Proteins : lectures given in the United States of America in 1924 / by S.P.L. Sørensen.

PROTEINS

LECTURES GIVEN IN THE UNITED STATES OF AMERICA IN 1924

S. P. L. SØRENSEN

Compliments of

THE FLEISCHMANN COMPANY

Fleischmann Laboratories
New York

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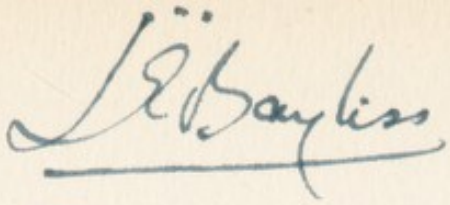
DR. S. P. L. SØRENSEN

Carlsberg Laboratory
Copenhagen



PROTEINS

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Lectures given in the United States
of America in 1924

BY

S. P. L. SØRENSEN

Director of the Chemical Department
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Copenhagen

(New York)

THE FLEISCHMANN LABORATORIES
For the Investigation of Fermentation and for
Industrial Research

1925

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PREFACE

PREFACE

It is indeed unfortunate for American scientists that so many of our distinguished European visitors leave our shores without the opportunity of giving us the pleasure of having personal contact with them. With this in mind, we take great pleasure in presenting these lectures to the scientific public. The author needs no introduction to American scientists, and it is hoped that a touch of his personality may permeate these printed pages, making it possible for those who have not been able to hear his voice, to feel, nevertheless, the spirit of the man whose mind has contributed so much to the progress of science.

The roots of every great industry are buried deeply in the science and art of man. A few men are able to create new industries by means of scientific discoveries; many men are able to make discoveries which contribute to the development and growth of industry; but many more must take their places as part of the vast mechanism without seeing the cause and purpose of it all. But the benefits and products of industry reach every individual in the civilized world.

As I stood in the laboratory of Prof. Sørensen at Carlsberg, I felt that I was indeed in the presence of those who were creating. Prof. Sørensen I found to be not only a great educator and scientist but a chemist who appreciates and recognizes the value of science to industry, and who actually applies it. I was animated by the desire to give to the American public, and especially to the students and teachers of America, a vision of what I had seen and something of the wonder I had felt.

Through the generosity and interest in science of the late Mr. Julius Fleischmann, President of The Fleischmann Company, I was enabled to extend an invitation to Prof. Sørensen to favor us with a visit and to give

some of his time while here to the scientists and students of America.

It is with the hope that we have contributed in extending the field of knowledge by making some of the recent work of Prof. Sørensen more easily available to scientists, and that in addition it will be possible for some to gain a glimpse into the science underlying great industries, that we present these lectures to the public. Prof. Sørensen has honored us by giving us this opportunity.

R. L. CORBY.

New York, N. Y.
March, 1925.

PREFACE

The suggestion of making a permanent record of the lectures as given during my stay in the United States in the autumn of 1924 marks one more beautiful thought and expression of co-operation between science and industry and makes my pleasure all the greater in complying with this wish.

It gives me an added opportunity of expressing my heartiest thanks to all of those, and they were many, who extended to Mrs. Sørensen and myself such friendly and genuine hospitality. Our stay was really a festival and an enlivening experience which we shall never forget.

My ever sincere thanks are due, in particular, to the late Mr. Julius Fleischmann, President of The Fleischmann Company, for the gracious and generous invitation which made our journey possible, and to Mr. R. L. Corby, Director of The Fleischmann Laboratories, for the never tiring amiability with which he conducted the tour for us.

S. P. L. SØRENSEN.

Copenhagen,
March, 1925.

INTRODUCTION

INTRODUCTION

The great development of colloid chemistry during the twentieth century has been of great significance for protein research and has, in special cases, shown the way for the elucidation of phenomena which hitherto had appeared incomprehensible.

Colloid chemistry applies particularly to behaviour which is dependent on the physical and not on the chemical nature of the substance in question. However, when properties and reactions in which the chemical nature of a substance takes part are being considered, the colloid chemical point of view is generally ineffective for giving any satisfactory explanation. As we are here concerned with a number of the most important and in the most exact sense, characteristic, properties of the proteins, it may be said in this connection, that colloid chemistry, frequently one-sided, has often restricted rather than advanced the study of these proteins. This is largely due to the conception that protein solutions, being of a colloidal nature, are so different from ordinary true solutions in their behaviour, that it is impossible to apply the theories based on the study of true solutions to protein solutions.

The research of the last ten years—not least that of American scientists—has effected a change in these views. It has

become evident that in order to understand the properties and reactions of protein solutions, the theories accepted for the state of true solutions must be applied. The reason this view has gained general recognition only recently may be attributed to the many great difficulties the minute investigation of proteins and of their solutions present.

Protein matter is not volatile, can only in special cases be crystallized, and on account of its amphoteric character, has a strong tendency toward mutual combination, with the result that purification is very difficult and the preparation of a well defined protein substance is therefore very complicated. Furthermore, the molecules or molecular aggregates of the proteins are so large, that only a very small amount of base or acid, respectively, is required to neutralize a single acidic or basic group in this large molecule. As such neutralizations are of the greatest significance for the properties of the protein solutions, it is essential that exact methods for the determination of even slight changes in the acidic or basic character of the solutions be applied. It might further be mentioned that protein substances, in dissolved as well as in precipitated state, are generally present in combination with water, the disperse phase consisting of protein matter in more or less loose connection with larger or smaller quantities of the dispersion medium, of which the chief constituent is water. This water content, which differs in amount according to the composition of the dispersion medium, and which is of essential importance for the properties of protein substances, makes investigation still more complicated.

These difficulties in addition to several others, instability, for example, place many obstacles in the way of an exact characterization and thorough study of protein solutions. In the

author's opinion, however, there is no doubt that should these obstacles be successfully overcome, it may be effected by the development and application of better and more exact methods of investigation, even if these require both time and toil, and the explanation of the results obtained may be worked out from the same points of view as those which have been proved applicable in the study of the behavior of true solutions.

It is from considerations of such a nature that the research on proteins at the Carlsberg Laboratory, Copenhagen, has been planned and carried out during the last fifteen years.

A part of these investigations on hen's egg albumin was published in the years 1915-1917. The lectures compiled in the present book constitute in all essentials a continuation of the earlier work. The research material besides egg albumin includes albumins and globulins of horse serum.

The contents of these lectures will not be summarized here, but certain important considerations will be indicated. It is one of the author's main points to emphasize strongly the importance and necessity of the best possible characterization of the protein under investigation, so that the substance in question can be considered well defined, thereby making repetition of the experiments and comparison with the results of other investigators possible.

One of the most important means for such a characterization, the solubility of protein substances in water and salt solutions, has been closely treated in one of the lectures. This property was studied especially for the elucidation of the strange and complicated solubility behavior of the serum globulins. Another lecture contributes to the enlightenment of that all important denaturation process which is characteristic of a number of protein substances.

In a third lecture the author has dealt with the determination of the magnitude of the protein molecules or molecular aggregates by the measurement of the osmotic pressure of protein solutions. On the basis of such determinations it can be decided how far differences in properties, such as different solubilities under the same conditions, can be explained as originating from a different degree of dispersion—the colloid chemical point of view—or must be explained in another way.

The author is convinced that the time has now come when it is possible to carry our knowledge of the chemistry of protein substances an important step forward, if only the necessary amount of time, toil and care can be sacrificed to this end. It is hoped that the research published in the present book may in some measure contribute to the solution of this great and important problem.

S. P. L. SØRENSEN.

SOLUBILITY OF PROTEINS

SOLUBILITY OF PROTEINS

One of the most important questions today concerning protein studies is how far it is possible to apply to protein solutions the theories derived from the study of true solutions; or whether such colloid solutions as those of protein are so diverging in their behavior from ordinary real solutions that to parallel the behavior of colloid solutions and true solutions must be considered inadvisable, or even warned against, as *Wolfgang Ostwald* has often done.

I believe there is a growing tendency to think that Ostwald is not right in his view of the matter; that the need is simply to make it possible, by improved technique, and particularly by the development of more exact analytical methods, to deal with protein solutions in accordance with general chemical and physico-chemical principles.

We cannot at present enter upon a more particular discussion of the great work already done in this direction. I must be content to mention a few names in order to throw some light on the nature of the researches under discussion.

The investigations of *Harriette Chick* and *C. W. Martin*[1] on the coagulation of protein have demonstrated, among other things, that the denaturation process is a monomolecular pro-

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[1] Chick and Martin, J. Physiol., **40**:404 [1910]; **43**:1 [1911]; **45**:61, 261 [1912].

cess, provided that the hydrogen ion concentration be kept constant.

The elaborate investigations of *Wolfgang Pauli and his collaborators* on proteins especially in acid or alkaline solutions should also be mentioned. Pauli has succeeded in showing that proteins form salts of the same nature and character as simple ampholytes.

Further should be mentioned *Jacques Loeb*, recently and too prematurely deceased, whose studies on the so-called "Donnan equilibrium" in connection with the physico-chemical properties of protein solutions have thrown light on so many hitherto unknown factors.

Finally, among all these examples, to which many more might be added, I may perhaps venture to mention the investigations on hen's egg albumin conducted at the Carlsberg Laboratory, by which we succeeded in demonstrating that in many and essential cases the theories derived from the study of true solutions may also be applied to the solutions of egg-albumin. This is true, for instance, in such important cases as the state of equilibrium by salt precipitation, the application of Gibbs' phase rule, the question of the osmotic pressure of albumin solutions, and in many more instances.

The present discussion concerns the precipitation of some proteins and the conditions of their solubility in water and salt solutions, these conditions being but imperfectly known and of no small importance.

I shall first mention briefly the equilibrium conditions of albumins when crystallized by means of ammonium sulphate, and then discuss somewhat more fully the solubility of the globulins of horse serum, more particularly that of euglobulin.

SOLUBILITY OF PROTEINS

A. Precipitation of Albumins by Ammonium Sulphate

If Gibbs' phase rule, which applies to true solutions, be applicable also to protein solutions, a precipitation of the solution by means of a salt must result in the same state of equilibrium between the precipitate and the surrounding mother liquor, irrespective of whether the initial solution be concentrated or diluted, if only care be taken that at the end of the process, the factors that influence the precipitation (temperature, salt concentration, hydrogen ion concentration) are alike in all cases. Under such conditions much albumin should be precipitated from a strong albumin solution; from a weak solution, on the other hand, only little; but the final albumin concentration in the solution must in every case be the same, provided that Gibbs' phase rule applies.

However, according to the generally accepted view, protein solutions do not behave in accordance with the phase rule; the general opinion being that under otherwise similar conditions, the higher the initial protein concentration, the more completely is the protein salted out. It is unnecessary to enter on a discussion of the voluminous literature published on this subject. The elucidation of a single example will suffice. For this purpose the otherwise most valuable work of *Harriette Chick* and *C. W. Martin*[2] on the precipitation of egg albumin by ammonium sulphate will serve, because the conditions of the experiments mentioned there, are analogous to those under which our experiments were conducted. In a series of experiments which are reproduced from their paper in Diagram 1, Chick and Martin investigated the influence of the initial albumin concentration on the precipitation of egg albumin by ammonium sulphate. In all the experiments of the series, the

[2] Biochem. Journal **7**:380 [1913].

ratio ammonium sulphate to water was constant, namely 31:100 but the amount of albumin in the mixtures varied from 3.3 to 27.4 g. egg albumin per 100 g. water. As may be seen from the sixth vertical column in the table, Chick and Martin find that the albumin is precipitated more completely, the higher the initial concentration.

DIAGRAM 1

Precipitation of Pure Egg-Albumin with Ammonium Sulphate. Influence of Concentration of Protein.

Ratio: salt/water = 31/100. Concentration of protein varying.

Albumin G.	Water G.	Salt G.	G. of albumin in 100 g. of total mixture	G. of albumin to 31 g. of salt and 100 g. of H_2O	G. of albumin in 100 g. of filtrate	G. of albumin precipitated from 100 g. of total mixture	Protein pptd. %
1.90	56.92	17.65	2.481	3.33	1.130	1.351	54.4
1.90	36.92	11.44	3.775	5.14	1.115	2.660	70.4
3.79	50.85	15.76	5.383	7.45	1.159	4.224	78.5
7.59	53.69	16.64	9.738	14.13	0.935	8.803	90.4
4.74	17.31	5.36	17.306	27.40	0.772	16.534	95.5

The error is due to the fact that Chick and Martin have taken it for granted that the proportion between ammonium sulphate and water does not change during the precipitation, and consequently they have made no determination of the amount of ammonium sulphate in the filtrates. It is, however, beyond doubt that an egg albumin solution behaves during precipitation just as during crystallization. In the latter case we have demonstrated that 1 g. of egg albumin contains constantly 0.22 g. of water. If, however, it is clearly realized that the egg albumin by its precipitation removes water from the solution, it is evident that the higher the initial concentration of egg albumin, the more water will be removed from the solution during the precipitation, and consequently the am-

monium sulphate concentration of the filtrate will be higher. As an increase of the ammonium sulphate concentration causes a more complete precipitation of the egg albumin, this accounts for the experimental results of Chick and Martin.

Our experiments made in order to throw light on this question have proved that the amount of albumin remaining in the mother liquor by the crystallization of egg albumin is independent of the initial protein concentration, provided care be taken that at the end of the crystallization the ammonium sulphate concentration of the mother liquor is the same in all the experiments. That this is really so, must of course be demonstrated analytically.

In Diagram 2 is shown graphically the result of one of our series of experiments. The content of egg albumin in the mother liquor is used as ordinate, and the crystallization time in days as abscissa. In all the experiments the ammonium sulphate concentration is the same, namely 26.658 g. ammonium sulphate per 100 g. of water, but the initial albumin concentration varies in the different experiments from 2.3 to 14.1 g. of albumin per 100 g. of water. There is a curve corresponding to each experiment, and it is plainly shown in the diagram that when the crystallization has proceeded for a few days, the amount of albumin in all the filtrates is practically the same. A more minute consideration of the experimental results will show that the small discrepancies between the results point in the opposite direction to what should be expected according to the previously mentioned generally accepted theory; moreover these small discrepancies can be easily explained.

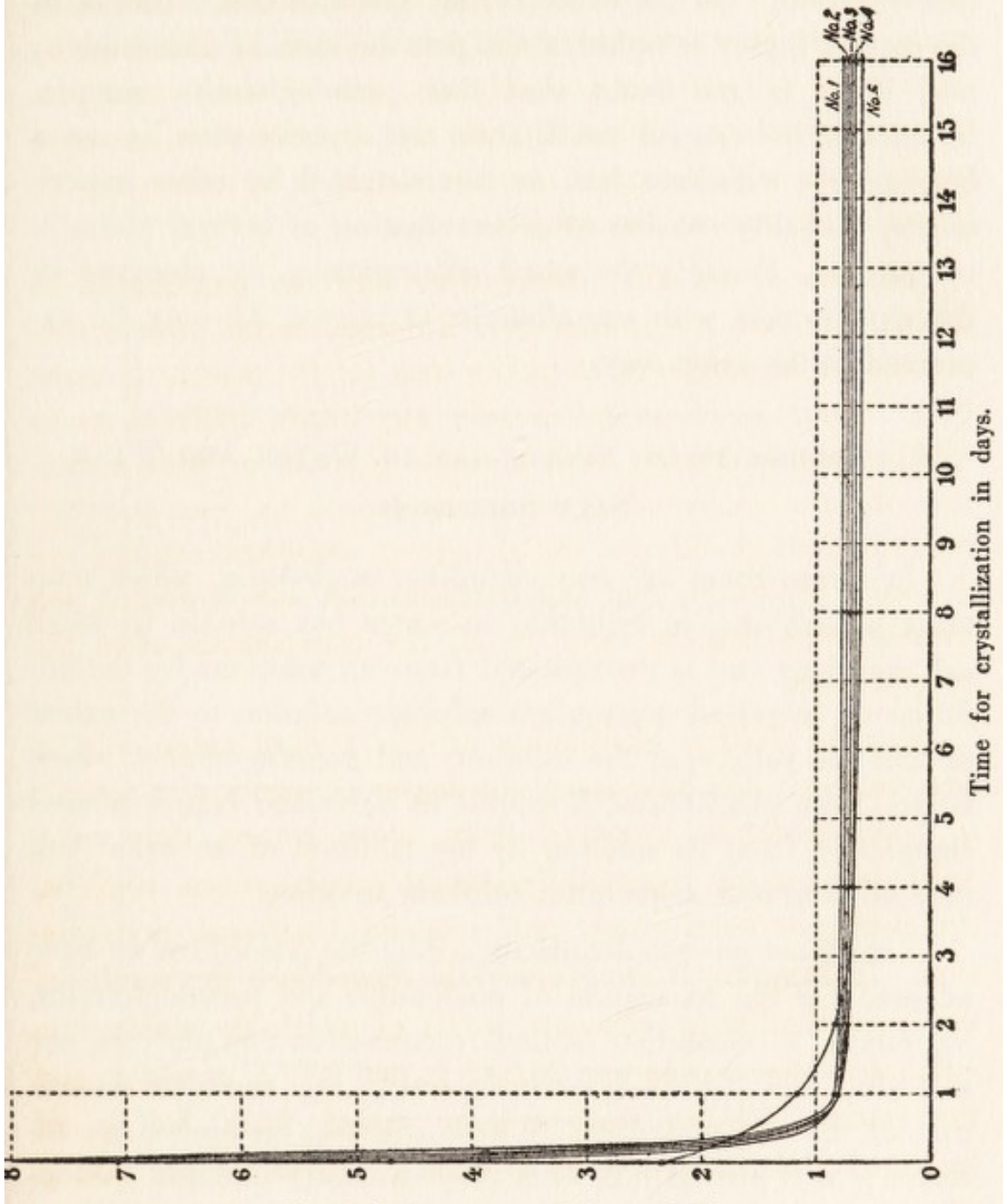
From what has been stated here, as well as from another series of experiments also including serum albumin, but which we cannot present now for lack of time, it is apparent that

the behavior of albumins when precipitated with ammonium sulphate does not exclude the application of the phase rule in its usual form and with its usual consequences. The statements made here concerning the precipitation of albumins by ammonium sulphate are probably also true of many other protein precipitations. When hitherto the opposite view has commonly been held, the cause must be sought in defective experimental treatment of the subject.

But this is not all. One further step can and should be taken: that such precipitations at different initial protein concentrations be employed as purity tests for the protein in question. There is at present hardly any better evidence as to whether one is dealing with a single protein, or with a mixture of, or a compound of two or more proteins, than just such precipitations at different initial protein concentrations and under such conditions that salt concentration and hydrogen ion concentration are the same in all the experiments after the precipitation.

For the further elucidation of this I shall refer only to one instance. We have tested the degree of purity of a sample of pseudoglobulin prepared from horse serum, very carefully purified by repeated fractional precipitations, dialysis, dissolving in pure water, and renewed fractional precipitation. In Diagram 3 are graphically reproduced the results of five precipitation experiments, in all of which the ammonium sulphate concentration was 24.533 g. per 100 g. of water, and the initial globulin concentration varied from 5.6 g. of hydrated globulin (curve I) to 0.28 g. (curve V) per 100 g. of water. As in the preceding diagram (Diagram 2) the precipitation time expressed in days is used as abscissa and the globulin content of the mother liquor as ordinate. It will

DIAGRAM 2


No.2
No.3
No.4
No.1
No.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Time for crystallization in days.
8
7
6
5
4
3
2
1
0
Egg albumin hydrate g. per 100 g. of H_2O

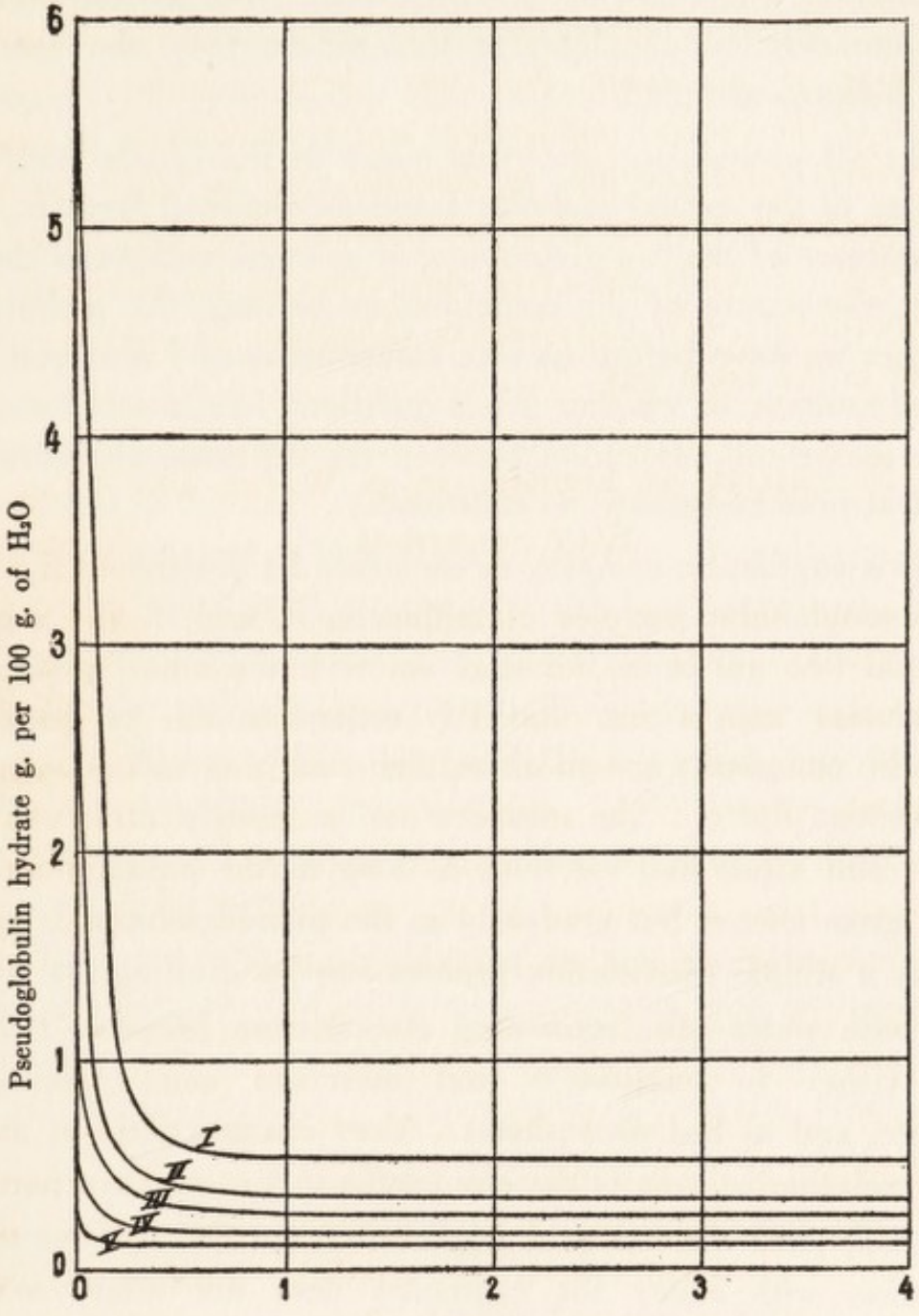
be seen from the diagram that the globulin concentration of the mother liquor is by no means lower, the higher the initial concentration. On the contrary, the globulin concentration in the mother liquor is higher, the higher the initial concentration; and there is no doubt that this pseudoglobulin sample, in spite of the careful purification and fractionation, is not a homogenous substance but, as demonstrated by other experiments, probably consists of a combination of several globulin complexes. Possibly the small discrepancies we observed in the experiments with egg albumin (Diagram 2) may be explained in the same way.

B. Solubility of Euglobulin in Water and Weak Salt Solutions

In serum there are two globulins: *euglobulin*, which contains phosphorus, is insoluble in water but soluble in weak salt solutions and is precipitated from its solutions by the addition of saturated ammonium sulphate solution to the extent of half the volume of the solution; and *pseudoglobulin*, which is free from phosphorus, is soluble in water and is precipitated completely from its solution by the addition of an equal volume of saturated ammonium sulphate solution.

I shall not go into details regarding the procedure we have adopted for the separation of euglobulin and pseudoglobulin, but merely mention that neither fractionation nor dialysis, nor a combination of the two methods, results in a complete separation of the two globulins. I have already mentioned an instance which proved that even very carefully purified pseudoglobulin is not a single substance and in that which follows I shall mention a series of experiments which serve to show that it is not possible to prepare a pure euglobulin in this

DIAGRAM 3


6
5
4
3
2
1
0
0
1
2
3
4
I
II
III
IV
V
Pseudoglobulin hydrate g. per 100 g. of H₂O
Time for precipitation in days

way either. In so doing I shall also have the opportunity of thoroughly elucidating the peculiar solubility conditions of euglobulin, which can be explained only from a viewpoint of the inter-relation of globulins that differs from the conception hitherto accepted.

In all probability globulins occur in the serum itself as well as in the special globulin fractions obtained from it, not as mixtures of the two globulins, but as combinations of them. As to the nature of the combinations between the globulins, whether we have before us true compounds, as I am most inclined to think, or whether it is a question of solid solutions, or of a reciprocal adsorption between the different globulins, I shall at present venture no statement.

If a euglobulin complex or molecule be designated *E*, and a pseudoglobulin complex or molecule, *P*, and if, for a provisional first guidance, we start out with the (most probably erroneous) supposition that the respective eu- or pseudoglobulin complexes are all alike, then such a combination may be written *EpPq*. The combination is readily dissolved in water and dilute salt solutions as long as the pseudoglobulin is in great excess, but gradually as the pseudoglobulin is split off by a simple dissociation process—as by diluting the solution with water—the remaining combination becomes richer and richer in euglobulin and therefore more sparingly soluble, and at last precipitates. Very characteristic of such a dissociation process is the circumstance that we have here a real reciprocal alteration of equilibrium, so that the reaction (dilution with water for example) does not affect *solely* the combinations present in the solution, but the precipitate already once formed takes part in the reaction until the new equilibrium is established between precipitate and mother

liquor, corresponding to the decreased salt concentration brought about by the addition of water. Of course, the order of the process is reversed if the salt concentration be increased. I shall not go further into this, but merely mention that the behavior of precipitation with greater salt concentrations may be explained on similar grounds.

The most elaborate researches on the solubility of euglobulin have been carried out by *I. Mellanby*[3] and *W. B. Hardy*[4]. In these classical papers it is stated that the solubility of euglobulin in dilute neutral salt solutions increases with the salt concentration, but it is also stated that the amount of globulin dissolved by any given salt concentration is approximately proportional to the total amount of globulin employed in the solubility experiment. This highly peculiar circumstance, which we have found corroborated by our experiments, can be explained according to our general chemical and physico-chemical theories only on the basis of the above mentioned assumption that the euglobulin employed is not a single substance, but can be dissociated by salt solutions. A brief account of a few of our series of experiments on these peculiar solubility properties will be given.

To begin with, I would mention that we have also found that the solubility of euglobulin increases with increased salt concentration, at any rate as long as fairly small concentrations are in question. Furthermore, a globulin obtained by dialysis and subsequent treatment with water, after several washings with water, showed the solubility in 0.02 *N* sodium chloride solution given in Diagram 4.

DIAGRAM 4

Total globulin used, mg. N.....	29.06	58.11	116.22
100 cc. of sat. soln. contains, mg. N.....	10.95	21.75	42.15

[3] Mellanby—J. Physiol. **33**:338 [1905].
[4] Hardy—ibid. **33**:251 [1905].

The solubility of this well-washed sample of euglobulin is thus very nearly proportional to the amount of globulin employed in the solubility experiment, just as stated by Mellanby and Hardy.

A really effective purification cannot, however, be obtained by washing with water, but only by dissolving the euglobulin in a weak salt solution and subsequent precipitation by dilution with water. To throw light on this question I shall mention a

DIAGRAM 5

Purification of the Globulin Fraction BIII

Nature of the Globulin Fraction	Solubility of the globulin fraction in 0.02 *N* NaCl solution		
	Total amount of globulin nitrogen per 100 cc. used for the experiment mg.	Amount of globulin nitrogen dissolved per 100 cc. of solution mg.	Mg. of P per g. of globulin nitrogen
BIII	26.22	3.23	4
	52.44	5.39	
	78.66	6.89	
BIV			
[BIII dissolved in 125 cc. of H_2O + 15 cc. of *N* NaCl, precipitated with 1 liter of H_2O; decantation of the mother liquor, washing with 250 cc. of H_2O, decantation of the washing water and suspension of Biv in 200 cc. of H_2O].	11.92	1.13	4
	23.84	1.64	
	35.76	2.05	
BV			
[BIV dissolved in 140 cc. of H_2O + 30 cc. of *N* NaCl, precipitated with 1 liter of H_2O; decantation of the mother liquor, washing with 200 cc. of H_2O, decantation of the washing water and suspension of Bv in 95 cc. of H_2O].	20.71	0.82	6
	41.42	0.87	
	62.13	1.28	
BVI			
[Bv dissolved in 45 cc. of H_2O + 35 cc. of *N* NaCl, precipitated with 850 cc. of H_2O; decantation of the mother liquor, washing with 190 cc. of H_2O, decantation of the washing water and suspension of BVI in 100 cc. of H_2O].	17.08	0.62	10
	34.16	0.62	
	51.24	0.72	

series of experiments in which the purification of a globulin fraction, B_{III}, was followed in all its details. In the experiment, B_{III}, which was taken as the primary substance, had already been reprecipitated once and was consequently rather sparingly soluble. It was further reprecipitated three times by solution in sodium chloride solution and precipitation by water. B_{III} itself, as well as the products, B_{IV}, B_V, B_{VI}, obtained by the reprecipitations, was subjected to an analysis by which the solubility in 0.02 *N* sodium chloride solution was determined, three different but known globulin amounts of each fraction being used in the solubility determinations.

The analytical results are grouped in Diagram 5 and graphically reproduced in Diagram 6 in which the total amount of globulin nitrogen is used as abscissa, while the globulin-nitrogen in 100 cc. saturated solution is used as ordinate.

From the table as well as from Diagram 6 it appears that the purification of the globulin not only causes a decrease of its solubility, but also makes it approximately constant and independent of the total amount of globulin employed in the solubility experiment. Graphically this is shown in Diagram 6 by the curves more and more approaching a parallel with the abscissa axis.

If it were really a question of a single substance, this substance should have a constant solubility in water or in salt solution of a given concentration, irrespective of whether the surplus of globulin left undissolved is large or small. As just mentioned, however, euglobulin does not behave in this manner, neither after thorough washing with water nor after several reprecipitations.

Euglobulin can be treated so long and so often with water that the pseudoglobulin complexes which can be dissociated

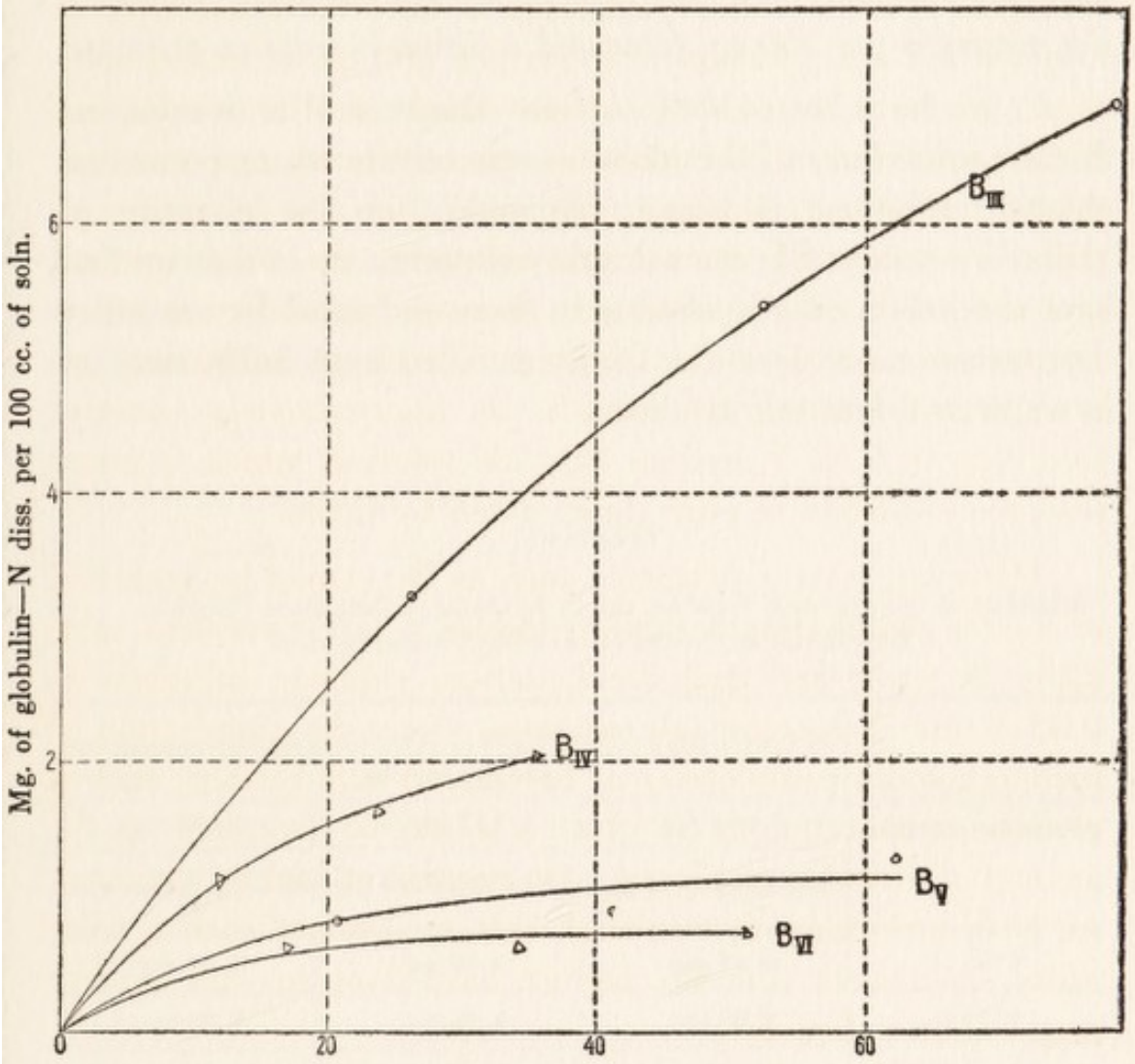
in pure water are really split off and the remaining substance will consequently show—at least approximately—a constant solubility in water, independent of the amount of globulin employed. Thus *Edwin J. Cohn*[5], in a series of carefully conducted and recently published experiments, has found that the solubility of well washed euglobulin in pure water corresponds to about 1.2 mg. globulin nitrogen per 100 cc. saturated solution. We have been able to obtain exactly similar results, but if the purification of the globulin was continued, and was conducted not only by washing with water, but also by reprecipitation by means of neutral salt solutions, the reprecipitated globulin exhibited a considerably decreased solubility in water. Furthermore, as shown in Diagram 5, such an adequately reprecipitated fraction B_{VI} exhibits an approximately constant solubility in 0.02 *N* sodium chloride solution, which is lower than the solubility in pure water stated by Cohn.

However, even a substance such as B_{VI} cannot be regarded as a pure euglobulin; it exhibits indeed a fairly constant solubility in water and such weak sodium chloride solutions as 0.02 *N*, but in stronger salt solutions, where the dissociation of pseudoglobulin complexes can be carried still further, such a globulin preparation as B_{VI} shows a solubility dependent on the amount of globulin employed. On account of lack of material, we have unfortunately not been able to conduct such a solubility experiment with B_{VI}, but we have done so with another preparation called α_{a4}.

The globulin sample α_{a4} was purified by means of a series of reprecipitations, and was consequently one of the most sparingly soluble globulin fractions with which we have had to deal. With this sample of globulin a series of solubility

[5] J. of Gen. Physiol. **4**, 697 [1922].

DIAGRAM 6



Total mg. of globulin-N per 100 cc.

experiments was conducted in rather strong potassium chloride solutions, and with three different quantities of globulin in each concentration of potassium chloride. The experimental results are grouped in Diagram 7 and graphically reproduced in Diagram 8, in which the potassium chloride concentration is used as abscissa, and the dissolved euglobulin in terms of mg. nitrogen per 100 cc. saturated solution is used as ordinate.

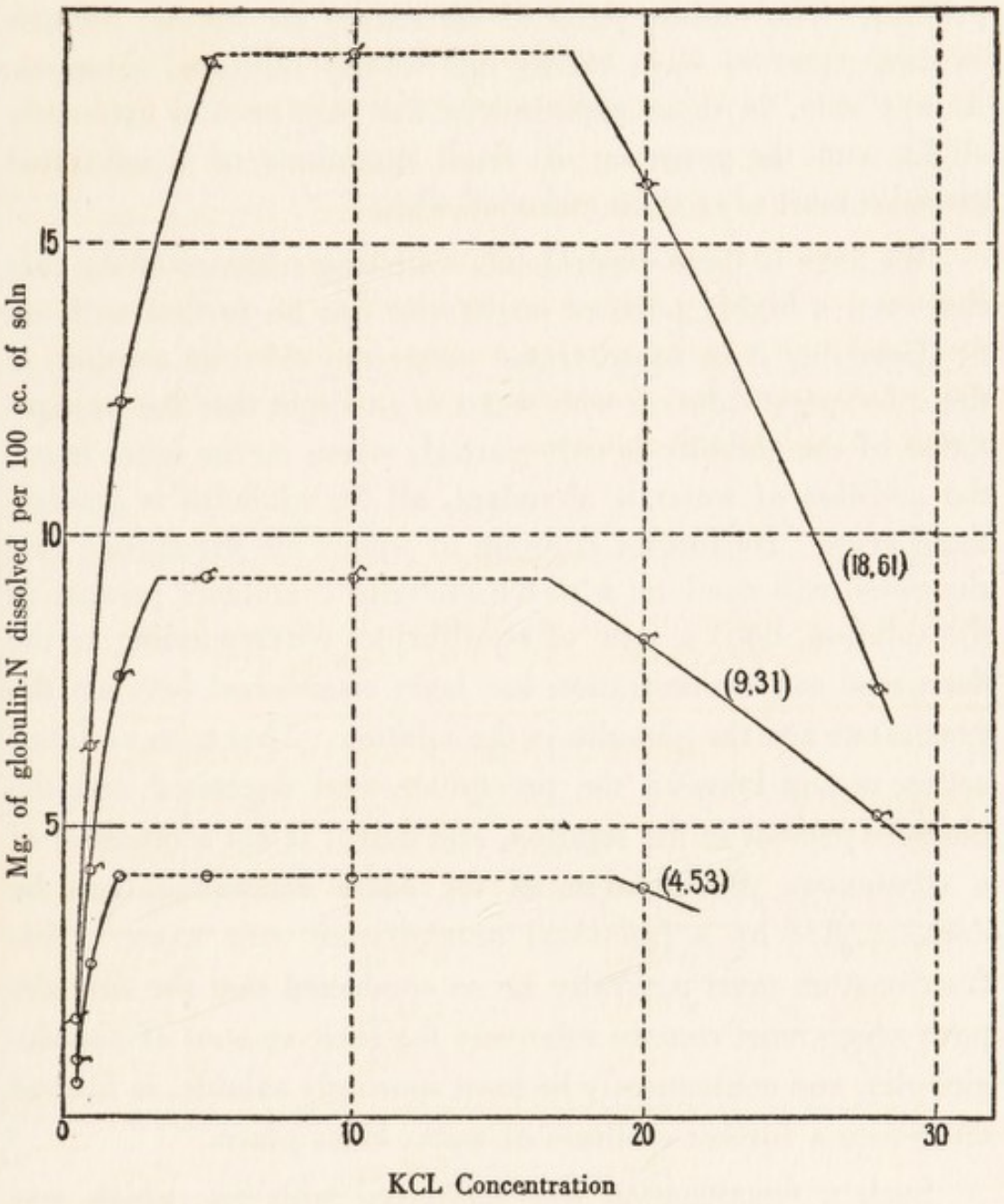
From the table as well as from Diagram 8 it is apparent that the solubility of α_{a4} in these comparatively strong potassium chloride solutions is largely dependent on the quantity of globulin employed in the solubility experiment, so that we find here conditions exactly similar to those indicated by solubility determinations of less thoroughly purified euglobulin samples in water or dilute salt solutions.

DIAGRAM 7

Solubility of the Globulin Fraction αa_4 in Solutions of Potassium Chloride. Equilibrium by Precipitation; 1 Day's Standing at 18°

Concentration of potassium chloride c	When the total of globulin-N used for the solubility experiment per 100 cc. was		
	4.53 mg.	9.31 mg.	18.61 mg.
	The amount of globulin-N dissolved per 100 cc. solution was		
0.05 N	0.62 mg.	0.98 mg.	1.70 mg.
0.10 N	2.63 mg.	4.22 mg.	6.33 mg.
0.20 N	4.12 mg.	7.57 mg.	12.25 mg.
0.50 N	4.17 mg.	9.27 mg.	18.07 mg.
1.00 N	4.12 mg.	9.27 mg.	18.27 mg.
2.00 N	3.91 mg.	8.18 mg.	16.00 mg.
2.80 N	....	5.20 mg.	7.36 mg.

DIAGRAM 8

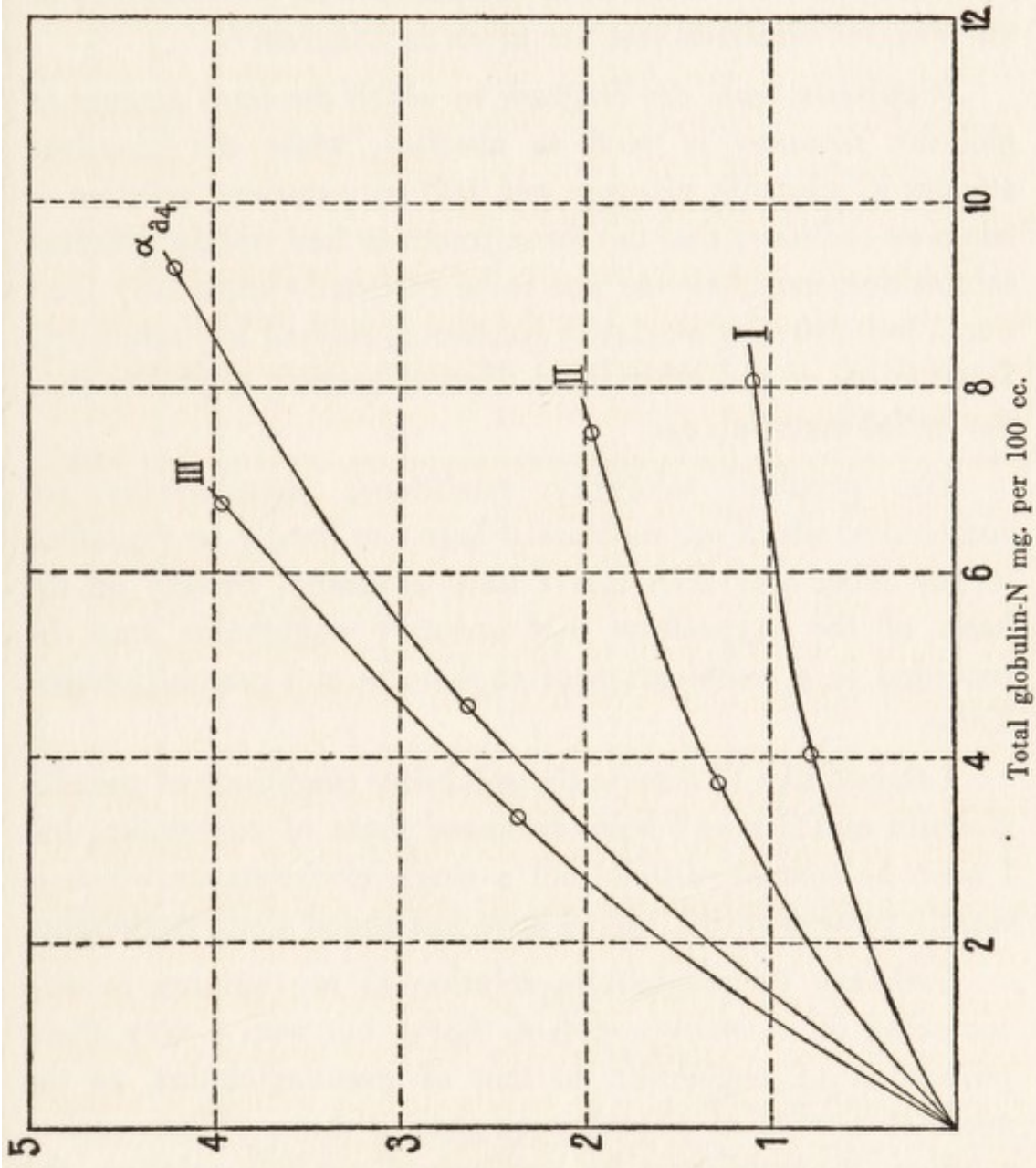

Mg. of globulin-N dissolved per 100 cc. of soln
15
10
5
0
10
20
30
KCL Concentration
(18.61)
(9.31)
(4.53)

Furthermore, in these experiments it appears that such salt concentrations have been used that the solubility of the globulin again decreases, no doubt owing to the precipitation of combinations between the globulin and the salt or one of its components. The middle parts of the curves are dotted, because in these cases we have hardly had wholly saturated solutions. At any rate, in these experiments the residue was extremely slight, and the presence of small quantities of denaturated globulin must always be reckoned with.

We have in these experiments a striking instance of the fact that even a highly purified euglobulin can be further split up by dissolving it in concentrated potassium chloride solution if the subsequent dilution with water is so slight that the precipitation of the globulin is only partial; when, on the other hand, the addition of water is abundant, all the globulin is precipitated anew. By further addition of water, the precipitate first deposited will combine with the globulin complexes present in the solution, until a state of equilibrium corresponding to the decreased salt concentration has been established between the precipitate and the globulin in the solution. That such an interaction occurs between the precipitate first deposited and the globulin present in the solution, and that it is not a question of a continuous precipitation of the same compound, can be demonstrated by a fractional precipitation with water. This fractionation must naturally be so conducted that the first deposit which must contain relatively the least amount of pseudoglobulin, and consequently be most sparingly soluble, is filtered off before a further addition of water takes place.

Such a fractionation was conducted with α_{a4}, which was dissolved in 0.5 *N* potassium chloride solution. From this solution $\alpha_{a4}I$ was precipitated by the addition of a little water;

DIAGRAM 9



Mg. of globulin-N diss. per 100 cc.

from the filtrate of this precipitate by addition of more water, α_{a4}II, and finally from the filtrate of this precipitate, α_{a4}III.

The solubility curves of these globulin fractions in 0.1 *N* potassium chloride solution as compared with the solubility of the original substance α_{a4}, are given in Diagram 9.

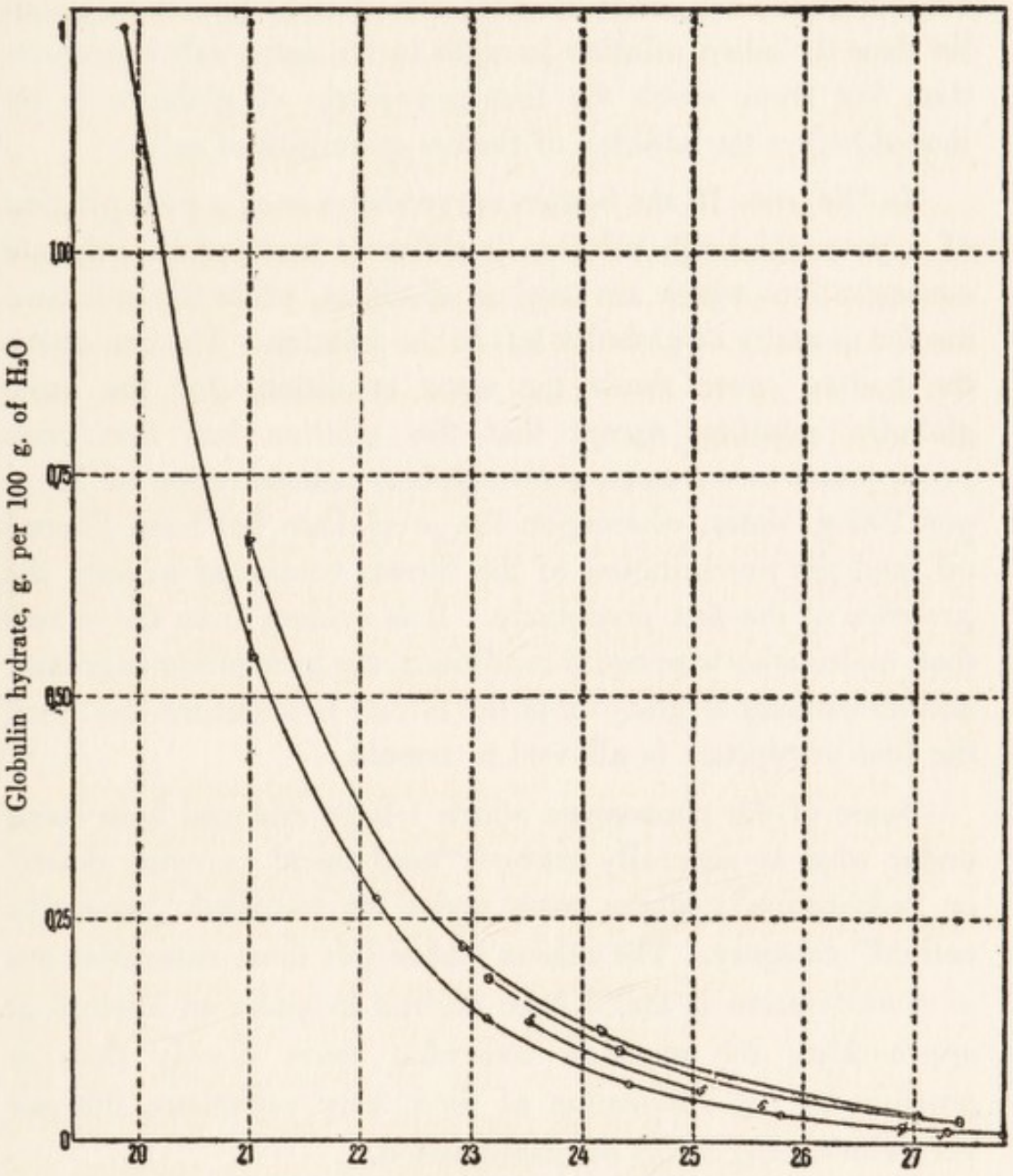
It appears from the diagram in which the total amount of globulin nitrogen is used as abscissa, while the dissolved amount of globulin nitrogen per 100 cc. saturated solution is taken as ordinate, that the three fractions had widely different solubilities, and that—as was to be expected—especially fraction I, but also very markedly fraction II, showed less solubility; fraction III, on the other hand, showed greater solubility than the initial material, α_{a4}.

The peculiar solubility conditions, characteristic for euglobulin, which are mentioned here can hardly be explained in any other way than that I have advanced, namely on the basis of the supposition that ordinary euglobulin must be regarded as a combination of euglobulin and pseudoglobulin, *EpPq*.

I should like to discuss the solubility conditions of pseudoglobulin as fully as I have discussed those of euglobulin, but I must be content to point out a single circumstance which is closely connected with what I have just stated about euglobulin.

Ordinary pseudoglobulin solution in my opinion is also composed of a complex such as *EpPq*, but with a very slight proportion of euglobulin to that of pseudoglobulin, as the minimal content of phosphorous goes to show. When such a solution is precipitated by ammonium sulphate solution, the precipitate first deposited, which is richest in euglobulin, will not remain passive during further precipitation, but will, when the state of equilibrium is altered by renewed addition of salt,

DIAGRAM 10


Globulin hydrate, g. per 100 g. of H_2O
1
0.75
0.50
0.25
0
20
21
22
23
24
25
26
27
$[NH_4]_2 SO_4$, g. per 100 g. of H_2O

combine with the pseudoglobulin of the solution. Therefore the solution from which the precipitate is not removed, as it is formed, on the addition of ammonium sulphate to a certain concentration, will, after the final filtration, be poorer in globulin than the same solution brought to the same salt concentration, but from which the first precipitate of globulin is removed before the addition of further quantities of salt.

In Diagram 10 the bottom curve shows such a precipitation of a pseudoglobulin solution to different ammonium sulphate concentrations which are used as abscissas, while the ordinates are the quantity of globulin left in the solution. The one above the bottom curve shows the same conditions for the same globulin solution, except that the solution has first been precipitated to an ammonium sulphate concentration of 20 g. per 100 g. water, whereupon the precipitate has been filtered off, and the precipitation of the filtrate continued *without the presence of the first precipitate.* It is evident from the curves that, under otherwise equal conditions, the precipitate is greater and the amount of globulin in the filtrate is therefore less when the first precipitate is allowed to remain.

Some of the phenomena which I have adduced here come under what is generally termed "mechanical carrying down" or "adsorption"; others come under the so-called "protective colloid" category. The reason I have left these categories out of consideration is that I have desired to make an attempt at approaching the question somewhat more closely than is possible by the application of these very capacious, but not very elucidating terms of classification.

Only by the application of purely chemical and physicochemical theories shall we succeed, I think, in bringing this very important problem of serum globulins, which occupies so

many minds but is still unsolved, one step further toward solution.

OSMOTIC PRESSURE OF PROTEINS

OSMOTIC PRESSURE OF PROTEINS

The question of the osmotic pressure of colloid solutions has occupied many investigators, and has produced an extensive literature. The comprehensive studies of the last few years in particular have thrown light on many hitherto doubtful points, but it must be admitted that no final solution of the problem has yet been found. The reason for this lies chiefly in the extreme difficulty of thoroughly adequate experimental research in this field. The difficulties arise partly from the methods required for accurate measurement of osmotic pressure, and partly from the difficulty of obtaining well-defined colloid solutions. In addition to this, the composition of such colloid solutions, or rather, the distribution of the substances contained between the disperse phase and the dispersion medium, depend on a series of different factors often acting in different directions. As the properties of a colloid solution—also its osmotic pressure—alter with the composition of the solution, the whole question is of a highly complex nature.

Consequently, only a very few of the earlier investigations have yielded really valuable results. The great majority of such researches present a more or less kaleidoscopic picture from which it is hardly possible to draw any definite conclusions as to the laws which hold good for this field.

It is hardly necessary to go into details here concerning the work hitherto carried out and the discussions to which it

has given rise. I will merely touch on a single important point, the question of the influence of the electrolytes present on the osmotic pressure of the colloid solution. It was *Donnan's*[1] theoretical treatment of this question, and the theory formulated by him on a sure thermodynamic basis, which first rendered possible the elucidation of this point, to which the comprehensive researches of *Jacques Loeb*,[2] whose early death is greatly to be deplored, greatly contributed.

The essence of Donnan's theory is best illustrated by a simple example. Let us take a solution containing both diffusible and indiffusible ions, a solution of protein chloride, Prot-Cl, with the chlorine normality N. This we place in a vessel with semipermeable membranes, a collodion tube, surrounded by an electrolyte solution, dilute hydrochloric acid of normality N. The collodion membrane may be denoted schematically by a vertical line, and we may use the terms "inside solution" and "outside solution" for the solution inside and outside the tube respectively. We can then, assuming the dissociation to be complete, express such a system as follows:

Inside	Outside
Cl^-	Cl^-
$Prot^+$	H^+

Donnan now shows by a simple thermodynamic consideration that this system cannot be in equilibrium, but that there will be a diffusion of hydrochloric acid from the outside solution to the inside solution until we have, in the state of equilibrium, the conditions of the following equation fulfilled:

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[1] Zeitschr. f. Elektroch. **17**:572 [1911].

[2] Proteins and the Theory of Colloidal Behavior [1922]. See also Niels Bjerrum: Zeitschr. physik. Chem. 110 [1924].

$$(H^+)_{ins.} \times (Cl^-)_{ins.} = (H^+)_{outs.} \times (Cl^-)_{outs.}$$

The unequal distribution of the hydrogen and chlorine ions in the inside and outside solution gives rise to a membrane potential, the magnitude of which can be calculated when the concentrations of the electrolytes are known. It can also be measured by a suitable experimental arrangement.

However, it is evident that the unequal distribution of the electrolytes between the inside and outside solutions must also be taken into consideration in measuring the osmotic pressure of the inside solution, as this is not due solely to the indiffusible protein compounds, but also to unequal distribution of the diffusible ions.

On closer consideration of the Donnan theory and its importance for the question before us at the moment, namely, the measurement of osmotic pressure in protein solutions, we find that *the unequal distribution of the electrolytes*, which of course ultimately lies in the ability of proteins to form positively or negatively charged ions at different concentrations of hydrogen ions, *is of less importance, the greater the concentration of salt, and the nearer the hydrogen ion concentration of the solution lies to the isoelectric reaction of the protein in question.*

If, on the other hand, we measure the osmotic pressure of a protein solution at a concentration of hydrogen ions essentially above or below that corresponding to the isoelectric reaction, then only a part, and often only a small part, of the osmotic pressure as measured will be derived from the osmotic pressure of the protein particles themselves, the remainder being due to the unequal distribution of the diffusible ions. It was reserved for *Jaques Loeb*, after comprehensive experimental

research, to make evident the important fact that the alteration in osmotic pressure which takes place in a protein salt solution on the addition of electrolytes, is, on the whole, parallel to the alteration in membrane potential, and, therefore, doubtless chiefly due to an alteration in the distribution of the electrolytes between inside and outside solutions.

In such experiments, then, the actual osmotic pressure of the protein will, as already mentioned, only amount to a portion of the measured osmotic pressure, and will be obtained as the difference between this and the pressure corresponding to the membrane potential as found. Such a determination of the true osmotic pressure of the protein itself as a difference is therefore subject to rather considerable errors, of which Loeb himself was well aware.

In order to avoid these errors, or at any rate, reduce them to a minimum, the measurement of osmotic pressure must be made under such conditions that the "Donnan Effect" can be disregarded. This again is equivalent to saying that the measurement must be made at or near the isoelectric reaction of the protein, and in solutions containing a fairly abundant quantity of salt. When the measurements are carried out under such conditions, and by a sufficiently accurate method, we may regard the results obtained as reliable enough to afford an estimate of the weight of molecules, or molecular aggregates, in the protein, under the circumstances in question. Obviously such experiments can only afford information as to the variation of molecular weight within the limits involved by the experimental conditions, but even this is a step forward, and may later on, when the method is further developed, be followed by measurements under conditions where the Donnan effect has to be taken into consideration.

At the Carlsberg Laboratory in Copenhagen we have, during the past ten years, carried out a series of experiments on the osmotic pressure of hens' egg albumin, horse serum albumin, and horse serum globulin at or near the isoelectric reaction of these proteins, which, at any rate in the case of the two first, corresponds approximately to pH=4.8. I shall describe the results of these measurements after first giving a brief account of the methods employed. A detailed description of these methods, as well as of the experiments with egg albumin, has already been published[1]; the experiments with albumin and globulin from horse serum on the other hand, have not yet been made public.

The Method Employed

Diagram 1 shows the apparatus we use for the measurement of osmotic pressure.

The osmometer consists chiefly of a collodion tube A, placed on a glass collar, B, into the neck of which a ground glass tube is fitted so as to be perfectly watertight. This glass tube is narrowed a little above the ground part to a capillary tube, divided into millimeters with zero at the glass collar B as starting point. The protein solution to be measured (the inside solution) is poured into the collodion tube, the ground glass is fitted on, and filled by means of a capillary pipette, until the inside solution reaches a little way up along the millimeter scale. The tap C is then closed and the osmometer placed in the outside solution, which is in a Dewar vessel standing in a cylinder glass, this again being placed in a well regulated water thermostat. The thermostat is not shown in

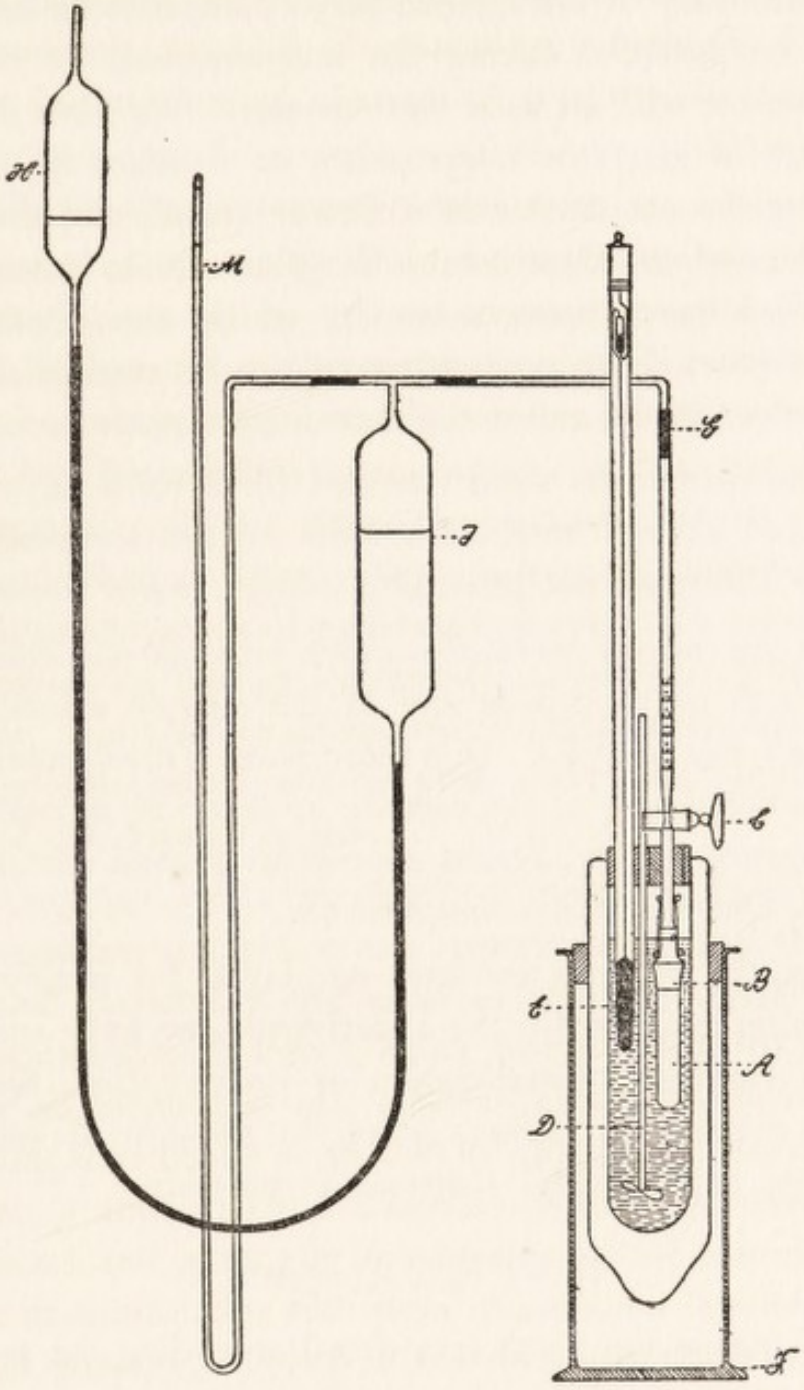
[1] Compt. rend. Lab. Carlsberg **12**, 262 [1917].

the diagram. The outside solution is so prepared as to have as nearly as possible the same hydrogen ion and salt concentration as the dispersion medium in the inside solution, but in order to get complete equilibrium of diffusion, the whole is left standing from one day to the next before the first measurement is made.

The measurement of osmotic pressure is a compensation measurement, the principle of which is simply to determine what counter pressure must be exerted on the inside solution in the osmometer in order to maintain equilibrium with the osmotic pressure of the solution. Now a counter pressure less than the osmotic pressure of the inside solution will permit a constant flow of the outer liquor through the membrane into the inside solution, whereas a counter pressure exceeding the osmotic pressure of the inside solution will give a flow of liquid in the opposite direction. The object of the measurement, then, is to determine that counter pressure at which no exchange of fluid takes place between the inside and outside solution, this counter pressure answering exactly to the osmotic pressure of the inside solution itself.

In the diagram, H represents the counter pressure apparatus which contains water. By lifting or lowering the container H, the counter pressure can be altered, and its magnitude in centimeters can be read off on the manometer M. The measurement is then made by simply producing a suitable counter pressure over the surface of the inside solution, opening the tap C and then, by means of a special scale-reading microscope, noting whether the liquid in the capillary rises or falls, and at what rate. Thus the counter pressure P_m at which the liquid in the osmometer tube neither rises nor falls can be found. On adding to this the difference in level between the inside and the outside solution, allowing for the capil-

DIAGRAM 1



Apparatus for measuring osmotic pressure

lary effect and the specific gravity of the solution, the total osmotic pressure measured is obtained.

The accuracy of the method largely depends on keeping a constant temperature during the measurement, as otherwise the osmometer will act as a thermometer. We have therefore taken care to keep the temperature as constant as possible, by placing the osmometer in a Dewar vessel, and this again in a well regulated thermostat. We place in the outside solution, a Beckmann thermometer, by which any alteration in the temperature during measurement can be read off in thousandths of a degree, and a slight correction made accordingly.

The measurements were repeated for several days in succession, and always until the results on two successive days showed a difference not exceeding 0.5 cm. water pressure.

When the measurement of osmotic pressure was completed, samples both of the inside and of the outside solution were weighed off for analysis. One more point I must note; *we indicate the magnitude of the osmotic pressure, π, in centimetres water pressure per milligram equivalent protein nitrogen per cubic centimetre of dispersion medium.*

As for the methods we have employed for purifying the protein solutions used for the experiments, we have spared no pains, by repeated crystallization or precipitation, fractionation and dialysis in various ways, to prepare as pure and well-defined experimental material as possible.

Hen's Egg Albumin

I will not go too much into detail regarding the measurements of osmotic pressure in egg albumin which we have already published, but will mention one or two of the results obtained.

We believe we have shown by these experiments, that the osmotic pressure of an egg albumin solution is well-defined and depends not only on the protein concentration, but also on the concentration both of ammonium sulphate and of hydrogen ions in the solution. *In a solution of a given composition, however, the osmotic pressure will always be the same—that is, within the limits of experimental error.*

In proof of this I will refer to Diagram 2 showing a series of measurements of egg albumin solutions of more or less uniform composition. These solutions were of different sorts, dialysed or non-dialysed, freshly prepared or kept for some time. The experiments were carried out by three separate investigators with five different osmometers, and with four different collodion tubes. Nevertheless, the osmotic pressure was, within the limits of experimental error, the same throughout, namely, an average corresponding to $\pi = 46.7$ cm. water pressure. As already mentioned, π denotes the osmotic pressure per milligram equivalent protein nitrogen per cubic centimetre of dispersion medium.

Without going into the details of the table, I will just draw your attention to the last two experiments noted there. We have here a dialysed egg albumin solution, which had been kept, saturated with toluol, in ice in an icebox for 690 days. Now a salt-containing egg albumin solution will keep for a very long time in ice in an icebox without any essential denaturation; a dialysed and therefore salt-free solution, on the other hand, will gradually denaturate. In the case of the solution in question, after 690 days' storage, no less than 58% of the total quantity of albumin was denaturated, but after filtering off the denaturated portion, the albumin remaining in the solution still showed, as you will see from the table, a completely normal osmotic pressure.

Diagram 2

Osmotic Pressure of some Egg-Albumin solutions

No. of experiment	Egg-albumin solution used: Mark	Egg-albumin solution used: Age, in days	Experimenter; No. of osmometer and No. of cap	Found on analysis: in 100 g. *outer* liquid: Ammonia nitrogen in g. (a_f)	Found on analysis: in 100 g. *inner* liquid: Ammonia nitrogen in g. (a_b)	Found on analysis: in 100 g. *inner* liquid: Protein nitrogen in g. (p_b)	Hydrogen-ion concentration of *inner liquid*, h. $h.\ 10^6$	Ammonium sulphate content of *outer* liquid, in g. per 100 g. water S
57	I.D.Æ. 1	15	Chr. I 3	2.7205	2.2693	1.9100	12.16	14.719
58	"	16	Chr. II 6	2.5848	2.1772	1.9090	13.15	13.883
59	"	20	Chr. II 6	2.4761	2.0933	1.8698	12.71	13.222
61	"	24	Chr. II 6	2.9169	2.5210	1.6319	13.58	15.951
87	"	175	Golds. II 6	2.8636	2.4884	1.6046	11.14	15.614
88	"	175	Golds. III 3	2.8559	2.4637	1.6534	10.96	15.566
89	"	182	Golds. II 6	2.8632	2.4778	1.6315	11.51	15.612
90	"	182	Golds. III 3	2.8659	2.4754	1.6429	10.45	15.629
137	I.D.Æ. 7	18	Golds. II 6	2.8319	2.4648	1.5064	13.43	15.415
138	"	18	Golds. III 3	2.8321	2.3651	1.9706	13.84	15.416
163	"	407	Pal. IX 5	2.8382	2.4432	1.6775	14.45	15.455
164	"	407	Pal. VII 7	2.8324	2.4468	1.6547	13.06	15.418
103	D.Æ. 4	80	Golds. II 6	2.9621	2.5983	1.4847	10.50	16.239
110	"	129	Golds. III 3	2.5747	2.2663	1.4299	11.59	13.822
111	"	174	Golds. II 6	2.5653	2.2577	1.4142	12.42	13.764
127	"	216	Golds. II 6	2.8880	2.5519	1.4061	4.85	15.768
165	"	690	Pal. IX 5	2.6501	2.3270	1.4927	4.54	14.284
166	"	690	Pal. VII 7	3.3234	2.9084	1.4732	4.61	18.588

Diagram 2

of nearly like composition

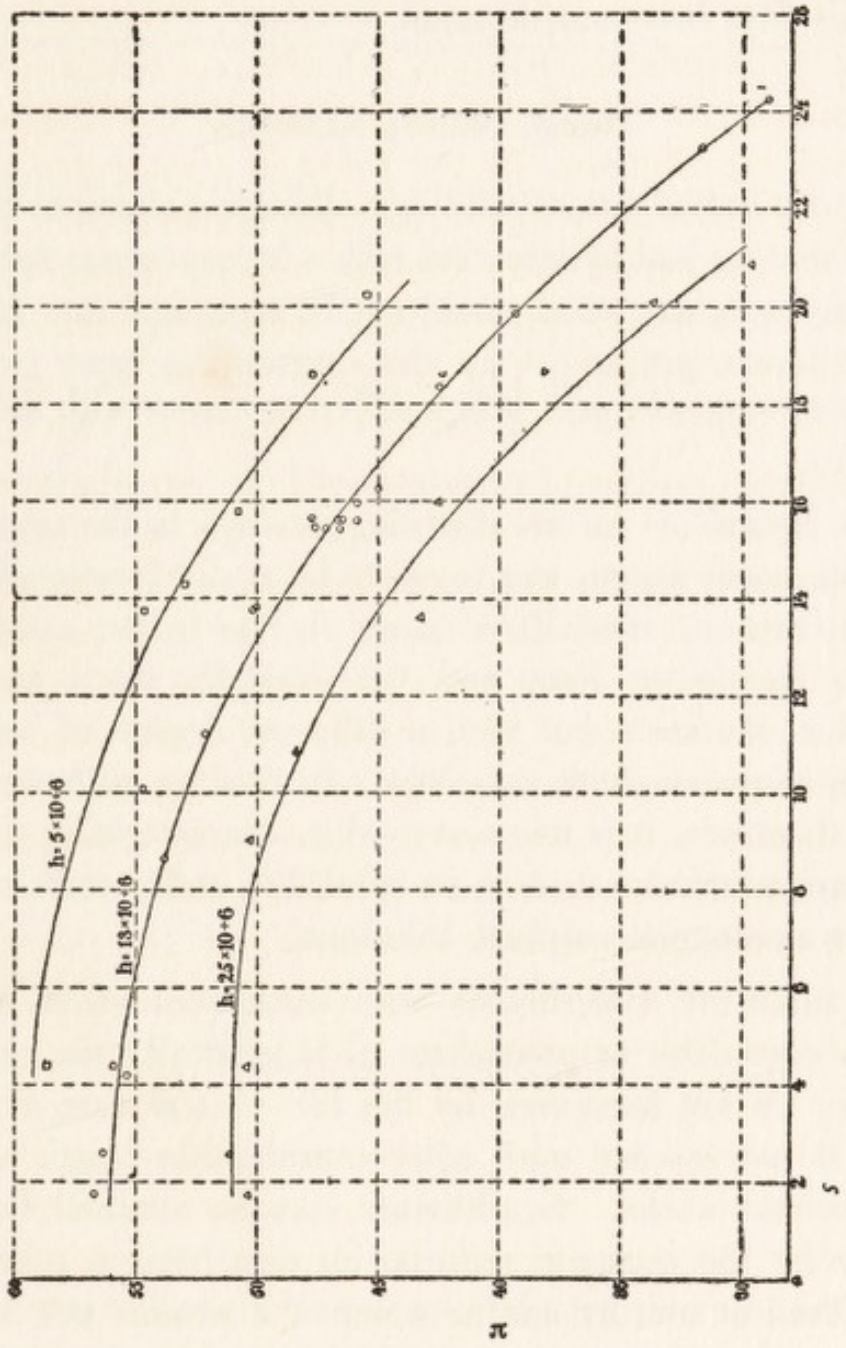
Values of z, y and Vs corresponding with found values of h and S			The *inner* liquid then contains per 100 g. water				*Osmotic pressure* in cm. pressure water				
z	y	V_s	Ammonium sulphate in g. S_i	Egg-hydrate in g. E_i	Milligr.-equival. protein nitrogen, $E = \frac{E_i \cdot 1000}{14.01 \cdot z}$	$e = \frac{E}{V_s}$	P (measured)	$\pi = \frac{P}{e}$	Correction, in cm., to be added to π in order to refer the measurement to $S = 15.6$	Correction, in cm., to be added to π in order to refer the measurement to $h = 13.10^{-6}$.	*Corrected* value of π (corresponding to $S = 15.6$ and $h = 13.10^{-6}$)
8.69	0.045	106.87	14.587	22.804	187.30	1.7526	86.16	49.16	—1.26	—0.40	47.50
8.69	0.045	106.45	13.908	22.660	186.12	1.7484	86.29	49.35	—2.45	+0.05	46.95
8.70	0.046	106.13	13.234	21.998	180.47	1.7005	84.18	49.50	—3.25	—0.12	46.13
8.68	0.044	107.48	15.967	19.137	157.37	1.4642	66.81	45.63	+0.50	+0.27	46.40
8.69	0.044	107.31	15.681	18.744	153.95	1.4346	68.00	47.40	+0.01	—0.92	46.49
8.69	0.044	107.29	15.586	19.394	159.29	1.4847	69.60	46.88	—0.02	—1.00	45.86
8.69	0.044	107.31	15.651	19.106	156.93	1.4624	69.28	47.37	+0.01	—0.70	46.68
8.69	0.044	107.32	15.654	19.262	158.21	1.4742	72.26	49.02	+0.02	—1.25	47.79
8.69	0.044	107.21	15.342	17.376	142.72	1.3312	61.69	46.34	—0.30	+0.20	46.24
8.69	0.044	107.21	15.414	23.849	195.88	1.8271	85.15	46.60	—0.30	+0.38	46.68
8.69	0.044	107.23	15.477	19.706	161.86	1.5095	70.95	47.00	—0.22	+0.60	47.38
8.69	0.044	107.22	15.464	19.391	159.27	1.4855	69.99	47.12	—0.30	0.00	46.82
8.69	0.044	107.63	16.272	17.224	141.47	1.3144	60.78	46.24	+0.95	—1.25	45.94
8.70	0.045	106.42	13.811	16.170	132.66	1.2466	63.19	50.69	—2.50	—0.68	47.51
8.70	0.046	106.40	13.724	15.956	130.91	1.2304	62.09	50.46	—2.58	—0.30	47.58
8.69	0.044	107.39	15.795	16.119	132.39	1.2328	62.77	50.92	+0.20	—5.27	45.85
8.69	0.045	106.65	14.330	17.042	139.98	1.3125	70.10	53.41	—1.92	—5.55	45.94
8.68	0.043	108.81	18.562	17.383	142.94	1.3137	63.02	47.97	+4.90	—5.42	47.45

Mean: 46.7

By way of illustrating how π varies with the ammonium sulphate concentration, I will refer to Diagram 3, where the value of π in cm. water pressure is taken as ordinate, while S, denoting grams of ammonium sulphate in the inside solution per 100 g. water, is taken as abscissa. Each of the three curves in the diagram corresponds to a different concentration of hydrogen ions, the middle one approximately to the isoelectric reaction of egg albumin, the one below to a somewhat higher, and the one above to a somewhat lower concentration respectively. All the curves show that the osmotic pressure of the egg albumin decreases greatly with increasing concentration of ammonium sulphate. The principal reason for this is, we think, to be found in an increasing condensation (with increasing ammonium sulphate concentration) of the albumin particles by means of the divalent sulphate ion, a condensation which ultimately results in a crystallization of the egg albumin sulphate. And it was also found, in the two experiments shown in Diagram 3, where S was greatest, that the inside solution, when removed from the collodion tube and stirred, precipitated a portion of the egg albumin in a crystallized state. These solutions must then, really have been supersaturated when the osmotic measurement was being made.

There is one more thing I must point out, and that is, that the magnitude of π is not, as one might suppose, independent of the protein concentration. This last is usually indicated by e which gives the number of milligram equivalents of protein nitrogen per cubic centimetre of dispersion medium. In egg albumin solutions containing ammonium sulphate, π will increase more or less with the protein concentration, but in egg albumin solutions without salt, the reverse will be the case. It is hardly possible at the moment to give any exhaustive explanation of these facts. I mention it, however, be-

DIAGRAM 3



cause we have found the same thing in other substances investigated, π dependent on the protein concentration. It is therefore necessary, for instance, in comparing the osmotic pressure of different proteins, under uniform conditions, to take this fact into consideration.

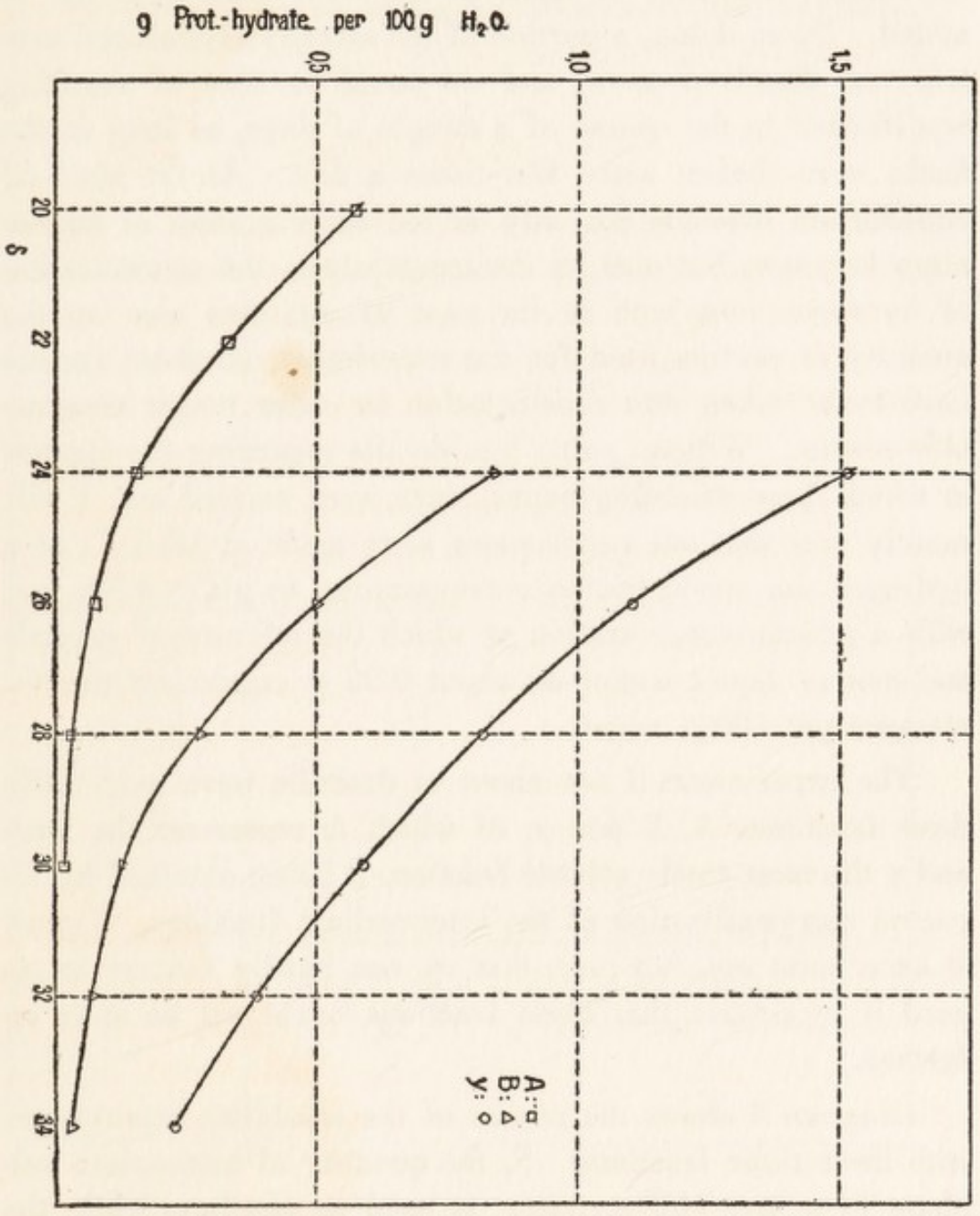
Horse Serum Albumin

All our investigations with egg albumin pointed to the conclusion that we had here to deal with a homogeneous substance, or at any rate, one which could not be separated into fractions with different properties by the crystallizing and purifying processes we employed. It is a different matter with the serum albumin.

The crystalline serum albumin, obtained in the usual manner from horse serum, was found to be easily fractionable and all the fractions crystallized more or less to the same shape forming handsome long and flat crystals, often somewhat pointed at the ends, but with a different degree of solubility in ammonium sulphate solutions. In dealing with serum albumin therefore, it is necessary, when characterizing the fraction more precisely, to note its solubility under certain conditions in ammonium sulphate solutions.

In solubility experiments with substances which take so long to crystallize or precipitate as is generally the case with proteins, we are hampered by the fact that a state of equilibrium is not reached until after considerable length of time, often several weeks. Equilibrium may be attained far more rapidly by the opposite process, by dissolving a precipitate, crystallized or not, by adding water or a weaker salt solution. We have, therefore, in our experiments with serum albumin, mostly proceeded as follows. A large portion of the solution

DIAGRAM 4

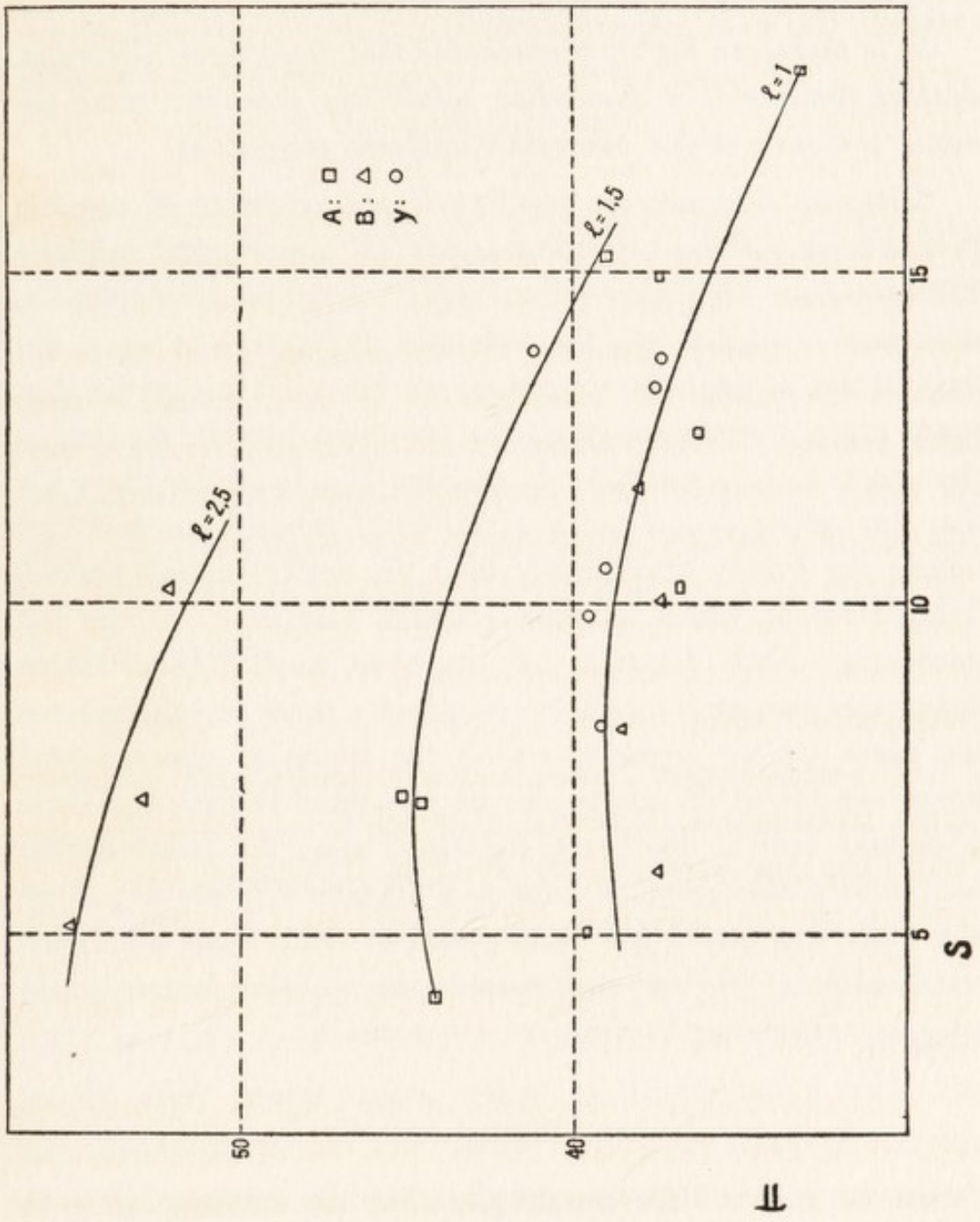


to be treated is first brought to full and abundant crystallization, and then, after distributing this mixture of crystals and mother liquor among a series of flasks, to each of these, dilute ammonium sulphate solution at *different* concentrations is added. By so doing, a portion of the already crystallized protein was dissolved anew, and we could be sure of reaching equilibrium in the course of a couple of days, as long as the flasks were shaken some few times a day. As the state of equilibrium depends not only on the concentration of ammonium sulphate, but also on the temperature, the concentration of hydrogen ions, and in the case of mixtures also on the quantity of protein used for the experiment, all these factors have to be taken into consideration in order to get comparable results. Without going into details regarding the manner in which these solubility experiments were carried out, I will merely note that our experiments were made at 18–19°, at a hydrogen ion concentration corresponding to pH = 4.80, and with a protein concentration at which the mixture of crystals and mother liquor contained about 0.02 g. equivalent protein nitrogen per 100 g. water.

The experiments I am about to describe were made with three fractions, A, B and y, of which A represents the least and y the most easily soluble fraction, B being obtained by repeated recrystallization of the intermediate fractions. I must at once point out, however, that we can hardly venture to regard it as certain that these fractions could not be split up further.

Diagram 4 shows the results of the solubility experiments with these three fractions. S, the quantity of ammonium sulphate in g. per 100 g. water, is used as abscissa, while the quantity of albumin hydrate in equilibrium in the solution in g. per 100 g. water is the ordinate. It will be clearly seen

DIAGRAM 5



from the curves that there is an enormous difference between the solubility of the serum albumins. At $S = 26$ for instance, A is practically insoluble, whereas B shows a considerable, and y a very high degree of solubility.

It is therefore highly remarkable that these three albumins, despite their widely dissimilar solubility, show the same osmotic pressure under otherwise uniform conditions.

Diagram 5 shows the results of measurements of osmotic pressure at different concentrations of ammonium sulphate (Temperature 18°, pH = about 4.8). S is used as abscissa, and π as ordinate. As the value of π depends to some extent on the protein concentration, we have drawn on the diagram three curves, answering to the three protein concentrations, $e = 1$, 1.5, and 2.5. It will be seen from the diagram, that the values found for π at different concentrations of albumin are evenly distributed about the corresponding curves, irrespective of which albumin fraction was used for the experiment. Each fraction has its own mark. Since these three fractions, under uniform conditions, must be said to have the same osmotic pressure within the limits of experimental error—and must therefore also be presumed to have the same molecular size or molecular-aggregate size, the great difference *in solubility cannot be due to a difference in the degree of dispersity*—as one might be disposed to think from a colloid-chemical point of view—*but must be due to other factors, probably to a difference in chemical composition.*

Horse Serum Globulin

In the case of the serum globulin, the situation is even more complicated than with the serum albumin. As I have had occasion to point out in an earlier lecture "On the Solu-

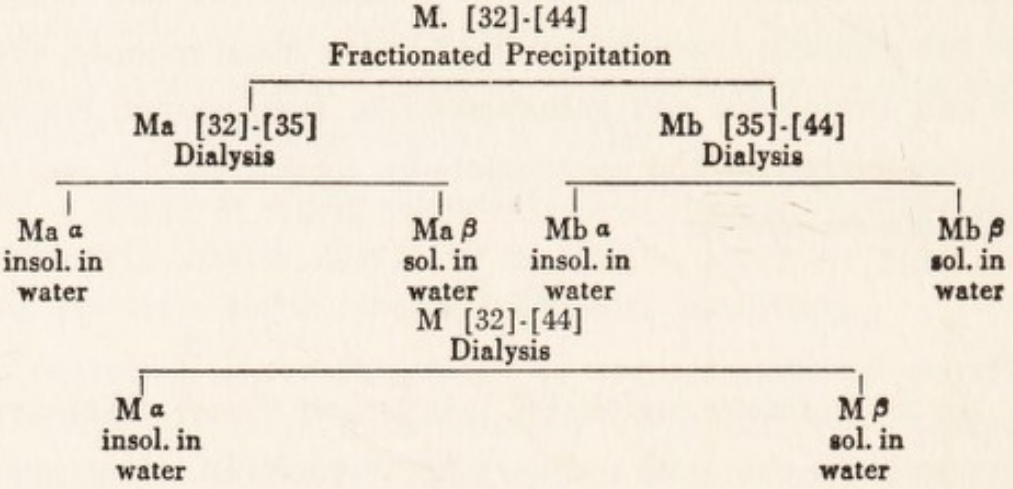
bility of Proteins" we have here to reckon with the fact that the two globulin fractions obtained in the usual manner, which we call *euglobulin* and *pseudoglobulin*, both contain euglobulin complexes (E) and pseudoglobulin complexes (P) and may therefore be written

$$Ep\ Pq.$$

In the ordinary euglobulin, p is greater than q, whereas the reverse is the case with ordinary pseudoglobulin. It is mainly the latter we shall now consider for the present. It is poor in phosphorus, soluble in water, and comparatively difficult to precipitate with ammonium sulphate, its precipitation limit being generally reckoned as from (33) to (45). These figures mean that each 100 cubic centimeters of solution contain 35 to 45 cubic centimeters respectively of saturated ammonium sulphate solution.

By way of illustration, we may further consider a fraction, M, obtained from a twice reprecipitated globulin preparation by thrice repeated fractionation with ammonium sulphate, the least and the most soluble fractions being separated each time. The intermediate fraction M thus obtained, corresponding approximately to the precipitation limits (32) to (44), was used for the experiments partly as such, partly after fractionation, either by fractionation with ammonium sulphate with (35) as the limit, or by dialysis and subsequent treatment of the remainder with water, or by using both processes. The fractions and their designations will be understood from the arrangement in Diagram 6.

DIAGRAM 6

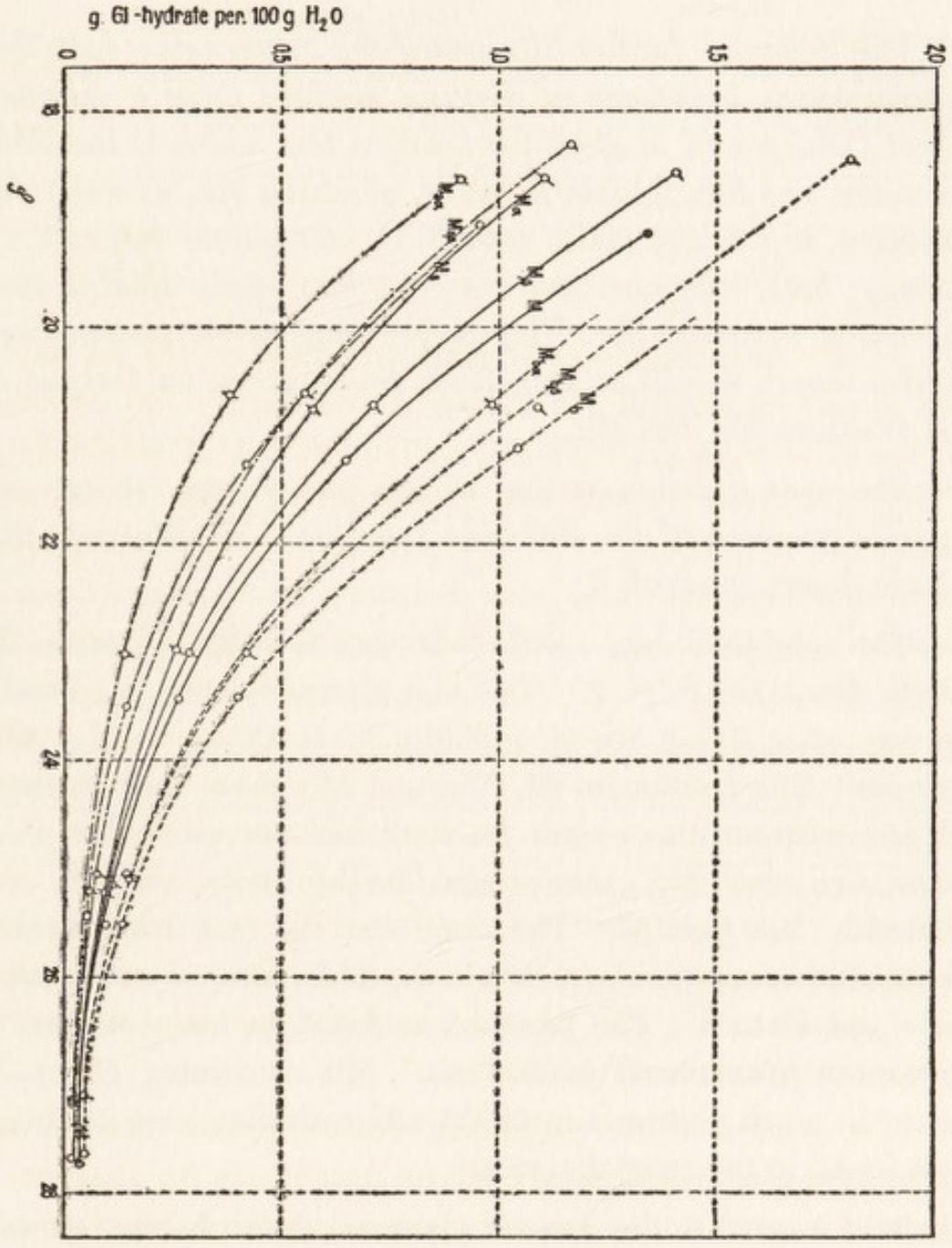


All the nine fractions obtained were used partly for analyses, including phosphorus determination, partly for measurements of osmotic pressure and determinations of solubility at different ammonium sulphate concentrations. The initial concentration of globulin is of essential importance in solubility determinations, and these were therefore, always carried out in precisely the same way. Solutions were so prepared as to give 10 g. Am_2SO_4 ($S = 10$) and 0.02 g. equivalents of globulin nitrogen per 100 g. water, and with a concentration of hydrogen ions corresponding to $pH = 4.8$. They were then precipitated at 18° with an ammonium sulphate solution diluted to (70) until the required ammonium sulfate concentration was reached. The precipitation was effected by slow dripping with automatic mixing and thorough stirring; then, after standing for 5 days at 18°, with repeated shaking, the state of equilibrium was determined by analyses.

The results of the solubility determinations carried out within the nine fractions are shown graphically in Diagram 7 where S is used as abscissa and the quantity of globulin hydrate per 100 g. water in the mother liquor as ordinate.

It will be seen from the diagram that the separation of

DIAGRAM 7



M into Ma and Mb really gave—as was to be expected—a fractionation indicated by the different solubility of the fractions, Ma being far less easily, and Mb far more easily, soluble than M.

The diagram further illustrates the remarkable fact that fractionations by means of dialysis produce quite a different effect. Dialysis of M gives the fraction Mα, which is insoluble in water, and Mβ, soluble in water, of which Mα, as was to be expected, is the less easily soluble in ammonium sulphate solutions; *both fractions, however, are less easily soluble than the initial material, M.* This highly remarkable phenomenon is also found, as will be seen from the diagram, on dialysis of the fractions Ma and Mb.

The next question is that of the phosphorus content and osmotic pressure of the different fractions. To illustrate this I have drawn Diagram 8.

The solubility data for S = 20 and S = 22 are taken directly from Diagram 7. The phosphorus content, expressed in mg. phosphorus per g. globulin-N, is shown in the next column. The figures for M, Ma, and Mb show, that *fractional precipitation also causes fractionation as regards the phosphorus content.* Ma contains considerably more, and Mb considerably less than M. The same was the case with another fractionation experiment shown in the table, where I corresponds to a and II to b. *The phosphorus fractionation, however, is far more pronounced in dialysis.* Mα containing about 50 times as much phosphorus as Mβ and a similar state of things was found in the other dialyses.

With regard to the osmotic pressure, shown in the last vertical column of the table, it is immediately evident from the figures, and even more plainly from the diagram which follows,

DIAGRAM 8

Globulin of horse-serum

Mark	*Solubility* g. globulin-hydrate per 100 g. H_2O		*Phosphorus* Mg. P per g. globulin-N	*Osmotic Pressure* [10 g.$[NH_4]_2SO_4$ 10 g. globulin-hydrate per 100 g. H_2O pH = 4.8] π
	S = 20	S = 22		
M	1.02	0.48	0.643	11.84
Ma	0.70	0.31	0.934	10.59
Mb	1.42	0.75	0.119	14.29
M	1.02	0.48	0.643	11.84
Mα	0.73	0.37	2.040	12.19
Mβ	0.92	0.44	0.0465	12.78
Ma	0.70	0.31	0.934	10.59
Maα	0.51	0.22	2.858	9.71
Maβ	0.68	0.33	0.0684	12.07
Mb	1.42	0.75	0.119	14.29
Mbα	1.18	0.63	0.697	....
Mbβ	1.30	0.68	0.0871	15.05
I	0.38	0.16	1.624	9.32
II	1.09	0.46	0.824	12.60

that *fractional precipitation* gives rise, not only to fractionation of solubility and of phosphorus content, but also to *fractionation of osmotic pressure. This cannot be said to be definitely proved in the fractionation effected by dialysis.*

Diagram 9 shows how the fractionation by precipitation of M into Ma and Mb, as well as of another globulin sample into I and II, occasions a fractionation of the phosphorus content, which is used as abscissa, and a decrease in the solubility and

DIAGRAM 9

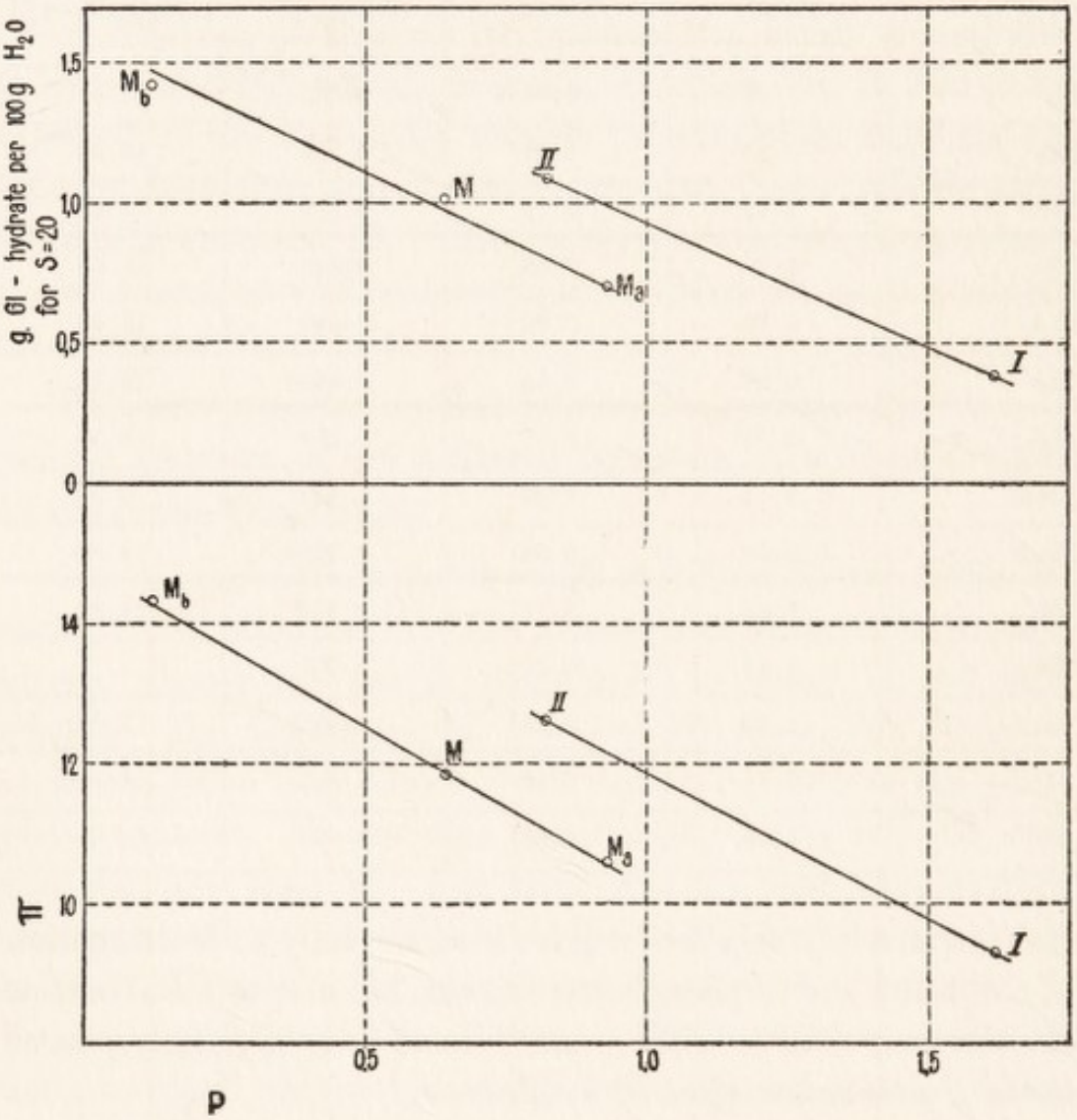
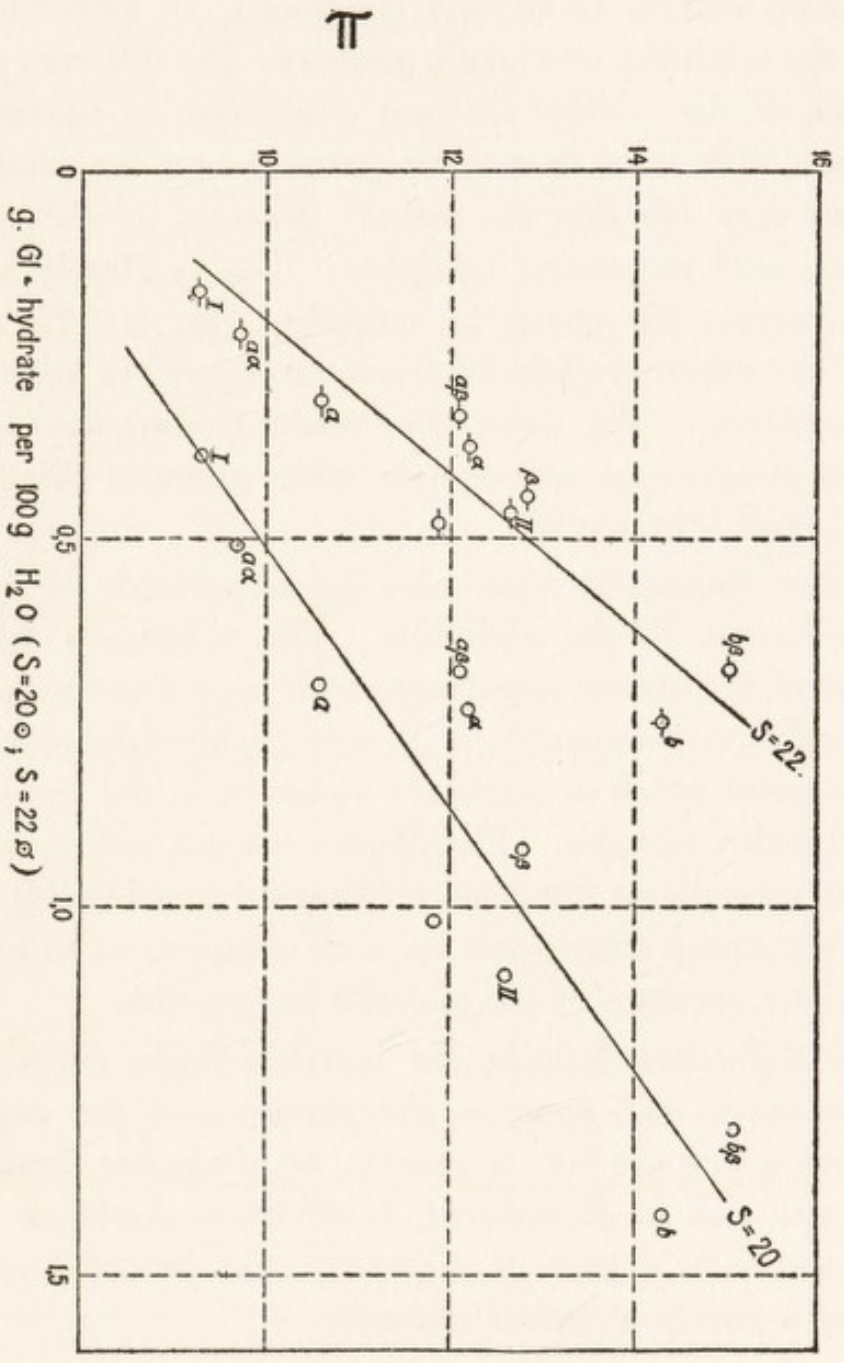

g. GI - hydrate per 100 g H_2O for S=20
1,5
1,0
0,5
0
M_b
M
M_a
II
I
14
12
10
π
M_b
M
M_a
II
I
0,5
1,0
1,5
p

DIAGRAM 10


π
0
10
12
14
16
0,5
1,0
1,5
g. Gl - hydrate per 100 g H_2O (S=20 ; S=22)
S=22
S=20
I
II

osmotic pressure with increasing phosphorus content.

Fractionation by means of dialysis, on the other hand, which gives such an enormous fractionation of the phosphorus content, makes, as already mentioned, no essential alteration in the solubility or osmotic pressure. The different globulin fractions do not—under uniform conditions, of course—show any great difference in osmotic pressure; *on the whole, however, we may say that the osmotic pressure of the globulins increases with increasing solubility.* This is clearly apparent from Diagram 10, where the solubility (for S = 20 and S= 22) of the different globulin fractions is used as abscissa and π as ordinate. We have also made measurements of the osmotic pressure in one or two other globulin solutions, to which I shall briefly refer.

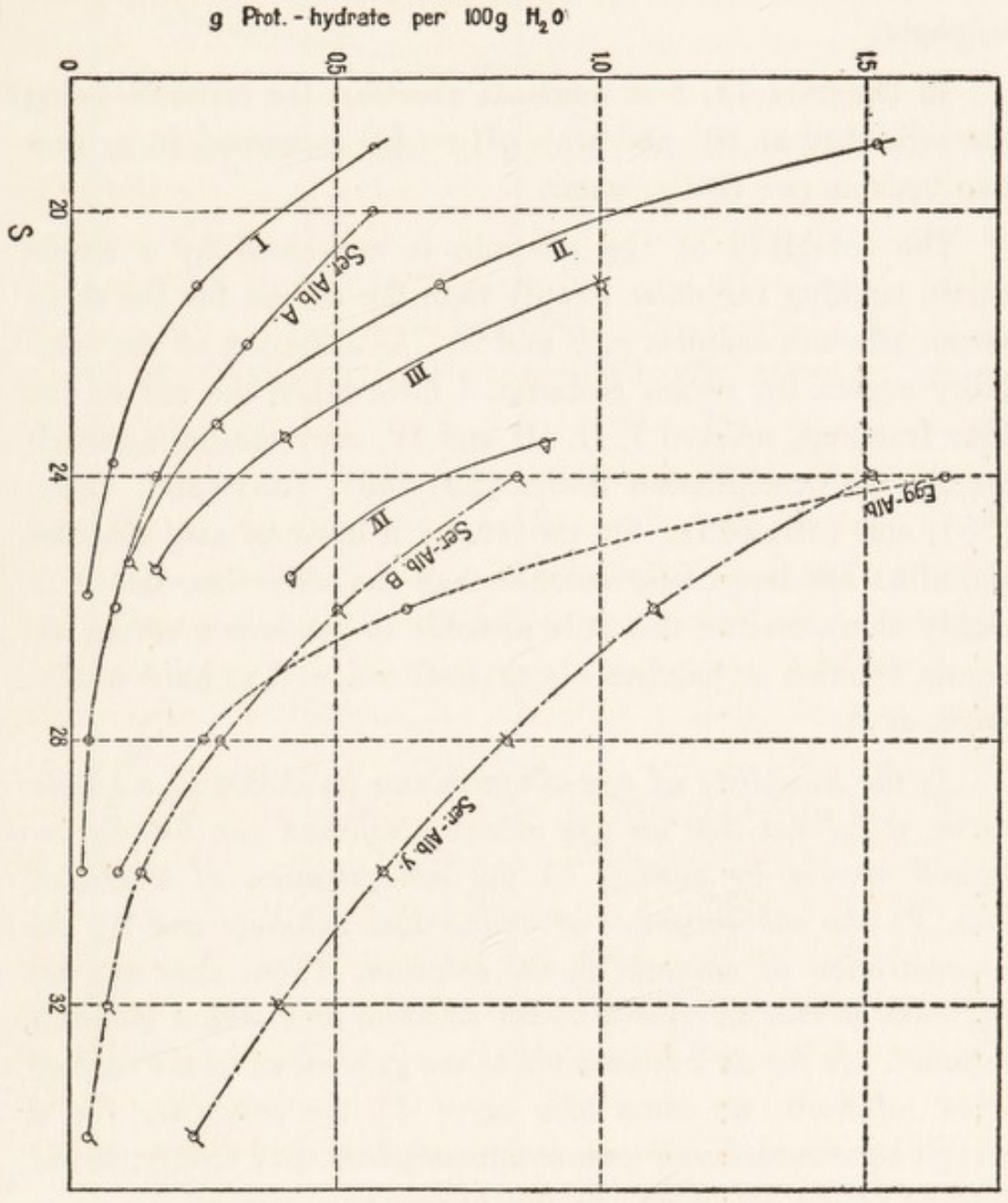
I must emphasize once more the importance of the phosphorus content of the globulins. The phophorus content is a factor of the utmost importance with regard to the solubility of globulins *in pure water,* or in very highly diluted salt solutions, a point which is markedly apparent in the investigation of euglobulin samples. Phosphorus content seems, however, to be of subordinate importance in regard to solubility in comparatively *strong ammonium sulphate solutions* or in regard to the osmotic pressure of the globulin in question.

The difference between the pseudoglobulin fractions, soluble in water and poor in phosphorus, and the euglobulin fractions, which are hardly soluble, or altogether insoluble, in water, and rich in phosphorus, is therefore doubtless not due to difference in degree of dispersity, but probably to differences of a purely chemical character.

General Survey

In order to give a general survey of the solubility and os-

DIAGRAM 11


g Prot. - hydrate per 100g H_2O
0
0.5
1.0
1.5
S
20
24
28
32
I
II
III
Ser. Alb. A
II
III
Ser.-Alb. B
Egg-Alb
Ser.-Alb. γ

motic pressure, and a comparative view of these two factors in the proteins we have been considering, I have drawn Diagrams 11 and 12 showing respectively the solubility and osmotic pressure in their relation to concentration of ammonium sulphate.

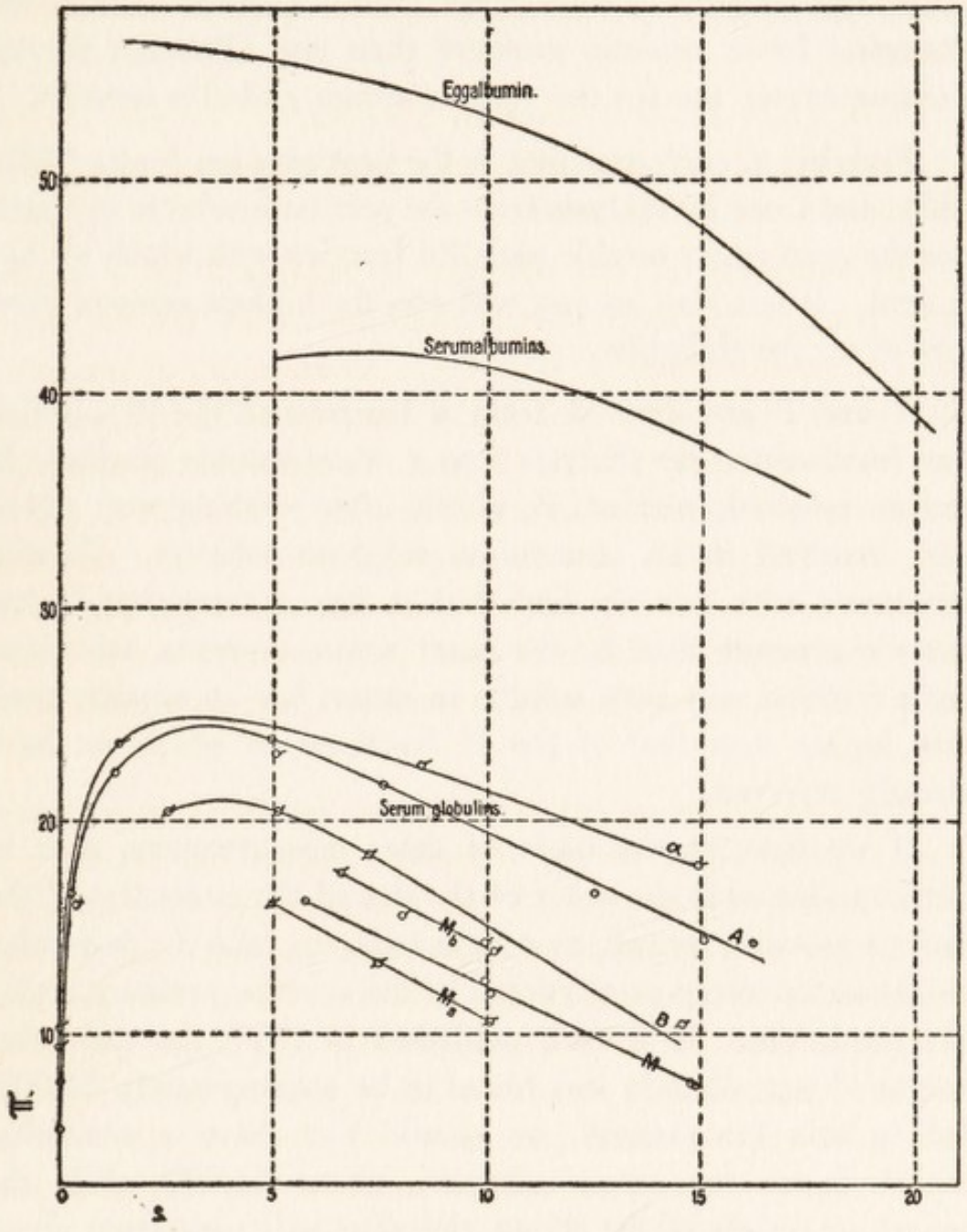
In Diagram 11, S is taken as abscissa, the ordinate being the solubility at 18° and with pH = 4.8 expressed in g. protein hydrate per 100 g. water.

The solubility of egg albumin is expressed by a single curve, running far more steeply than the curves for the three serum albumin samples y, B and A. As examples of the solubility curves for serum globulin, I have taken the curves for four fractions, marked I, II, III and IV, representing approximately the precipitation limits (28)-(33), (33)-(35), (35)-(38), and (38)-(45). On the whole, it must be said that the globulins are less easily soluble than the albumins, but it is highly characteristic that it is possible to produce a serum albumin fraction so handsomely crystallized, and so hard to dissolve, as A.

As the solubility of egg albumin can be shown in a single curve, it follows that an egg albumin solution can be characterized merely by noting: 1) *the concentration of hydrogen ions*, 2) *the concentration of ammonium sulphate and* 3) *the concentration of albumin in the solution. These data are not sufficient to characterize a serum albumin or a serum globulin solution. As far as it is possible to see at present, in the case of these solutions, we must also note*: 4) *the solubility for a certain concentration of ammonium sulphate, and finally, in the case of serum globulins*, 5) *the phosphorus content.*

Diagram 12 shows the variation in osmotic pressure with the concentration of ammonium sulphate, S being used as ab-

DIAGRAM 12


Eggalbumin.
Seruma/bumins.
Serum globulins.
50
40
30
20
10
0
5
10
15
20
π.
c.
A
B
M
M1
M2

scissa and π as ordinate. The curves correspond to measurements made under as nearly as possible uniform conditions (pH = 4.8–4.9; e = about 1.25). The uppermost curve is for egg albumin, the next for serum albumin, which has somewhat lower osmotic pressure than egg albumin; the remaining curves are for the various serum globulin samples.

Fraction α, corresponding to the precipitation limits (40)–(45), and freed by dialysis from the portion insoluble in water, was the most easily soluble globulin fraction with which we had to deal. It has also, as you will see, the highest osmotic pressure of all the globulins.

A and B are derived from a fraction (35)-(40), which was fractionated by dialysis into a water-soluble portion, A, and an insoluble portion, B, which, after washing with water, was dissolved in an ammonium sulphate solution. In nice agreement with this we find that A has a somewhat higher osmotic pressure than B; the latter again, however, representing a fraction very little soluble in water, has an osmotic pressure higher than that of the M fractions, to which we have already referred.

If we now, on the basis of these measurements, seek to form an idea as to the order of the size of the molecules of the various proteins, we can, as a mere estimate, take the molecular weight as inversely proportional to the osmotic pressure under like conditions. In a work published in 1917, the molecular weight of egg albumin was found to be approximately 34,000. Taking this for granted, we should then have a somewhat higher figure for serum albumin, about 45,000 while the molecular weight of the serum globulins will vary from about 80,000 to about 140,000.

Of course, the material herein presented will by no means

suffice to give a final answer to the question of osmotic pressure of the albumins and globulins. I hope, however, that this may be a good beginning on which to work further towards the ultimate solution of a problem so important in the chemistry of proteins.

COAGULATION OF PROTEINS BY HEATING

COAGULATION OF PROTEINS BY HEATING

The question of the coagulation of proteins by heating is of ancient date, and has been made the subject of numerous researches and much speculation. Up to the present no final and decisive answer to the question has been found, nor have I such a one to offer. I wish to put before you merely the results of some experiments throwing light on a single phase of this question, which is of great importance in the chemistry of proteins.

Coagulation consists of two distinct processes: a denaturation of the protein, and subsequent precipitation or flocculation of the denatured protein. This has already been noted by *W. B. Hardy*[1] and later by *W. Pauli and H. Handovsky*,[2] but this side of the question was for the first time really made clear in the series of excellent researches by *Harriette Chick and C. J. Martin.*[3]

Experiments we have made at the Carlsberg Laboratory, independently of the work of *Chick and Martin*, and at about the same time, as well as more recent researches by *W. W. Lepeschkin*,[4] in all essentials confirm the results of *Chick and Martin.*

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[1] J. of Physiol. **24**, 182 [1899].
[2] Beitr. zur. Chem. Physiol. u. Pathol. **11**, 425 [1908].
[3] J. of Physiol. **40**, 404 [1910], **43**, 1 [1911], **45**, 61 and 261 [1912]; Resume in Kolloidchemische Beihefte V, 49 [1913].
[4] Kolloid-Zeit. **31**, 342 [1922], **32**, 42, 44 and 100 [1923]; Biochem. J. **16**, 678 [1922].

It will be natural, then to regard the latter part of the coagulation,—the precipitation, or flocculation, of the denatured protein—as proceeding, at any rate in the main, from a discharging of the electrically charged particles of denaturated protein which, after denaturation, behaves like a typical suspensoid.

It is far more difficult to give an explanation, supported experimentally, of the first part of the coagulation process, the denaturation itself. It is at once obvious that we have here an alteration of the entire character of the protein, and it is highly probable that it is a question, not merely of an alteration in the degree of dispersity or similar physical qualities, but also of a true chemical change in the constitution or composition of the protein.

What then can we imagine to be the nature of this process in the course of which the protein is denaturated?

Chick and Martin are of the opinion that the denaturation is brought about by a reaction between the protein and water. They particularly stress the fact that dry, or nearly dry, proteins can be heated to a degree far beyond the boiling point of water without denaturation. But they do not venture to express any opinion as to whether in denaturation it is a question of hydration or dehydration, of a condensation or a decomposition of the protein molecule. They have endeavoured by means of formol-titrations to prove the existence of hydrolysis, but without success. In these experiments, they used slightly alkaline solutions of crystallized egg albumin, or purified pseudoglobulin from horse serum. The results of these experiments are, as we shall see later on, not altogether in agreement with those of our own.

In the opinion of *Lepeschkin* also, everything seems to

suggest that denaturation is an incipient, weak hydrolysis. He, however, makes no attempt to confirm this by experiments, but regards any demonstration of the hydrolysis by pure analysis as impossible, owing to the size of the protein molecules.

At the Carlsberg Laboratory, we have for many years had this problem under consideration, and in particular, we have sought for some means of answering with experimental proof these two questions:

1. Does the protein on denaturation give off any ammonia or other nitrogenous substances?

2. Does the protein give off or take up water during the process of denaturation?

I shall now describe the experiments we have made—for the most part with egg albumin—with a view to finding out if possible, an answer to these two questions.

Does the Protein on Denaturation Give off any Ammonia or Other Nitrogenous Substances?

a) In the work published in 1910[1] it was shown that an egg albumin solution, not subjected to any purification, but acidified with hydrochloric acid to the optimal concentration of hydrogen ions for coagulation, required heating for a considerable time in a boiling water bath before the minimum value for nitrogen content of the filtrate was reached.

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[1] S. P. L. Sørensen and E. Jürgensen: Compt. rend. Lab. Carlsberg **10:49** [1910].

TABLE 1.

Time of coagulation in hours	40 cc. solution					Concentration of hydrogen ions
	Consumption for neutral reaction with litmus paper a cc. of 0.2 n. sodium hydroxide solution a	Consumption for faint red with phenolphtalein b cc. of 0.2 n. sodium hydroxide solution b	Contained c mg. total nitrogen c	Contained d mg. formol-titrable nitrogen d	d % of c.	P_H
Non-coagulated solution	1.86	2.08	181.7	8.34	4.59	4.70
1 hour	0.13	0.15	11.83	0.48	4.06	5.41
3 —	0.14	0.18	10.58	0.64	6.05	5.43
6 —	0.15	0.20	10.49	0.98	9.34	5.53
12 —	0.21	0.28	12.95	1.79	13.82	5.52
24 —	0.22	0.40	17.18	2.94	17.11	5.60

Table 1 shows the results of one series of our experiments. It will be seen that the nitrogen content of the filtrate did not reach its minimum and the coagulation thus its maximum, until after heating three or six hours. But it will also be seen, that the content of formol-titratable nitrogen in the filtrate—and more or less parallel with this, the consumption of sodium hydroxide for neutral reaction with litmus and phenolphthalein respectively—rise steadily with the period of heating, whether the coagulation be completed or not. The explanation must doubtless be, that we have here a dual process, partly a precipitation of coagulable proteins, and partly an hydrolysis, which results in a re-solution of a part of the precipitated protein. It will further be seen, from the last vertical column but one in the table, that the quantity of formol-titratable nitrogen increases far more than the total nitrogen content of the filtrate.

This is naturally explained by the fact that it is not only the precipitate but in all probability, specially the substances present in the solution, which undergo further hydrolysis.

In this experiment, however, we had to deal with a mixture of the different proteins present in the white of hens' eggs, and a clear answer to the question involved could hardly be expected. This would doubtless require investigations with solutions of *pure* proteins.

b) We have therefore resumed these studies, using for the protein solution a solution of egg albumin several times recrystallized by means of ammonium sulphate, and then completely freed from ammonia by washing the crystallized precipitate with a saturated solution of potassium and sodium sulphates, and then recrystallizing again repeatedly, with the potassium-sodium sulphate solution as precipitant. Such a protein solution has the great advantage that the entire quantity of nitrogen present should be coagulable, so that determinations of the ammonia-N and the total-N in the filtrates would give direct measurements for any decomposition taking place on coagulation.

In several of these experiments, we had to make a quantitative determination of very small amounts of nitrogen. The analytical methods used, therefore, had to be adapted to this purpose (ammonia determination according to *Nessler*, and total nitrogen determinations according to *Kjeldahl-Nessler*). Also, care had to be taken to use pure distilled water and reagents particularly free from ammonia or as nearly so as possible. Any quantity of ammonia or other nitrogenous matter, however slight, in the chemicals used, had also to be determined by test experiments, and taken into account in the calculations. I will not go into details regarding the manner

of these analyses or of the coagulation experiments. One point should be noted, however. The heating, that is, the denaturation itself, was carried out at various concentrations of hydrogen ions, but after cooling, the hydrogen ion concentration was brought to the optimum for flocculation, that is, pH = 4.7–4.8 This was done in the case of all the samples by adding a suitable solution of acetic acid and sodium acetate. The heating was effected in conical flasks, covered with watch glasses and placed over, not down in, a boiling water bath. The heating conditions were, therefore, not absolutely uniform for all the experiments, but nearly enough for the result to give a quantitative idea.

Table 2 shows the results of a series of experiments with different concentrations of hydrogen ions and different periods of heating. Most of the samples, which were only heated for one hour, were found not to have coagulated out completely. This was shown by the fact that the filtrates turned cloudy on adding strong sulphuric acid. The analytical results from

TABLE 2.

Each experiment contained 1.295 g. egg-albumin nitrogen.

Concentration of hydrogen ions in the solution after heating PH	Time of heating							
	1 hour	3 hours	6 hours	12 hours	1 hour	3 hours	6 hours	12 hours
	Ammonia-N liberated in mg.				Total-N liberated in mg.			
abt. 3.8	0.18	0.52	0.67	1.12	0.19	0.73	3.26	6.30
abt. 4.4	—[1])	0.44	0.58	0.98	—[1])	0.58	1.17	3.89
abt. 4.65	—[1])	0.54	0.60	0.98	—[1])	0.63	1.39	2.98
abt. 4.9	—[1])	0.50	0.65	0.99	—[1])	0.61	1.60	2.47
abt. 6.1	—[1])	0.83	0.94	1.46	—[1])	1.45	2.45	3.28

[1] Coagulation incomplete.

these experiments, being too high, are not included in the table.

It will clearly be seen from the table that decomposition was least in samples with hydrogen ion concentration approaching the isoelectric reaction of egg albumin (pH = about 4.8); also, that the quantity of ammonia liberated was greatest in the least acid samples, whereas the total nitrogen content was greatest in the most acid samples.

There can be no question of any proportionality between time of heating and quantity of ammonia or total nitrogen. But it will easily be seen from the figures given, that an extrapolation would give at any rate very low values for the quantities formed in 0 hours' heating. In other words, the results obtained may easily be explained on the supposition that the actual denaturation does not cause any formation either of ammonia or of other nitrogenous substances, but that the decomposition in question takes place by the action of the hot water on the denaturated protein. The decomposition will therefore be the greater the longer the heating lasts, and the more of the denaturated protein there is in the solution, that is to say, the more the concentration of hydrogen ions diverges from the isoelectric reaction of egg albumin.

These experiments, however, do not give any decisive proof that no decomposition takes place in the process of denaturation itself. And we have therefore approached the problem from another aspect.

c) A solution of egg albumin entirely similar to that above described, was coagulated by means of 93% alcohol, which was added to the egg albumin solution a little at a time, with thorough shaking. The samples with alcohol were then left to

TABLE 3.

50 cc. egg-albumin solution, containing 1.050 g. egg albumin-N, used for each experiment: (pH = 4.7—4.8).

Experiment No.	Coagulation with alcohol				Heating of precipitate in boiling water bath		
	Quantity of alcohol used cc.	Standing for hours	100/250 of filtrate contained		Time of heating hours	100/500 of filtrate contained	
			Ammonia N in mg.	Total N in mg.		Ammonia N in mg.	Total N in mg.
Test I.....	50	—	0.04	0.07	—	0.01	0.04
Test II.....	100	—	0.04	0.07	—	0.01	0.04
1.....	50	½	0.06	0.09	1	0.05	0.14
2.....	50	2	0.04	0.08	1	0.04	0.10
3.....	50	5	0.04	0.06	1	0.05	0.17
4.....	50	32	0.05	0.06	4	0.13	0.24
5.....	50	72	0.05	0.06	4	0.13	0.31
6.....	25	32	0.04	0.06	18	0.29	0.60
7.....	100	32	0.04	0.07	28	0.43	0.70

stand for different periods, as will be seen from Table 3, where the results of this series of experiments were shown.

After standing, water was added to make a total volume of 300 cc. After thorough stirring, repeated and careful shaking, and standing till the next day, the precipitate was filtered off, and washed several times in cold water.

The filtrate and the washing water were then, after adding a little dilute hydrochloric acid, concentrated in a water bath to a small volume, in order to remove the alcohol, and the remainder then diluted to 250 cc., of which 100 cc. was used for ammonia determination, and 100 cc. for determination of the total nitrogen content.

The washed precipitates were transferred to conical flasks and heated with 300 cc. of water on a boiling water bath for different lengths of time, as shown in Table 3. After being

filtered and washed, the content of ammonia-N and total-N in the filtrates was determined.

Simultaneously with the experiments proper, two blank experiments [tests I and II] were made without egg albumin solution, in order to determine the content of ammonia and total nitrogen in the water and reagents used.

Now the experimental error in an ammonia determination by *Nessler's* method can hardly be taken as less than 0.01–0.02 mg. N. And in a *Kjeldahl-Nessler* determination, it will perhaps hardly be as low as this.

We think, then, that the experimental results in the fourth and fifth vertical columns of Table 3 clearly show that during the coagulation of egg albumin with alcohol, neither ammonia nor other nitrogenous substances are formed, at any rate, not in such quantity as could be determined by the method here employed. Taking the smallest quantity of nitrogen demonstrable by this method as 0.02 mg., this would give a value for the whole filtrate of $0.02 \times 250/100 = 0.05$ mg., or, in proportion to the total quantity of egg albumin nitrogen used in each experiment (that is, 1.050 g.) about 1:20,000.

We have shown in a previous work that the egg albumin molecule hardly contains more than about 380 atoms of nitrogen. Consequently, a simple calculation shows that the amount of nitrogen separated, if any, in our experiments, is at any rate less than the equivalent of one atom of nitrogen per 50 molecules of egg albumin.

We think then, that these experiments warrant the conclusion that *during the denaturation of egg albumin with alcohol, there is no formation of ammonia or other nitrogenous substances.*

From the last three vertical columns of Table 3 it will be seen that as soon as the denaturated egg albumin is heated with water, decomposition sets in, and on continued heating, the quantity both of ammonia and of total nitrogen in the solution increases. This is entirely in agreement with the results of the previous series of experiments.

Under these circumstances, then, it seems to us most natural to assume that *the actual denaturation itself by heating is, like the denaturation with alcohol, not accompanied by any decomposition, but, that the decomposition is a secondary process, whereby the egg albumin during and after denaturation is decomposed by the action of the hot water.*

d) In the case of egg albumin the decomposition which can be supposed to take place on ordinary coagulation, as in the quantitative determinations of egg albumin, will hardly be perceptible. If we take, for instance, experiment No. 3 in Table 3, we find that after the precipitate has been boiled for one hour, the amount of total nitrogen separated is only 5 x 0.17 mg. in all, or hardly $1_{0/00}$ of the whole amount of nitrogen present.

In the case of other proteins, however, as for instance serum albumin and serum globulin, this source of error is not without importance, and analytical determinations of these substances by heat coagulation must therefore be corrected for the error as far as determinations not of comparative, but of absolute values are concerned. It would take too long to describe in detail the test experiments of this sort which we have made at one time or another. I will content myself with noting the principles on which the experiments were arranged, giving a single example by way of illustration.

When a coagulable protein, under optimal coagulation con-

ditions, is not completely precipitated by heating, this may be due to the following causes:

α) That the denatured protein is not absolutely insoluble in water, or in the salt solution in which the coagulation has taken place, or at any rate, that the precipitation is not quantitatively absolute;

β) That the heating, as we have just learned, causes a decomposition accompanied by the formation of nitrogenous substances soluble in water and in salt solutions.

With regard to the source of error noted under (α) we have presumed that even though the denaturated protein did not precipitate completely from the solution in which coagulation takes place, the precipitate once deposited will in any case not dissolve again on subsequent washing with hot water. This assumption contains no essential error in itself, and permits us to regard the correction originating from the source of error here noted—x— as constant for the same protein, provided the coagulation is always effected in the same volume, without regard to the absolute quantity of protein. In our experiments, the volume was generally 100 cc.

And with regard to the source of error noted under (β) we have assumed—though this is only approximately correct —that the decomposition of the protein is a process which takes place uniformly at a constant temperature, so that the amount of "soluble nitrogen" will be directly proportional both to the time of heating and to the quantity of protein nitrogen present. If we call this last, expressed in milligrammes, p, and the period of heating in hours, t, using y to denote the quantity, in milligrams, of "soluble nitrogen," formed per milligram protein nitrogen by one hour's heating, then the correction for this source of error will be p.t.y.

The total correction, n, will then be

$$n = x + p \cdot t \cdot y$$

where the values of x and y must be determined by special test experiments with solutions of the protein in question, the amount of "coagulable nitrogen" and the amount of "non-coagulable nitrogen" being determined under different conditions of coagulation.

In these test experiments, it must be borne in mind that the protein solution used for them may possibly even before the heating, already contain some non-coagulable nitrogenous substances. Such substances are extremely easily formed from the coagulable proteins, through the action of bacteria, or merely by allowing the solution to stand at ordinary temperature, as for instance, in lengthy dialysis. The error originating from this source is of course proportional to the quantity of protein solution used in the experiment, but independent of the time of coagulation and the volume during coagulation. It can therefor be expressed as $p \cdot z$, where z denotes the quantity of "non-coagulable nitrogen" expressed in milligrams, contained in the protein solution used per milligram of "coagulable nitrogen." In the test experiments therefore, the quantity of "non-coagulable nitrogen" found, m, must be expressed as follows:

$$m = x + p \cdot t \cdot y + p \cdot z$$

and by means of the value found, or given, for m, p and t, the values for x, y and z can be calculated.

I will not go into the question of analytical methods, but merely mention that the test experiments fall mainly into two series. In one series, the time of heating, t, is varied, all other factors remaining constant; in the other, the quantity of protein p, is varied, the remaining factors being kept constant. The first series enables us to determine y and $x+p.z$, whereas the second gives us x and z.

Table 4 and Figure I show the results of two such series of experiments with a readily soluble fraction of serum albumin.

It will be seen from Table 4 series I, that the quantity of "coagulable" N found decreases, and that of "non-coagulable" N increases with increasing time of heating. It will further be seen, from series II, that the quantity of "non-coagulable" N though not proportional to the quantity of protein, nevertheless increases with the same.

TABLE 4.

Test experiments with an easily soluble fraction of serum albumin

Correction term: $m = x + p.t.y + p.z$ $\begin{cases} x = 0.11 \\ y = 0.0076 \text{ (t. in hours)} \\ z = 0.0009 \end{cases}$

Series of Experiments	Time of heating	Total content of serum albumin N	Found			Calculated correction m = x+p.t.y +p.z			
			Coagulable N	Non-coagulable N	Total content of serum N	x	p.t.y	p.z	m
	minutes	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
I Influence of time of heating	10	25.50	25.31	0.22	25.53	0.11	0.03	0.02	0.16
	15	do.	25.29	0.20	25.49	0.11	0.05	0.02	0.18
	20	do.	25.28	0.24	25.52	0.11	0.06	0.02	0.19
	25	do.	25.17	0.23	25.40	0.11	0.08	0.02	0.21
	30	do.	25.20	0.20	25.40	0.11	0.10	0.02	0.23
	45	do.	25.10	0.30	25.40	0.11	0.15	0.02	0.28
	60	do.	25.09	0.37	25.46	0.11	0.19	0.02	0.32
	120	do.	24.89	0.55	25.44	0.11	0.39	0.02	0.52
II Influence of quantity of protein	45	5.10	4.88	0.13	5.01	0.11	0.03	0.00	0.14
	do.	10.20	9.84	0.12	9.96	0.11	0.06	0.01	0.18
	do.	15.30	14.94	0.18	15.12	0.11	0.09	0.01	0.21
	do.	20.40	20.01	0.21	20.22	0.11	0.12	0.02	0.25
	do.	25.50	25.10	0.30	25.40	0.11	0.15	0.02	0.28
	do.	30.60	30.19	0.30	30.49	0.11	0.17	0.03	0.31
	do.	51.00	—	0.44	—	0.11	0.29	0.05	0.45
	do.	102.00	—	0.76	—	0.11	0.58	0.09	0.78

Figure 1 shows the results of the experiments in graphical form, and it is at once evident that the accuracy of these de-

terminations is not quite so high as could be wished, the error in some cases evidently amounting to 0.05 mg. of "non-coagulable" N. We do not think, however, that a higher degree of accuracy could be obtained without great inconvenience. It is a matter of no great difficulty, on the basis of the experimental results, to draw the two straight lines by means of which the calculation of x, y and z can be made as above described.

The calculation gives, in the present instance, $x = 0.11$, $y = 0.0076$ (for t in hours) and $z=0.0009$. It is evident from Table 4, on comparing the fifth and the last vertical columns, that the correction calculated by this formula agrees well with the quantities of "non-coagulable" N found in the experiments.

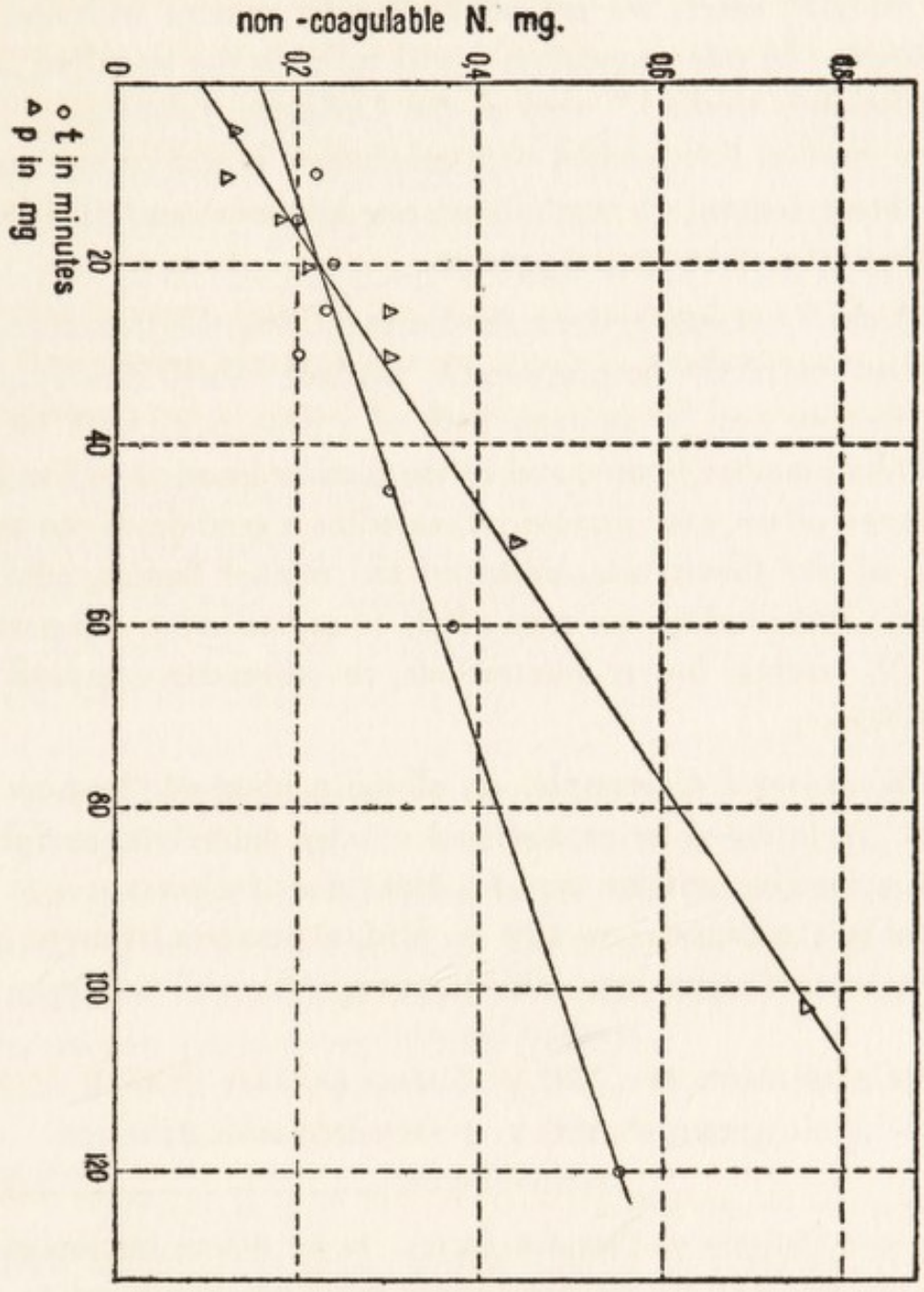
The magnitude of the correction in proportion to the total quantity of protein nitrogen is, as will be seen from the table, somewhat different according to the time of heating and the concentration of the protein. But it may easily reach a value of 1% and at low concentrations, the correction is considerably higher.

In accurate determinations of the content of "non-coagulable" N in the solution beside its "coagulable" N, as for instance, in determining the so-called "non protein nitrogen" of blood by the heat coagulation method, this source of error must therefore be taken into consideration.

Does the Protein Give Off or Take Up Water During the Process of Denaturation?

a) To the second question before us it is far more difficult to give a definite and final answer. Direct determination of the water content of the protein before and after denaturation cannot be carried out, as the evaporation of a protein solution, or the dessication of crystallized, precipitated or denaturated pro-

FIGURE 1


non -coagulable N. mg.
0
0.2
0.4
0.6
0.8
o t in minutes
▵ p in mg
20
40
60
80
100
120

tein usually results in a complete dehydration which renders it impossible to determine whether any of the water given off—and if so, how much,—was an original constituent of the protein.

In such cases, we are obliged to be content with indirect methods. In this connection I will refer to the so-called "proportionality method," used at the Carlsberg Laboratory. We have applied this method with advantage in determinations of the water content of crystallized egg albumin[1] and also in the study of the denaturation process.

I shall not go far into details regarding the principle and application of the proportionality method. I will merely point out that we can, by analysis both of a precipitate with its surrounding mother liquor, and of the mother liquor itself without the precipitate, gain certain information regarding the composition of the precipitate, provided the mother liquor adhering to and surrounding the precipitate is of the same composition as the mother liquor filtered off or otherwise removed for analysis.

We have, for example, an albumin precipitate which has been precipitated or crystallized out by ammonium sulphate. The respective weights may be denoted as follows:

Ammonia nitrogen per 100 g. filtrate (mother liquor) ..... a_f
Ammonia nitrogen per 100 g. precipitate with adherent mother liquor ..... a_b
Protein nitrogen per 100 g. filtrate (mother liquor) ..... p_f
Protein nitrogen per 100 g. precipitate with adherent mother liquor ..... p_b

We can then show, that the factor, x, by which the weight of protein nitrogen in the precipitate must be multiplied to give

[1] S. P. L. Sørensen and Margrethe Høyrup: Compt. rend. Lab. Carlsberg **12**:164 [1917].

the weight of the hydrated protein, may be expressed as follows:

$$x = \frac{100\ (a_f - a_b)}{a_f \cdot p_b - a_b \cdot p_f}$$

By means of analyses of this kind we have succeeded in showing that x for crystallized albumin has a value independent of the conditions of crystallization, 7.86, while the factor by which the weight of protein nitrogen must be multiplied to give the weight of non-hydrated egg albumin is 6.4. This means, as a simple calculation will show, that crystallized egg albumin contains 0.22 g of water for every gram of water-free egg albumin.

The formula here used for x is valid only in cases where the protein does indeed contain water, but has no ammonia or ammonium sulphate in combination. In the great majority of cases, however, we have to take into account the fact that the protein, both in solution and as a precipitate, holds in combination some sulphate of ammonia [or other electrolytes present]. The formula then becomes more complicated. If we take z and y respectively for the factors by which the weight of protein nitrogen must be multiplied to give the weight of protein + water + ammonium sulphate [factor z] in one case, and the weight of ammonium sulphate therein [factor y] in the other, we can apply the following formula:

$$z = x + y = r + s \cdot y$$

where $r = \frac{100\ [a_f - a_b]}{a_f \cdot p_b - a_b \cdot p_f}$ and $s = \frac{100\ [p_b - p_f]}{4.7163\ [a_f \cdot p_b - a_b \cdot p_f]}$

Now experiments show, as was also to be expected, that y is always quite small in comparison with x, but y is usually not equal to 0 and only in this case is $z = x = r$. On the other hand

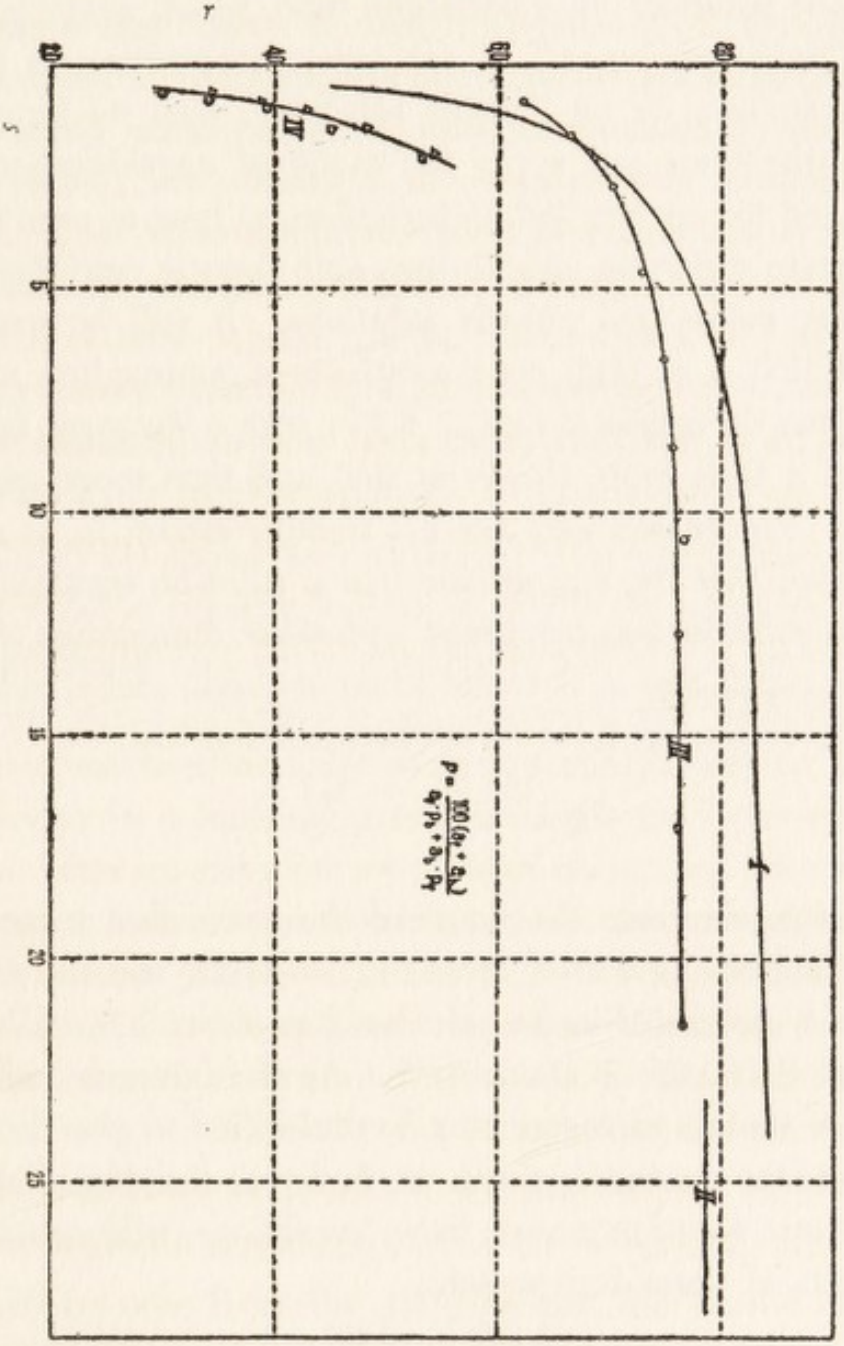
s·y need not be small in comparison with r; s·y will naturally be of greater importance, the greater is y, or in other words, the greater the amount of ammonium sulphate bound by the protein, and the greater s is. This is equivalent to saying, as examination of the formula for s will show, the lower the concentration of ammonium sulphate. The value found by experiment for r thus will only be equal to z for $y = 0$, and for the rest, it will be found to be less, the greater s·y becomes, or the smaller the ammonium sulphate content in the mother liquor [a_f] and the greater y is. Under otherwise uniform conditions, r thus alters with a_f, and the more strongly the less a_f becomes, so that from these alterations in the magnitude of r, we may form an idea as to the magnitude of y. I will not go further into the considerations on which this approximate determination of y is based; it must suffice here to mention the variation of r for varying values of the concentration of ammonium sulphate.

The curves in Figure 2 show the results of some series of experiments of various kinds which we must now consider more closely. All the curves express the relation between the value of r and the concentration of ammonium sulphate in the mother liquor, r being used as ordinate, while the quantity of ammonium sulphate S in the mother liquor per 100 g. water is used as abscissa. The concentration of hydrogen ions in all experiments corresponded to pH=4.7–4.8.

b) Curve I is borrowed from a previous paper[1] and expresses the relation between r and S for ammonium sulphate containing aqueous solutions of egg albumin with pH=about 4.8. In such solutions the disperse phase, that is, the hydrated protein, corresponds to what we have above termed the precipitate, whereas

[1] S. P. L. Sørensen: Comp. rend. Lab. Carlsberg, **12**:362 [1917].

FIGURE 2



the dispersion medium, the aqueous ammonium sulphate solution surrounding the disperse phase, corresponds to the adherent mother liquor. If an egg albumin solution of this kind be placed as "inside solution" in a collodion tube, which again is placed in an "outside solution" of the same composition as the dispersion medium in the "inside solution", then we have a system to which we can apply the mode of consideration noted above, and the corresponding formulae, as long as care is taken to maintain diffusion equilibrium and osmotic equilibrium between the inside and outside solutions. It will be seen from curve I that r at high concentrations of ammonium sulphate is very nearly constant [about 8.4]; with a decrease in S, the value of r falls quite slowly at first and then more and more rapidly. The reason why r is not found constant is, as already mentioned, that the egg albumin in a solution containing ammonium sulphate has combined with some ammonium sulphate with the result that a_b is found to be too high, and r, calculated from the following formula is therefore too low.

$$r = \frac{100\ [a_f - a_b]}{a_f \cdot p_b - a_b \cdot p_f}$$

We can see from the shape of the curve that y must have a value increasing with S from 0.01 to 0.05, and the maximal value of z can therefore be calculated as about 8.7, while x will be about 8.7–0.05 or about 8.65. As the factor by which the weight of protein nitrogen must be multiplied to give the weight of water-free protein is 6.40, we find that the egg albumin in an aqueous solution under these conditions will contain, for each gram of water-free protein,

$$\frac{8.65 - 6.40}{6.40}$$

or 0.35 g. of water. The question is now whether this water

will be wholly or partly given off on denaturation or whether a further quantity of water will be taken up by this process.

c) Before describing our denaturation experiments, I must just mention that Curve II, which appears as a straight line corresponding to $r = 7.86$, represents the crystallized egg albumin, the water content of which we have, as already mentioned, found constant, independent of the concentration of ammonium sulphate. This curve comprises only the high concentration of ammonium sulphate, as egg albumin, of course, does not crystallize out at a lower concentration. $r = x = z = 7.86$ corresponds to a water content of 0.22 g. water per gram of water-free egg albumin, so that even on crystallization of the egg albumin a certain amount of water is already given off.

d) Curve III shows the results of a heat coagulation experiment, in which a series of solutions, all at the same concentration of hydrogen ions corresponding to $pH = 4.7$, and all containing the same amount of egg albumin, but with different contents of ammonium sulphate, were coagulated by heating for one hour in a boiling water bath with good and repeated shaking. After standing for a couple of days, they were filtered and samples both of the filtrate and of the precipitate with adherent mother liquor were weighed. In the former the amount of ammonium sulphate was determined, in the latter both the amount of ammonia nitrogen and of egg albumin nitrogen, after which r was calculated according to the formula given above. The results of the experiments are tabulated in Table 5 and shown graphically in Curve III (Figure 2).

It will be seen from the diagram that the results of these experiments can easily and naturally be arranged in a curve, and there is hardly any doubt, therefore, that this curve can be used to elucidate the question at issue. At high concentrations

TABLE 5.

Experiment No.	100 g. filtrate contained Ammonia-N in g. a_f	Filtrate's content of Ammonium sulphate in g. per. 100 g. water S	r (found)	Mean of r
1	0.1729	0.822	6.23_9	6.24
2	0.3272	1.567	6.58_0 6.69_8 6.63_5	6.64
3	0.5595	2.710	6.96_1 6.99_1 7.08_1	7.01
4	0.9393	4.635	7.20_3 7.29_7 7.33_5	7.28
5	1.3074	6.571	7.54_0 7.41_1 7.48_0	7.48
6	1.6722	8.562	7.64_2 7.53_0 7.47_5	7.55
7	2.0369	10.628	7.65_6 7.69_3 7.66_3	7.67
8	2.3882	12.693	7.67_3 7.58_8 7.55_6	7.61
9	3.0957	17.096	7.59_6	7.60
10	3.7563	21.530	7.61_2 7.75_8 7.59_3	7.65

of ammonium sulphate, r is very nearly constant [7.6–7.7]; with decreasing S, the value of r falls quite slowly at first, and then faster and faster. The value of r for the coagulated egg albumin is, as will be seen from the figure, still less than that for the crystallized egg albumin and far less than that for the dissolved egg albumin. This may be due to the fact that the denaturated egg albumin contains less water than the dissolved, but it can also be explained if we may assume that the denaturated egg albumin has bound more ammonium sulphate than the dissolved at the same concentration of ammonium sulphate. But a closer examination of the formula given above shows that the quantity of ammonium sulphate combined per gram-equivalent of egg albumin nitrogen must be the greater, the steeper the course of the curve. It is also very plainly evident from the figure that Curve III is far less steep than Curve I. The experiment shows then that the denaturated egg albumin, under otherwise equal conditions, has bound less ammonium sulphate, and contains considerably less water than the dissolved. On the other

hand, at a fairly high concentration of ammonium sulphate, r has a far higher value than 6.4, from which we may conclude that only a portion of the water was lost by the denaturation process.

e) Curve IV, Figure 2, shows some of the results from a series of denaturation experiments with egg albumin by means of alcohol.

On comparing Curve IV with the other curves in Figure 2 it will be seen that the same ammonium sulphate concentration gives values of r still lower for the egg albumin denaturated with alcohol than for that denaturated by heating. The curve, however, is steeper than Curve III but less steep than I, which suggests that the egg albumin denaturated by alcohol has, under otherwise equal conditions, bound more ammonium sulphate than the heated sample, but less than the dissolved. Curve IV, however, does not embrace such high concentrations of ammonium sulphate that we can draw absolutely certain conclusions from its course, but a closer study of the results of the experiments leaves very little doubt that the alcohol denaturation also has caused a considerable loss of water.

f) The main result of the experiments dealt with in this section can then be expressed as follows:

In the denaturation of egg albumin, both by alcohol and by heating, the egg albumin loses water, but it has not been possible to determine how far the giving off of water extends, or whether it is in all cases to the same extent. It must be considered as proved, however, that the denaturated egg albumin always retains some water.

I shall not now go into the very important question of the influence of the various electrolytes on denaturation and on

flocculation. It is a highly complicated question, and we are at present occupied with its investigation.

I am quite aware, as I mentioned at the beginning of my lecture, that this important question, the problem of proteins and their coagulation, is far from being solved by the investigations I have had the honour to present. Nevertheless, I hope I have made some slight contribution towards a better understanding of what takes place.

CHARACTERIZATION OF PROTEINS

Address to the National Academy of Sciences
Boston, Nov. 11, 1924.

CHARACTERIZATION OF PROTEINS

May I be permitted to express to you my hearty thanks for the great honour you have shown me in inviting me to present a communication to your National Academy of Sciences? I am very glad to have this opportunity to speak to you about a question, which has much significance for me in my studies, and which I hope may be also of some interest to you, and this is, the "characterization of proteins."

The proteins are, as you know, essential constituents of all living cells. They are a most important part of the matter derived from plants and animals, such as seeds, eggs, blood, brain, internal glands, muscles, etc.

It is therefore not surprising that these substances have been the subject of numerous investigations. As the result of extensive research by many investigators, we have at present some knowledge of the properties and nature of the proteins, the so-called genuine proteins and also their decomposition products. The main portion of these products is found to consist of amino acids, which therefore may be regarded as the real and essential constituents of the proteins.

Above all, in this field of protein chemistry, the qualitative and quantitative determination of the content of the different amino acids in the various proteins has been the subject of research work on a large scale during the past thirty years.

In addition to this work, the studies on the genuine proteins have given us noteworthy information, and in this connection I need only refer to Thomas B. Osborne's excellent investigations on the plant proteins, and the extensive work of Jacques Loeb, of Wolfgang Pauli, of Chick and Martin, and now recently, of Edwin J. Cohn.

In certain other branches of protein chemistry, which are very important, it seems to me that only very slight progress has been made. I refer here to the characterization of the different proteins and their classification on an exact and systematic basis. The main reason for this lack of progress may be traced, I believe, to the conception that protein solutions—being of colloidal nature—are so different in their behaviour from ordinary true solutions, that it is impossible to apply the theories based on the study of true solutions to protein solutions. This conception is in my opinion not tenable. I mean by this that the essential point is, that with improved technique, and, particularly, by the development of more exact analytical methods, it should be possible to deal with protein solutions from general chemical and physico-chemical viewpoints. I think that a consistent application of the modern physico-chemical methods in the studies of proteins will make it possible to give us a better characterization of the different proteins and to elaborate a more satisfactory classification of these substances than we have at present.

Allow me to illustrate by some simple examples:

1) All the proteins are of an amphoteric character, but the acid and basic nature respectively are ordinarily present in a different degree, according to which the proteins are classified as being acid, basic or neutral proteins.

This classification and characterization of the proteins is fundamentally sound as a principle, but it is too vaguely formu-

lated and may be replaced by a more exact statement of the acid and basic character of the protein in question. The final goal of our endeavors should be to determine the constants of acid and of basic dissociation of the proteins, but for the present we must be content with the determination of the acid- and base-binding capacity of the proteins at different hydrogen ion concentrations, and under other known and fixed conditions. Such determinations can easily be made, and a graphic representation of the results will give us a curve which very exactly illustrates the acid and basic character of the protein in question, taking for instance the pH values as abscissae and the corresponding binding capacities as ordinates. By means of such determinations we can further obtain information about the isoeletric reaction of the protein, the magnitude of this latter being of the greatest importance for the classification of the proteins.

2) In the description of some proteins, for example, the albumins and the globulins, the temperature of coagulation has often been stated to be a characteristic of the protein in question.

However, the denaturation, as well as the flocculation of the denaturated protein are influenced by many other factors than the temperature alone, for instance, by the concentration of hydrogen ions and by the nature and the concentration of the salts present in the solution. A simple statement of the coagulation temperature without any indication of all the surrounding conditions during the coagulation process is, therefore, of very little value. If, on the other hand, the coagulation process is carried out under properly controlled conditions, which must be exactly stated, there is probably no doubt that the coagulation temperature can be used for the characterizing of proteins.

The most exact way, however, in which to express the difference in the denaturation tendency seems to me to be the indica-

tion of the different rate of velocity of the denaturation by the coagulation of the different proteins under exactly equal conditions. The denaturation being a monomolecular reaction—as shown by Chick and Martin—it seems that it should be possible to find a rational term for this property, so important for the characterizing of certain proteins.

3) As a last example I shall mention the precipitation of proteins by means of salts. You know that such precipitations within the usual limits of salt concentrations are ordinarily used in characterizing, as well as in separating, the different groups of proteins.

However, the completeness of a protein precipitation, or, in other words, the solubility of a protein in a salt solution, depends not only on the salt concentration, but also on a number of other factors—the temperature, the hydrogen ion concentration and under certain circumstances, the intital protein concentration and the carrying out of the precipitation. By using such a precipitation process in characterizing or separating proteins or groups of proteins, all these factors must be taken into consideration, and the process itself must therefore be defined much more exactly than has hitherto been the case.

We ordinarily separate the albumins and the globulins by half saturating the solution with ammonium sulphate, the albumins being considered soluble, and the globulins insoluble in such a salt solution. In this simple form, without any indication of the hydrogen ion concentration of the solution, the process of separation is completely undefined, the result of the separation varying with the hydrogen ion concentration.

Further, the solubility or insolubility, respectively, in a half-saturated ammonium sulphate solution can scarcely be used as a general method of separation of albumins and

globulins. It is, for instance, very easy to prepare from horse serum a well crystallized serum albumin which at 18° C.—and at the isoelectric reaction—is very slightly soluble in a half-saturated ammonium sulphate solution, and in every case much less soluble than most of the serum globulins prepared in the usual way under equal conditions.

Other kinds of well crystallized serum-albumins are easily soluble in half-saturated ammonium sulphate solution, and we have in the solubility, exactly defined, and, making proper allowance for the other factors which are of significance for the state of equilibrium between the precipitate and the mother liquor, an excellent means of characterizing the different kinds of serum albumins.

Still more informative is a complete solubility curve showing the variation of the solubility with the ammonium sulphate concentration, all other factors of influence being kept constant. The more or less steep shape of the curve is very characteristic for the protein in question.

May I say one thing more? In the studies on proteins we should always have in mind, that the protein under consideration is possibly not a single substance, but a mixture or a combination of two or several different substances. In characterizing such mixtures, solubility determinations are of the greatest value. In such cases solubility experiments at different initial protein concentrations will give valuable information about the character of the protein mixture. It is particularly of significance in the use of protein mixtures in physiological, serogical or other biological studies. In such cases an exact characterization of the original material is of the greatest importance, because it will be impossible to repeat the researches or to compare the results with those obtained by other investigators, unless the material used is exactly defined.

There are, of course, many other questions, which I should like to discuss, if time permitted, because they are of importance in characterizing the proteins. Some of these are hydration of protein, the size of the molecules, or the molecular-aggregates, and the stability of protein solutions.

I hope however, that by these few simple examples, that I have had the honor of bringing to your attention, I have made evident the importance and the necessity of a more exact characterization of the proteins and of the behaviour of their solutions.

BREADMAKING
HYDROGEN ION CONCENTRATION
PROTEINS

Address to the American Bakers' Association
27th Annual Convention, Atlantic City
September 22, 1924.

BREADMAKING
HYDROGEN ION CONCENTRATION
PROTEINS

Baking has been the last branch of the fermentation industries to institute a rational and systematic scientific study of the materials used in its work, and the processes which take place in the preparation of those materials: the flour, the dough and bread itself.

I do not mean to say that no labour or attention has been devoted to flour investigation, for example; it would be quite sufficient to mention *Thomas B. Osborne* and his pioneer work in connection with the vegetable proteins, including those of wheat and rye. What I do mean is that such work was formerly carried out without that close association and intimate co-operation with the working industry which are essential if the industry itself is to profit fully by the results obtained. And, on the other hand, such co-operation is of the greatest importance to science. The scientist, one might say, is constantly being spurred on by the questions put to him by the masters of industry, which force him to look further into old problems, and occasionally present him with some new ones.

The importance of this co-operation in the bakery business, as well as in others, has during the last ten years become more keenly appreciated throughout the world, not least in Amer-

ica. I will not refer to individual investigators; I must, however, mention the steady and purposeful work which has already been carried out for some years at the Minnesota Agricultural Experiment Station, and the work commenced both at the American Institute of Baking and the Fleischmann Laboratories. I might also add the Canadian Institute at the Ontario Agricultural College at Guelph, which I understand is being opened this year.

I venture to say that America in this respect is better equipped than any other country, both with regard to its scientists and laboratories and the organization of their work.

You will understand, then, that it was not without great hesitation that I undertook to deliver an address to the convention of the American Bakers' Association. I thank you heartily for the great honor you have done me in inviting me to speak, but I cannot help thinking to myself: I have never had anything to do with bakeries directly; what can I find to tell the bakers of America? I feel sure that one of your own skillful investigators could put before you much more interesting matter than I am able to do myself.

However, since I am to speak, I propose to take as my subject:

Breadmaking—Hydrogen Ion Concentration—Proteins

and I would ask you at the outset not to be frightened by the title. On the other hand, I must beg your indulgence for having to touch upon some points which many of you know well enough already.

You all know, of course, that the degree of sourness in flour, its *acidity*, affects its baking qualities, and that it is often advisable, when preparing the dough, to add sour milk or buttermilk. But if you ask me *why* it is advisable to sour the

dough, if you want to know what part or parts of the dough will be favorably affected by the acid—then you are posing a difficult question. And it is just such questions that I propose to deal with today.

That property of the flour which we have just referred to as *acidity* is really, to give it a more rational, scientific term, *the concentration of the hydrogen ions it contains.* We will now consider what effect this property has on the baking quality of the flour.

It is often thought that acidity and concentration of hydrogen ions are merely two different expressions, if not for one and the same thing, at least for two parallel qualities, so closely connected that it might seem unnecessary to bother oneself—at any rate in practice—with the newer and less familiar term. This, however, is altogether incorrect, and I may as well begin by pointing out the difference between the two.

The acidity of a solution is measured, as you all know, by *titration;* in other words, by finding out how much soda of a given strength must be added to neutralize the acid. Suppose we have a solution of hydrochloric acid, and one of vinegar, one of sour milk and one of an extract of flour, etc., all requiring the same quantity of soda to neutralize the same amount of solution, then we say that all the solutions are of the same degree of acidity, or sourness.

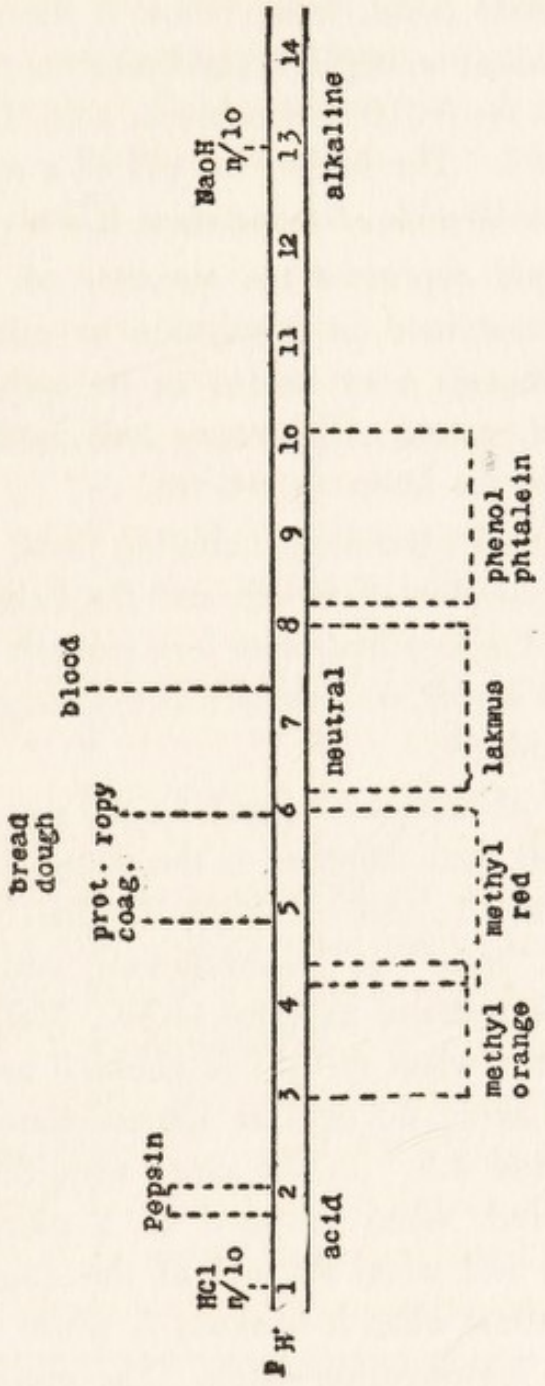
But *are* they all equally sour? Is one as acid to the taste as another? Not by any means. A solution of hydrochloric acid tastes much more sour than an equally strong solution of lactic acid or acetic acid. The reason is this. According to the theory of the Swedish scientist, *Arrhenius,* different acids differ in their tendency to throw off hydrogen ions, and it is only these hydrogen ions that *taste sour;* in other words, affect

the nerves of taste. An acid such as hydrochloric acid, for instance, will at once throw off all its hydrogen ions when dissolved in water; lactic acid, on the other hand, and more particularly acetic acid, keep the greater part of theirs in reserve. The hydrochloric acid, therefore, is very sour to the taste, the other acids only slightly so, though all three are present in the same degree of strength, the same acidity.

As with the sensation of taste, so is it with all the biological processes, from the growth of the seed in the cornfield or the growth of yeast in the tub or its action on the dough, to the process of digestion and all other physiological functions in the human organism. Throughout all these processes the hydrogen ions play a certain part; they are indispensable, but on the other hand, there must not be too many of them. Each process has its optimal concentration of hydrogen ions, the one at which it works best. And you will understand that it is therefore of importance, if we wish to understand the biological process in question, that we should be able to determine whether the concentration of hydrogen ions is as it ought to be. There is no need for us to taste these ourselves; we can let suitable indicators do the tasting for us. One of these indicators is litmus, often used in the form of litmus paper, which can taste whether a solution is acid or alkaline. There are a number of these indicators, each useful in its particular sphere, according as the solutions are strongly or slightly acid, or strongly or slightly alkaline. You may be aware for instance, that *Cohn, Cathcart and Henderson* have proposed and successfully used the indicator known as *methyl red* for determining the concentration of hydrogen ions in dough and bread.

In measuring the concentration of hydrogen ions, the actual magnitude is denoted by means of the figure, pH. Its magnitude

DIAGRAM 1


pH
HCl n/10
Pepsin
bread dough
prot. ropy coag.
blood
NaoH n/10
acid
neutral
alkaline
methyl orange
methyl red
lakmus
phenol phtalein
1
2
3
4
5
6
7
8
9
10
11
12
13
14

increases as the concentration of hydrogen ions decreases.

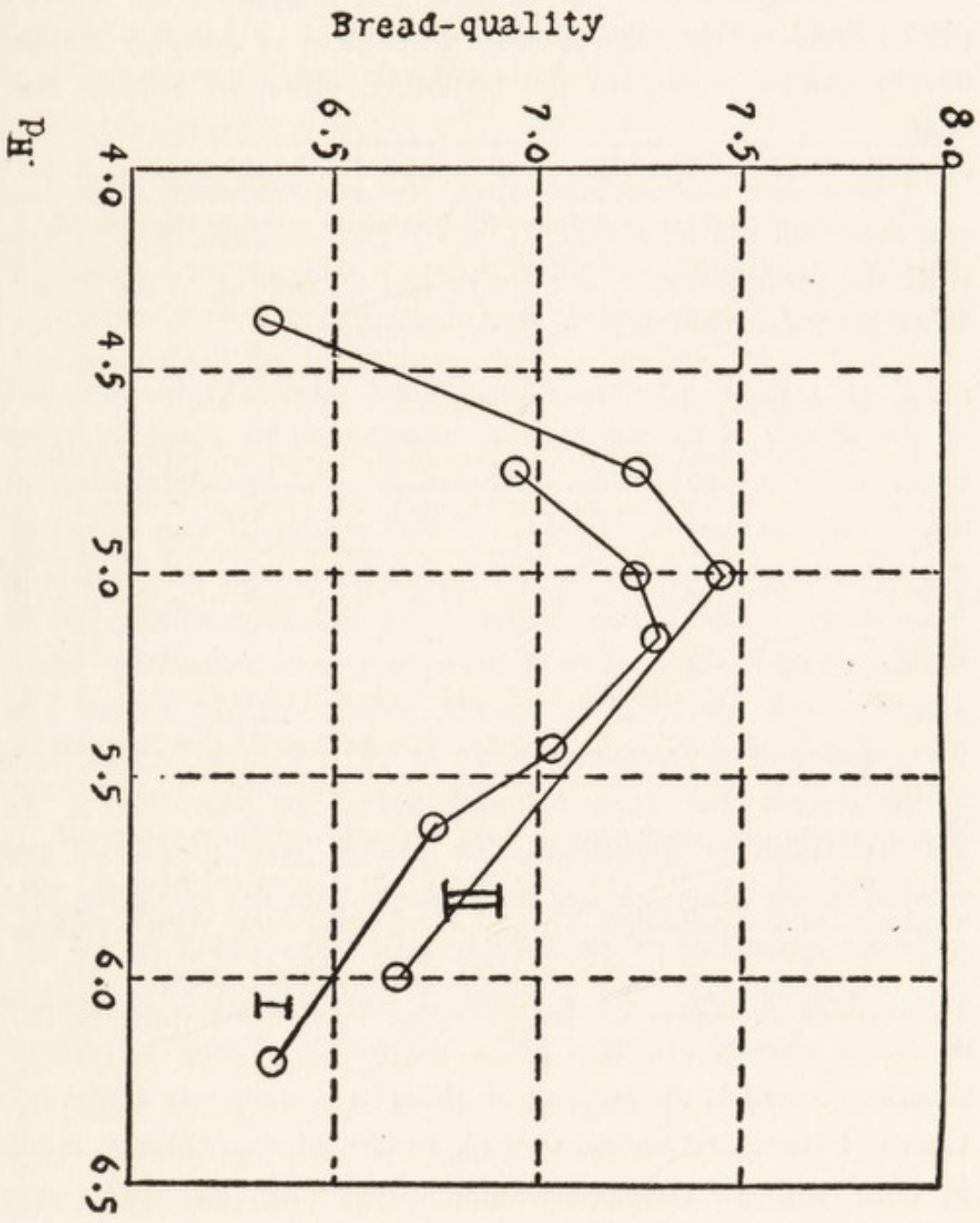
In Diagram 1, you will find the concentration of hydrogen ions expressed as pH in numbers from 1 to 14. The figure 7 denotes the neutral point, those below it the acid region and those above the alkaline region. The lower the pH of a solution, the more sour it is: *N*/10 hydrochloric acid, for instance, has its pH = about 1. The higher the pH of a solution the more alkaline it is; *N*/10 soda, for instance, has a pH = about 13.

The value pH expresses the quantity of active, effective hydrogen ions contained in a solution or mixture. The old term, *acidity*, though very useful in its own way, only expressed the total content of hydrogen ions, both the active and the inactive ions, the latter in reserve.

In all biological processes, including those we have to deal with in the preparation of dough and the baking of bread, it is the quantity of active hydrogen ions present that is most important; in other words, it is the pH of the dough and the bread we have to consider.

The manner of determining pH I need not enter into here. I will merely call your attention to the names of the indicators shown in Diagram 1, each of which is able, in its own particular field, to "taste" as I said before, what the pH of a given solution or mixture happens to be. Methyl orange, for instance, turns red when the pH is about 3 and turns yellow when the pH is about 4.5 and an intermediate shade when it lies between 3 and 4.5. In the same way, the important indicator methyl red, when dropped on a piece of dough or bread, will turn red when the pH of the dough or bread is about 4.5 but yellow when it is about 6, while an intermediate pH will give an intermediate shade. The optimum pH of the dough or bread is shown in Diagram 1 as pH = 5. It may vary a little, but if the pH be essentially higher than 5, then

DIAGRAM 2



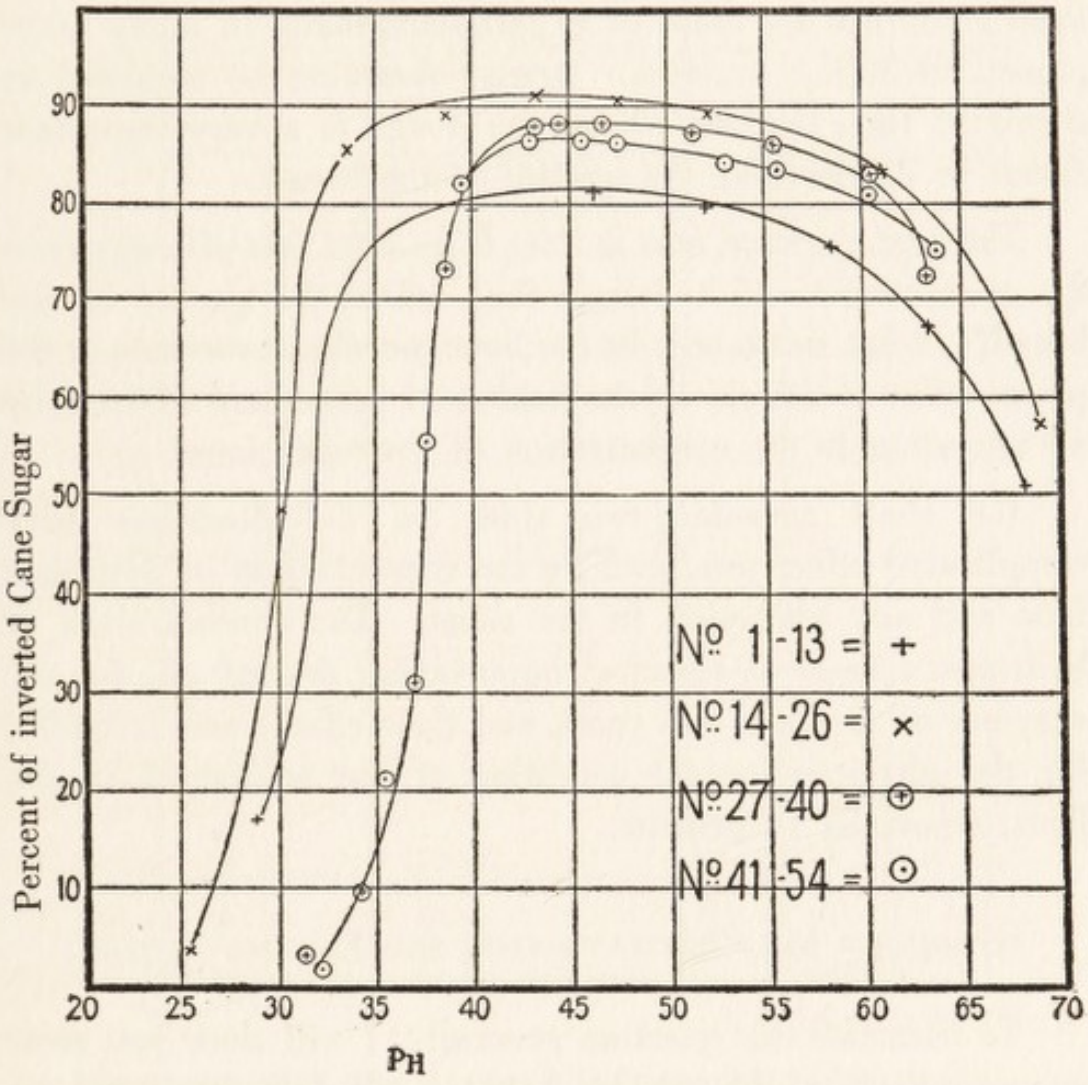
the dough is not in the best condition. The fault in dough when it is what is called "ropy" is a point which has been specially studied by *Morison and Collatz*, as well as by *Cohn and his collaborators*. It is generally indicated by a high pH (5.5–6), as the rope-producing bacillus or *Bacillus mesentericus* cannot withstand the combined effect of acidity and heat.

I have now said enough about the concentration of hydrogen ions and pH in a general way. But we have then to consider the preparation of the dough and the baking of the bread. What part does pH play in these processes?

It is, I think, generally recognized nowadays that the pH of the dough is of the highest importance in ensuring good bread from an otherwise good dough. As an illustration of this I have shown in Diagram 2 the results of two series of experiments carried out by *Dr. Jessen-Hansen* at the Carlsberg Laboratory. The curves indicate the influence of the pH in the dough upon the quality of the bread, as expressed by "characters" given according to certain definite rules. Curve 1 is for a series of experiments made in 1910 with a Danish flour of the second class (note the high pH of the pure flour). In the individual experiments, the dough was prepared and treated in the same manner throughout, with the exception that different quantities of phosphoric acid were added in the different experiments. As a result of this, the different batches of dough showed different pH's, this being, of course, proportionately lower as the amount of phosphoric acid was increased. Curve II shows, in the same way, results of experiments made in 1922 with an unmixed Danish wheat flour, but with lactic acid used to sour the dough.

You will see from the shape of the curves that the quality

DIAGRAM 3


Percent of inverted Cane Sugar
90
80
70
60
50
40
30
20
10
20
25
30
35
40
45
50
55
60
65
70
PH
No 1-13 = +
No 14-26 = ×
No 27-40 = ⊕
No 41-54 = ⊙

of the bread rises with increasing amount of acid added, until pH reaches about 5, after which, any further souring again impairs the quality of the bread.

These two series of experiments are but two of many which have been made with the most widely dissimilar sorts of flour; and the results arrived at in the Carlsberg Laboratory have been confirmed by those of experiments made in many other places, including America. It may therefore be regarded as a proved fact, that the pH of the dough is a very important factor in determining the quality of the bread.

The next question now is this: *Why* does this pH, why does the concentration of hydrogen ions, affect the quality of the bread? What substances in the flour, or what processes in the preparation of dough, or the baking of bread, are affected by an alteration in the concentration of hydrogen ions?

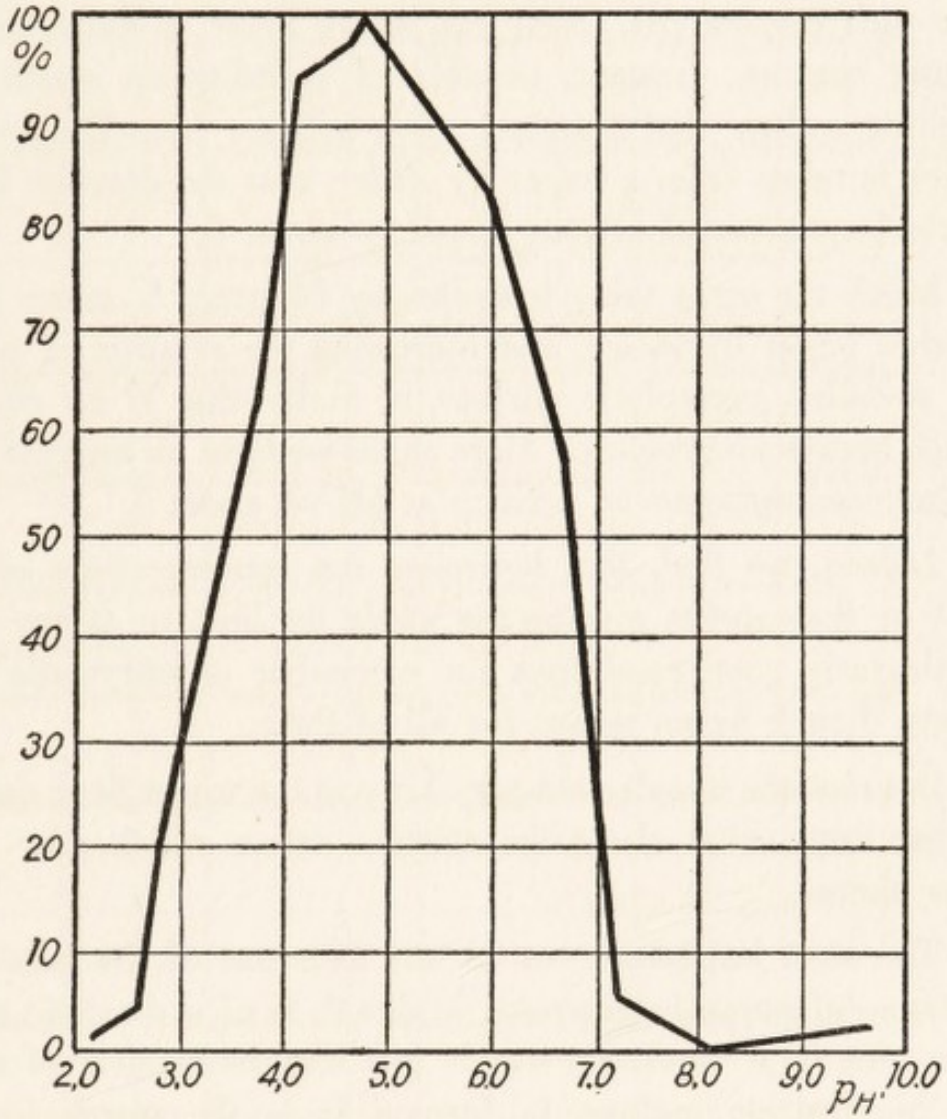
We shall consider two sides of the doubtless very complicated effect produced by the concentration of hydrogen ions and any alteration in the same. The concentration of hydrogen ions is of essential importance; *first* of all, for the enzymes of the flour and yeast, and their effect; and *secondly*, for the physico-chemical condition of the substances in the flour, especially its proteins.

Hydrogen Ion Concentration and Enzyme Action.

To illustrate this question generally, I will show you some curves indicating the relation between pH (abscissa) and enzyme action (ordinate).

Diagram 3 illustrates the invertase action. Invertase is an enzyme found in yeast, and is capable of splitting up ordinary cane sugar into the two sugars of simpler composition, glucose

DIAGRAM 4



Formation of maltose at various hydrogen ion concentrations with acetate buffer.

and fructose. This diagram, which is taken from one of the works published by the Carlsberg Laboratory, shows that the enzyme in question has an optimal zone at pH = 4.5–5, its action decreasing very strongly with decreasing pH.

Diagram 4 shows in the same way the action of malt diastase with varying pH. Malt has, as we know, a starch-decomposing enzyme, diastase, capable of splitting up starch and forming maltose and dextrine. You will see from Diagram 4, which is taken from a paper by *Adler*, that the diastase has a marked optimum of activity at pH = about 5.

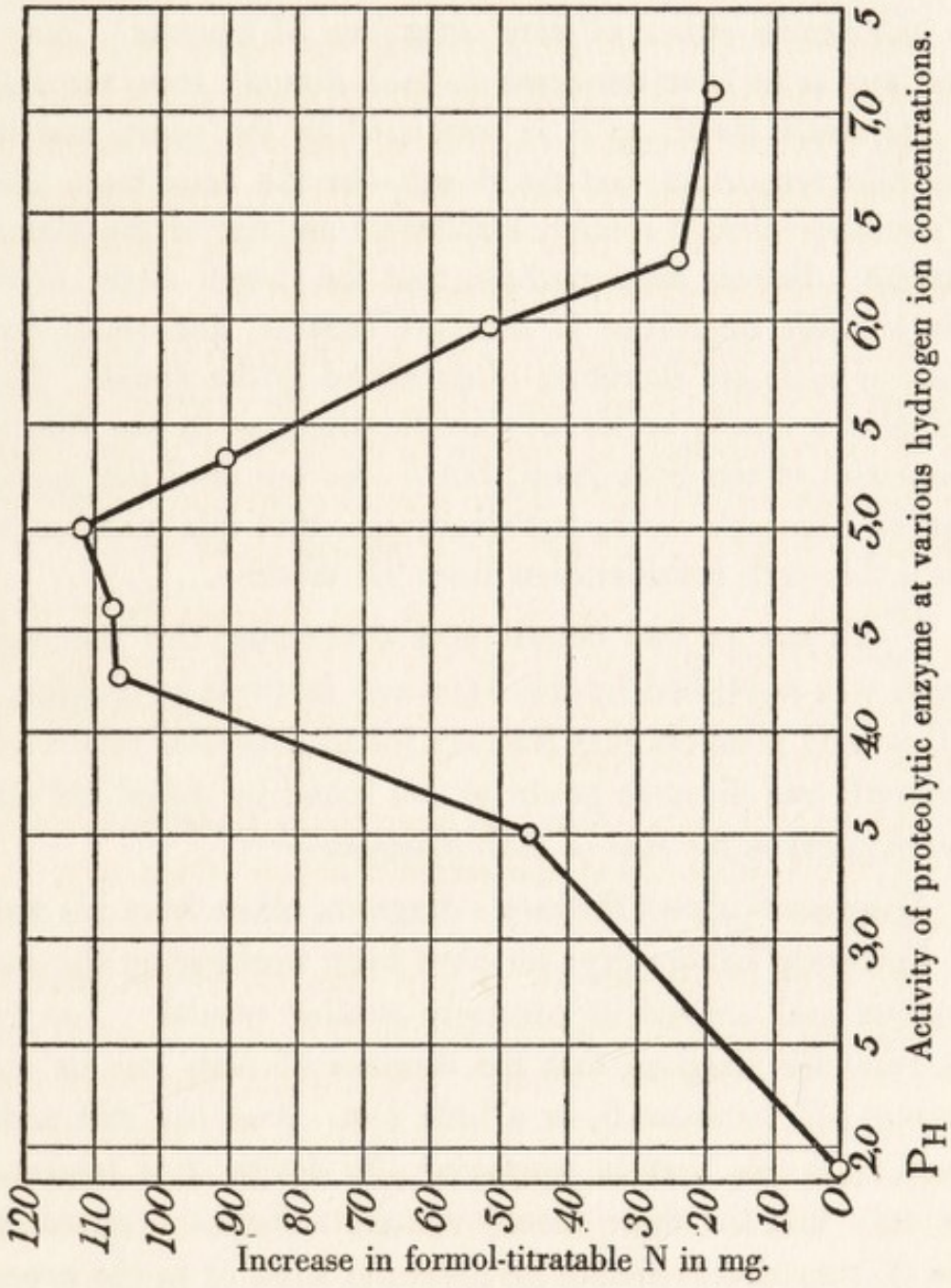
Much the same thing is shown by Diagram 5, taken from another paper by *Adler*, and indicating the relation of pH to the so-called proteolytic enzyme in malt—that is an enzyme which breaks up proteins. Here again we have an enzyme with pronounced optimum of activity at pH = about 5.

Indeed, we find, that hydrogen ion concentrations of pH = 5 or thereabouts give on the whole the best, or at any rate, particularly good conditions for a number of enzymatic processes, though by no means for all of them.

But now the dough—we may keep to the wheat flour dough, by the way—what about the enzyme action which may take place there?

The most important—or at any rate one of the most important—of enzymic processes in dough is of a diastatic character, that is, it breaks up starch, just like the diastase of malt. By this process maltose is formed from the starch in the dough, and the maltose again is split up by the yeast into glucose. This in its turn is decomposed by the yeast, in the process of alcoholic fermentation, into alcohol and carbonic acid. The importance of this last named substance for the raising of the dough is one into which I need not go further here.

DIAGRAM 5

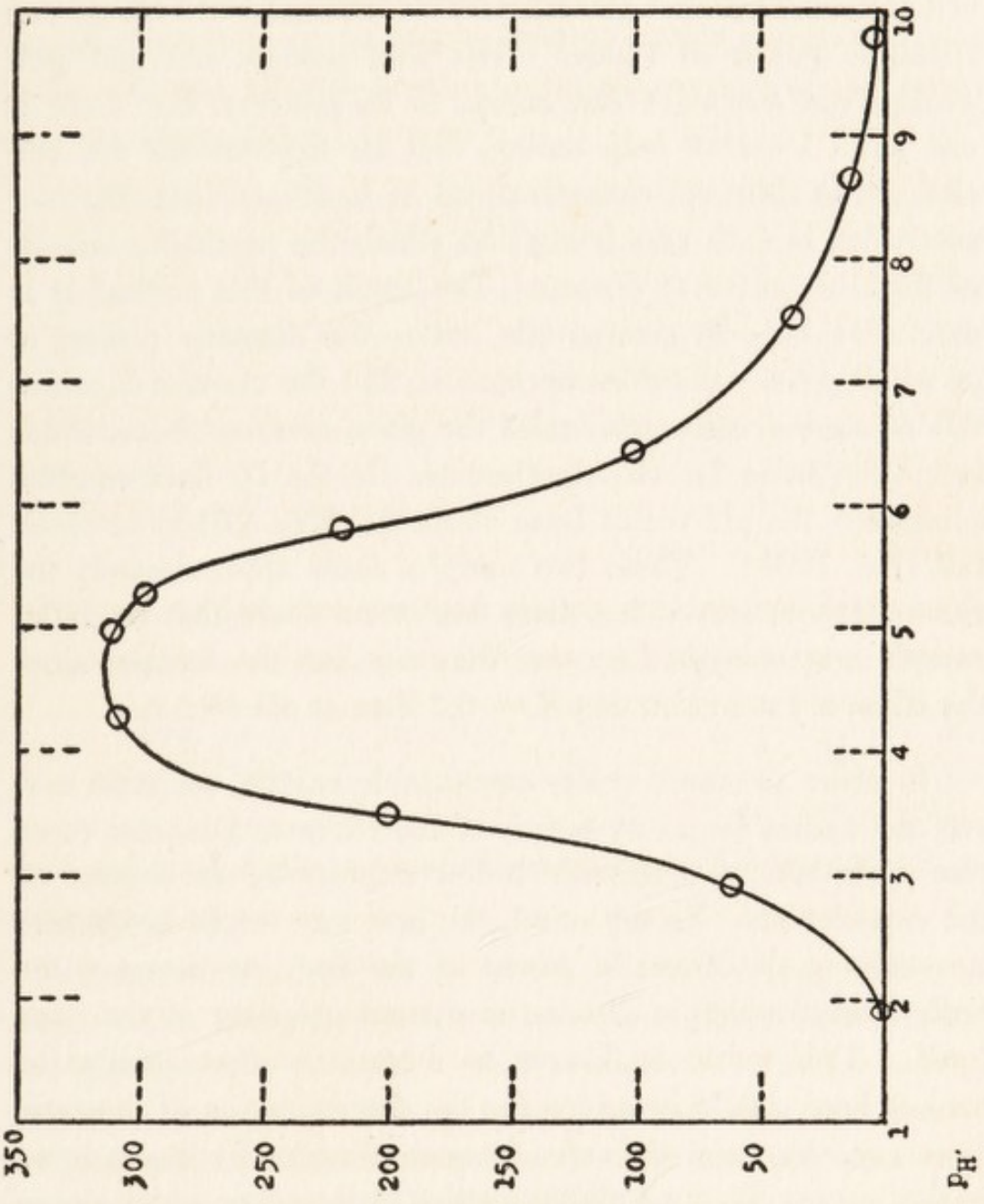

120
110
100
90
80
70
60
50
40
30
20
10
0
Increase in formol-titratable N in mg.
2,0
3,0
4,0
5,0
6,0
7,0
P_H
Activity of proteolytic enzyme at various hydrogen ion concentrations.

You realize that in order to develop the necessary quantity of carbonic acid we must have a sufficient amount of sugar to produce the carbonic acid by fermentation. This sugar can, of course, be mixed with the dough, and it is often done by an admixture either of cane sugar or of glucose. But the best way is to have the sugar formed directly from the starch in the dough itself, as it is consumed by the yeast, and it is therefore important that the dough—or the flour from which it is made—should contain a sufficient amount of the diastatic enzyme. Bakers have realized that the dough ought to contain suitable quantities of diastatic enzyme, and small quantities of malt are therefore often added to the dough. There are also a whole series of investigations as to the diastatic properties of the flour itself, but it was not until the publication of *Rumsey's* work, two years ago, that this question was given thorough consideration from all sides.

I will not go into details here concerning this fine work, which was published by the American Institute of Baking. I will merely mention, that *Rumsey* found a similar relation between pH and diastatic power to that found by *Adler* and other investigators in the case of malt diastase.

Diagram 6 shows *Rumsey's* diagram, taken from his work. At Carlsberg Laboratory, we have been working on the same problem and arrived at precisely similar results. You will see from the diagram that the diastase of flour has its optimum at pH = about 5, or a little less. And you will understand from this that in preparing the dough it is important not only that the flour should contain sufficient diastase, but also that the dough should be prepared with, or in the process of fermentation attain, a concentration of hydrogen ions at which the diastase can act as it should. You will see from

DIAGRAM 6


350
300
250
200
150
100
50
1
2
3
4
5
6
7
8
9
10
$p_{H'}$
Maltose in Milligrams

the curve that at pH = 6 the effect is barely half that at pH = 5.

I should like to take this opportunity of making just one critical observation on *Rumsey's* "Method of Measuring Diastatic Power of Flour." His well thought out and well worked out method I can accept in its entirety, but there is one point I cannot help noting, that his experiments are carried out at different concentrations of hydrogen ions, the concentration in each case being that which the particular sample of flour happened to possess. The result of this method is to determine not—in comparable units—the diastatic power, or as we might say the diastase content, but the manner in which this diastase content acts under the given circumstances, which is not the same for all experiments. In the 14 flour samples compared, the pH varies from about 5.6 (No. 1013) to about 6.2 (No. 1004). These two samples show approximately the same diastatic power, but there can be no doubt that the latter sample must contain far more diastase than the former, since the effect is far weaker at pH = 6.2 than at pH = 5.6.

In order to obtain really comparable results, we must correct the values found by means of the curve in Diagram 6, or else make use of a suitable buffer mixture in the course of the experiments. To my mind, the best way would be, before determining the diastatic power of the flour, to measure the buffer value which is also an important property of the flour itself. This would enable us to determine what amount of acid or base should be added for the determination of diastatic power, at, say, three different concentrations of hydrogen ions, so that we can draw a suitable portion of the curve which shows the diastatic power of the flour sample in question at different pH. This is the method we used at Carlsberg Labora-

tory, and in comparing different flour samples we used the values for diastatic power at pH = 6 or pH = 5.5.

You will, I hope, pardon this little digression. I have mentioned the point because I think it is of some importance that we should all work on the same lines wherever we happen to be, and thus be able to give the results obtained more weight and more exactitude.

In the fermentation of dough there are also other enzymic processes taking place, which I shall not have time to discuss at present. I should like, however, to direct your attention to fields of particular interest, and one which, as far as I am aware, has been very little investigated as yet. And that is, the question as to how, and to what extent, the protein of the flour is affected during fermentation of the dough by the proteolytic enzymes of the yeast.

Hydrogen Ion Concentration and the Physico-Chemical Properties of the Proteins in the Dough.

In wheat flour the substance known as *gluten* is far the most essential part of the nitrogenous ingredients. But apart from this there are also smaller quantities both of an albumin and a globulin, and of decomposition products of these substances.

Gluten which consists of a mixture, or perhaps rather a more or less loose association between two substances, gliadin and glutenin is of the very greatest importance in the manufacture of bread. It takes up water and forms a tough, elastic and extensible mass, able to retain the carbonic acid developed during fermentation and thus "raising" the dough. It is therefore very natural that the question of the relation between quantity and quality of the gluten on the one hand, and the

baking value, or strength, of the flour on the other, should have been made the subject of numerous investigations. According to recent work of *Gortner and Sharp*, and also *Woodman*, the gliadin seems to be of the same composition whether obtained from strong or weak flour, whereas the glutenin differs in character from one flour sample to another. There is therefore every reason to follow with the greatest interest the researches made with a view to studying alterations in the physico-chemical condition of the glutenin, the gluten and the entire dough, produced by alterations in the concentration of hydrogen ions for example. In this field of work, a great deal has already been done by American flour and bakery chemists, who are unquestionably the leading experts on this question at the moment.

I will not therefore go further into this side of the question, but will approach another side of it which, as far as I am aware, has not received the attention it deserves. I refer to the question of the coagulation and transformation of the proteins in the dough during baking, and especially to the part played by the hydrogen ion concentration in these pro cesses.

In a coagulation process of this sort we must be careful to distinguish between two different phases of the process, each of which is affected in a different way by the concentration of hydrogen ions. The one phase is the so-called denaturation of the protein by heating, which occasions a chemical change in the substance and often draws off water from it; sometimes also an incipient decomposition takes places simultaneously with the denaturation. The second phase is the flocculation or precipitation of the denaturated protein. In both of these processes, the concentration of hydrogen ions is a very important factor. But while denaturation and the accompanying de-

DIAGRAM 7A

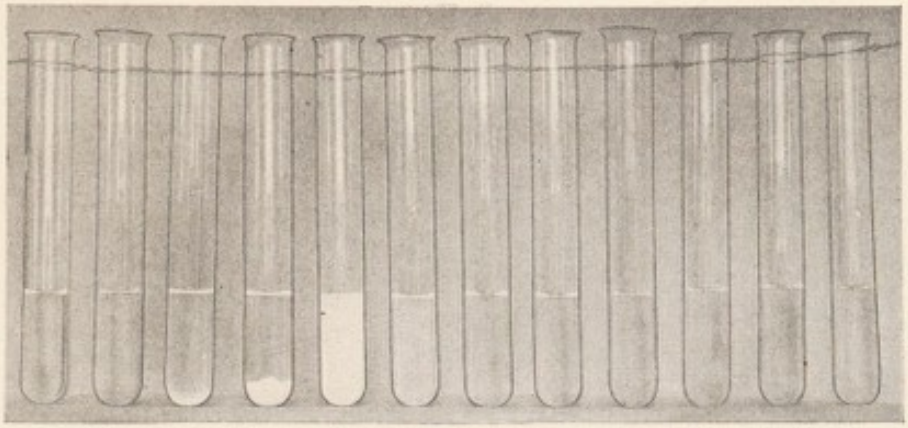


DIAGRAM 7B

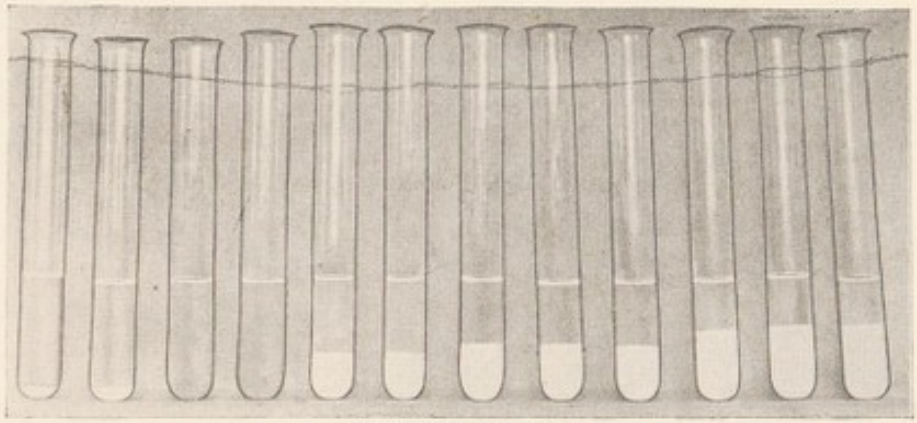
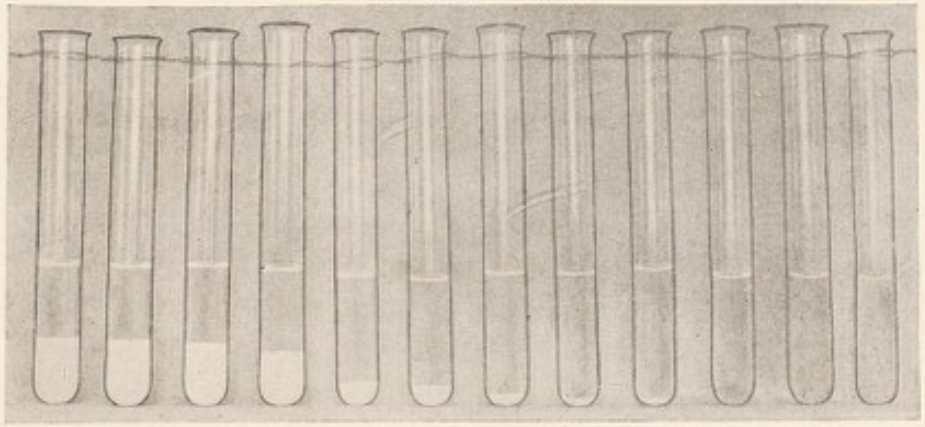


DIAGRAM 7C



composition proceed more rapidly and strongly the higher the concentration of hydrogen ions, it is quite a different matter with the flocculation.

There is for each individual protein a certain characteristic hydrogen ion concentration at which the denaturated protein is best precipitated, and in the least gelatinous form. This hydrogen ion concentration, which for albumins lies at about pH = 4.8, is called the isoelectric point of the protein, and the more the concentration of hydrogen ions diverges from this isoelectric point, the more difficult it becomes to precipitate the denaturated protein, and the more gelatinous it will be.

These features are of great importance in all transformations of proteins by heating. I am unable to illustrate them for you by referring to experiments made with the proteins of flour—I do not think any have been made—but I think it would be useful if such researches were made. I can, however, illustrate the position by describing an experiment we have made with another protein, pure egg albumin. In so doing, I will add a few remarks on the importance of this characteristic feature in connection with another industry, that of the breweries.

In each of the twelve test tubes in Diagram 7A, there are placed five cubic centimetres of a pure egg albumin solution containing forty to fifty milligrammes of egg albumin nitrogen and fifteen cubic centimetres of water and hydrochloric acid together, in such a manner, that the tube on the extreme left of the picture is given fifteen cubic centimetres of water without hydrochloric acid, the quantity of hydrochloric acid increasing until we reach the glass on the extreme right, which is given fifteen cubic centimetres of pure n/100 hydrochloric acid. After heating for fifteen minutes in a water bath, at a

temperature of sixty to sixty-five degrees, the samples present the appearance shown in Diagram 7A. Nos. 1 and 2 will be quite clear; there is a precipitate in Nos. 3 to 6, most in No. 4, while the rest have no precipitate, but only an opalescence, decreasing from left to right along the row. It looks, then, as if coagulation had only taken place in the middle samples, in which the concentration of hydrogen ions lies near the isoelectric reaction of egg albumin. Actually, however, the case is quite different. When the samples are filtered, Nos. 1 to 6 filter clearly and easily, the others with difficulty. On adding a buffer mixture (sodium acetate and acetic acid) at ordinary temperature, which brings all the filtrates to the optimal hydrogen ion concentration for flocculation corresponding to pH = 4.8, we get a precipitate in all the samples except Nos. 3 and 4 and most in the samples containing most acid, as shown in Diagram 7B.

As all the samples now have the optimal concentration of hydrogen ions for flocculation, all the denaturated egg albumin will have been precipitated, and if we now again filter the samples and heat the filtrates as before, any non-denaturated egg albumin still remaining in the filtrates will now be denaturated and precipitated.

Diagram 7C shows that this last heating forms precipitates only in tubes Nos. 1 to 9, and the quantity is greater in proportion as the samples have had less acid.

The position then, is this. The egg albumin in the most acid samples (those farthest on the right) was completely denaturated by the first heating, but no precipitation took place, because the concentration of hydrogen ions was far higher than the optimum for flocculation. On the other hand, the

sample which showed most precipitate on the first heating namely No. 4, was by no means completely denaturated, since it yields an abundant precipitate on the second heating. But all that was denaturated by the first heating was also precipitated (see Diagram 7B) because the concentration of hydrogen ions in this sample was at the optimum for flocculation.

Other things being equal, then, the rate of denaturation will be greater the higher the concentration of hydrogen ions in the solution, but the flocculation of the denaturated protein takes place only at the isoelectric point or near it.

In the boiling of the wort at a brewery it is of the highest importance that the coagulable protein should not only be denaturated, but also precipitated, as it would otherwise later on be deposited quite slowly, and thus cloud the beer. The brewer has therefore to see that the hydrogen ion concentration of the wort lies somewhere near the isoelectric point. Now the water used in brewing contains certain alkaline ingredients; the malt, on the other hand, certain acid ingredients, which neutralize them. During the war, when breweries were obliged to reduce the quantity of malt, while the quantity of water remained the same, the alkaline ingredients gained the upper hand, and the wort became too alkaline; the precipitation of protein therefore less complete, and the beer in consequence less able to keep for any length of time. This difficulty, however, was obviated by slight but systematic acidulation of the wort until it reached the desired concentration of hydrogen ions. The method was found so excellent in its effect on the keeping quality of the beer that it is still used in many places, even now that normal conditions have been restored.

This is a slight digression from my subject proper, but I wished to show you an example of how knowledge gained on

a purely scientific basis can be adopted and applied directly in the sphere of practical work.

I have the more reason for doing so, since practical bakers may very reasonably ask: What is the use of all these highly specialized scientific investigations to us? I grant at once, it would be ever so much simpler if we could, by a single line of research, find a method of determining the baking value of a given sample of flour. In this connection I would direct your attention to *Bailey's* fine and valuable work on the connection between the baking value of the flour and the extensibility of the dough.

On the other hand, the question of the baking value of a flour is one of so complicated a nature because the strength probably represents the result of the co-operation of many different factors, of which I have here touched on only a few. To get to the bottom of the question we shall, I think, first have to study the importance of each factor separately. And this means a great deal of work, and a great deal of time.

It will need, on your part, Gentlemen, as representing the practical side, a great amount of patience. And on the part of the scientific investigators, not only skill, but also perseverance. And finally, it calls for the most intimate co-operation from both sides.

It has been a great pleasure to me to see the way in which this co-operation has been taken up in America on a broader basis than anywhere else. And I believe, that in the case of such a comprehensive task as this, where individual effort would never suffice, co-operation is the only way.

Such co-operation as has already existed for some time past between Dr. Barnard and Dr. Morison at The American Institute of Baking and on the other side, Dr. Bailey at The

Division of Agricultural Biochemistry of the University of Minnesota is, therefore, a most welcome sign.

I have no doubt that this co-operation like other great American efforts—not least the important investigations of flour and yeast, commenced by Mr. R. L. Corby at The Fleischmann Laboratories—will produce a weighty contribution to the solution of the great and important question of the baking value of flour.

LOUIS PASTEUR

Address at the unveiling of the bronze bust of Pasteur,
gift of Helge Jacobsen, Vagn Jacobsen and the
Carlsberg Breweries, to the American
Institute of Baking, Chicago,
Sept. 16, 1924.

LOUIS PASTEUR

The great French chemist, Wurtz, in his well-known "Dictionary of Chemistry," published in 1869, prefaces in this manner: "Chemistry is a French science. It was created by Lavoisier".

The German author, Hermann Kopp, in his book "The Development of Modern Chemistry", published four years later, has expended more than fifty pages to prove that the claim made by Wurtz is not in accordance with reality.

Kopp's meaning is that chemical science has existed before Lavoisier's time, but that the latter has effected a radical reform therein.

There is certainly no doubt that Kopp is right, but there is just as little doubt that Kopp entirely misunderstands Wurtz's proud sentence. Lavoisier, "The Father of Chemistry", who during the French Revolution suffered death at the guillotine, has indeed not created chemistry, but he has revolutionized its principles, and he has established the foundation upon which modern chemistry is built.

If I should say to you today, "Microbiology is a French science. It was created by Pasteur," then neither would that be in accordance with the truth, but Pasteur has revolutionized microbiology. His life's work has been the basis for the whole of modern bacteriology and microbiology.

The gigantic edifice of these sciences rests on that firm foundation Pasteur has created—in a domain, where everything before then was unstable and baseless, and as far as these sciences and their practical application extend—from medicine, veterinary science, antiseptics, and hygiene to agriculture, dairy-farming and every kind of fermentation industry —so far has Pasteur's work left its mark on our daily life.

Louis Pasteur was born on the 27th of December, 1822, in the little town Dole in the East of France, but the family removed a few years later to Arbois, the home of Pasteur's childhood, to which during his whole life he was greatly attached.

His father, like his grandfather and great grandfather before him, was a tanner, and had taken part in the Napoleonic wars as a member of the valiant 3rd Regiment, called brave among the brave. Pasteur's mother was lively and of an easily animated disposition, while his father was of a reserved and somewhat melancholy nature.

Little Louis had a gift for drawing, and at one time art was seriously considered as his future career, to which his father was quite averse. Otherwise Louis was not an ordinary "clever boy"; as his biographer, Vallery Radot, says, his childhood might be compared to the almost hidden source of a mighty river.

Pasteur was trained as a chemist, and among his teachers and patrons, Dumas, Balard and Biot can bear special mention. His first task was therefore of a purely chemical nature, in that his doctoral dissertation dealt with the subject of arsenic. By far the most important of Pasteur's purely chemical or physico-chemical work is his famous separation of racemic tartaric acid into its two optically active forms. This work, published

in 1848, when Pasteur was only 26 years old, has been of the greatest significance for the development of organic chemistry. I will not go further into the matter, except to mention that young Pasteur himself felt that his observations were just as significant as they were surprising, for he asked his old friend Biot if he could do the experiments over again for him. It is well known how Biot doubted his young colleague, how he himself supplied the necessary chemical preparations, how he himself did most of the work according to Pasteur's directions, and how when the experiments completely proved the correctness of Pasteur's statements, he folded him in his arms exclaiming, "My dear boy, I have loved science so much during my life that this touches my very heart."

In the year 1849 Pasteur became Professor of Chemistry at Strassburg, where he won his bride, Maria Laurent, a daughter of the Rector of the University. They were married the same year, and Madame Pasteur throughout life was not only a most devoted wife but also her husband's faithful friend and co-worker.

Pasteur continued his studies of tartaric acids in Strassburg by taking up the action of the organism of fermentation on these substances. It was a happy chance, one might say, that caused Pasteur to give his mind to these studies which formed a link between chemistry and microbiology.

It was also a happy chance that as early as 1854 Pasteur was transferred to Lille as Professor of Chemistry and Dean of the new Faculty of Science, for thereby he came to live in one of the centres of the fermentation industry in France. And finally it was a happy chance, when in 1856 a Lille vinegar manufacturer instigated by his son, who was inspired by Pasteur's lectures, applied to Pasteur for advice concerning

serious difficulties in the manufacturing of vinegar. Pasteur obligingly complied, took up the task, and thus began the investigations which led to a complete revolution in microbiology and in the application of this science.

The position of fermentation industries was not an enviable one at this time. One had only experience and tradition to go by from the one generation to the other, but no one had any comprehension of what happened during the fermentation. The fixed methods of working generally gave the required results, but suddenly there might occur the most peculiar irregularities, which quite changed the character of the fermentation and occasioned great loss.

The fermentation industry could not expect help from science, as the prevailing theory—first and foremost defended by Liebig—held that the chemical actions taking place during fermentation and allied changes could be explained "in terms of molecular physics" alone, and without "direct co-operation" of yeast. In close connection herewith rested the whole view of the past on the question of "generatio æquivoca," the question of how far living bodies, or in a narrower sense, how far yeast or bacteria could generate "of themselves."

When it is known that in the fermentation of grape-must or beer wort, in the souring of milk, or the putrefaction of foodstuffs, a great number of microbes make their appearance, while it is not usual or possible to identify them in fresh bodies, it can be assumed that they generate "of themselves."

One of Pasteur's greatest achievements was to show that all the micro-organisms which are the cause of fermentation, putrefaction and a great number of diseases which we now call "infectious diseases" are to be found in the air, in the ground, in the water and so on, in short, in the outside world sur-

rounding us. They can therefore force themselves in everywhere and, under favorable conditions for their development, unfold their characteristic action.

On the other hand, if such micro-organisms are kept out, nothing happens; must does not ferment, milk does not turn sour, meat does not putrefy, because microbes cannot generate "of themselves." Fermentation, decomposition, putrefaction, all are "acts of life," and in the absence of life do not take place. A liquid really sterile will remain sterile forever, and, in that condition, will neither ferment nor putrefy. Germs are not exempt from the general law of life; they cannot come into the world without germs, without parents like themselves.

Pasteur's many and troublesome tasks in this domain were carried out partly in Lille and partly in Paris, to which place he was transferred in 1857. Pasteur's views met with violent opposition, and gave rise to excited debates in the French Academy, particularly in the Academy of Medicine. To many of the old doctors it seemed in fact to be bordering on sacrilege that a chemist and not a medical man should be teaching them the origin of disease. At a very early stage, about 1860, Pasteur became fully aware that it would be exceedingly desirable to make use of the experience he had reaped by the study of the fermentation organism in order to make the origin of the different infections evident.

Pasteur gradually overcame all opposition. He disarmed every objection by fresh experiments, which were carried out under the control of commissions appointed by the French Academy. Besides, which was of even greater importance, it came to pass that his theories could be carried out in practice.

He taught men of the fermentation industry that when the fermentation of vinegar, wine, beer, alcohol, etc. went wrong,

it was because the fermentation was "unclean," viz., that foreign ferments or bacteria, or in short, foreign micro-organisms, had forced their way into the fermenting liquid, and these foreign organisms developed the "disease" in vinegar, wine, etc., against which the manufacturers had to fight. He further showed them how these diseases could be prevented by keeping out the injurious foreign organisms as far as possible, and if they had got in, how they could be made harmless, killed by heating, through that process which in our time still bears the name "Pasteurization."

It was therefore very natural that when the silk crop in France was threatened with destruction on account of the steadily increasing attacks of silk worm disease that Pasteur was applied to for help. This was in the year 1865. Pasteur took up the question at once and with it came to the last and indeed the most significant stage of his life's work, the pathological researches.

From 1865 to 1869 Pasteur was grappling with this problem. It was complicated by the fact that there were two diseases, pebrine and flachery, to be investigated, and the hostility or scepticism of the very people whom his discoveries would benefit increased the difficulty. Pasteur identified the two diseases, proved them to be contagious as well as hereditary and he showed how to detect and stamp them out. Even in March, 1869, the silk trade was still only half convinced of the absolute value of Pasteur's results. "They are absolute," he insisted, and to prove his point, sent to the sceptical Lyons silk commission four lots of seeds. "Lot 1 is healthy," he said, "and will succeed; lot 2 will die of pebrine only; lot 3 will die of flachery only; and out of lot 4 some will develop pebrine and some flachery," and everything he predicted came to pass. "That is," says one of his biographers, "the story of how the

silk trade was saved by an "unpractical" man of science."

I will only add that this involved a considerable sum of money, as the disease in the course of a few years had reduced the proceeds of the silk crop from 100 million francs to less than 10 millions, and as it was in France, so was it in other silk producing countries.

It was a characteristic of Pasteur's research that with impressive ease and ingenuity he so adapted his great scientific discoveries that they could be made use of in practical life. He is an ideal representative of co-operation between science and practice. We have seen that his purely scientific, chemical and biological investigations with regard to fermentation and diseases of the silkworm gave results of the greatest importance to practical industry.

With the same peculiar ability Pasteur was able to investigate the infectious diseases of animals and the microbes which were the cause of the diseases and to make use of these investigations to combat them. His research in this went on without intermission, and in the space of a few years (1880-1883) communications were issued by Pasteur regarding methods for battling against three widely-spread and considerable diseases, chicken-cholera, anthrax or splenic fever, and red sickness—a contagious disease of the pig.

These discoveries aroused the greatest sensation everywhere, not only in scientific circles, but also in the minds of the public, as many hoped that it would be possible to combat human diseases in the same way. The hope was not disappointed, for in 1884 at an international congress of doctors held in Copenhagen, Pasteur produced results which substantiated the possibility of an effectual treatment of one of the most dreaded diseases of humanity namely hydrophobia.

All modern vaccine and serum treatment of a number of infectious diseases among human beings and animals is in reality a direct consequence of Pasteur's work, which opened the way. Let us consider a little more in detail Pasteur's investigation of anthrax and hydrophobia.

The anthrax or splenic fever is a dreaded disease of sheep and of cattle, which at that time—it was in the year 1877—was destroying five per cent of the cattle and ten per cent of the sheep in France. The principle which Pasteur applied to combat this, and the other infectious diseases mentioned, was similar to that already previously applied by Jenner against smallpox. Just as Jenner, by the inoculation of cow-pox rendered a person safe against the actual smallpox, so Pasteur protected an animal against a really severe attack of the disease by inoculation with contagious matter which had been weakened in some way, and which only developed such a mild form of the illness that it presented no danger. In this Pasteur was successful in the highest degree.

To begin with, he conducted a number of splendid investigations as decisive evidence that the cause of anthrax disease was the stave-formed bacteria, *Bacillus anthracis,* also observed by former scientists.

Pasteur followed this up by showing that with the help of heat, by cultivating this microbe some few degrees above the temperature of the blood, a weakening of the anthrax culture could be attained, a weakening, the process of which one could follow by inoculating animals of different size—white mice, guinea-pigs and rabbits.

While an unenfeebled culture by inoculation under the skin had a deadly effect on all the three classes of animals, a culture held at a temperature of 42°–43° C, would gradually lose its

virulence and thus its deadly capacity, first for the rabbits, next for the guinea pigs and lastly, with a sufficiently long treatment, for the mice.

With such an enfeebled culture Pasteur vaccinated sheep and cattle against anthrax, and it is well known how, with an experiment planned on a large scale in 1881 he swept away all doubt as to the correctness of his views.

On a farm close to Melun there were collected altogether 58 sheep, 2 goats, and 10 horned cattle. The experiment began on the 5th of May, 1881. Half of the animals were inoculated with a greatly weakened anthrax culture, while the other half which should serve as means of control, were not inoculated. Twelve days later, on May 17th, the vaccinated animals were inoculated afresh, but this time with a somewhat less weakened culture than the first. This finished the vaccination, and a fortnight later on May 31st its effect was tested, in that all the animals, vaccinated and unvaccinated, were inoculated with a fresh strong poisonous culture of anthrax bacilli. Some days must pass before the disease broke out, and the experiment was followed with thrilling interest, both by Pasteur's partisans and opponents. Already on June 2nd, Pasteur could show the result of the experiment, before the large interested circle that had streamed to the trial farm, and this result left nothing to be desired in the way of plainness. All the vaccinated animals were perfectly healthy, without a sign of disease; the unvaccinated sheep and goats were all dead except 3, which died in the course of the day; the unvaccinated cows were certainly not dead, but they were all very ill, very feverish, and were very much swollen.

I shall not dwell on the immense significance in purely economic respects effected by Pasteur's anthrax vaccination inves-

tigations. I shall only mention an enlightening fact, that according to the statistics up to the year 1900, over 11,000,000 animals were vaccinated.

The best known and most conspicuous research of Pasteur is his battle against rabies or hydrophobia. It will be impossible here to go into details concerning these experiments which were begun in 1880 and which were of a particularly difficult nature, because Pasteur did not succeed, and neither has anyone else succeeded, in isolating the causative micro-organisms. The experiments were made still more difficult by the long incubation of the disease, as several weeks elapse from the time a person or a dog is bitten until the sickness breaks out; and by the extraordinary large mortality from the disease. In spite of all difficulties, however, Pasteur succeeded in finding a mode of procedure by which he could make a dog immune to the disease, and, also prevent the sickness from breaking out, even if the treatment had first been begun after the dog in question had been bitten by a mad one.

Pasteur, however, was fearful of applying his results on a human being, even if he felt ever so safe in his treatment of dogs. "I take two dogs," he wrote in September, 1884, "I have them bitten by a mad dog. I vaccinate the one and I leave the other without treatment. The latter dies of rabies, the former withstands it, but however I may multiply my cases of protection of dogs, I think that my hand will shake when I have to go on to mankind."

This anxious pondering came to an abrupt end when on the 6th of July, 1885, a mother with her nine year old, Joseph Meister, came to the laboratory. The boy had been bitten two days previously by a rabid dog, and the mother begged Pasteur to save the child's life. After most serious consideration,

Pasteur complied, and his anxiety and uneasiness heightened from day to day, according as the vaccine employed became stronger and stronger, but all went well, and this first human case was a complete success. Shortly after, a young shepherd, Jupille, was treated. He had rescued some children from a mad dog, and had been himself badly bitten before managing to kill it. To make matters worse, he arrived in Paris six days after the attack; yet inoculation saved him also.

The news of the successful treatment of these two cases rang through the world and in the next six months 350 patients were treated. The only failure was a little girl, Louise Pelletier, who was brought for treatment thirty-seven days after being bitten. In March, 1886, nineteen Russians, badly torn and mutilated by a mad wolf, were brought by their doctor to Paris. The bite of a mad wolf is even more deadly than that of a mad dog, and it was fifteen days since the wounds had been inflicted. It was almost certainly too late, but Pasteur could not deny these forlorn fellow creatures even the faint hope of life that his treatment offered. Of the number, only three died—a really surprising result, considering the conditions.

Conviction of the value of inoculation against rabies grew steadily in the minds of unprejudiced doctors and men of science, and a commission appointed by the Academy of Science unanimously voted that subscriptions should be asked for in France and abroad to found a "Pasteur Institute" in Paris for the preventive treatment of hydrophobia.

It is well known what a success this subscription was, and it is equally well known how the "Pasteur Institute" opened in Paris in 1888, was followed by the establishment of similar institutes in other parts of the world, which have continued Pasteur's fight against diseases of widely different natures, and which have been a blessing to mankind.

Pasteur was a marked man himself when he moved into the large new institute which bears his name, and he was never able to take an active part in the new laboratory. In 1868 he had had a serious apoplectic stroke, the after-effect of which never left him. He never gave up, however, but continued his beneficial work as long as he could, with the usual "Pasteur" energy and sense of duty. In this respect it is characteristic of Pasteur's view of his work for the welfare of humanity that when a colleague once spoke to him of the danger connected with some investigations he carried out during an epidemic of cholera, and exclaimed "Courage is needed for this sort of work," Pasteur's simple answer was "and the sense of duty."

On September 28th, 1895, Pasteur passed peacefully away. He rests in a crypt under the main building of the Pasteur Institute above the entrance of which is written in gold the few words, "Here reposes Pasteur."

In conclusion, if we should ask what to our knowledge gives the name of Pasteur such a special lustre, I do not think we would give most preference to the great discoverer who has paved new roads in science. Neither do I think we would give our attention to the exceedingly great practical significance the work of Pasteur has had for industrial life. I think that what would be first and last with us would be the rich and warm humanity in the whole of the blessed realm of Pasteur's life work. As the great benefactor of humanity will Pasteur's name be preserved throughout ages. It will be able, like a standard emblem, to gather together all races and all classes to peaceful co-operation for the good of humanity.

Let then this bust of Louis Pasteur we unveil today be as a symbol, that also this branch of the fermentation industry housed within these walls is built on the foundation Pasteur has created. Let it stand as a symbol that the work accomplished

here be in the same spirit as Pasteur's, and in reverence to his memory.

INFORMAL ADDRESS

Before the Chicago Chemical Club
Chicago, October 13, 1924

CARLSBERG LABORATORIES

Allow me to express my heartiest thanks for the kindly words which Mr. President has addressed to Mrs. Sørensen and myself. The reception which has been prepared for us in America, not least in the circle of American Chemists, has touched us very much. Quite especially, I express my cordial thanks to the President and the Board of The Chicago Chemical Club, and to all present, for the welcome opportunity given me to make the acquaintance of so many of the chemists of America.

I need not say, that I have had the warmest interest and the greatest pleasure visiting the excellent institutes and the extremely well equipped laboratories in your country. This was not unexpected, but I may say, that what I have seen has greatly surpassed my expectations.

However there is another matter which weighs upon my mind and in regard to which I hope to profit by my visit in the United States. I refer to the question of co-operation between science and practice. Several times in lectures at home in Denmark, I have strongly emphasized the importance and necessity of the most intensive co-operation between theory and practice, and it has greatly interested me to see how far you have accomplished this in America.

Naturally, I speak especially about that branch of industry in which I have had some experience, the fermentation

industry. I have had the best opportunity of closely studying your technical scientific institutes for the investigation of all the substances and all the processes which have any significance for the manufacture of flour and bread—this branch of the fermentation industries which only recently has taken up the rational and systematic co-operation between science and practice.

I admire your Institute of Baking in Chicago, as well as The Fleischmann Laboratories in New York—and I venture to say that America is in this respect better equipped than any other country, both with regard to its scientists and its laboratories, and the organization of their work.

I believe that this state of things is not without relation to a fact which often meets us in America. Your great business men desire to make a great deal of money, but when they have gained this money they also know the old saying "Noblesse oblige," [Rank imposes obligation]. It is of the greatest importance for your science, for your industry and for the progress, welfare and prosperity of this country, that it is so. I know how to fully appreciate this, because The Carlsberg Institution in my homeland is founded upon the same basis.

I have had several inquiries about the nature and character of The Carlsberg Laboratories, and therefore I think it will not be amiss at this time to tell you a little about the Carlsberg institutions.

The Carlsberg Brewery in Copenhagen was founded in 1847 by the brewer, Captain J. C. Jacobsen, and later on enlarged by union with "The New Carlsberg Brewery," founded by his son, the "young" Jacobsen, Carl Jacobsen. A son of Carl Jacobsen, Vagn Jacobsen, is now a Director of The United Carlsberg Breweries, the managing Director being P. C. Poulsen.

The "old" Jacobsen, the Captain, was a remarkably clever and industrious man, interested not only in his business, but also in science and art, in humanism and politics, and he rendered his country and his city the greatest services.

His most important contribution was the foundation of The Carlsberg Fund and, as a part of this, The Carlsberg Laboratories were established in the year 1876. The initial fortune of The Carlsberg Fund consisted of one million Danish Crowns, but by several gifts of Jacobsen this fortune was greatly increased, and finally at his death, he bestowed upon The Carlsberg Fund the property in full of the Carlsberg Brewery itself. Later on his son, the great lover of the arts, Carl Jacobsen, gave the New Carlsberg Brewery to The Carlsberg Fund, so that for the present, The Carlsberg Fund is the owner of a capital of about twenty million Danish Crowns and of the United Carlsberg Breweries.

Properly speaking The Carlsberg Fund is given to, and belongs to our Academy of Science in Copenhagen. The Board of Directors of the Fund are chosen by "The Academy of Science" from among its own members. Jacobsen took these measures to secure for the Fund a governing body elected upon a purely scientific basis.

The income of the Fund after deducting expenses for purposes of enlargement and stabilization is divided into four parts according to certain rules: part A is used for the management of The Carlsberg Laboratories; part B serves as a fund for further scientific research work in general; part C is used for our national historic museum at Frederiksborg Castle, (this museum was also founded by J. C. Jacobsen); part D is reserved for the "Carlsberg Glyptotek" founded as a museum of art by the young Jacobsen and now directed by his son, Helge Jacobsen.

The Carlsberg Laboratories themselves are divided in two departments, the physiological and the chemical department.

The first Director of the Physiological department, Emil Chr. Hansen [1876-1909] is well known by his important research work on yeast, especially on the cultivation of pure yeast. His successor, my colleague for the present, Dr. Johannes Schmidt, is known particularly for his investigations on the migration of the eel.

The first director of the chemical department was Johan Kjeldahl [1876-1900], whom you all know as the father of the famous method for the determination of nitrogen after Kjeldahl. Since his death in 1900, I have been the director of this department.

You will note from this short survey of some of the work done in the Carlsberg Laboratories, that the field of our researches is not limited to the fermentation industries. The Carlsberg Breweries themselves have their own working laboratory.

However, even if we at The Carlsberg Laboratories have nothing directly to do with the daily work at the Breweries, we are—if I may say so, proud of our common name. We feel a part of the whole Carlsberg Institution, which has had and steadily has so great a significance for the development both of science and of industry in our little country and for the co-operation between them both.

Let me then finally—apologizing if I have spoken too long—express the wish, that the excellent co-operation between science and practice you have established here in America on a broader basis and on a larger scale than I have seen anywhere else, may always be inspired by the true scientific spirit for the benefit and usefulness of your industry and for the honor of American Science.