldehyde, analysed for

Albumin 4.29% Globulin 2.5%

From these values it can be calculated that 100 grams of

serum will contain protein amino groups equivalent to

4.75×10^{-3} grams lysin in albumin

and 1.684×10^{-4} grams lysin in globulin.

The molecular weight of lysin, $H_2N.(CH_2)_4 CH(NH_2)COOH$ is 146. To each gram molecule of protein lysin, one gram molecule of formaldehyde (30 grams) can join to give a methylene derivative. The protein content of the normal serum used was therefore equivalent to

3.46×10^{-4} grams HCHO corresponding to globulin

and 9.76×10^{-4} grams HCHO corresponding to albumin,

or a total of 1.322×10^{-3} grams formaldehyde. Assuming, for the moment, that the specific gravity of the serum was 1, it seems that the minimum quantity of formaldehyde found to give a gel is actually equivalent to about 2 1/3 times the total protein content of the specimen of serum used. When the serum was concentrated (in vacuo, at room temperature) to 1.77 times its original strength, the minimum quantity of the aldehyde giving a gel in 24 hours was equivalent to about 1½ times the serum protein content. Yet no gel has yet been obtained involving the whole of the serum proteins. When a very large excess of formaldehyde solution is added to serum the proteins are practically all slowly coagulated but while they form a somewhat

gelatinous precipitate, no gel is obtained analogous to those produced with a small quantity of the aldehyde.

Napier found that the optimum concentration of formaldehyde to gel kala-azar serum was 1 part of HCHO to 200 parts of serum. According to the results tabulated above, the minimum concentration of aldehyde giving a gel in 24 hours, in the concentrated serum, was 1 part of HCHO to 290 parts of serum.

VI

The action of formaldehyde upon serum is accompanied by increasing viscosity of the mixture. The original limpid fluid slowly becomes viscid, though, in the case of serum of cases of syphilis, malaria, or in sera heated at 56° C., evaporated at room temperature in vacuo, or treated with alcohol, there is no notable change in the refractive power. Gel-formation commences first at the liquid-air interface, so that the serum does not flow on careful inversion of the test tube. But this surface film is easily broken by vigorous shaking. Gradually the whole mass of the serum sets to the gel, which becomes firmer and more elastic as time passes. Tuberculous sera, and sera treated with inorganic salts

(and, possibly leprous and kala-azar sera) after addition of formaldehyde, gradually become opalescent and finally opaque. The gel seems to form evenly throughout the fluid and never attains the firmness or elasticity of the transparent gels. Several days after the formation of a gel in the presence of ammonium sulphate, this gel can be easily broken by shaking. A gel of this type, so broken, has the appearance of the bulky, flocculent precipitate, obtained by precipitating the proteins with salts, though it is somewhat more gelatinous. Gels formed in the presence of sodium nitrate appear to be intermediate between these two forms. They are opalescent, never become quite opaque, and are as firm and elastic as are the transparent gels.

Under the ultramicroscope, the process of gelification of tuberculous serum appears very like the clotting of casein. At first the field contains a few large particles, slightly motile, and a large number of small particles in rapid Brownian movement. As the formaldehyde acts upon the proteins, larger nuclei begin to form and gradually lose their movement. This process spreads through the whole field, until it contains only coarse, motionless masses. "The gelatinisation ... appears to be due to the formation of an insoluble precipitate which does not

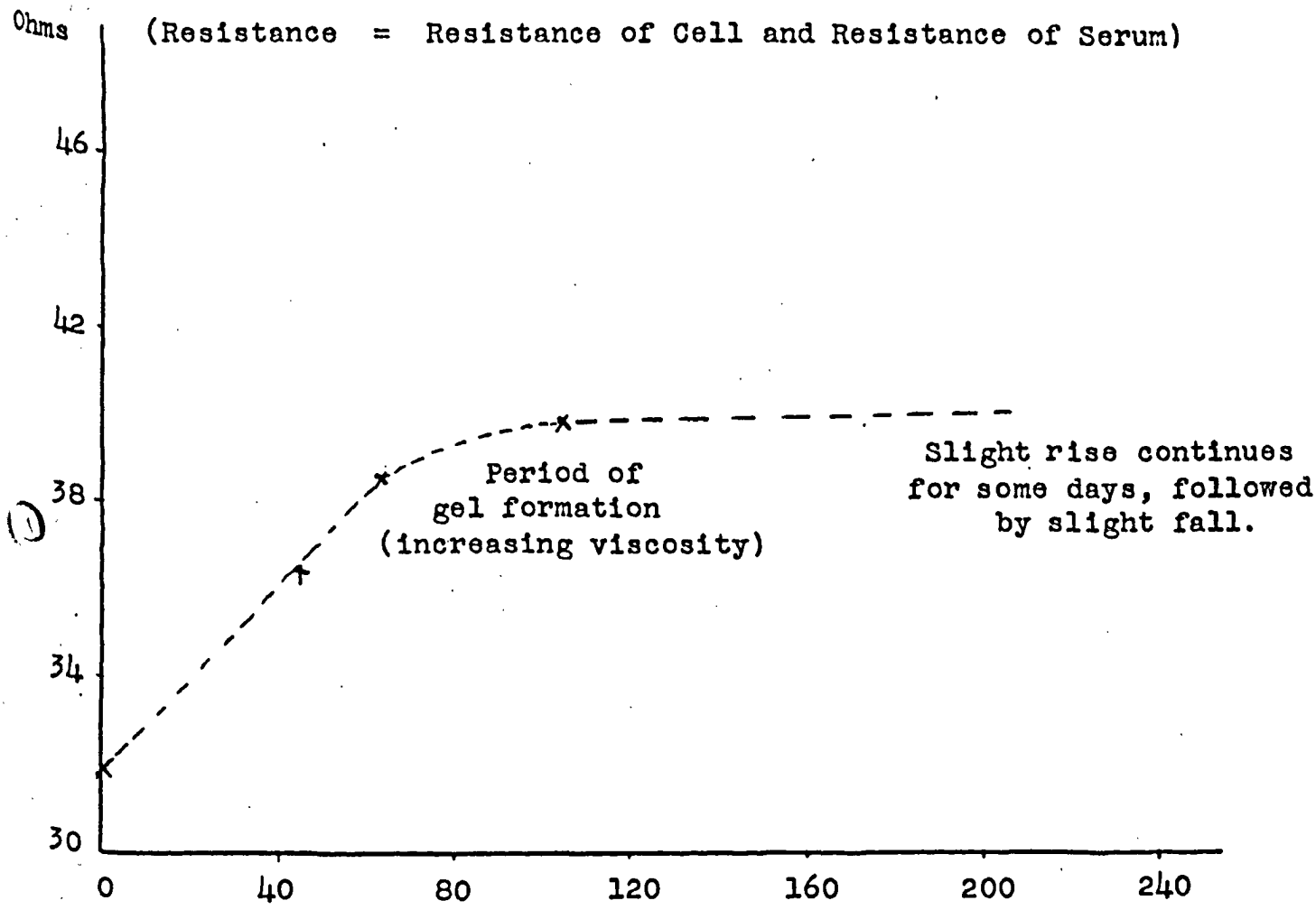
flock out of the solution, but which remains in situ and which holds the liquid between the particles" (Mathews, Physiological Chemistry, 3rd Edition, page 231). In the particular sample of serum studied, the whole process occupied only 5 to 10 minutes. The field finally became opaque, scarcely any light being transmitted.

The viscosity of an evaporated serum, after treatment with formaldehyde, shows a gradual rise with time, increasing rapidly, as gelification sets in, to a value not measurable with the apparatus available. This viscosity change is shown in the accompanying figure.

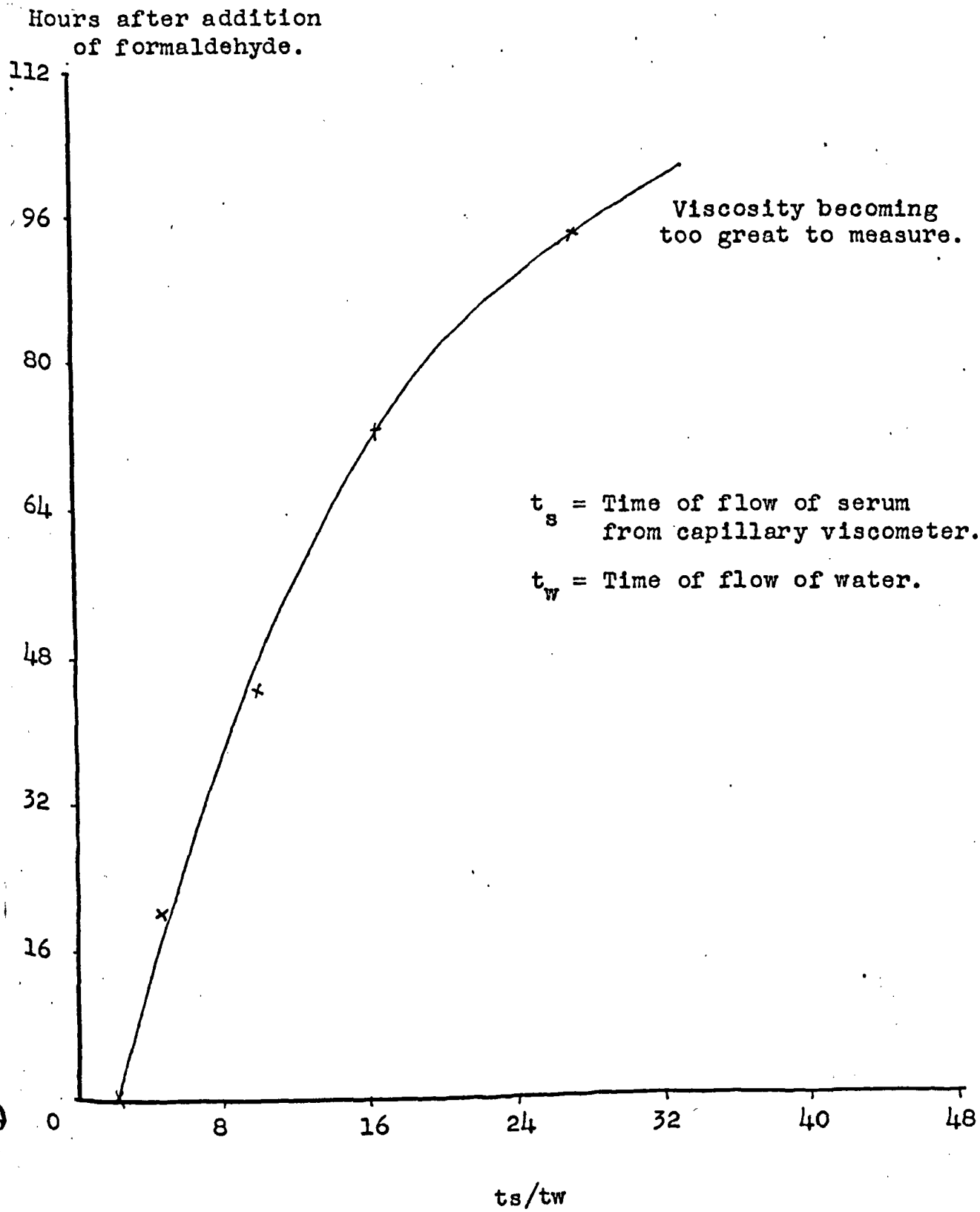
The electrical resistance of a serum, heated at 56° C. for about 12 hours, and treated with formaldehyde, shows a fairly uniform rise to the point at which the mixture becomes viscid. Then the rise of resistance is slower and finally reaches an almost constant value, not greatly different from the original value. There is actually a very slight rise, extending over several hours, followed by a very slight fall. But at this stage the gel breaks away from the electrodes, preventing the taking of further readings. Serum formaldehyde gels appear to be similar to the soap gels obtained by McBain. The increase in electrical resistance during gel formation is small compared with that which accompanies the process of reversion

of phase in an oil-water emulsion. The process of gel-formation in serum appears to be rather a settling out of less soluble constituents of the system to form an open, net-like structure, rather than a complete reversal of the arrangement of dispersing and dispersed phases.

Change of Resistance of Serum during Gel Production with HCHO



Change of Viscosity of Serum during Gelification with Formaldehyde.



Attempts to measure the velocity of action of formaldehyde upon either serum or serum globulins have not been successful. The most satisfactory method would be one in which the unused aldehyde was estimated. But the methods of estimating formaldehyde are not very reliable. Addition of iodine and liberation of the iodine which has not been taken up by aldehyde cannot be employed, for proteins themselves take up iodine and the quantity which they can take up appears to vary irregularly during the course of action of formaldehyde. The study of the rate of reaction between formaldehyde and a pure protein would not throw much light upon the conditions existing in a mixture of serum and formaldehyde, since in this latter case one must consider the effect of salts in addition to the mutual effects of the two proteins, albumin and globulin.

As already stated, the formaldehyde gel appears first at the liquid-oil interface, producing a definite film. If the gel be treated with an alkaline solution of sodium hypobromite (prepared from caustic soda and bromine) no reaction occurs, no gas is liberated. But if this surface film is broken and the interior of the gel is exposed, an immediate evolution of gas is noticed.

From this fact it would seem that the surface film contains no free -CONH- groups with which the hypobromite can react. The interior of the gel, of course, will contain a certain quantity of protein not linked to formaldehyde. Because of this, a formol-gel will give both biuret- and diazo- reactions. These reactions are still given by the insoluble formaldehyde-protein compound, when washed as free as possible from unaltered albumin and globulin, but this residue does not react so early with hypobromite as do unchanged proteins. It is difficult to determine whether the aldehyde reacts with all, or some, of the -CONH- groups of the protein. The ease with which the aldehyde-protein compound dissolves in alkali would indicate that, apart from the conversion of $-NH_2$ to $-N = CH_2$ groups, the protein molecule is but little changed during gel formation.

VII

The action of formaldehyde upon proteins was, apparently, first noticed by Trillat (1892) and Hauser (1903). The former found that on addition of concentrated formaldehyde solution, egg-white was converted into an opaque, gelatinous mass, whilst the latter noticed that

gelatin was changed by formaldehyde into a hard, insoluble substance. Blum (1896) found that addition of a small quantity of formaldehyde to egg-white solution caused the latter to lose its capacity for coagulating on heating: but it remained clear after the addition of formaldehyde. Later Schwarz (1901) showed that dilute solutions, especially in the absence of salts, remain clear on addition of formaldehyde, and lose their coagulability, whereas more concentrated solutions become turbid but can be made to coagulate by the addition of salts. Benedicenti (1897) studied the reaction more closely. He added dilute (2 %) solutions of formaldehyde to protein solutions and estimated, quantitatively, at given intervals, the amount of formaldehyde which had not entered into reaction. For this purpose he used hydroxylamine hydrochloride, which reacts with the aldehyde according to the equation

$$NH_2OH.HCl + H.CHO = CH_2:N.OH + HCl$$

By titrating the hydrochloric acid with standard potassium hydroxide, using methyl orange as indicator, the amount of formaldehyde in a solution could be estimated. When methyl orange was employed as indicator the proteins themselves acted as bases; a certain amount of acid was therefore necessary to neutralise the solution to this indicator before

the addition of the hydroxylamine. This alkalinity decreased as the action proceeded. It was concluded that the alkalinity was due to the presence of amino groups, and gradually diminished as the formaldehyde condensed with them to form methylene derivatives. The reaction with dilute formaldehyde solutions (e.g. 4 c.c. of a 2% solution added to 10 c.c. of a protein solution) was rather slow; usually the maximum amount of aldehyde had not entered into reaction until after two or three weeks. After this time it was found that 1 gram of gelatin combined with 0.0135 gram formalin; 10 c.c. fresh egg-white combined with 0.375 gram; 2 grams powdered egg-white with 0.0360 gram, 10 c.c. blood serum with 0.315 gram, and 5 grams caseinogen with 0.0294 gram formaldehyde. The compounds formed were no longer digestible with pepsin, but could be decomposed when steam distilled, and a digestible protein thereby recovered. The formaldehyde could also be recovered quantitatively in the distillate. Similar results were obtained by Treves and Salomone (1907). Schiff (1901) also investigated the action of formaldehyde upon proteins. He added a concentrated solution (40%) of the aldehyde to a solution of protein and then estimated the acidity of the mixture. The reaction which took place

was assumed to be similar to that occurring with the amino acids. The amino group, with the formaldehyde, forms methylene derivatives. The alkalinity due to the amino groups is therefore removed and the mixture becomes strongly acid and can be directly titrated with alkalis, with the use of phenol-phthalein. By this method Schiff found that 1 gram molecular equivalent of potassium hydroxide neutralised 3,231 grams of egg-albumin and 4,680 grams of gelatin, after solutions of these proteins had been treated with formaldehyde. The titrations were carried out in some cases immediately after mixing the proteins with the aldehyde, and in others after the mixtures had stood for twenty-four to forty-eight hours. The same amount of alkali was required for neutralisation in each case. Thus, in contrast to the findings of Benedicenti, with dilute aldehyde, the reaction with strong formaldehyde is very rapid.

The testing of serum with formaldehyde depends upon the slow action of a dilute solution of the aldehyde. A normal serum will always, eventually, produce a gel. The abnormality of the serum is, therefore, indicated by the relative increase in the speed of the reaction. The visible sign of this increased speed is the earliness of

gel-formation.

In the preceding sections we have seen that a serum which reacts but slowly with formaldehyde can be brought to react rapidly, in some cases instantaneously, by various methods of treatment. These are

(1) Concentration by evaporation in vacuo at room temperature, (18° - 20° C.).

(2) Prolonged heating at temperatures above 56° C. but below the point of heat coagulation.

(3) Treatment with various salts.

A serum which would normally give a gel with formaldehyde can be prevented from doing so by dilution with water or aqueous solutions.

Since these activating agencies produce a common result there must be some common effect of their action upon serum. To discover this common effect we must consider the properties of the protein or proteins involved in gel formation.

As mentioned above, Napier was able to trace the active element of serum to that fraction of the serum proteins known as the eu-globulins. These proteins are

characterised by their insolubility in water, in carbon dioxide and very dilute salt solutions. They comprise the least soluble fraction of the serum proteins. But it is difficult to determine whether the eu-globulins are normally present in serum as such, for there is much evidence that the other serum globulin fraction, the pseudo-globulins are converted into eu-globulins with great ease. For example Banzhaf found that by simply heating serum for 12 hours at 56° C. 50% of the eu-globulins is converted into pseudo-globulin , the conclusion being based upon the only known method of differentiating eu- from pseudo-globulins, namely, the precipitation of the former on prolonged dialysis of the serum against distilled water. One is only justified in arguing, therefore, that by dialysis a portion of the serum proteins is rendered less soluble in dilute salt solutions. There is one outstanding property of the proteins, however, which may be considered, by analogy with inorganic compounds, to be considerably modified by this process of heating. This is, the property of taking up water, the property known as 'hydration'. Of the actual manner in which this water is held in the protein molecule, or of the actual amount of water held, we are as yet ignorant. But there is no valid reason

for imagining that water is held by complex organic compounds in any manner differing essentially from the manner in which it is held by such salt hydrates as sodium sulphate $Na_2SO_4.7\ H_2O$. We may expect therefore that the hydration of proteins will be affected by heat in the same way as is the hydration of salts, that is, it will, in general, obey the law of mobile equilibrium.

On the basis of the work of Henderson and Ryder (1907) demonstrating that tryptic digestion (i.e. hydration) of casein is accompanied by evolution of heat, Robertson (Physical Chemistry of the Proteins, 1920) concludes that a rise in temperature produces a decrease in hydration of proteins, reaching a critical point at the temperature of heat coagulation. Chick and Martin (1910) find that heat coagulation is not an instantaneous process but that it proceeds with a definite velocity, increasing very markedly with rise in temperature. It seems therefore that by heating serum for some time at 56° C. we are reducing the hydration of some, or all of the serum proteins and, in so doing, are rendering a portion of the globulins less soluble and, at the same time, more open to attack by formaldehyde. So much for one of the methods of activating a normal serum toward formaldehyde. It remains for us to discover whether other methods also result

in dehydration of globulins.

Evaporation in vacuo has proved the best method of treating serum to obtain a gel with formaldehyde. This obviously does not involve any notable heat change. But it results in an increased concentration of all the constituents of the serum, with the exception of the free carbon dioxide. The removal of free CO_2 does slightly increase the speed of action with formaldehyde. A sample of serum giving a gel in 11 days, will gel in 7 days if free CO_2 is removed by a stream of nitrogen. (In such an experiment, however, it is difficult to make sure that slight concentration of the serum does not occur). However this increase in activity is but slight compared with that produced by the concentration of the serum. Apart from the well known dehydrating action of high concentration of salts, (which we shall consider later) an increase in concentration of the proteins will, (again by analogy with inorganic salt hydrates), diminish the hydration of these complex substances. (Nernst, Theoretical Chemistry, 1923, page 450). That is, the effect of evaporation will be the same as that of heat.

The tendency of a serum to gel with formaldehyde is increased to a greater or less extent, by the addition of salts. Inorganic salts are noted for their effect upon

the hydration of proteins, which effect varies with the nature of the ions composing the salt and the concentration of these ions. For this reason an extensive list of salts has been employed and concentrations have been varied. It is therefore possible to compare the relative potency of the members of this list with the known power of these members to decrease the hydration of proteins, either as shown by their influence on the swelling of proteins or by their power of precipitation or of changing (raising) the temperature of heat coagulation.

The action of inorganic salts upon the serum proteins has probably received more study than any other factor in the behaviour of serum. It was found quite early that certain salts, sodium chloride and magnesium sulphate were capable of precipitating the "globulins" while ammonium sulphate could remove both globulins and albumins from solution. Halliburton (1884) systematically applied salts to the separation of natural proteins of serum, finding that sodium nitrate and sodium acetate, in addition to the salts already mentioned would separate the globulins. Kauder (1886) studied the precipitating action of ammonium sulphate upon diluted serum. He showed that globulin precipitation, in a serum solution,

commenced when ammonium sulphate was present to the extent of 24 to 29 per cent of complete saturation and ended when the saturation amounted to 34 - 46 per cent. Addition of more salt gave no further precipitate until the degree of saturation reached about 64 per cent. The limits of saturation for commencement and completion of precipitation of globulins (and albumins) varied slightly, being to a small degree dependent upon the concentration of the serum used. Lewith (1887) found that certain salts, sodium chloride, potassium chloride and sodium nitrate, will not produce complete precipitation of even the serum globulin (from a solution) when present to the point of complete saturation. Hofmeister (1887) examined the phenomenon quantitatively. Schryver (The General Characters of the Proteins, 1909, p. 11) tabulates Hofmeister's results in a form extremely useful for the examination of the effect of salts upon the speed of the formaldehyde reaction in serum. Only those salts employed to accelerate this reaction are mentioned in the table.

Factors of Normal Solutions necessary to Start Precipitation of Globulins.

1.5 - 1.69 N	2.03 N	2.51 - 2.72 N	3.53 - 3.63 N	5.42 - 5.62 N
Na_2SO_4	$(NH_4)_2SO_4$	$MgSO_4$	NaCl	$NaNO_3$
CH_3COONa			KCl	

These figures were obtained by the study of the precipitation of globulin from a solution in egg-albumin, the concentration of the globulin being 2 grams in 100 c.c. of the mixture albumin-globulin solution plus salt solution. Since the precipitation of any one protein in serum involves the protective action of the other protein, Hofmeister's experiments fulfil the conditions existing in serum more adequately than do measurements made with solutions of individual proteins.

The order of power of salts to accelerate the gelification of serum by formaldehyde was found to be CH_3 COONa, NaCl, Na_2SO_4, $MgSO_4$, KCl, $(NH_4)_2SO_4$, $NaNO_3$ (see p. 16 b) when all of these salts were present in "normal" solution i.e. 1 gram molecule of salt to 1000 c.c. of serum. At "half normal" concentration sodium chloride appears to activate gel formation as efficiently as does sodium acetate, while at normalities above unity its power of acceleration is reduced. With the exception of ammonium sulphate and sodium chloride the order of the power of these salts to accelerate gel formation is the same as the order of their power to precipitate globulin. The anomalous position of ammonium sulphate is quite possibly

due to its power of reaction with formaldehyde. That of sodium chloride is, at present, difficult to explain. Henley (1923) found that all the serum proteins became less soluble in ammonium sulphate after treatment with formaldehyde. But the concentration of formaldehyde used by him was much greater than that employed in the experiments recorded here and he did not treat the proteins with the salt until after they had been subjected to the action of the aldehyde. The interaction of the two reagents was, therefore, not possible in his experiments.

The action of inorganic salts is a rather more complex one than the mere removal of bound water from the protein molecule. It seemed desirable to try the effect of a reagent which would cause dehydration, yet would have no tendency to combine with the protein to produce complex salts. Such a reagent is found in alcohol. A series of tests was carried out using a 90% solution of ethyl alcohol. The serum used had been evaporated until it gave a gel with the stock formaldehyde within 24 hours. Three tubes were set up as follows: -

Tube 1, 2 c.c. evaporated serum.

Tube 2, 1.9 c.c. evaporated serum 0.1 c.c. 90% alcohol.

Tube 3, 1.8 c.c. evaporated serum 0.2 c.c. 90% alcohol.

To each were added 2 drops of the stock formaldehyde solution. The readings obtained were:-

Tube 1, no gel within 12 hours after adding formaldehyde.

Tube 2, slightly viscid in 4½ hours. Gelled within 6 to 7 hours.

Tube 3, viscid in 3 hours. Gelled in 4 hours.

Thus, in spite of the dilution of the serum by alcohol, the reaction was greatly accelerated in both tubes containing the dehydrating agent. The gels formed were all clear and transparent.

It seems, then, that all the agents accelerating the gelification of serum by formaldehyde have this property in common - that they dehydrate proteins. But if dehydration of proteins merely accelerates the reaction, then it follows that the slow action of formaldehyde upon normal serum is a combination of two processes,

(a) a dehydration of the proteins (globulins),

(b) reaction with the amino groups of the proteins to give methylene derivatives.

Now, in order to account for the basic properties of the amino group, it is necessary to assume that it is present, in aqueous media, as the hydrated group $-NH_2.H_2O$.

Reaction (a) then becomes

$$R.NH_2\ H_2O + H.CHO = R.NH_2 + H.CHO + H_2O$$

But formaldehyde is itself capable of combining with water. Hence we may write this equation

$$R.NH_2.H_2O + H.CHO = R.NH_2 + H.CHO.H_2O$$

and reaction (b) will be

$$R.NH_2 + H.CHO = R.N = CH_2 + H_2O$$

The velocity of reaction (a) appears to depend upon the concentration of H.CHO, being small with low H.CHO concentration and great with high concentration; in fact, in 40% formaldehyde solution, as used by Schiff, the reaction is instantaneous. (a) and (b) must be consecutive reactions, (b) proceeding at a very great velocity, once (a) is complete. The removal of free $-NH_2$ groups, by combination with the aldehyde, would tend to make reaction (a) proceed to completion.

The fact that dilution influences the speed of the reaction rules out the possibility of (a) being a monomolecular reaction. It is necessary to know the exact proportion between the concentration of H.CHO and the speed of the change, in order to classify it as mono-, di- or n-molecular. Such a relationship it is impossible to determine in the complex system which we term "serum". But

its determination may be possible if a pure protein be used. Such a study will be carried out later.

The conditions governing the type of gels obtained are interesting, in view of Hardy's suggestion regarding the state in which the proteins exist in blood serum. That author, finding that the proteins of the serum are non-ionic (by cataphoretic experiments) believes them to be electrically neutral. He says: "The balance of probability is in favour of there being in serum some (possibly one) complex proteid, which breaks down into fractions, whose composition and properties depend upon the degree of dilution and the reagents used". (Hardy 1905) The equilibrium of the serum contents is disturbed by such a process as dialysis, and "globulins" appear. On removal of this insoluble fraction the remaining solution becomes homogeneous, but is non-ionic (Hardy). Robertson interprets this observation as meaning that the protein complex in serum is formed by the union of a number of alkali protein compounds. "If such a complex were non-ionic mere dilution of its solution or the removal of dehydrating agents (salts) by dialysis might result in the splitting off of fraction after fraction. A very faintly alkaline reaction would favour its stability.

A mere trace of acid might be expected to disrupt this complex." (Robertson, Physical Chemistry of Proteins, 1918, p. 153)

If the removal of dehydrating agents (i.e. salts) rendered the complex unstable, then, obviously, the normal stability of this complex is due to the slight degree of dehydration produced by the salt normally present. In addition, since no globulin precipitate or turbidity is noticeable when serum is evaporated in vacuo at room temperature (18° to 20° C.) the dehydration thus produced cannot result in rupture of the complex; nor, for the same reason, can heating the serum at 56° C. for several hours. Sera, so treated, remain perfectly clear and from such sera transparent gels are always obtained by the action of formaldehyde.

But the common laboratory method of obtaining the serum protein fractions, by precipitation with salts, shows that by this treatment the serum complex is broken up. Whenever the gelification of serum by formaldehyde is accelerated by salts, or by acid (which, according to Robertson, also ruptures the protein complex) the gel obtained is cloudy.

Hardy also observed that "fresh ox-serum has an extraordinary power of dissolving globulin" and "in ox-serum so saturated there is not a trace of alkali globulin or any

other globulin". This power of dissolving globulin I have observed with horse serum. But, despite the fact that Hardy's results indicate that this extra globulin combines with the serum protein complex, horse serum so saturated with globulin and then treated with formaldehyde gives a cloudy gel, firmer and more opaque, even, than the gels produced in the presence of salts. If the excess globulin combines with the serum complex, then its combination must be weaker than is that of the original albumin and globulin in the serum complex, otherwise a clear gel should be produced.

In normal serum there is not sufficient salt concentration to split up the protein complex. In some pathological sera - syphilitic, malarial - presumably this condition also holds, for clear gels are given with formaldehyde. But in sera from cases of tuberculosis, leprosy and kala-azar the production of a white, opaque formol-gel leads one to suppose that there are globulins present either outside the serum complex, or so weakly linked to the complex that, like the added globulin in experiments mentioned above, they are split off under the action of formaldehyde. If outside the complex, then they may either have been broken off by an excess of salts or

increased acidity or they may have entered the blood stream from the tissues. If weakly joined to the complex, then also, they may have entered from the tissues.

In this connection, one is reminded of the suggestion of Herzfeld and Klinger (1917) that "by breaking up of body cells there arise protein constituents with the physico-chemical properties of fibrinogen; they are gradually resolved into smaller units of the character of globulin"; and of the idea of Kollert (1923) that "if numerous cells of an organ are damaged and a correspondingly large number of coarsely divided proteins are set free in the tissues at the point of injury, these proteins cannot but cause an alteration of the blood protein content, when they arrive in the plasma from the tissues". Frisch (1921) speaking of tuberculosis, says, "The more acute and progressive a lung tuberculosis is, the more drastic is the cell destruction, the richer is the blood in fibrinogen".

Is the opaque formol-gel in tuberculosis a sign of this fibrino-globulin influx from the tissues? Serum is but a by-product of the clotting of blood. Foster (Text Book of Physiology, 1898, page 1174) states that "when a fluid containing purified fibrinogen is made to yield fibrin by the action of fibrin-ferment, the amount of fibrin formed is always less than that of the fibrinogen which

disappears at the same time. The deficit thus observed is at least partly accounted for by the appearance of a globulin". This globulin must, in Hardy's scheme, remain in the serum as part of the serum protein complex. But suppose that the total fibrinogen in the plasma is made up of blood fibrinogen and tissue fibrinogen. Our methods of detecting fibrinogen are not sufficient, in the course of ordinary blood analyses, to differentiate between fibrinogens from these two sources. Yet both take part in the clotting of blood, and, presumably, in clotting, each yields fibrin and a globulin, which globulin remains in the serum. It is not unjustifiable to suggest that the globulin produced from the tissue fibrinogen will act in the same manner as does any excess globulin added to the serum. It will be dissolved and will combine with the blood protein complex. But this combination will be so weak as to be broken down by formaldehyde and thus such a serum would give a white, opaque gel.

The presence of such tissue proteins in the blood stream could be demonstrated by the method of Retinger (1918). This worker argued that since tissue proteins do appear in the blood in the course of tissue necrosis and since they do not accumulate there, then

specific enzymes must appear also capable of digesting the tissue protein. By isolating proteins from various tissues of the brain and testing out the power of sera to digest these proteins, Retinger was able to diagnose the site of many brain lesions by a purely serological process. It is hoped that such a method can be applied to the study of the tissue proteins, which cause the particular type of formaldehyde gel found in leprosy, tuberculosis and kala-azar.

N.B. The effect of increase of albumin upon the speed of gel formation has not yet been investigated. A preliminary experiment appears to indicate that excess of albumin will delay the reaction with formaldehyde. Although the result needs careful confirmation it is not unexpected. It is known that serum albumin is less easily precipitated than is serum globulin, which implies that it is less easily dehydrated. It is known, also, that albumin and globulin differ in their behaviour toward such a substance as congo red - albumin tending to protect this dye from precipitation by neutral salts while globulin tends to sensitise it, i.e. to accelerate precipitation, (Bonsmann, 1921). The albumin content of serum is usually greater than the globulin content. It is

not improbable that the highly hydrated albumin will tend to protect the less hydrated globulin from the dehydrating action of formaldehdye. Therefore an increase in the albumin content would quite possibly appreciably retard the dehydration of the globulin.

VIII

The formaldehyde test has not been applied to a very large number of diseases, yet, from consideration of the effect of protein concentration on the speed of gel formation, in conjunction with analyses of serum in various pathological conditions, one might predict gel production with many types of diseased sera.

In quite a number of cases, blood analyses show either a change in the total protein content - a dilution or concentration of the serum - or a change in the ratio globulin/albumin, often in the direction of an increase in the numerical value of this ratio. At present there is no really satisfactory method of estimating blood proteins. The refractometric method, developed by Robertson, is a difference method, and the most important fraction of the serum proteins, at least for the formaldehyde reaction, namely, the serum globulins, can only be

estimated by difference. So that one can only emphasise those cases in which there is a large departure from the normal serum protein content.

The formaldehyde reaction has given positive results in the following diseases: -

Syphilis, kala-azar, malaria, tuberculosis (human and bovine), leprosy, coccidiosis (rabbits), taenia (dogs), erysipelas, scarlatina and eruptive diseases, trypanosomiasis (camels), gonorrhoea and in anaphylactic conditions, both sensitisation and shock.

In very few of the diseases studied have observers recorded the type of gel, transparent or opaque, produced.

For statistics regarding blood analyses, reference has been made to papers by Rowe (1916). These statistics are recorded in the following table.

Concentration of Serum Proteins in Disease, Immunity etc.

Disease	Total Protein	Albumin	Globulin
Normal Serum	6.7 - 8.7 % (Average 7.94)	4.95 - 7.7 % (Average 6.2)	0.98 - 1.91 % (Average 1.74)
Tuberculosis	increased		increased
Syphilis	7.5%	5.0%	2.5%
Nephritis, Chronic	increased	Two cases gave 5.44% & 3.06% i.e. low	3.6% & 1.8% i.e. increased
Pneumonia	6.2%	37%	2.5%
Diabetes	4.8 - 3.6%		3.5 - 3.0 % variable
Pernicious anemia	7.4%		slightly increased
Angina Pectoris			increased
Leukemia	7.6%		
Chlorosis	8.25%		
Cholera	increased		increased
Arteriosclerosis	normal		slight increase
Acute infections	generally much reduced		high
Immunisation			
Animals immune to castor oil	may be slightly increased		eu-globulin increased
Animals immune to various proteins	may be slightly increased		increased
Animals immune to diphtheria	5% lowered to 3.7%		increased

This list is,of course, very far from complete and probably many of the observations need confirming, yet there seems to be an abnormally high content of globulin in the serum in many pathological conditions. And in very few of those listed has the formaldehyde reaction been tested. In many diseases, for example, tuberculosis, there is a marked change in the concentration of the serum in the course of disease. According to Wells (The Chemistry of Tuberculosis, Wells, Witt and Long, 1923, Section 2, p. 185) tuberculosis is divided by some writers into three stages, in the first of which there is dilution of the blood, followed in the second stage by an increase of organic solids and by another period of dilution in the third stage. Upon these facts there is not complete agreement. But the extent of dilution is a great factor in the study of the formaldehyde reaction and therefore, before the reaction can be either accepted or rejected as a clinical test, it must be carefully studied in all stages of disease. There are many conflicting statements as to the success of the reaction even in syphilis and tuberculosis - two of the infections to which the test was first applied.

Bessemans found that the number of positive tests (i.e. the number of tests in which gels were obtained) increased with time, no matter what the type of serum used. That being so, it is necessary to define an exact time limit - the period, after addition of aldehyde, during which the appearance of a gel can be considered as a "positive" result. The most convenient time period is 24 hours. In this connection the experiments, quoted above (p. 16 b) on the effect of dilution on the speed of gel production in serum, are of interest. Here a gel was obtained in 24 hours when the serum was concentrated to 1.293 times its original strength, while a serum 1.222 times the original strength showed viscidity. - the stage previous to actual gel formation - in that time. The minimum concentration at which a gel would form lies between these two values. The serum used had, before concentration, a protein content of 4.86% albumin and 1.74% globulin. The arithmetic mean of the values for concentration of the serum, i.e. $\frac{1.293 + 1.222}{2} = 1.257$, which gives a protein content of 6.11% albumin and 2.19% globulin. These figures, of course, hold strictly only for horse serum. The normal protein content of human blood serum as estimated by Thanter and Rowe (1915) averages albumin 7.94%, globulin

1.74%. The horse serum used in the concentration experiments was therefore low in albumin, compared with a human serum. The influence of albumin upon the gel reaction has not yet been completely investigated. An increase in albumin may possibly retard the reaction. A decrease would then probably have the reverse effect. But, roughly, one can say that a gel will be formed in a serum containing about 2.2 per cent globulin, especially since, in disease, when the globulin figure rises, that for albumin usually falls. The figure for globulin in syphilis is 2.5%, the same in pneumonia and a higher figure in diabetes and chronic nephritis. Rowe (1916) mentions one case of tuberculosis of the pleura in which the globulin fraction increases from a normal average of 25% of total proteins to a value of 35%, i.e. from 1.74% of total blood serum to 2.64%, and Hurwitz and Meyer (1916) found that, after intravenous inoculation with bovine tubercle bacilli there was an increase of globulin in serum from 1.3 to 4.5 per cent - the albumin figure falling greatly.

The formaldehyde reaction has been successful in both syphilis and tuberculosis, thus adding additional weight to the results of the experiments on horse serum.

IX

The suggestion of Gate and Papocostas (1920) that the formation of a gel, on addition of formaldehyde to syphilitic serum, might be used as a diagnostic test, has led to many observations on the comparative values of the formaldehyde and the Bordet-Gengou and Wassermann reactions. These observations have shown no regularity of results. In summarising these results Bessemans (1922) concluded that the agreement between the formaldehyde and the other tests was purely relative, depending upon the number of comparisons made and upon the time elapsing between the addition of formaldehyde and the final readings of gel formation. The number of positive formaldehyde reactions increases with time, no matter what may be the infection of the serum tested. In another article on this subject, Bessemans and Van Boeckel (1922) question whether the two types of reactions are not due to entirely different serum constituents.

The Bordet-Gengou and Wasserman tests are carried out with a system containing an "immune" serum (that is, a serum in which, in course of disease, there have been elaborated substances opposing the action of the infecting parasite) a so-called "specific" substance toward which the

serum is immune (this is replaced in the Wassermann test by lipoidal tissue extracts), a hemolytic serum and blood corpuscles. The tests depend upon the power of the immune serum, in presence of the "antigen" (or in the Wassermann test, the lipoid extract), to inhibit hemolysis by taking up that particular serum constituent, called "complement", which is essential for the process of laking of blood. "Complement" is present in both immune and hemolytic serum, but it is necessary that this should first be destroyed by heating at 56° C. for twenty to thirty minutes. The "complement" necessary for studying the hemolytic power of the serum is supplied by the addition of normal serum.

The experiments recorded in previous sections indicate that, as Napier suggested, the formaldehyde reaction involves principally the serum globulin fraction known as "eu-globulin".

Gibson (1906), Banzhaf (1908-9) and Heinemann (1910), believing that antitoxins exist in the pseudo-globulin fraction of the serum proteins, have elaborated a method of concentrating the antitoxins of tetanus, diphtheria and hog-cholera by prolonged heating of the serum at 56° C. followed by removal of the eu-globulins.

Banzhof states that this heating will, in 12 hours, convert fifty per cent of the pseudo- into the eu-globulins (Hess-Zinsser, Text Book of Bacteriology, 1922, p. 272).

Experiment has shown that normal serum will gel with formaldehyde after heating at, or better, slightly above 56° C. and that the longer the serum is heated, the quicker is the process of gel formation.

As far as the Wassermann reaction in syphilis is concerned it is not decided just what fraction of the serum proteins is responsible for the test. Rowe (1916) examined the relationship between variation of globulin content and the success of the Wassermann test and concluded that the globulins are not responsible for the test. But Mackie (1923) considered that the serum constituent responsible for the reaction is the globulin fraction not soluble in CO_2 - i.e. the eu-globulins. The actual concentration of eu-globulin necessary for the success of the test has not yet been determined.

The original Bordet- Gengou test depended upon the presence of specific antibodies in the immune serum. The presence of the antibodies in the pseudo-globulin fraction in diphtheria, tetanus and hog-cholera may be considered as evidence that other antibodies also exist in the protein fraction.

Since the eu-globulin content of serum is increased by the simple process of heating and since also the formaldehyde test is obtainable in an otherwise negative serum, if that serum be heated at 56° C. it is highly important, in comparing "complement fixation" tests with the formaldehyde reaction, that the samples of serum used be kept separate and that the sample tested with formaldehyde be not heated. While even thirty minutes heating at 56° C. is not sufficient to cause a normal serum to give a gel with formaldehyde, yet Bessemans found that it is sufficient to cause a strongly syphilitic serum, originally not influenced by aldehyde, to give a gel. It is said also that the Wassermann reaction is weakened by too prolonged heating of serum.

The importance of the time factor in the formaldehyde reaction is understandable when it is remembered that even a normal serum, gives sufficient time, will gel with formaldehyde in the concentration used in the Gate and Papocostas test.

Conclusion.

Upon the basis of the experiments recorded above, it is concluded that the gelification of serum by formaldehyde is a process of gradual partial dehydration of a portion of the serum proteins - the globulins - and the conversion of these proteins to methylene derivatives by combination of the aldehyde with the free amino groups. This process involves only a small fraction of the proteins present. But since the reaction of formaldehyde is retarded by the presence of water, it can only attain to the speed noted in the Gate - Papocostas test when the blood serum is abnormally concentrated or when such conditions obtain that the degree of hydration of the globulin is abnoramlly low.

It is suggested that the two types of gel produced correspond to two types of serum conditions accompanying disease; the one, leading to formation of a transparent gel, involves either increased concentration of the serum proteins or increase of the ratio globulin/albumin; the other, giving rise to a white, opaque gel, is accompanied by the appearance in the serum of tissue globulins, not closely bound to the serum protein complex.

It is believed that the formaldehyde reaction might give positive results in many diseases, especially if the serum globulin content rises above 2.2%. Few have yet been tested. Attempts will be made to extend it to others.

Its wide spread application makes the test useless as a specific diagnostic method, but, with refinements, it might possibly be employed as an index of changes in the protein content of the blood or of necrosis of tissues. But before it can be so used, the test must be studied during the whole course of such diseases as tuberculosis or syphilis.

The discordance between the results of the Wassermann Bordet-Gengou and Formol-Gel tests is due to the fact that, at least the two latter, involve different these tests, fractions of the serum proteins. But since both these fractions are globulins, and since there seems to be little difference between the two serum globulins - pseudo and eu-globulin - other than the extent of their hydration, it is suggested that, in comparing the applicability of the two tests to a particular disease, great care must be taken to avoid undue disturbance of the relations between the proteins and the other constituents

of the serum. Heat or evaporation are capable of converting a serum negative to the formaldehyde test into one which would be positive.

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