STUDIES ON THE PRECIPITATION OF EGG ALBUMIN

THESIS FOR THE DEGREE OF M. S.

Edwin G. Donahue

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Albumin

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A THESIS

SUBMITTED TO THE FACULTY OF

MICHIGAN STATE COLLEGE OF AGRICULTURE

AND APPLIED SCIENCE IN PARTIAL

FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

By

EDWIN G. DONAHUE

June, 1934

ACKNOWLEDGMENT

My sincere thanks are offered for the help and guidance of Professor C. D. Ball, who understands and forgives human frailties, but at the same time gives a challenge for hard work and clear, straight scientific thinking.

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INTRODUCTION

Changes in the state of aggregation of the substance making up protoplasm is known to be intimately related to life, activity and death of an organism. In this connection one need only refer to the works of Chambers (1), Addoms (2), Marinesco(3), Heilbrunn(4), and Bancroft and Richter (5), as a few of the papers on the subject. These workers draw their conclusions after making direct observation upon the protoplasm of living organisms.

Such studies on systems as complex as protoplasm give us highly significant information. However, it is believed that more fundamental knowledge of living processes must come primarily from investigations on simpler systems containing pure substances whose concentrations are known and controllable.

Protein systems possess many properties of protoplasm, especially when we consider opalescence, viscosity, ease of precipitation, etc. And when we realize, (I) that proteins occur as a major constituent of all living protoplasm, (II) that most enzymes are known to be proteins or at least always associated with them, and (III) that the science of immunology is largely a study of protein reactions, it becomes

clear why careful studies on pure proteins are important.

Lloyd (6A) states that the proteins of the cytoplasm belong always to the classes of albumins and globulins.

It was with the above point of view that the present work on precipitation of crystallized egg albumin was begun.

DEFINITION OF TERMS

The terms, precipitation, agglutination, denaturation, flocculation and coagulation are considerably employed throughout the literature on the chemistry of proteins, and unfortunately they have often been used rather loosely.

For clearness the following definitions of these terms will be strictly adhered to in this work.

Precipitation is a general term that refers to any separation of material from the colloidal state or from true solution. Whenever doubt is held as to whether the aggregated mass is reversible or not, the process of formation will be called precipitation. A precipitate may vary in properties from a faint turbidity in solution, to a stiff gel.

Agglutination is defined as the formation of larger aggregates after precipitation has taken place, the aggregates being plainly visible to the naked eye.

Denaturation is a change in properties of a native soluble protein, brought about by numerous agencies (heat, acids, bases, alcohol, urea, etc.), such that the protein loses its property of being soluble in water or salt solution at the isoelectric point of the modified protein. This change does not cause precipitation unless the pH is adjusted to the isoelectric point, and only water or salts

are present.

Flocculation is that special kind of precipitation which must always be preceded by denaturation. The flocculated protein then, is insoluble in water at the isoelectric point, but can be dissolved in acids or bases, the protein separating out from the latter solutions by adjusting to the isoelectric point again.

Coagulation refers to the complete process: denaturation plus flocculation.

HISTORICAL

The treatment of the literature reviewed may conveniently be divided into the following subjects:

(I). Agencies Causing Precipitation of Proteins.

(II). Stabilizing Factors; Protection against Precipitation.

(III). Adsorption and Chemical Combination as Factors in Precipitation and Stabilization.

(IV). Use of the Nephelometer in Studying Precipitation and Stabilization.

For a more unified discussion in this review reference will be made chiefly to observations on the albumins. Other proteins will be brought in only where it is thought that the data have some bearing on albumin.

(I). Agencies Causing Precipitation of Proteins.

Heat.

Probably as long as eggs have been cooked and used as food the coagulation of egg white by heat has been observed. Certainly it is common experience that when egg white is heated to nearly the boiling point of water, the "whites" are changed from a slimy transparent liquid to a white, opaque, nearly solid gel. If the albumin solution is more dilute, the heat will form merely a turbid suspension.

The classical work of Chick and Martin (7) in 1910 on crystallized egg albumin, established the fact that the heat precipitated protein is truly coagulated. Also by measuring the rate of denaturation by heat at constant pH, the reaction was shown to be a monomolecular one for both egg albumin and hemoglobin. The rate of the denaturation was a function of the concentration of the protein only. These workers considered that the usual method of measuring the temperature of coagulation was of value only if the conditions of heating, time, protein concentration, etc., were accurately standardized.

Lloyd (63) quotes Lepeschkin, who reported in 1922 that denatured egg white flocculates in ten seconds, while two thousand, two hundred seconds are required to coagulate the native product under the same conditions.

That the specific optical rotation of egg albumin is increased by heat denaturation, was shown by Barker (8) in 1933. He found that the measurement was a constant for a given degree of denaturation. It was his belief that the protein denatured by various agencies was very probably a different substance in each case.

Later the same worker (9) gave evidence that the refractive index of pure egg albumin is increased slightly as measured by the Zeiss interferometer, which gives the refractivity out to the seventh place. He considered that the change in the re-

fractive index supports the contention that a change in chemical structure has taken place. Citing this and previous work of others, he listed nine properties of egg albumin which are known to change upon heat denaturation: the solubility, and affinity for water decrease; viscosity of the solution, specific optical rotation, refractive index, digestibility by pepsin and trypsin, are all increased; nitroprusside reaction for -SH groups becomes positive; and immunological reactions, and absorption spectra are known to change. Properties which do not change are cataphoretic mobility and molecular weight, the latter being measured in urea solution.

Bancroft and Hutzler (10) claimed in 1931 to have succeeded in reversing the denaturation of egg albumin, by heating the coagulated egg white in the presence of ether, and further subjecting this to the peptizing action of potassium thiocyanate. The reversed product was soluble in water after dialysis, was coagulable by heat, and gave the same immunological reactions as the original egg white.

Freezing.

In 1923, Heiner (11) subjected egg white to repeated freezing and thawing and found that the protein began to

gather at the bottom of the container. If the process was not repeated too many times the protein could be redissolved when melted, but eventually an irreversible product was obtained.

D'yachkovskii (12), in 1932 observed that albumin, hemoglobin, and vanadium pentoxide sols are precipitated after thawing the sols frozen at -5, -15, and -20 degrees, but that they are completely stable if frozen at -182 degrees. The suggestion was made that the stabilizing effect at the lowest temperature was due to the higher rate of freezing.

Pressure.

In 1914, Bridgman (13) found that egg white could be precipitated by pressure. Upon subjecting the material to a pressure of 3000 atmospheres for 30 minutes, he observed a noticeable stiffening; 6000 atmospheres gave it an appearance like curdled milk; at 7000 atmospheres apparently complete precipitation resulted. The process was accelerated by lower temperatures, but appeared to be independent of the time of application of the pressure.

Basset, Macheboeuf and Sandor(14) published the results of their work in 1933, in which they stated that the serum of horse blood could also be precipitated by high pressures. The minimum pressure for gelification was 13,000

atmospheres. Serum globulin jellied completely at 15,000 atmospheres, while serum albumin remained limpid at all pressures up to the same pressure.

Radiation and Vibration.

From the literature one finds that practically all known forms of vibration can play a part in precipitating albumin from solution. This section will treat these from the lower frequencies to the higher, and not chronologically.

Hopkins (15) proved in 1900 that coagulation of pure egg albumin occurred from solution when the system was shaken violently. He observed that the precipitated product gave a reddish violet color with sodium nitroprusside as did that coagulated by heat. Three years later, Ramsden (16) brought out that adsorption at a surface or in a film results in denaturation, and that the stiff foam of egg white formed when the material is beaten is an irreversible one, composed of the coagulated albumin adsorbed in the film of the air bubbles.

Audible sound was found by Flosdorf and Chambers (17) to bring about precipitation of albumin. Frequencies of 1000 to 15,000 v.p.s. precipitate albumin from solution almost instantly at 30 degrees.

That mechanical agitation and sound waves may be related

in their precipitating action is indicated by the work of Wu and Liu (18), in 1931, in which is reported the precipitation of egg albumin from solutions by exposure to supersonic waves. When the solution was saturated with air, hydrogen or oxygen the albumin was precipitated in fine shreds enclosing gas bubbles. No precipitation resulted when in gas free solution, or when saturated with carbon dicxide or hydrogen sulfide; incidently in the latter cases no gas bubbles were formed. The theory was that gas bubbles caused precipitation at the interface similar to that occurring when albumin solutions are mechanically shaken.

Young (19), in 1922, showed that light rays of the visible spectrum , denature albumins in many ways similar to heat. Denaturation by ultraviolet light was demonstrated by Dryer and Hanssen (20) in 1907.

Clark (21) reported the results of her studies on the action of ultraviolet light toward egg albumin, in the year 1922, in which it was found that at pH values below the iso-electric point the light did not precipitate the protein. At the reaction of pH 5.6, 6.2, and 6.8, precipitation did take place, but at pH 7.8 the solution once more remained clear. The conclusion was that the action of ultraviolet light is one of emission of electrons from the charged protein particles, since greater dispersion results when the protein is positive,

and greater aggregation when the protein is negative. Miss Clark showed that a change has resulted in all the tubes of varying pH, by the action of the light, since half-saturation with $(NH_4)_2SO_4$ caused precipitation in all of them. Before the exposure, such treatment caused no such change.

Fernau and Pauli (22) observed in 1915 that radium emanations precipitated albumin. In 1923 , Fernau reported (23) that X-rays precipitate both albumin and ceric hydroxide sols.

Acids and Bases.

Chick and Martin (7) in 1910, showed that the H-ion concentration greatly influences the velocity of heat denaturation, there being a minimum velocity at about the neutrality point of water, pH 7, and a very rapid increase of the velocity as the H -ion concentration is increased. However in the acid , part of the denatured protein was soluble, the total amount precipitating out only when the reaction was adjusted to the isoelectric point.

In 1912, the same workers (24) showed that alkali also increases the velocity of denaturation in a manner analogous to that in which acid increases it.

Lloyd (60),after reviewing the work of Chick and Martin,

and of Wagner (73) on the effects of acids, points out that if the temperature is sufficiently high, denaturation takes place in neutral solutions; and if the H -ion concentration is sufficiently high denaturation can take place at 20 degrees or less.

Concentrated acid can also crowd the protein out of solution. Schoorl (25) in 1924 observed that 5 percent egg albumin in strong acid (2.5% HCl), became opalescent after a half hour in the cold, became very viscous after several hours, and precipitation took place in about five hours. This is similar to the action of salts in high concentration, which subject will be discussed later.

The coagulated protein obtained after denaturation by mineral acids is called by the special name acid metaprotein, and that obtained after denaturation by alkali is called alkali metaprotein, according to Hawk and Bergeim (26).

In a series of qualitative experiments on the behavior of egg albumin towards a large variety of ordinary laboratory reagents, Hunter (27) reported in 1913 that the protein was precipitated by concentrated H_2SO_4 and by the sulfonic acids of the following: α-naphthol, dimethyl aniline, salicylic acid, resorcinol, quinol, and fluorescein.

Vallery (28) in 1913 presented data indicating that heating with least dissociated acids gives the greatest amount of protein precipitate from serum albumin solutions or from albumin in the urine. The reason given for this was that during coagulation by heat the dissociated acids induce

hydrolysis of the protein giving rise to soluble peptones. Thus acetic acid was found to give more coagulated protein from serum albumin in the urine than did trichloracetic acid, the latter being more dissociated.

Labes and Jansen (29) in 1930 studied the effect of substitution in acetic acid upon the precipitating power of the acid toward denatured serum albumin. The isoelectric point of denatured serum albumin is shifted toward a more acid reaction by β-iodopropionic, dichloracetic, phenylacetic, benzoic, trichloracetic, and tribromoacetic acids; at the same time the zone of precipitation is broadened.

At the same time, Labes , collaborating with Schuster (30), reported an interesting study of the effect of substitution in aromatic acids upon the optimum precipitation zone of denatured serum albumin. The effect of substitution depends upon the position of the groups. Thus a nitro group on the para position in benzoic acid causes a much greater amount of precipitation of the protein, and a greater shift in the optimum zone than a similar group in the ortho or meta position. With hydroxyl or amino groups the reverse relationship holds. The effect of position is explained on the basis of the polar character of the benzoic acid molecule. Sulfonic acid groups substituted into aromatic radicals increase the precipitating power of the compounds.

Salts.

In a review of the subject of precipitation of proteins by inorganic salts, Robertson (31) restates the findings of Hardy (32) that precipitation of proteins and colloids in general may be of two kinds: I. One is accompanied by a decomposition of the precipitating agent, occurring only if the protein possesses a charge. In this case relatively small quantities of the precipitating agent are required to bring about precipitation.. II. The second kind, whether accompanied by decomposition of the precipitating agent or not, occurs even at the isoelectric point of the protein and requires relatively large amounts of the protein. The first type of precipitation is brought about only by electrolytes, and it appears to be chemical in nature, but the second type may be actuated by certain non-electrolytes, for example, alcohol. The mechanism of the latter type seems to be a dehydration of the protein by the precipitant, which has greater affinity for the water than has the protein.

Robertson, continuing with the subject of salt precipitation, again cited Hardy (33). The latter noted that denatured egg albumin can be made into an anion or cation simply by changing the reaction of the solution. In acid the protein was positive, migrating to the cathode; in alkali it was negative, going to the anode. In both cases the protein was precipitated at the electrode toward which it migrated. Hardy (33) observed that when the denatured

protein was electrically charged, it was very readily precipitated by traces of electrolytes (1 gm. mole in 80,000 cc), the cation being effective when the protein was negative, and the anion when it was positive. This work of Hardy's shows precipitation of the first type, noted above.

Similarly, Bodansky (34) quotes Loeb, showing that gelatin combines with silver cations only when the pH of the solution is above its isoelectric point, the combination being such that it cannot be reversed by washing with water. Also it is only below the isoelectric point that gelatin combines with ferrocyanide anions. Both of these ions were readily detected by color reactions.

Pauli (35) in 1899 showed that as a rule the precipitating power (second type of precipitation) of a mixture of salts is the algebraic sum of the separate effects of its components, in other words the effect is additive. Also he noted that the presence of salts lower the temperature of heat coagulation. However in a later paper, 1903, he noted that a number of salts that will not precipitate egg white by themselves will either increase or decrease the precipitating power of other salts. This was considered by Pauli to be due to the antagonistic effects of the anion and cation.

Citing Hardy, Robertson (31) stated that both types of precipitation can take place by the same salt (of alkali

metals or of heavy metals). The salt will precipitate the charged protein; higher concentration of salt will redissolve the precipitate, and finally with still more salt, precipitation will take place again.

Cervello and Varvaro (36) studied the egg albumin solution made by precipitating with salts of Fe, Cu, Hg, Zn, and Mn, then adding more until the precipitate had just redissolved. The solution gave no characteristic tests for the metals. The temperature of heat coagulation was raised in all cases except the Zn and Hg solutions. In the case of the Fe, heat coagulation is inhibited altogether.

Before we leave the subject of precipitation by electrolytes, it should be noted that the isoelectric point (point of maximum precipitation) is specifically influenced by different ions, as one might expect from a knowledge of the Hofmeister series. Lloyd (6H) gives a review of the literature, showing that with casein solutions, sulfosalicylate ions greatly shift the isoelectric point toward the acid side; and thiocyanate, iodide and bromide, less so. Acetate ions move this point only slightly to the acid side, nearly coinciding with the accepted value. Cations move the isoelectric point toward the alkaline side.

Oxidizing Agents.

In 1923, Bernau (23) noted that hydrogen peroxide and ozone precipitate albumin and ceric hydroxide sols, and hydrolyze sucrose, and that X-rays, ultraviolet rays, and α-rays do all of these.

The work of Hunter(27) recorded the fact that potassium dichromate and bromine water will precipitate egg albumin (1923).

Cossu (38) reported in 1924 that iodine causes precipitation of albumins.

Of possible interest in this connection is the finding of Effront (37), in 1911, that the action of sunlight on proteins and amino acids in alkaline solution, gave rise to the formation of ammonia, volatile acids and nitric acid. Starch-iodide paper indicated the presence of hydrogen peroxide in solution. To test if hydrogen peroxide was the cause of the decomposition, alkaline solutions of various proteins and amino acids were distilled in its presence. All the nitrogen was converted into ammonia and a slight amount of nitric acid and fatty acids were formed. Effront suggested that the peroxide oxidizes the amino groups to hydroxyl groups.

Organic Compounds.

Precipitation of proteins by methyl and ethyl alcohols has long been known.

Lepeschkin (39) in 1923 made a study of precipitation of albumin solutions, reporting that alcohol precipitates the albumin reversibly, noting that the action is similar to that produced by salts. Non-soluble substances such as ethyl ether, chloroform, and benzene, which form emulsions in water, precipitate the protein but do not form an insoluble precipitate of albumin on the surface of the dispersed fluid particles.

However, Pauli and Weiss (40) reported in 1931 that alcohol precipitates gluten reversibly and ovalbumin non-reversibly.

Jacobson (41) observed that benzyl alcohol precipitates egg albumin and peptone irreversibly even in solutions containing 1 p.p.m.,(1923).

The precipitation of hemoglobin by KCl was found by Jirgensens (42) to be aided by the presence of methyl and ethyl alcohols in all concentrations, while propyl alcohol sensitizes the precipitation only when present in small concentrations. At higher concentration the latter acts less and less as a sensitizing agent, (1927)

Kruyt (43) in 1925, pointed out that the alcohol concentration necessary to precipitate a protein is at a minimum

at the isoelectric point. He considered this to be a necessary consequence of the lower degree of hydration of the protein at that point.

In 1929 Utzino (44) reported that the opalescence of albumin solutions containing alcohol is increased by raising the temperature or by increasing the amount of alcohol.

The first to report that acetone precipitates albumin from neutral solutions was Weyl (45), in 1910.

Lloyd (6F) states that both alcohol and acetone cause denaturation of proteins; also that temperature doubtless plays an important role in the denaturation, since Hardy and Gardiner demonstrated in 1910 that this reaction does not occur at temperatures lower than 5 degrees.

The effect of other non-electrolytes on the precipitating power of ethyl alcohol was studied by Mitolo (46) in 1930. He reported that in general there was an enhancing effect. Urea slightly increases precipitation, while glucose greatly increases it. The precipitating powers of various alcohols were in the order of benzyl alcohol and phenol, the strongest, then ethyl alcohol, and methyl alcohol the weakest.

Labes and Jansen (29) studied the effect of substitution in phenol, on its precipitating power toward denatured serum albumin. When one group is introduced of methyl, nitro or chloro, a more rapid precipitation occurs over a wider range of pH than with phenol alone. When two groups are substituted this action is even stronger. However, the substitution

of OH in the ortho position markedly decreases the precipitating action, the para slightly less, and in the meta position (resorcinol), the precipitating effect is about the same as with phenol.

Marie (47), using ethyl acetate, developed a very sensitive test which is positive for egg white at a dilution of 1: 10,000. The ester is superposed upon the liquid in a test tube, and if albumin is present the white ring forms at the junction of the two surfaces.

The first to report that urea denatured albumin was Spiro(57), in 1900. Later it was studied by Mansden (48) in 1912, who stated that coagulable proteins are converted at room temperature into substances having properties of acid- or alkali albumin, depending on the reaction of the medium at the time. Strong solutions of egg white are changed into a stiff jelly at ordinary temperatures, the jelly remaining even after the urea is washed away. Another interesting point was that NH_4OCN and NH_4SCN produce many of the effects of urea.

Hopkins (49) in 1930 observed the striking fact that denaturation by urea had a negative temperature coefficient, if the temperature was kept below that of heat denaturation. Thus at 0 degrees after 1 hr., a given experiment yielded 86.2% of the albumin in the denatured form; at 23 degrees, 69.2%; at 37 degrees, 61.0%. In the same paper, Hopkins considered that the red-violet color produced by sodium nitroprusside had been proved to a specific test for a denaturation.

Accordingly he used the test to observe the denaturing action of various substances on crystallized egg albumin solutions. Those which were positive were methyl-, ethyl-. and butyl urea, unsymmetrical dimethyl- and diethyl urea, thiourea, acetamide, formamide and urethane. The following were negative: symmetrical diethyl urea, acetyl- and methyl acetyl urea, biuret, allantoin, semicarbazide, alanine, phenylalanine, valine, leucine, cysteine, benzamide, creatine, caffeine and asparagine.

Hopkins pointed out that although urea denatures albumin, it also peptizes the denatured protein even at the isoelectric point. Precipitation is observed only upon diluting with water.

(II). Stabilizing Factors; Protection Against Precipitation.

It is well known that albumin heated with sufficient acid or base will not flocculate at all. Although these agencies increase the velocity of denaturation, they also stabilize the denatured protein until the reaction is adjusted to the isoelectric point.

The work of Hopkins just discussed above shows that urea also denatures, but stabilizes at the same time.

Munaretto (50) observed that both sulfur dioxide and formaldehyde increase the viscosity of albumin sols at 25 degrees and prevent their coagulation by heat. Similarly, Cubin

(51),found in 1929 that the pH range over which flocculation occurs from heat denaturation is sharper in the presence of formaldehydeThis was held to be a confirmation of the view that flocculation involves the amino groups.

The work of D'yachkovskii (12) has already been mentioned on freezing of albumin solutions. Precipitation occurred when frozen at temperatures a few degrees below zero, but at -182 degrees the remelted solution was completely clear.

Fairbrother (52) in 1929 stated that the viscosity of egg albumin is decreased to a minimum value sometimes as much as 40 percent, by an X-ray dose of definite strength. This decrease is permanent and no appreciable subsequent coagulation occurs. Such albumin coagulates more slowly than the original when brought to 61 degrees.

Mitolo (46) reported that caffeine inhibits alcohol precipitation of protein from concentrated solutions of ovalbumin, but that in dilute albumin solutions it aids precipitation.

The effect of temperature on stability to alcohol and acetone has already been noted (6F). Below 5 degrees, these solvents do not denature albumin at all.

Chick and Martin (6G) showed in 1910 that egg albumin crystals pressed dry until they contained only 20 percent water could be heated for five hours at 120 degrees in a current of dry air, and still be soluble in water. However when heated with steam they became completely insoluble in a few minutes.

Lepeschkin (53) reported (1922) that a high salt concentration slowed up the rate of denaturation by heat. Jirgensens (54) noted that high salt concentrations stabilized egg albumin against precipitation by alcohols.

Teorell (55) in 1930 observed that methyl, ethyl and propyl alcohol caused a lower turbidity in serum albumin which was heated in its presence than when heated in aqueous solution only. This was considered to be a protective action exerted by the alcohols. Protection was in the order of decreasing strengths: propyl, ethyl and methyl alcohol. No influence was observed on egg albumin. Teorell found that turbidity by heating decreased with increasing acetate buffer concentration. He suggested that the nephelometric measurement did not necessarily give the amount of albumin precipitated, but may be partly or wholly a measure of difference in the state of dispersion.

Beilinsson (56) showed in 1929 that the denaturation of serum albumin was greatly prevented by the presence of sucrose or of glycerol in high concentrations. The method was to bring the heated solution to pH 5.3 and titrate with saturated ammonium sulfate to a standard turbidity. The protective action with sucrose was also checked by nitrogen determinations on the centrifuged precipitate.

Neuberg was stated by Beilinsson in the above paper, to have reported that many of the hydrotropic salts exert a

protective action on proteins toward heat coagulation.

Iwanowsky (58) in 1933 studied the effect of glycerol on the formation of alkali albuminate from egg albumin, and also on relative precipitation of the albumin from solution by ammonium sulfate, the latter being measured by the nephelometer. He concluded that glycerol does not hinder the formation of the alkali albuminate, but that it does protect against precipitation by the salt.

In 1932, Duddles (59) reported that solutions of crystallized egg albumin were stabilized to heat coagulation by glucose, fructose, mannose, sucrose and mannitol; and that glucose protected the albumin from coagulation by ultraviolet light. His method was to run nitrogen determinations on the filtrate from the heat coagulum, after heating for ten minutes at 70 degrees.

Pauli and Schön (60) applied conductivity measurements in studying binding of zinc chloride on serum albumin (1934). It was observed that between salt concentrations of 2×10^{-3} and 8×10^{-3} N., the mobility of the Zn -ion was greater than was expected and that it was within this range that the zinc chloride markedly protects albumin against coagulation by heat.

Observations have been made on the stabilizing effects of sugars on colloidal systems other than the proteins. For example it may be of significance to note the observations

of Joshi and Lal (61) that manganese dioxide sols were rendered more stable by the presence of sucrose (1933). Also sucrose was found by Sen (62) in 1928, to stabilize the hydroxides of zirconium, lanthanum, yttrium, and uranium, when their precipitation is attempted by alkalies.

(III). Adsorption and Chemical Combination as Factors in Precipitation and Stabilization.

Water relationships in Albumin systems appear to be very important. The work of Gayda (63) in 1912 showed that there was a contraction in volume of the system, albumin-water, when the dry albumin was dissolved in water. He studied the changes in volume of an egg albumin-water-sodium chloride system upon heating slowly from 15 degrees to 95 degrees, and observed that the albumin solution had a more rapid expansion than the salt solution or water alone.

Baker (64) showed in 1933 that the rate of denaturation of a partially dried egg albumin is greatly decreased by decreasing the water content. This confirms the work of Chick and Martin (60) already discussed. Baker further observed that the heat coagulated albumin takes up about 80 percent as much water at all relative humidities as does native egg albumin.

The minimum alcohol number at the isoelectric point of a protein solution was considered by Kruyt (42) to be a nec-

essary result of the lower degree of hydration of the protein, corresponding to a lower charge, since the two are interrelated.

Weber and Versmold (65) in 1931 reported the results of their work on cryoscopic measurements on egg albumin systems containing non-electrolytes. By calculation of the volume of space occupied by the solvent, protein and solute, it was determined that the hydration space was found to give the value, 1.33 to 1.36 gms. of hydrated matter per gm. of protein. The binding of glucose and glycerol attains a maximum with rising crystalloid concentration, when about 0.084 moles of glucose and 0.048 moles of glycerol are bound by 1 gm. of the albumin. Under similar conditions urea is bound to a much larger extent, and even at 2 molar concentration its binding does not reach a maximum value. On heat coagulation the hydration space diminishes 0.1 gm. per gm. of protein. The authors thus believe that Sørensen's idea of denaturation being incomplete dehydration is substantiated.

In 1927, Lundén (66) reported that the sweetness of sugar solutions was decreased by the presence of 0.005% to 3% egg albumin.

Strong evidence that glucose is adsorbed by egg albumin, was brought out by de Anciaes and Trincao (67), who established in 1928 that the reducing power of the mixture of the sugar and albumin was less than the sum of the reducing powers of each one tested separately. This difference was ascribed to adsorption of the glucose on the albumin. The reducing power

was determined by the method of Hagedorn. If the albumin was first dissolved in a 5 percent sodium chloride solution, the adsorption was almost completely prevented.

In the same year Boutaric and Banes (68) studied the binding of eosin on mastic resin, gold sol, casein, and albumin. It was found that when the micelles were thrown down by centrifugation, no eosin was found in the precipitate, but when they were precipitated by aluminum chloride or by heating, and then separated, the greater part of the eosin was found in the colloidal material.

Chick and Martin (7) reported a decrease in H -ion concentration during heat coagulation of egg albumin when below the isoelectric point, and a decrease in OH -ions when above the isoelectric point. This was attributed to a binding of the acid and base, respectively.

The work of Cervello and Varvaro (36) , already discussed, points to a combination of ions of the heavy metals with albumin when the salts are added to the point of redissolving the initial precipitate. Such solutions did not give the usual tests for the metals.

Ito (69) in 1927, studied the conductivity of potassium chloride, zinc chloride, and calcium chloride solutions upon the addition of egg albumin. In all cases the added protein brought about a lowering of conductivity. The author concluded that this was caused by the adsorption of the salt.

Galeotti (31) showed that at the moment when precipitation begins, upon the addition of silver nitrate, the precipitate is of constant composition, containing silver and the protein.

(IV). The Use of the Nephelometer in Studying Precipitation and Stabilization.

Kober (70), in 1913 published a nephelometric method of determining proteins in milk. He used a three percent solution of sulfosalicylic acid as the precipitating agent.

Mention has already been made of the work of Teorell (55), on the use of the nephelometer in studying the protective action of alcohols on serum proteins to heat; also, that of Iwanowsky (58) on the stabilization effect of glycerol on egg albumin toward precipitation by ammonium sulfate. Neither of these workers reported any studies on the nature of the protective action: whether the nephelometric measurements show differences in actual amounts of protein in the precipitate, or merely changes in the degree of dispersion of the same amount of precipitate in all cases, or a combination of both factors.

The value of the nephelometer was discussed in 1927 by Wells (71), in a critical review of the literature on turbidity measurements. He stated that turbidity is a measure

of other factors in addition to concentration. However it was suggested that there is good reason to expect that the other variables would be of interest also. If the limitations of turbidity measurements are recognized, he considered that the use of the turbidimetric instruments can be invaluable, especially in work that requires quick observations without disturbing sensitive adjustments in the system. Then too, minute amounts of material can be estimated which cannot be weighed on the most sensitive balance.

Wells further considered that certain limitations must be recognized. In the absence of suitable standards of turbidity, which can be prepared in any laboratory, it is now impossible for different workers to duplicate turbidity measurements. Wave length and intensity of light, shape of instrument, size of cups, rate of mixing of solutions, etc., have been found to effect readings considerably.

Among the "other variables" which Wells mentioned was an observation made by Bechhold and Hebler (72) in 1922, who found that particle size has an influence in the turbidity as measured in the nephelometer. They prepared barium sulfate sols in glycerol and ethyl alcohol, the particle size ranging from 2.5μ to 4 mμ. With increasing size of micelles, the turbidity increases rapidly up to particles 800 mμ in size, then it falls off rapidly at first, then more slowly.

EXPERIMENTAL

Egg albumin was prepared in the pure crystalline form by the method of Sørensen (74), and kept in concentrated solution under toluene in the refrigerator. The more dilute solutions were prepared by dilution of the stock solution, and then checked by duplicate nitrogen determinations using the micro-Kjeldahl method, modification by Allen (75).

For nephelometric studies the Bausch and Lomb nephelometer attachment was used, with clear-glass flanged cups, to be placed upon a black movable base in the Dubosque colorimeter, with fixed plungers.

(I). Standardization of Nephelometer.

A solution of egg albumin was prepared, containing 0.1500 mgms. N per ml.

The standard suspension was prepared always as follows: From a 10 ml. micro-burette, 1 ml. of the above albumin solution was measured into a clean, dry 50 ml. Erlenmeyer flask. Then, 9 ml. of water was added to this from another burette, and the contents were mixed well by gentle rotation, care being taken to prevent the formation of air bubbles

which cause denaturation. Then 5 ml. of a 0.2000 molar solution of sulfosalicylic acid was added from a pipette by allowing it to run down the side of the flask. Again the contents were mixed by rotation. A turbid suspension resulted which was stable for about 10 minutes, after which time a gradual agglutination developed.

The "unknown" suspension was prepared in the same way, except that the volume of albumin solution was varied, and that of water adjusted so that the final volume of the suspension was the same: 15 ml.

To keep constant any gradual changes in turbidity after the addition of the sulfosalicylic acid, the standard was made up new with each "unknown" and the acid was added by means of two 5 ml. pipettes, simultaniously in the standard and the "unknown".

The nephelometer cups were rinsed out once with the suspension, then filled to the flange. After wiping off the outside of the cups with a dry cheesecloth they were placed in the nephelometer and adjusted so that the plungers were in the centers of the cups. The standard was placed on the left and set at 20.0 mm.; the unknown, on the right.

From preliminary study it was found that in case the standard setting had to be lowered, say, to 2.0 mm. in

order to take a reading of a very dilute "unknown", the reading (within experimental error) corresponding to the standard at 20.0 mm. can be obtained by multiplying the observed reading by 10. The observed reading for each sample tested was taken as the average of five different settings of the "unknown".

In Table I are the results of the standardization. Each recorded nephelometer reading is the average of four separate determinations on entirely new suspensions. The pH was measured in a few of the suspensions, by the quinhydrone electrode, and it was found to be pH 1.35. It was a constant, regardless of the amount of albumin present.

Fig. 1 shows the relationship between the total mgms. N in the suspensions and the nephelometer readings. It is obvious that there is no linear relationship between the two. The logs of both values were then tabulated, Table II, and plotted as shown in Fig. II. A perfectly straight line results.

The equation for the curve in Fig. II was obtained in the following manner. A straight line curve is of the general form:

1). $a = nb + c$

If we let,

$a = \log R$; $b = \log W$; and n and c be constants,

Table I.

Standardization of the Nephelometer.

Standard suspension.	"Unknown" suspension.
1 ml. alb. soln., (0.15 mgms. N).	x ml. alb. soln.
9 ml. water.	10 − x ml. water.
5 ml. 0.2 M. sulfosalicylic acid.	5 ml. ss. acid.

Set at 20.0 mm.

Series No.	x (ml. alb. soln.)	10 − x (ml. H_2O)	W (total mgms. N)	R (neph. read.)	No. Detns.	Ave. Deviat. of R.
#1	0.15 ml.	9.85 ml.	0.0225 mg.	167.0 mm.	4	±0.7 mm
2	0.25	9.75	0.0375	98.8	4	±3.3
3	0.50	9.50	0.0750	42.2	4	±0.7
4	0.75	9.25	0.1125	27.5	4	±0.3
5	1.00	9.00	0.1500	19.3	4	±0.3
6	1.25	8.75	0.1875	14.3	2	±0.4
7	1.50	8.50	0.2250	11.7	4	±0.4
8	2.00	8.00	0.3000	8.2	3	±0.2
9	3.00	7.00	0.4500	5.5	3	±0.1
10	5.00	5.00	0.7500	3.5	1	---

Table II.

Derivation and Use of the Formula.

(Data from Table I).

Series No.	$\log_{10} W$	$\log_{10} R$	W (known mgs. nitrogen)	Wcalc. (mgs. N by formula).	% Error by Formula.
#1	8.352-10	2.223	0.0225 mgs.	0.0240 mgs.	+6.55%
2	8.574-10	1.995	0.0375	0.0374	-0.33
3	8.875-10	1.685	0.0750	0.0763	+1.70
4	9.051-10	1.439	0.1125	0.1093	-2.87
5	9.176-10	1.286	0.1500	0.1474	-1.75
6	9.273-10	1.155	0.1875	0.1905	+1.60
7	9.352-10	1.068	0.2250	0.2247	-0.16
8	9.477-10	0.914	0.3000	0.3023	+0.77
9	9.653-10	0.740	0.4500	0.4270	-5.86
10	9.875-10	0.544	0.7500	0.6225	-17.0 *
				By formula, ave. % dev.	±2.4

* This figure was omitted in the calculation for the percent average deviation. Agglutination was very marked.

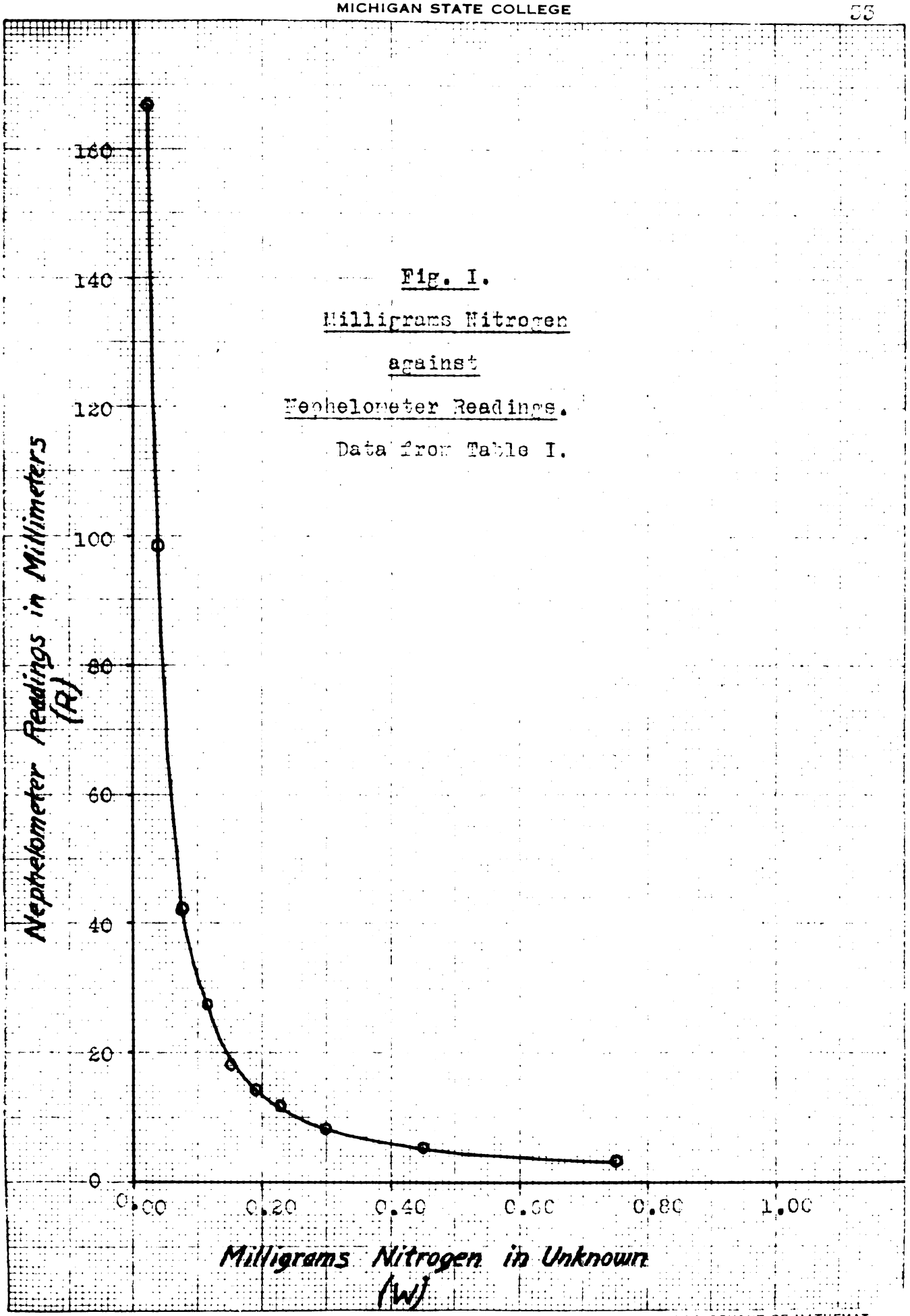


Fig. I.

Milligrams Nitrogen

against

Nephelometer Readings.

Data from Table I.

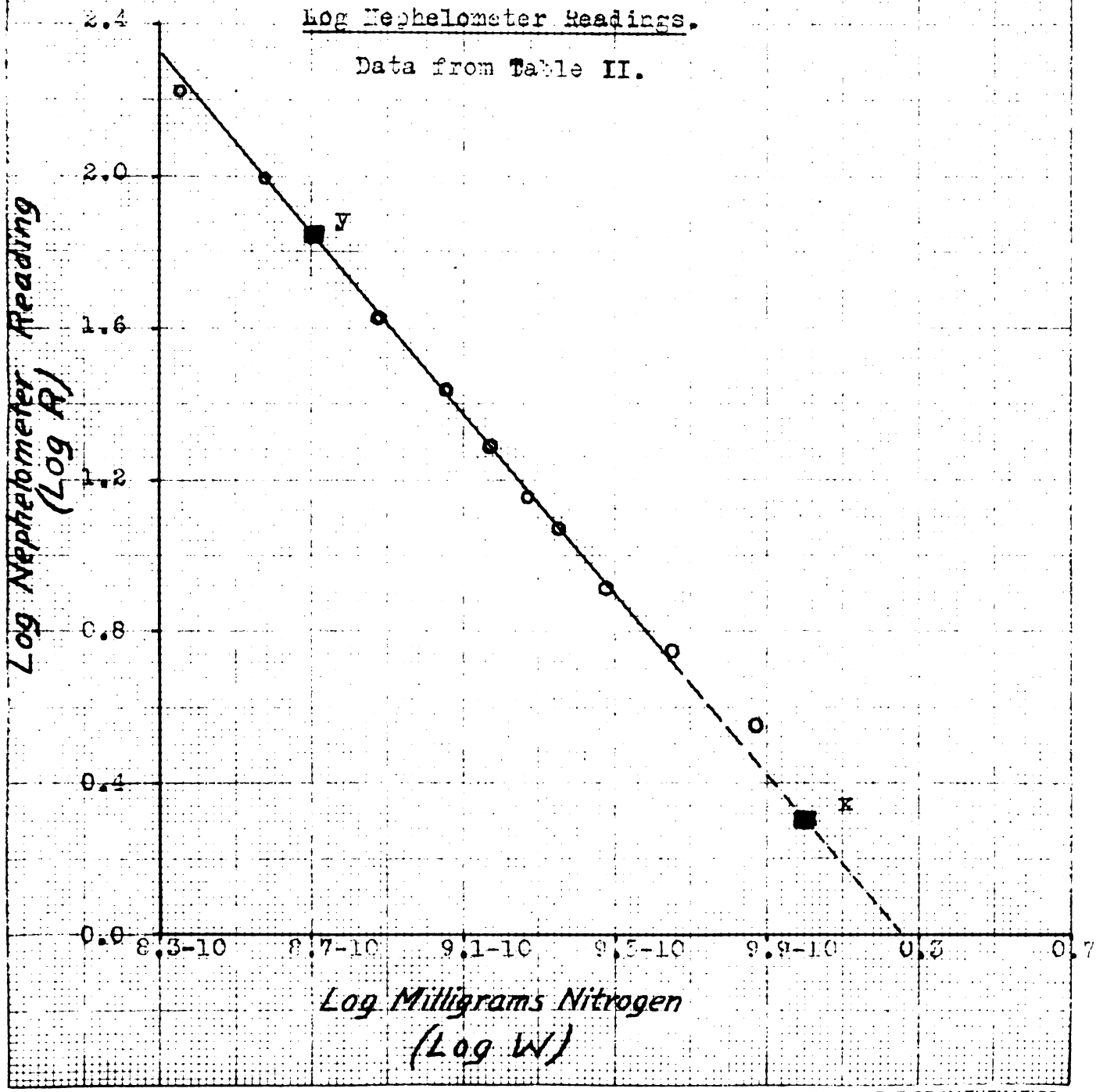
Fig. II.

Log Milligrams Nitrogen

against

Log Nephelometer Readings.

Data from Table II.



Where R is the observed nephelometer reading of the unknown in mm., and W is the known mgms. N in the total unknown suspension. Then, substituting,

2). $\log R = n \log W + c$

To solve for c, the curve extrapolated through the point (x) on the graph, where $\log W = 0$, gives

3). $c = \log R = 0.300$

Point (y) on the graph gives

4). $\log R = 1.840$; $\log W = 8.700\text{-}10 = -1.300$

Substituting 3), and 4) in equation 2),

5). $n = -1.185$

Equation 2) then becomes

6). $\log R = -1.185(\log W) + 0.300$

This gives for Fig. I,

7). $R W^{+1.185} = 1.995$

A somewhat more convenient modification is the following:

8). $W = \text{antilog}\left(\frac{0.300 - \log R}{1.185}\right)$.

which was found to hold to ±2.4 %, over the range of 0.02 to 0.45 mgms total nitrogen in 15 ml. of suspension, as will be observed in glancing at the last three columns in Table II. Using the same instrument and cups, there is no doubt that the formula will hold for determination of albumin in pure aqueous solutions of the crystallized product. A standard solution made up from a new batch of crystals and compared in the nephelometer with a standard of the old, gave the same reading within limits of error.

(II). "Protective" Action of Alcohols on Nephelometric Turbidity of Albumin Suspensions, when Precipitated by Sulfosalicylic Acid.

Qualitative experiments indicated that the turbidity of albumin suspensions (made as in the standardization experiments), was greatly decreased by the presence of methyl- , ethyl-, and propyl alcohols, glycerol, glucose, fructose, mannose, galactose and mannitol. Since it is

known that the sugars and mannitol protect egg albumin from heat coagulation (56), (59), that the alcohols decrease the turbidity from heat precipitation of serum albumin (55), and that glycerol decreases the turbidity upon the addition of ammonium sulfate to albumin (58), these qualitative observations were thought to indicate a new angle of approach to the same problem of protective action.

The author believes that previous work points to the importance of the hydroxyl group in the stabilizing action of the added substances. Consequently the simpler alcohols were studied in the present work.

The action of methyl alcohol was studied first. The standard suspension was prepared as in the standardization experiments. The "unknown" suspensions all contained the same amount of albumin as the standard (0.15 mgms. N in 15 ml. of suspension), but varying amounts of alcohol. The mixing was always carried out in the following order: 1 ml of the albumin solution(0.15 mgms. N/ml), was added to the flask; x ml. water was added, mixed by rotation of the flask; (9-x) ml. of 10 M. alcohol added; mixed by rotation; at once 5 ml. of 0.2 M. sulfosalicylic acid was pipetted into the standard and "unknown" simultaneously, mixed, and read.

The results for methyl alcohol follow in Table III.

Table III.

Effect of Methyl Alcohol on

Nephelometric Turbidity.

(Pptn. by sulfosalicylic acid).

Standard suspension.	"Unknown" suspension.
1 ml. alb. soln., (0.15 mgs. N).	1 ml. alb. soln.
9 ml. H_2O.	x ml. H_2O.
5 ml. 0.2 M. sulfosalicylic acid.	9-x ml. 10 M. CH_3OH.
Set at 20.0 mm.	Contains 0.15 mgs. N.

$W_{calc.}$ is obtained by the formula,

$$W = \text{antilog}\left(\frac{0.300 - \log R}{1.185}\right)$$

Susp. No.	Final * molarity CH_3OH	R (neph. read.)	$W_{calc.}$ (mgs. N)	$W - W_{calc.}$ (mgs. N "protection")	% (protection)	No. Trials	pH of
#1	0.17 M.	20.0mm.	0.1433mg.	0.0067mg.	4.47%	3	1.35
2	0.33	19.3	0.1477	0.0023	1.53	2	----
3	0.67	20.1	0.1424	0.0076	5.07	1	----
4	0.84	19.0	0.1492	0.0008	0.53	1	----
5	1.33	20.9	0.1378	0.0122	8.13	1	----
6	1.67	22.5	0.1292	0.0208	13.87	1	1.35
7	2.67	26.6	0.1126	0.0374	24.9	2	----
8	4.00	33.6	0.0923	0.0577	38.5	1	----
9	4.67	36.8	0.0854	0.0646	43.1	1	----
10	6.00	56.8	0.0593	0.0907	60.5	2	1.27
11	12.00 (a)	75.6	0.0465	0.1135	75.7	1	----
12	14.7 (b)	65.6	0.0525	0.0925	65.0	1	1.22

Notes concerning the table.

*"Molarity" is probably the incorrect term because of expected volume changes. However the assumption is made that the difference would not be over 2 %, which is within the error of the nephelometric measurement.

(a) Made by adding 9 ml. of 20 M. CH_3OH to the suspns.; slightly turbid before adding the sulfosalicylic acid.

(b) Made by adding 9 ml. of the C.P. CH_3OH to suspns.; very turbid before adding the acid.

(c) Measured by the quinhydrone electrode.

Assuming that the turbidity as measured in the nephelometer gives the actual amount of protein in suspension by use of the formula, methyl alcohol shows marked protective action against the precipitation of pure egg albumin by sulfosalicylic acid, the action increasing with increasing amounts of alcohol up to 12 molar. The change in pH is hardly sufficient to account for the decrease in turbidity.

In exactly the same manner, ethyl alcohol, propyl alcohol, ethylene glycol and glycerol were studied. The results are shown in the next four tables.

Table IV.

"Protective" Action of Ethyl Alcohol.

(Pptn. of alb. by sulfosalicylic acid).

Standard and unknown suspensions, the same as in Table III.

Suspension No.	Final Molarity C_2H_5OH	R (neph. read.)	Percent "Protection"
#1	0.17 M.	20.2 mm.	5.40 %
2	0.33	20.0	4.73
3	0.67	23.3	15.81
4	2.00	34.0	39.0
5	4.00	56.6	60.4
6	6.00	65.2	64.8
7	9.7 *	74.0	68.4

* Nine ml. of 95 % C_2H_5OH was added to the suspns.; slightly turbid before adding the ss. acid.

These results show a slightly greater rise in "protective" action at the lower concentrations than those with CH_3OH. However, the maximum "protection" is slightly less, with the concentrations used.

Table V.

"Protective" Action of Propyl Alcohol.

(Pptn. of alb. by sulfosalicylic acid).

Standard and unknown suspensions the same as in Table III.

Suspension No.	Final Molarity C_3H_7OH	R (neph. read.)	Percent "Protection"
#1	0.17 M.	20.1 mm.	5.06 %
2	0.33	22.3	13.0
3	0.67	31.3	34.3
4	1.33	54.8	59.2
5	2.00	37.0	43.2
6	2.67	29.4	31.1
7	3.33	24.9	20.4
8	3.67	23.0	16.0
9	3.87	36.0	41.9
10	4.00	41.6	48.5
11	4.67	167.0	84.3
12	6.00	400.	91.9
13	8.0 *	1000.	96.4

* Nine mls. of C.P. propyl alcohol used to give this concentration.

One observes that propyl alcohol has a maximum "protective" action at about 1.33 molar alcohol, then a minimum at 3.67 molar, followed by a very steep rise, reaching almost complete "protection" at the highest concentration used. The suspension # 13 was practically water clear.

Table VI.

"Protective" Action of Ethylene Glycol.

(Pptn. of alb. by sulfosalicylic acid).

Standard and unknown suspensions the same as in Table III.

Suspension No.	Final Molarity CH_2OH-CH_2OH	R (neph. read)	Percent "Protection"
#1	0.67 M.	21.0 mm.	8.47 %
2	2.00	21.9	11.6
3	4.00	26.8	25.8
4	6.00	43.8	50.7
5	10.7 *	190.	85.7

* Nine mls. C.P. glycol was used to give this concentration.

Here again, the results show the same phenomenon. In general glycol is weaker in its action than any of the alcohols studied so far, except at the highest concentration.

In Table VII, on the next page, are shown the data for the effect of glycerol on the albumin suspension. Slightly greater "protection" is observed in the case of glycerol than in that of glycol, when equal molarities are compared. The effect of glycerol and of methyl alcohol are similar.

All the results in the last five tables (III -VII, inclusive), are plotted in Fig. III, giving a perspective on all the data.

Table VII.

"Protective" Action of Glycerol.

(Pptn. of alb. by sulfosalicylic acid).

Standard and unknown suspensions the same as in Table III.

Suspension No.	Final Molarity $CH_2OH-CHOH-CH_2OH$	R (neph. read)	Percent "Protection"
#1	0.67 M.	20.7 mm.	7.40 %
2	2.00	25.3	21.4
3	4.00	32.3	36.4
4	6.00	59.2	61.8
5	8.2 *	115.	78.2

* Nine mls. of the C.P. glycerol was used to give this concentration.

From an inspection of the curves in Fig. III, it is plain that the factor which is being measured, increases with increasing concentration of alcohol, as a general rule. The peculiar curve shown by propyl alcohol has no explanation that the writer can offer. If the curve is continued from x to y, a smoothe curve results.

The experiment with methyl alcohol was repeated, using this time twice the amount of albumin (0.3000 mgms. N in the total suspension). The results are shown in Table VIII

Fig. III.

Concentration of Alcohols

and

"Protective"Action.

From Tables III-VII.

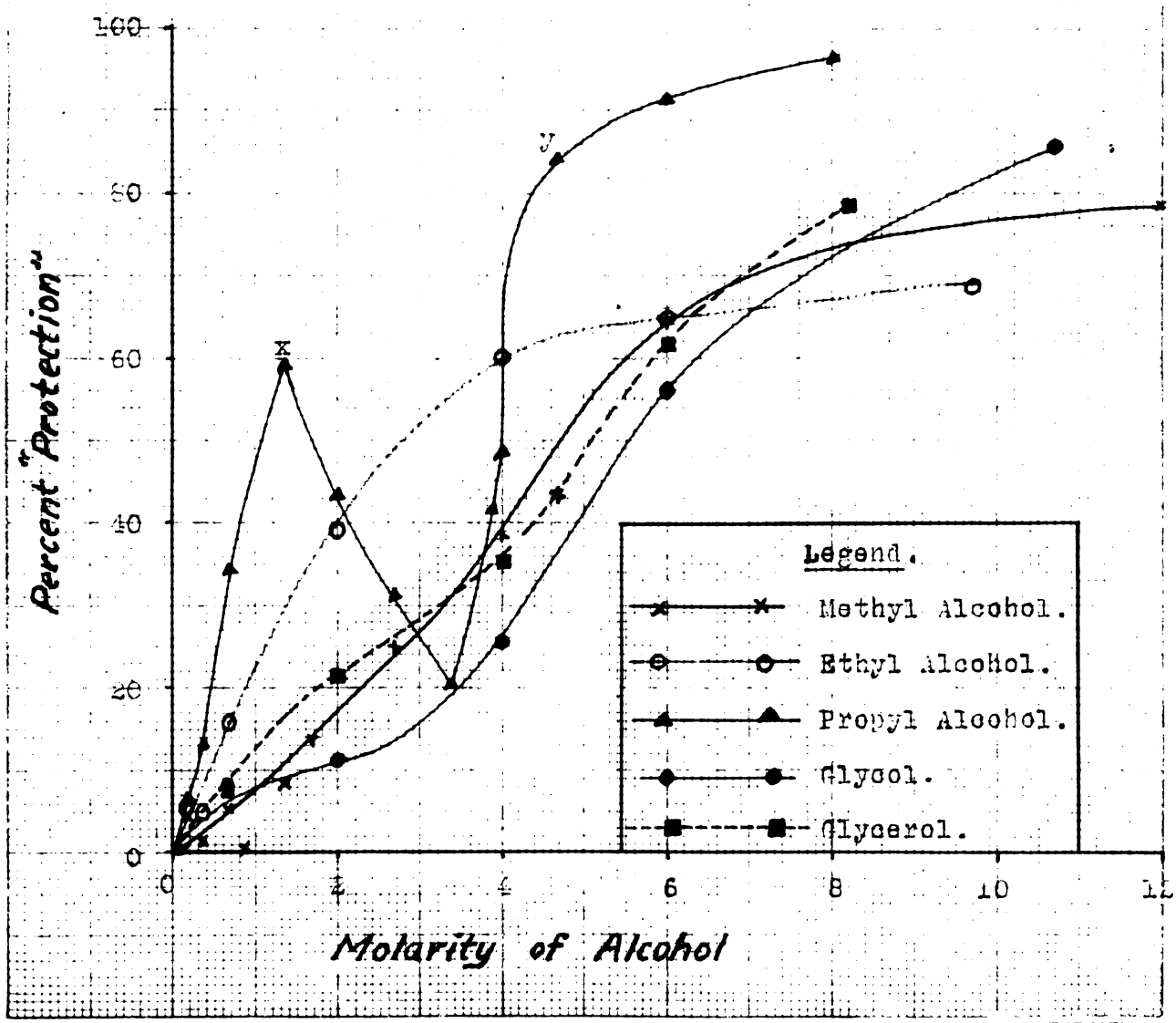


Table VIII.

Effect of Higher Concentration of Albumin on the "Protective" Action of Methyl Alcohol.

Standard Suspension.	"Unknown Suspension.
1 ml. alb. soln.(0.15 mgs N/ml.);	2 ml. alb. (total, 0.3 mgs N)
9 ml. H_2O.	x ml. H_2O.
5 ml. 0.2 M. sulfosalicylic acid;	8-x ml. of 10 M. CH_3OH.
	5 ml. 0.2 M. ss. acid.

Set at 20.0 mm.

Suspension No.	Final Molarity CH_3OH	R (neph. read.)	Percentage "Protection"
#1	0.67 M.	8.8 mm.	4.60 %
2	2.00	11.0	21.0
3	4.00	16.6	44.2
4	5.33	21.8	55.6
5	10.66 '	36.8	71.5
6	13.1 *	43.0	75.0

' Eight mls. of 20 M. alcohol used to make this.

* Eight mls. of C.P. alcohol to make this.

Upon comparing the above table with Table III, it is seen that the same percentage"protection" occurs, regardless of the different amounts of albumin in the two series of experiments. The effect, then, is independent of the albumin concentration for these two concentrations used. The above results are not plotted in Fig. III, because they correspond closely to the CH_3OH curve already plotted.

To obtain additional information, the attempt was made to check the nephelometric studies by the determinations of total nitrogen in the filtrates from the suspensions prepared as for the nephelometer, both with and without the alcohols.

The method was as follows: A suspension containing 5.063 mgms N in the total 15 ml. was made up in each case. The suspension was allowed to stand five minutes (the nephelometric readings required an average of about 8 min.), then filtered through qualitative filter paper. The first 5 ml. of the filtrate was discarded, and a 3 ml. aliquot of the rest analyzed for N by the micro-Kjeldahl method.

The results of the experiments follow. Each recorded value for % mgms. N in the filtrate is an average of two separate determinations on entirely new suspensions.

Table IX.

Comparison of N detns. of the Filtrate with Nephelometric "Protective"Action in the Presence of Alcohols. (ppt. by ss. acid)

5.063 mgms. N in total suspension; 3 ml. aliquot for detn.

Suspn. No.	Alcohol	Molarity	Kjeldahl % N in flt.	Nephelometer %"protection"
#1	None	0.00 M.	1.24 %	0.00 %
2	Methyl	5.33	0.85	50.0
3	Ethyl	5.33	0.28	64.0
4	Propyl	7.1	8.48	95.0
5	Glycol	9.5	0.84	81.5
6	Glycerol	7.3	8.16	72.2
7	Control		100.0	100.0

The "control" in the table consisted of only the aqueous solution of 5.063 mgms.N in the albumin, no alcohols or sulfosalicylic acid present. The"% protection by the nephelometer" was obtained by inspection of the Fig. III.

From Table IX, it must be concluded that the "protective" action of the alcohols, as measured by the nephelometer, is only apparent. The albumin is almost quantitatively precipitated by the sulfosalicylic acid, both alone and in the presence of the alcohols, but the alcohols lower the turbidity considerably. Propyl alcohol and glycerol do show protective action by the N determinations, but the actual protection is only 10 % of that calculated by the nephelometer method.

Some doubt may justly arise as to the fairness of comparison on the two suspensions; the Kjeldahl determinations were made on those containing 5.063 mgms. N per 15 ml., while the nephelometer was used on those having only 0.15 mgms. N per 30 ml. However, the author attempted a few N determinations on suspensions containing 0.300 mgms. N per 15 ml., taking 20 ml. aliquots, with similar results, though such small amounts of N were difficult to check.

Another criticism may also be brought up, in that the time of filtering for suspensions containing the more viscous alcohols was very long, taking upwards of an hour in some instances. In the continuous presence of the sulfosalicylic acid, it is possible that there is a gradual increase

in turbidity, and also in actual amount of albumin in the precipitate after such a long time.

Therefore a check was made on the effect of time on the turbidity. A single suspension was prepared, containing 6.00 molar glycol, 0.15 mgms. N in the 15 ml. of suspension, and precipitated by the usual strength of sulfosalicylic acid, and was read at once in the nephelometer, compared with the standard. The reading was 44.4 mm. After half an hour a new standard was prepared, and the glycol suspension read again. The reading was exactly 44.4 mm. as before.

Therefore, the writer believes he is justified in concluding that the nephelometric observations on the effect of the alcohols are measurements almost entirely of factors other than true protective action. Differences in particle size may have something to do with the observed phenomena, as was suggested by the work of Bechhold and Hebler (72). However this seems doubtful, since the filtration readily removes the suspended particles in all cases. It appears to be a tenable hypothesis that the degree of swelling or hydration of the suspended particles would explain the facts more closely: the more the swelling, the clearer the suspension.

(III). Heat Precipitation Studied by the Nephelometer and Nitrogen Determination Methods.

The experiments were extended to heat precipitation in checking the nephelometric measurements with nitrogen determinations on the filtrate or precipitate. It was found that a pH of 5.2 gave a stable suspension, when a solution containing albumin at the concentration of 0.7050 mgms. N in 20 ml. of total volume (experiments of Dudiles (59)), was heated at 70 degrees for 10 minutes.

In each of twelve large test tubes was placed 5 ml. of sodium acetate-acetic acid buffer solution (0.157 moles NaAc. and 0.051 moles HAc. per liter), sufficient albumin solution so that there was 0.7050 mgms. N introduced, then water was measured into the tubes so that with a given volume of the 10 M. alcohol added the total volume was 20 ml. The solutions were prepared in triplicate, A, B, and C. After heating all the tubes at the same time in a water bath at 70 degrees for 10 minutes, A was determined in the nephelometer, compared with the usual sulfosalicylic acid standard suspension; and B and C were filtered, and the filtrate or precipitate analyzed for nitrogen by the micro-Kjeldahl method.

The results for methyl alcohol are shown in Table X. The column headed "mgms. N in ppt. by neph. eq." gives values calculated by means of the equation,

9). $$W = 1.333\left[\text{antilog}\left(\frac{0.300 - \log R}{1.185}\right)\right]$$

where W is the mgms. N in the ppt.; R is the observed nephelometer reading in mm.; and the 1.333 is the dilution factor which corrects for the difference in volumes of the standard and the unknown.

Table X.

Effect of Methyl Alcohol on Heat Precipitation.

(Measured by N detns. and by nephelometer).

pH 5.2 ; 0.7050 mgms. N in 20 ml. ; heated at 70° C. for 10 min. Each value ave. of duplicates.

Expt. No.	Series No.	Final Molar Alc.	R (neph. read.)	Mgms. N in ppt.		Percent of total	
				by Kjeld.	by Nephel.	by Kjeld.	by Nephel.
#1	#1	Control H_2O	11.1 mm.	0.3441	0.3135	48.8%	44.5%
	2	M/4	8.4	0.4560	0.3967	64.6	56.2
	3	M/1	5.7	0.6654	0.5497	94.4	77.9
	4	2.5 M.	5.8	0.7062	0.5424	100.	77.0
2	1	H_2O	16.4	0.2960	0.2255	42.1	31.0
	2	M/10	16.3	0.2952	0.2265	41.9	32.2
	3	M/4	10.1	0.3570	0.3387	50.6	48.1
	4	M/1	6.1	0.6339	0.5190	89.8	73.6
3	1	H_2O	11.0	0.3206	0.3155	45.5	44.8
	2	M/10	10.5	0.2870	0.3275	40.7	46.5
	3	M/4	8.8	0.3200	0.3805	45.4	54.0
	4	M/1	5.9	0.6323	0.5335	89.7	75.7

In the suspensions containing 1 M and 2.5 M. alcohol, agglutination was observed at the end of the heat treatment.

The table shows two new facts: the first is that CH_3OH accelerates the heat precipitation of egg albumin, the action increasing(with concentrations above 0.1M), with increasing amounts of alcohol; the other is that in the case of heat precipitation the nephelometric method agrees fairly well with the Kjeldahl method. The latter is surprising, since the suspensions of albumin are prepared by two entirely different methods. Where disagreement occurs in the table, it is a question whether the nephelometer or N detn. method is more accurate. In filtering for the N detn. it was often found that it was difficult to get a clear filtrate; furthermore, filtration often caused slight mechanical denaturation, which would introduce considerable error where such small quantities of N are present.

The disagreement between the two methods at the higher amounts of albumin in the precipitate is to be expected. The figures in Table II show that when the formula is applied to suspensions containing 0.75 mgms of nitrogen in 15 ml. the calculated value is 17 % too low.

The same experiments were carried out using glycerol, as a different type of alcohol. The results follow in Table XI..

Table XI.

Effect of Glycerol on Heat Precipitation.

(Measured by N detns. and by nephelometer).

pH 5.2 ; 0.7050 mgms. N in 20 ml. ;

Heated at 70° C. for 10 min. Each value

the average of duplicates.

Expt. No.	Series No.	Final Molar. Alc.	R (neph. read.)	Mgms. N in ppt.		Percent of total in ppt.	
				Kjeld.	Neph.	Kjeld.	Neph.
#1	#1	H_2O	11.4 mm	0.2999	0.3061	42.5 %	43.4 %
	2	M/4	9.6	0.2894	0.3540	41.0	50.1
	3	2.5 M.	24.5	0.2580	0.1644	36.6	23.3
	4	5. M.	56.8	0.1648	0.0789	23.4	11.2
2	1	H_2O	12.7	0.3370	0.2795	47.7	39.6
	2	M/4	14.1	0.2950	0.2621	41.8	37.2
	3	2.5 M.	23.8	0.2342	0.1643	33.2	23.3
	4	5. M.	47.0	0.1599	0.0925	22.6	13.1

These results confirm Beilinsson's observation (56) that glycerol stabilizes egg albumin against heat coagulation. The nephelometer gives the same conclusion, however the absolute values do not agree by the two methods. In high concentration of glycerol, the nephelometer gives lower results. This may be the same type of phenomenon as was observed in the experiments with sulfosalicylic acid, since the turbidity increases more slowly than the absolute amount of albumin in the precipitate.

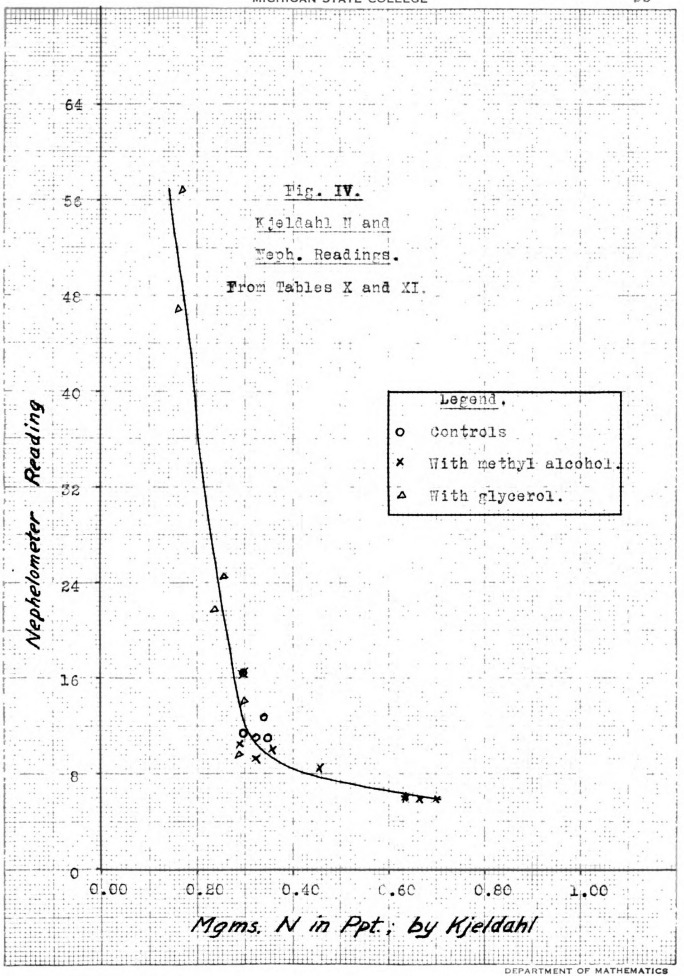


Fig. IV.
Kjeldahl N and
Neph. Readings.
From Tables X and XI.
Legend.
Controls
With methyl alcohol.
With glycerol.
Nephelometer Reading
0
8
16
24
32
40
48
56
64
0.00
0.20
0.40
0.60
0.80
1.00
Mgms. N in Ppt.; by Kjeldahl

When the nephelometer readings are plotted against the observed mgms. N by the Kjeldahl method, in the presence of methyl alcohol and of glycerol, Fig. IV is the result. Perhaps if a larger number of experiments were carried out using other non-electrolytes, such as glucose and acetaldehyde, the curve may be of great value in studying, by the nephelometer, the effects of such non-electrolytes on heat precipitation. The nephelometer method is by far the easier of the two methods.

A single experiment was carried out with ethylene glycol on heat precipitation. Table XII contains the results, obtained by use of the nephelometer, only. The assumption is made that glycol follows the same curve as in Fig. IV, the N values being taken directly from the curve.

Table XII.

Effect of Ethylene Glycol on Heat Pptn.

(Detnd. in duplicate by the neph.)

pH 5.2 ; 0.7050 mgms. N in total of 20 ml. ; heated at 70° for 10 min.

Series No.	Final Molarity Glycol.	R (neph. read.)	Mgms. N in Precipitate.	Percent N in Ppt.
# 1	H_2O	12.4 mm.	0.297 mgs.	42.1 %
2	M/4	14.1	0.286	39.2
3	M/1	9.8	0.330	46.8
4	2.5 M.	6.5	0.590	83.7

Glycol increases the amount of heat precipitation, as judged by the nephelometric method. The accelerating effect, however, is less than that for methyl alcohol.

(IV). Conclusions.

1. The nephelometer can be used in determining minute amounts of pure, aqueous egg albumin, when sulfosalicylic acid is used as the precipitating agent. The formula has been worked out, and found to be accurate to 2.4 percent, over the range of albumin concentration containing 0.02 to 0.45 mgms. nitrogen in 15 ml. of suspension.

2. When alcohols are present in amounts above 1 molar the formula is valueless for determining actual amounts of albumin, with sulfosalicylic acid as the precipitant.

3. In the presence of about an 8 molar concentration of methyl-, ethyl-, and propyl alcohols, glycerol and glycol, the turbidity shown by sulfosalicylic acid precipitation decreases about 68 to 97 percent, depending on the alcohol. At the same time, the protein is nearly quantitatively precipitated. With propyl alcohol and glycerol, slight protective action is observed by nitrogen determinations on the filtrate, but the amount of protection is far below that which would account for the decrease in turbidity. The suggestion is made that differences in particle size may explain the decrease, but more likely

a difference in the degree of hydration of the suspended particles is the cause.

3. Methyl alcohol and glycol accelerate the heat precipitation of egg albumin at pH 5.2. The effect is weaker with glycol than with methyl alcohol.

4. Glycerol protects the albumin from precipitation by heat at pH 5.2 . This confirms the report of Beilinsson (56),who obtained his results by a different method.

5. A comparison of the nephelometer method of measuring heat precipitation of albumin, and the micro-Kjeldahl method , was made.. The methods agree, in that a high turbidity corresponds with a high amount of precipitate, and conversely. However, the absolute values by the two methods do not check as well as one would like.

The findings in the comparison of the two methods make it probable that the observations of Teorell (55), who found a decrease in turbidity in heat precipitation of serum albumin in the presence of alcohols, revealed true protection of the actual amount of albumin precipitated.

6. It is probable that with a few additional experiments on the comparison of the nephelometer readings and nitrogen determinations, the nephelometer can be made very useful in studying the protective action of non-electrolytes on proteins.

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