THE BINDING OF ACIDS AND BASES BY GLUTENIN.

A THESIS

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MANUEL SHAW, B. Sc., (Hons.)

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K. S.

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INTRODUCTION

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Among the great number of chemical substances existing in nature, none offer as peculiar a problem for research as do the proteins. There can be no direct application of such basic chemical laws as the law of mass action or Avogadro's hypothesis. Neither can synthesis be utilized in the study of this complex group of substances, nor can the standard preparation of derivatives be carried out in the study of chemical structure. Suffice to say that when the pure mathematics of thermodynamics cannot be applied directly to protein study, there is created a mass of controversial evidence based mainly upon hypothetical assumptions.

Enhancing the peculiarity of protein research is the fact that purely chemical methods cannot be applied with disregard of physical methods. Neither can physical study be employed without the inclusion of the pure laws of chemistry. In illustration of this, proteins fall into that division of chemical matter known as colloidal. As colloids they can therefore be studied in respect to their surface properties, such as osmosis, viscosity and surface tension. However, it is definitely known that the chemical groupings inherent in the protein itself have also an imposing influence upon its environment.

For the above reasons the literature on protein research has become voluminous and diversified. Occuping a large portion of this literature is the study of proteins in their reactions with acids and bases. It is largely in this terrain that the physical and chemical forces meet with controversial data, for

there are three separate factions each offering a description of the reaction between proteins and acids or bases. One faction led by such men as Jacques Loeb, E. J. Cohn, and D. I. Hitchcock, argue that proteins combine with acids or bases in definite and measurable proportions. A second group, of whom Wilder D. Bancroft is the main supporter, is of the opinion that acids and bases are bound by proteins in a manner that can only be explained by the physical phenomenon of adsorption. W. F. Hoffman and R. A. Gortner express the third view that both physical and chemical factors must be included to explain satisfactorily the reaction of proteins with acids and bases.

Contributing to these opposing views are the individual and different methods of attack. Thus Loeb employs titration to substantiate his claims, whereas Bancroft bases his opinions on the results of Phase Rule studies. The success of scientific research depends upon the fact that the theoretical explanation of any particular problem can be firmly established only when different workers employing different methods obtain results that justify the same conclusions. It is therefore in this demand that the study of acid or base binding by proteins fails to a considerable extent.

In later researches however, it was attempted to enhance the study of acid and base binding by proteins under varying conditions in the hope that the added information would contribute to the knowledge of the acid-or base-protein system itself. A paper published by A. D. Robinson, R. A. Gortner, and L. S. Palmer (1932) on "The Effect of Salts on the Casein-Sodium Hydroxide and Paracasein-Sodium Hydroxide Equilibria" applied the

principle originated by the work of I. M. Kolthoff and W. Bosch on the influence of neutral salts on acid-base equilibria, to the protein system on the basis that proteins, being amphoteric, possessed both acidic and basic properties. Their method of attack was to study the increased binding brought about by neutral salts to determine whether this increase was due to adsorption or to chemical combination or to both.

It was deemed desirable to repeat the work of Robinson, Gortner, and Palmer, employing a new protein, and to study as well what effect temperature has upon its alkali-binding. Because glutenin is a simple protein, thus eliminating the possibility of the presence of mineral acid split off due to hydrolysis as might be the case with the phosphoprotein, casein, and because its reaction with acids and bases has not been studied as extensively as have those of other proteins, it was chosen for this work.

II

HISTORICAL

HISTORICAL

Part I

BINDING OF ACID AND ALKALI BY PROTEINS

In view of the great accumulation of literature on research of the protein-acid or -alkali system, a comprehensive review cannot easily be given here, and indeed has no place in writing of this sort. Among others, Loeb (1924), Hoffman and Gortner (1925), and Bancroft and Barnett (1930), present a sufficiently concise summary of this field of work. In their review, Hoffman and Gortner mention that it was Platner, who in 1866 stated for the first time that individual proteins have the property of combining with both acids or bases. Substances having this property are now referred to as being "amphoteric".

A.

Methods of Measuring the Binding of Acid and Alkali by Protein

Whatever the mode of combination may be, it has been firmly established that reaction between proteins and acids or bases certainly exist which cause evident changes in the reactants. When methods were ultimately introduced which permitted the measurement of hydrogen ion concentration, undeniable evidence was obtained which showed that a certain amount of hydrogen ions in the case of acids, and hydroxyl ions in the case of bases, was removed from free activity in the presence of a protein. Prior to the introduction of physico-chemical methods, however, various other means were devised for the quantitative determination of the acid and alkali binding of proteins.

One of these methods applied the use of indicators, but

this proved unsatisfactory and misleading. Being amphoteric substances, proteins gave a very poor indication as to the exact point of neutrality. Another drawback was that only a very meagre representation could be obtained of the acid or base binding by the protein, since the only concentration that could be measured was that at the indicator change. Van Slyke and Hart (1905) found that widely different results were obtained according to the indicator used. Thus 1 gram of casein was found to neutralize 55 x 10^{-5} gram equivalents of alkali when litmus was employed. However using phenolphthalein, 90 x 10^{-5} gram equivalents were required. Another fault of indicators lies in the fact that most of them are either acids or bases and therefore they may combine chemically with the proteins.

Another method employed frequently was the precipitation or coagulation of protein salts by the use of alcohol or other precipitants. It has since been shown by Robertson (1910) that the coagulation of alkaline caseinates through the addition of alcohol is preceded by a decrease in the degree of dissociation of the protein complex. He also pointed out (1910) that by using potentiometric methods, twice as much alkali was bound per gram of casein as that calculated by Spiro and Pemsel (1898) from experiments where ammonium sulfate was used to precipitate the sodium caseinate.

Solubility methods, in which the combination has been reported in terms of the amount of acid or alkali bound when the acid or alkali is "saturated" with the protein, have been reported. Such values are obtained when an excess of the insoluble protein is added to the aqueous system. Thus Robertson (1910) found that 1 gram of casein required 32 x 10^{-5} gram equivalents

of hydrochloric acid and 11.5 x 10^{-5} gram equivalents of alkali.

Hoffman and Gortner (1925) mention that the existence of protein salts has been demonstrated by the solution of otherwise insoluble substances. They refer to the work of W. A. Osborne who showed that casein, when treated with calcium carbonate, carries a definite proportion of the calcium into solution in the form of soluble calcium caseinate with the evolution of carbon dioxide. They refer also to a method employed by Cohnheim and Krieger in which phosphotungstic acid is used in determining the acid binding capacity of proteins. This method is based upon the fact that phosphotungstic acid forms insoluble salts with proteins. If the calcium salt of phosphotungstic acid is combined with protein and acid a double decomposition is said to take place in the following manner:

Protein hydrochloride + Calcium phosphotungstate

↓

Protein phosphotungstate + Calcium chloride

The amount of hydrochloric acid bound is then calculated from the quantity of calcium chloride present in the filtrate.

The measurement of the electrical conductivity of a protein solution to which is added varying amounts of acid or alkali was the first instance of the application of physico-chemical methods to the study of acid and base binding by proteins. Hoffman and Gortner (1925) relate that Sjöqvist in 1895 and Lagueur and Sackur in 1903 were the first to apply this method of attack. Since the conductivity of protein-acid or-base systems is appreciably lower than that of the free base or acid, a measure of the change in specific conductivity will give a measure of the amount of acid or alkali bound.

Greenberg and Larson (1935) employed the electrical conductivity method in the study of casein, edestin, and gelatin. They found that the conductivity of these proteins in anhydrous lactic and glacial acetic acids was not much greater than that of the acids themselves. However, the conductivity of the solutions of these proteins in anhydrous formic acid was nearly equal to that of the alkali and alkaline earth formates in formic acid. From these observations they concluded that the proteins in formic acid had formed ionizable salts having electrochemical properties that would be expected of formic acid salts of rather high valency.

It is also mentioned by Hoffman and Gortner (1925) that Bugarsky and Liebermann were the first to employ cryoscopic methods in measuring the binding of acids and alkali by proteins. In their experiments these two workers found that the difference between the freezing point of a solution of 6.4 grams of egg albumin in 100 cubic centimetres of 0.05 normal hydrochloric acid or sodium hydroxide, and that of water was reduced approximately 50 per cent. This indicated a decrease of nearly 50 per cent in the total number of ions plus molecules. This was compared with the temperature effect of a protein-salt solution in which there was no appreciable depression of the freezing point. Since this method involves the necessity of maintaining a temperature in the vicinity of the freezing point, a true protein-acid or -base relationship at room temperature cannot be obtained.

Methods involving velocity of reaction have also been employed in the study of acid and alkali binding of proteins. Hoffman and Gortner (1925) refer to the methods used independ-

ently by Hoffman and Cohnheim in which the measurement of the concentration of hydrogen ions in solution was accomplished by determining their catalytic effect upon the inversion of cane sugar. Hoffman and Gortner also describe the methods of Hardy in one instance and Winton and Krüger in another, in which the rate of saponification of methyl acetate by hydroxyl ions was studied in a measurement of the amount of alkali bound by protein.

Almquist and Greenberg (1931) employed optical methods, in which the determination of the rotary power of protein solutions gave a measurement of acid and alkali binding.

The potentiometric method of measurement of the acid or alkali binding capacity of proteins was introduced, as is related by Hoffman and Gortner (1925) by Bugarszky and Liebermann in 1893, and is the method that was chosen for our work. Being up to date the most satisfactory method, many papers have been published on the protein-acid or -base system in which measurements were obtained from electromotive force results. From the measured pH_g the amounts of unbound acid or base may be calculated, and the amounts bound be determined readily by difference.

B.

CALCULATION OF ACID AND ALKALI BINDING BY PROTEIN

Various formulae have been originated by which the amount of acid or alkali bound by protein may be calculated, and they all include the concentration of the hydrogen ion which can be measured directly by the potentiometric method. Using the latter method, Manabe and Matula (1913) found that the amount of hydrochloric acid bound by a definite amount of protein increases

to a maximum at which it remains even if more acid is added. Hoffman and Gortner (1925) mention that Blasel and Matula introduced the following formula for calculating the amount of acid or alkali bound by protein:

$$n = N - \frac{cH}{\alpha}$$

where n = the amount of acid or alkali bound,

N = the original normality of acid or alkali,

cH = the measured hydrogen ion concentration of the protein-acid or -base solution at equilibrium,

α = the dissociation constant as calculated from specific conductivity data.

Pauli and Hirschfeld (1914) employed the same formula as that used by Blasel and Matula and obtained results that agreed with those of Manabe and Matula in regard to the maximum of acid binding by protein, which can take place only in the presence of excess acid.

Lloyd and Kaye (1922) in studying the binding of hydrochloric acid by gelatin, used the formula

$$n' = N - \frac{cH\ corr.}{\alpha}$$

where n' = the normality of acid bound,

N = the original normality of the acid,

cH corr. = cH x cCl

α = degree of ionization as determined from conductivity data.

Lloyd and Kaye were of the opinion that their formula gave a better approximation for cH than did the one used by Blasel

and Matula. c_H is determined electrometrically, and c_{Cl} is obtained by assuming that the gelatin chloride is completely ionized so that $c_{Cl^-} - c_H = n$.

Robinson, Gortner, and Palmer (1932) recount the opposing existant views with regard to the question of chloride ion binding by protein. Proctor and Wilson assume that none of the chloride ions are bound, while Bugarszky and Liebermann show that almost as many chloride ions as hydrogen ions are bound. Hanabe and Matula (1913) show that only a small quantity of chloride ions are bound at lower concentrations of acid, but the binding increases with increasing acid concentration until at the higher concentrations of acid an equal amount of hydrogen and chloride ions are bound.

Hoffman and Gortner (1933) have shown that the values for α as used by Lloyd and Mays bear a marked difference with those obtained by them from potentiometric measurements. Therefore Hoffman and Gortner argue that values for the degree of ionization as determined from conductivity data cannot be applied to the formulae for calculating the amount of acid or alkali bound by protein when potentiometric methods are used. On the basis of this view they offer a correction to the formulae of Blasel and Matula and Lloyd and Mays respectively. That is, the corrected Blasel and Matula formula will be

$$n = N - \frac{c_H}{\alpha'}$$

and that of Lloyd and Mays

$$n' = N - \frac{c_H \text{ corr.}}{\alpha'}.$$

where α' is the degree of ionization as determined from potentiometric data.

By means of the hydrogen electrode, Hitchcock (1922) determined the pH values of various concentrations of gelatin-acid solution, and then estimated the amount of acid which was necessary to give these same values of pH. Subtracting the former from the latter he was able to calculate the amount of acid bound at various pH levels.

Robinson, Gortner, and Palmer (1932) calculated the amount of sodium hydroxide bound by paracasein by the method evolved by Cohn and Berggren in which the use of the activity coefficient is introduced. Since this is the method of calculation employed by us, a detailed account of it will be given in the section on "Apparatus and Methods."

C.

Types of Combination of Acid and Alkali with Proteins

The question of whether the combination of alkalies and acids with proteins is one of a chemical nature, or one that is purely adsorptive has not yet been satisfactorily settled, even though years of research have been spent in studying the problem.

As a propounder of the chemical viewpoint of protein-acid or -base combination, Loeb (1922) emphasizes that above the isoelectric point a protein can combine chemically, and only chemically, with cations, and below the isoelectric point combination takes place only with anions. He gave proof of this belief in showing that gelatin, which has an isoelectric point whose pH is 4.7, combined with the Ag cation of silver nitrate only at pH values greater than 4.7, and with the anion $Fe(CN)_6$ of potassium ferrocyanide only at pH values less than 4.7.

Thus a protein in sodium chloride solution will form a sodium proteinate as soon as the pH of the solution is greater than the isoelectric point of the protein, and a protein chloride when the solution exists at a pH value lower than the isoelectric point of the protein.

According to Loeb, the quantity of anion or cation in combination with protein is always the same for the same pH, with a given mass of isoelectric protein. From this it follows that when acid is added to isoelectric protein an equilibrium is established between free acid, protein salt, and isoelectric protein. When an alkali is added, the equilibrium established is between metal proteinate, non-ionized protein, and hydrogen ions. Combination between proteins and acids is analogous to that between acids and ammonia; combination between alkalies and proteins compares with that between acetic acid and alkali.

It is known that a weak dibasic or tribasic acid gives off one hydrogen ion more readily than both or all three, as the case may be, while in a strong dibasic acid such as sulfuric, both hydrogen ions are held with a sufficiently small electrostatic force as to be easily removed. Loeb argued that if forces which determine the reaction between these acids and proteins are purely chemical, it would follow that three times as many ccs. of 0. 1. normal phosphoric acid would be required to bring 100 ccs. of a 1% solution of isoelectric protein to a given pH, as are required in the case of nitric or hydrochloric acids, while twice as many ccs. of 0. 1. normal oxalic acid as of nitric acid would be required. He showed this to be the case with gelatin and egg albumin, and concluded therefore, that the ratios in which ions can combine with proteins are identical with the

ratios in which these same ions can combine with crystalloids. From this it follows, that the forces by which gelatin and egg albumin combine with acids or alkalies are the purely chemical forces of primary valence.

Loeb treated granulated gelatine with salt solutions and concluded that no salt was formed at the isoelectric point, while sodium gelatinate was formed at a pH higher than 4.7, and gelatin chloride at a pH lower than the isoelectric point. Bancroft (1922), in reviewing Loeb's work, states that the latter overlooks the fact that there is no proof of the formation of either the sodium or protein salts, and that the same results would be found if he had postulated adsorption of hydrogen and hydroxyl ions instead of formation of sodium gelatinate and gelatin chloride. Bancroft uses this same argument in discussing Loeb's conclusions as to the ratios in which ions combine with proteins, remarking that again such results would follow if it were assumed that the hydrogen ion was adsorbed. He emphasizes that such experiments as carried out by Loeb do not enable one to distinguish between the two conflicting hypotheses. Bancroft mentions that Loeb's work assumes that the chemistry of protein solutions is merely the orthodox chemistry of hydrolyzed salts, and he asks why orthodox methods are not used to show the existence of these salts.

Calugareanu (1907) found that the electrical conductivity of solutions containing acids and protein was less than that of similar solutions containing no proteins. A curve, resembling the adsorption curve, was obtained by plotting bound acid against the non-bound acid. However, a similar curve was obtained when glycine was substituted for protein, and Calugar-

eann therefore concluded that adsorption in the case of proteins was doubtful, the form of the curve being due in large effect to the hydrolytic products of the reaction, which were formed in the mixture, protein and acid.

Results obtained by Weber (1927) pointed to a chemical linkage between protein and acid, rather than mere adsorption. He found that the hydrogen ion binding reached a well-defined end point beyond which the acid binding could not be increased by further addition of acid. A negative temperature coefficient was not observed for the acid binding by insoluble protein, and the titration curves for insoluble protein showed no deviation from those of soluble protein. It was found that the hydrogen ion binding followed the same curve as the increase in free carboxyl groups.

Lloyd (1923), supporting the chemical theory of acid and alkali binding by protein, stresses that while it is true that the adsorption equation fits the acid titration curve of the proteins nearly as well as the stepped curves drawn through the experimental points, the divergencies from the adsorption curve are nevertheless real since they have been repeatedly found at the same pH values by different workers. She mentions that the alkali titration curve shows even more marked divergencies from the adsorption equation.

Hardy (1907) agrees as to the chemical action of proteins with acids and bases, but emphasizes that the protein ion does not resemble a true ion in that no valency can be assigned to it. He originates the expression "pseudo-ion" and suggests that this terminology be given to the protein ion. Wood and Hardy (1909) admit that proteins react with acids and alkalies

to give salts, but state that the reactions are not precise, as an indefinite number of salts may be formed.

In the study of the acid binding capacity of haemoglobin, Cohn, Green, and Blanchard (1937) obtained evidence which suggested the existence of 99 basic amino groups in the haemoglobin molecule. Analysis of the hydrolysates of haemoglobin showed 85 such groups, or 93, when the method of nitrogen distribution was employed. It was suggested that this discrepancy was due to the splitting of the haemoglobin molecule whereby haeme was set free, which accounted for, in part, the excess acid combining capacity. In a study of the acid combining capacity of globin and applying corrections, results were obtained which showed the presence of 92 free basic groups, agreeing very closely with the original number of 93.

Although many workers adhered to the chemical explanation of the reactions between proteins and acids or bases, still many others objected to this hypothesis, some denying any existence of chemical phenomena at all, and others insisting that the existence of adsorptive forces should not be wholly disregarded by the adherents of the chemical view.

Bancroft and Barnett (1930) were of the opinion that the methods employed in the past for studying the binding of acids and bases by proteins were unable to discern between chemical and adsorptive forces. They decided that only by studying the problem from the point of view of the Phase Rule, could a clear insight into the type of combination between proteins and acids or bases be obtained.

The phase rule can be stated in the equational form:

$$F = C - P + 2$$

C represents the number of components in the system, and since we are dealing with a protein-acid or protein-base system, there will always be two components. P represents the number of phases which can co-exist in the system, and F, the degrees of freedom, or the variability of the system. That is, F refers to the number of available choices of the independently variable factors, such as temperature, pressure, or concentration. Suppose we have a system which consists of two components present in two co-existing phases, then from the phase rule equation

$$F = 2 - 2 + 2$$

or

$$F = 2$$

That is, there are two degrees of freedom which implies in effect, that we are able to make any two choices of temperature, pressure, and concentration, and still retain the system of 2 phases. Thus if we consider the temperature constant, we are still able to set a value for the pressure, or for the concentration (but not both) without destroying the system.

In studying the protein-acid or -base systems we have two components, protein and acid, or protein and base (the acid or base existing in the gaseous form). If it is assumed that the reaction is one of adsorption, there will always be two phases, that of the solid protein and the gaseous acid or base. Therefore F will equal 2, or that is to say, there will be two available choices of temperature, pressure, or concentration. If the temperature is kept constant, we are left with a univariant system or the availability of one choice only. Therefore for every value of pressure chosen, there will be one

definite value of concentration (concentration being that of the gas in the solid phase). The pressure will then vary continuously with the concentration, giving a smooth curve resembling that of an adsorption curve.

However, if it is assumed that a chemical compound is formed, there will be three phases, that of the gas (acid or base), the protein, and the protein salt. If the temperature be kept constant, the system will become invariant, meaning that there are no available choices for the experimenter, the system setting itself according to physical laws. Therefore, when ammonia or hydrogen chloride is added to a protein, and assuming compound formation, the pressure of the gaseous component will increase until a point is reached at which chemical combination takes place causing the introduction of a third phase making the system invariant. According to the phase rule, the pressure will then remain constant until all the protein has been converted into the compound. This constancy will show itself in a straight line parallel to the axis of concentration. When all the protein has been used up, the system will revert to the two-phase stage again, the solid protein compound and the gas, returning it to the state of univariancy. Therefore the pressure will continue to rise parallel to the axis of pressure until a possibility of the formation of a second compound may occur. Thus in the case of compound formation between proteins and acids or bases, plotting a curve of pressure of ammonia or hydrogen chloride against concentration of gas in the solid protein phase, will give a step-wise curve.

Bancroft and Harnet used this principle in their studies of the protein-acid or-base systems. Their procedure was to add

ammonia or hydrogen chloride to a quantity of powdered protein, noting the amount taken up at equilibrium, and the corresponding pressures. Thus according to the phase rule, if a solid ammonium proteinate is formed, the pressure will remain constant but if the gas is adsorbed, a smooth curve characteristic of adsorption will result. From their results they concluded that casein, zein, arachin, fibrin, and gliadin, adsorbed ammonia but did not form compounds. They also found that casein, arachin, fibrin, gliadin, and edestin, formed definite compounds with hydrogen chloride. Zein did not form a compound with hydrogen chloride.

However, even the phase rule method is not free from controversial evidence as supplied by various workers. Czarnetsky and Schmidt (1934), employing the same methods of study as did Bancroft and Barnett, reported definite compound formation in the case of casein, gelatin, edestin, and zein, in the presence of ammonia, disagreeing with the latter's findings of an absence of such a formation with ammonia. Czarnetsky and Schmidt conclude, also in opposition to Bancroft and Barnett, that zein does form a chemical compound with hydrogen chloride. Their results are also in disagreement with those of Holden (1931) who concluded that the reaction between gelatin and ammonia was purely one of adsorption.

Czarnetsky and Schmidt not only state that the reactions between proteins and hydrogen chloride or ammonia are chemical in nature, but that they even combine in stoichiometric proportions in certain ranges. They conclude that the combining capacity of certain proteins for hydrogen chloride is approximately determined by the presence of the ϵ -amino N of

lysine, the imino N of tryptophane, the guanidine group of arginine, and the tertiary and imino N of histidine.

Parks and Nelson (1937) proposed a mechanism for the adsorption of acids and bases by protein materials, based upon the polypeptide theory of protein structure. It is considered from the standpoint that polypeptides are linked in the protein molecule by secondary or residual valency forces, in contrast to the primary valence existing in simpler organic compounds. The polypeptide residues are said to have a preferred orientation while the protein molecules themselves have a random orientation with respect to each other. The probability of adsorption taking place is ruled by the possible arrangement of the molecules in different positions so that opposite or similar valence force groups are adjacent to one another, thus creating an area capable of adsorption or prohibitive of adsorption. In this way the binding power of various protein groups will be determined by the type and number of surrounding groups.

Buttner (1933) employed the method of ultrafiltration in studying the combination of gelatin with histamine or adrenaline. He found that the least amount was bound at the isoelectric point, but increased gently with rising pH, reaching a maximum at 6.4. At a constant pH, the distribution of base in the intermicellar fluid, and in the protein micelles was found to follow the Freundlich adsorption isotherm.

Tolman and Stearn (1918), in an explanation of the swelling of fibrin in acids, consider adsorption to be the acting force in protein reactions with acids. They define a colloidal

gel as a fibrous sponge-like structure with many minute pores full of water. Addition of acid gives adsorption of hydrogen ions on the surface of the pockets, which consequently increase in size owing to electrostatic repulsion, being accompanied by imbibition of solution. Addition of a neutral salt decreases the swelling because the ions of the salt arrange themselves in a way as to neutralize the electrostatic repulsion. Adsorption is increased by the addition of salt, since the ions of the salt neutralize the electrical field of the adsorbed acid, hence making it easier for more acid to get to the surface of the pockets, thus leading to increased adsorption. The same was found in the case of alkalies by Tolman and Bracewell (1919).

The adsorption mechanism for the explanation of swelling put forward by Tolman and Stearn is in disagreement with the work of Procter and Wilson (1916) who give a chemical explanation to the phenomenon of swelling in gels. According to the latter, when gelatin is added to a dilute acid solution, combination takes place between the gelatin molecules and the hydrogen ions to give a highly ionizable gelatin salt, the anions of which, in tending to diffuse, exert an outward pull on the jelly mass increasing its volume, thus causing the phenomenon of swelling. Wilson and Wilson (1918) support Procter and Wilson's claim that swelling is due to a chemical phenomenon.

Langmuir (1918) found that Freundlich's formula for adsorption did not hold for the reaction of gases with mica, glass, and platinum, possessing a smooth surface, and suggested therefore that the latter reaction was determined by chemical forces of primary and secondary valencies. Working

from this point of view. Bracewell (1919) introduced a new conception of the type of combination between acids and bases with proteins. He considered the attraction exhibited in the phenomenon of adsorption was not produced by physical forces, but was more chemical in its nature, arising from stray fields of "chemical force" around atoms whose chemical affinity has not been completely satisfied.

Hoffman and Gortner (1925) working with the prolamines, concluded that the chemical nature of a protein to bind acid and alkali in stoichiometric relationship depends upon the chemical groups within the molecule, and is therefore limited to the range between pH 2.5 and pH 10.5. They state that their findings afford a logical explanation for the divergent views of those who hold that acid and alkali binding are of a stoichiometric chemical nature, and those workers who hold that colloidal adsorption is the predominating factor. Thus according to Hoffman and Gortner, both beliefs are correct, and they have shown in what regions (in terms of hydrogen ion concentration) one or the other phenomenon may be expected to predominate.

A potentiometric method of investigation was employed. Hoffman and Gortner found that the degrees of ionization of acids and bases had a different value when measured with potentiometric apparatus than they had when measured with conductimetric apparatus. Therefore standard tables of dissociation constants that had been obtained conductimetrically were considered useless, and Hoffman and Gortner altered the formula for calculation of the amount of acid or alkali bound to make it compatible with potentiometric methods.

They found that approximately equivalent amounts of hydro-

chloric, sulfuric, and phosphoric acids were bound by a unit amount of protein. This was in disagreement with the conclusions of Loeb, who found that more phosphoric acid was bound than was hydrochloric. The acids were compared on a normality basis. A negative temperature coefficient was obtained by carrying out experiments at 15^0, 25^0, and 35^0 in pH ranges greater than 10.5 and less than 2.5, thus offering evidence for adsorption in these regions.

Hoffman and Gortner conclude by suggesting that "there are two types of combination between proteins and acid or alkali: (1) a chemical type of combination which takes place between a hydrogen ion concentration represented by pH 2.5 and pH 10.5 and (2) and adsorption type of combination which takes place when the hydrogen ion concentration is greater than pH 2.5 or the hydroxyl ion concentration is greater than pH 10.5.

The evidence of a chemical type of combination between a hydrogen ion concentration of pH 2.5 and pH 10.5 is presented by:

1. The logarithms of the amount of acid or alkali plotted against the original concentrations do not form a straight line.

2. The Buffer curves do not form a smooth regular line.

3. The amount of acid or alkali bound at any hydrogen ion concentration between pH 2.5 and pH 10.5 depends on the chemical composition of the protein. This is not true where the pH is less than 2.5 or greater than 10.5.

4. When the hydrogen ion concentration is below about pH 2.5, the protein chloride is highly ionized.

Evidence of the adsorption type of combination is furnished by:

1. At the higher concentrations of acid and alkali, all of the proteins used in this work regardless of their chemical composition, bind approximately the same amount of acid or alkali.

2. There is a marked negative temperature coefficient of the acid or alkali binding at the higher concentrations of acid or alkali.

3. The logarithm of the amount of acid or alkali bound plotted against the logarithm of the original acid or alkali concentration or against the final pH form a straight line.

4. There is more alkali bound, when the original concentration is 0.500 normal, than can be accounted for by chemical combination assuming that there is an available carboxyl group for each nitrogen atom, an assumption far in excess of the possibility.

5. When the hydrogen concentration is greater than about pH 2.5, there is no increase in the ionization of the protein chloride."

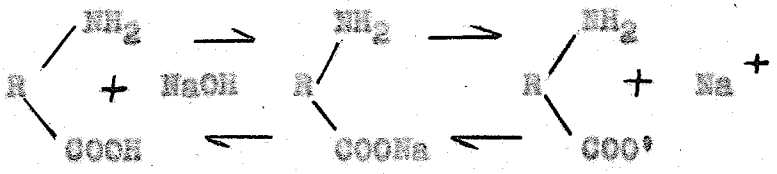
Evidence for a chemical type of combination between proteins and acids or bases has been furnished by Sandstrom (1930) who studied acid and alkali binding by native and deaminised proteins. He reports that the acid bound by casein, edestin, arachin, fibrin, and duramin, is roughly proportional to the lysine content. In the case of the deaminised proteins the acid binding capacity was greatly decreased. However, they found that at values below pH 2.5 and above pH 10.5, the phenomenon of adsorption appeared to predominate, which is in agreement with the theory of Hoffman and Gortner (1925).

Further evidence for chemical forces in protein-acid or -base reactions has been supplied by the optical study of Almquist and Greenberg (1931). They found that the instantaneous change of rotation, the ready reversibility of the change, and the well-defined maxima, indicated rapid interreaction between protein and reagent ions with typical salt formation. On these observations they discredit the adsorption theory of protein combination with acids or bases. They found evidence for extensive chemical combination in the higher alkaline regions, in contrary to the views of Hoffman and Gortner (1925) who assume that the binding in the extreme pH ranges is due to colloidal forces.

D.

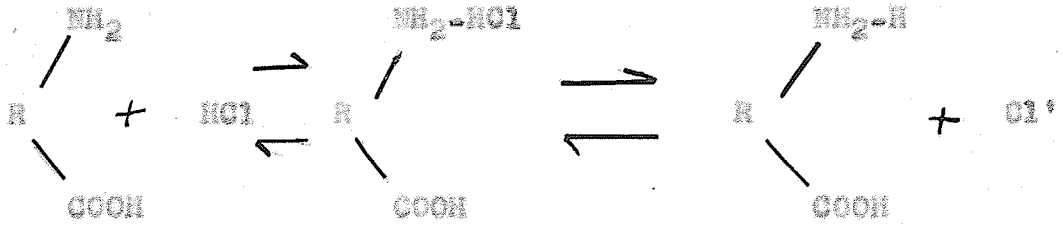
Mode of Acid and Alkali Combination with Proteins.

Assuming that the combination between proteins and acids or bases is of a chemical nature, workers have endeavored to theorize the actual mechanism of union. The oldest theory is that proteins form typical salts with bases as in the following manner:



Isoelectric protein

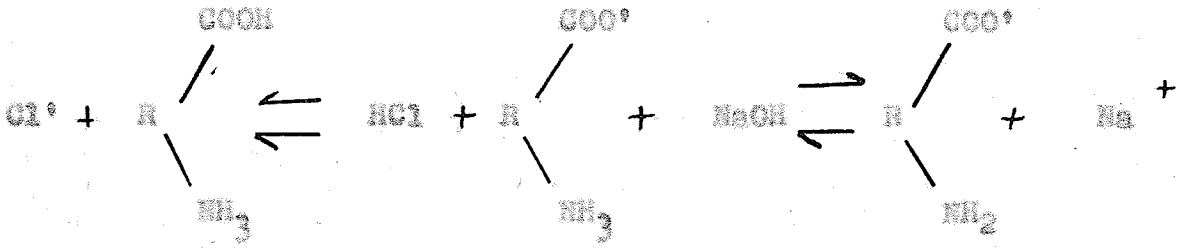
In this case, a negative protein ion results. In an acid reaction, proteins form positive ions as follows:



Isoelectric protein

The above represents the classical view of salt formation by proteins. According to this conception the protein is dissociated into ions either on the acid or basic side of a certain critical hydrogen ion concentration, the "isoelectric point." Thus the isoelectric protein molecule as represented in the usual fashion, owes its neutrality to the absence of dissociation, or at most to minimal and equal acid and basic dissociation.

In a modern hypothesis, Birch and Harris (1930) have brought forward evidence to show that amino acids, polypeptides, and proteins, possess the "Zwitterion" structure. In this newer view the isoelectric protein or amino acid owes its neutrality to the complete and simultaneous ionization of the acid and basic groups. Thus the formation of a negative protein salt by the addition of alkali, and the formation of a positive protein salt by the addition of acid can be represented in the following manner:



Isoelectric protein

Hoffman and Gortner (1925) mention that Robertson empha-

sizes the importance of the peptide linkage as the source of basic and acidic valencies. He assumes that the protein undergoes a keto-enol transformation, and that the peptide group reacts in the enol form. According to Robertson's theory the protein is ionized by rupture of the peptide linkage with formation of two oppositely charged protein fragments. The keto-enol transformation and the peptide rupture occurs as in the following manner:

$$-CO-NH \rightleftharpoons -C(OH)=N-$$

Keto form Enol form

Rupture occurs at the peptide linkage:

$$-C(OH)=N- + NaOH \rightleftharpoons -CONa^{++} + -NH-OH$$

M.

The Effect of Salts on the Alkali Binding Capacity of Proteins

A new method of attack in the problem of acid and alkali binding by proteins was originated by Robinson, Gortner, and Palmer (1932) by studying the effects of salts on the alkali binding capacity of casein and paracasein. These workers mention that this method of attack was suggested by the work of Kolthoff and Bosch who studied the influence of neutral salts on the acid-base equilibrin. The latter calculated the dissociation constant from dilution data, and then noted the effect of neutral salts on the activities and dissociation constants of the components. Changes in pH were noted which were shown to be due to an increase in the activity coefficient of the undissociated acid. Robinson, Gortner, and Palmer realized that such a thermodynamic study as

applied to weak acids, would present difficulties when applied to proteins, since the calculations of Kolthoff and Reach involved the degree of basicity of the acid, its molecular weight and its average ionic size. Such values, in the case of proteins, are not known with any degree of certainty. However, Robinson, Gortner, and Palmer were of the opinion, that using a protein as a weak acid, it would be possible to record whether a protein "behaves as a true electrolyte, or whether it reacts in a manner that can be explained only on the assumption that colloidal behaviour is involved."

They found from their dilution data, that a theoretical, mathematical interpretation could not be applied to the protein system, and stated that such a mathematical failure of applica- may be due to the absence of constant proportion between the protein and the alkali. If this were the case, their results would tend to show strong evidence for the inclusion of adsorptive forces in protein-base reactions.

According to Robinson, Gortner, and Palmer, the presence of salts such as KCl, KBr, and KI, tend to increase the alkali binding capacity of the proteins with which they worked. They concluded also that the magnitude of the "salt effect" was the same for all the salts employed in the study. They stress that the "salt effect" must be taken into consideration when interpreting the electrometric titration curves of proteins. Thus, if one titrates proteins electrometrically with either acid or alkali and then back-titrates the resulting system, the two curves will not coincide due to the salt effect. Hoffman and Gortner (1925) found a discrepancy in such curves.

Although Robinson, Gortner, and Palmer found a definite

"salt effect" on the alkali combining capacity of casein and paracasein, Cohn, Green, and Blanchard (1937) found no such effect in the case of haemoglobin and sodium hydroxide. They state that although the titration curve is very sensitive to ionic strength, the maximal base combining capacity was shown to be the same in protein solutions having no salt present as it was in protein solutions containing salts of normal concentration.

From the above it is obvious that there is need for further study of the salt effect on acid or base binding by proteins. We therefore chose to study the acid and alkali binding capacity of glutenin, to notice how this binding was effected by the addition of neutral salts, and to determine whether the effect, if any, was due to adsorption or to chemical combination.

Part II

PREPARATION AND ANALYSIS OF GLUTENIN

Many papers have been published with regard to the nature of the wheat proteins, and since there is a good deal of discussion as to the identity of glutenin itself, a short account of its history seems in order at this point.

Osborne (1907) was the first to characterize wheat gluten as being separable into two distinct proteins. These he categoried as to their solubility or insolubility in alcohol, "gliadin" being the alcohol-soluble component, and "glutenin", the alcohol-insoluble. Osborne mentions that the name "glutenin" was suggested by C. H. Johnson, but was known as "zymon" in 1820 when it was first described and named by Taddei.

The classical method of preparation, being that of Osborne's, was to extract the gluten with 10% sodium chloride, and then with dilute alcohol to remove the soluble proteins. The residue was then extracted with 0.1% potassium hydroxide solution whose action was to disperse the glutenin into its colloidal particles. Precipitation of the protein was brought about by neutralizing the alkaline solution with 0.2% hydrochloric acid. The product was again dispersed with alkali and again precipitated with acid. This procedure was repeated a few times and finally the glutenin was dried in ether and alcohol. The nitrogen content of a number of preparations varied from 15.51 to 17.57%. Osborne admits the glutenin prepared by this method is far from being pure.

A simpler preparation designed to be less-laborious and time-consuming, was introduced by Hlynka and Sandstedt (1925) which removed the difficulty of filtering alkaline solution

containing starch, a troublesome procedure in the Osborne method. Normal sodium hydroxide was added to the flour-water mixture, followed by a dilution with methyl alcohol. In this case the starch settled to the bottom of the container leaving in solution all of the flour protein. The glutenin was then precipitated by adding 0.2N normal hydrochloric acid from a burette. The optimum pH for precipitation was found to be between 6.0 and 6.8. After centrifuging, the glutenin was left at the bottom of the tube.

Although Osborne found no evidence for loss of nitrogen due to the prolonged contact of glutenin in alkaline solution during its preparation , Blish and Sandstedt (1929) were of the opinion that such did occur, and devised a new method of preparation under conditions avoiding exposure to alkaline reaction. In this improved preparation the gluten was dispersed in large volumes of very dilute acetic acid, and then diluted with methyl alcohol. Normal sodium hydroxide was again employed as the precipitating agent, never allowing the solution to become alkaline at any time. The glutenin prepared in this manner was both physically and chemically different from products whose preparation involved the use of alkaline dispersion agents. In their own words, "Glutenin as prepared by customary methods involving extraction with, or temporary solution in, alkali, is a product resulting from an irreversible alteration by the action of alkali on a more complex body." They insist that in the usual preparation of glutenin, in which alkali is used as the dispersion agent, both the yield and the chemical constitution will vary with the concentration of the alkali employed.

Larmour and Sallans (1932) prepared glutenin from one flour

by various methods including that of Osborne. Their preparations showed marked difference in nitrogen distribution, especially in the ammonia and basic nitrogen content. They recommended the method employed by Blish and Sanstedt (1929) where dilute acetic acid is used.

An entirely new method in the preparation of glutenin was devised by Cook and Alsberg (1931) who employed urea instead of alkali or acid. They argued that the preparation of glutenin involved a temporary dispersion in acid or alkali, including an extraction with alcohol in removing the gliadin, and that these reagents were known to denature other proteins. They concluded therefore that the glutenin thus prepared had undergone both physical and chemical changes. In their search for a neutral dispersion medium they found that concentrated solutions of urea were best suited. In this case precipitation was brought about by the "salting out" effect of magnesium sulfate.

Since Osborne published his method of preparation of glutenin, doubts arose as to the individuality of this protein. Woodman (1932) prepared glutenin from two different types of flours, and concluded that the resulting proteins were two distinct substances. He based his views on a study of their different optical behaviour. The gliadins, were found to be identical.

Blish and Finckney (1934) prepared seven samples of glutenin from widely different types of wheat and examined them as to their respective racemization rates in the hope of discovering a difference in their chemical configuration as was suggested by Woodman. Six out of the seven glutenins prepared were found to be identical. They insisted since Woodman had based his conclusions on only two types of wheat, their work on seven

samples would tend to show there is no basis for Woodman's theory of a difference in chemical configuration.

Kondo and Hayashi (1931) working with five different wheats, concluded all glutenins to be identical, supporting the view of Blish and Finckney. The nitrogen content of each of their glutenins was substantially the same, varying from 17.3 to 17.5%.

Halton (1924) brought about a separation of glutenin into two distinct fractions. He dissolved a sample of purified glutenin in sodium hydroxide and slowly added normal hydrochloric acid until a precipitate was obtained which settled to the bottom. He then drew off the supernatant liquid and added more acid, drop by drop, and in this manner obtained a second precipitate. The latter, when dissolved in alkali, had a slightly different specific rotation.

Blish (1926), in repeating Halton's work, found no fractionation as the latter had claimed. However, when slightly racemized glutenin was used Blish was able to obtain a second fraction. His conclusion was that racemization of glutenin gives rise to a second "fraction" with a different iso-electric point from that of natural glutenin. This racemized protein must be considered as a protein derivative however, and not as a protein itself, since racemization appeared to be accompanied by partial hydrolysis. Evidence of hydrolysis of protein when undergoing racemization has been found by Bakin (1912) and Underhill and Hendrix (1915).

However, the evidence of fractionation of an "individual" protein became more frequent, and doubts arose as to the reliability of the system of protein classification. Hoffman and Gortner (1927), in isolating proteins from wheat flour, noticed a differ-

ence in amount and character of protein obtained with both type and concentration of salt employed in the extraction. Furthering this point, Gortner, Hoffman, and Sinclair (1923), protesting against the definition of protein types, showed that the average percentages of protein extracted from a series of wheat flours varies with the type and concentration of the salt used. On this basis they argued that the prescribed classification of proteins was meaningless.

Blish and Sandstedt (1933) showed that in varying the methods of extraction, wheat gluten can be roughly divided into three fractions. These were designated as the "glutenin" group, the "gliadin" group, and the "mesonin" group, the last-named having properties intermediate with respect to those of the other two. They conclude that the gluten proteins may be characterized in a manner that is compatible with Sorensen's theory that proteins can be regarded as "reversibly dissociable component systems" where each component system is said to consist of a series of "complexes". Sorensen's theory continues:

"Within each complex all the atoms or atom groups are interlinked by main valencies, whereas the various complexes or components are reversibly interlinked by means of residual valencies. The linkage between the components is comparatively feeble and of such a character that alterations in the composition of the solution (salt content, hydrogen ion activity, alcohol content, temperature) can cause reversible dissociations of, and exchange of components between, the component systems present. When these alterations in the composition of the solution are so adapted as to render possible the formation in sufficient amount of a component system insoluble or sparingly soluble under the new conditions, this system will be formed and precipitated."

In application of Sorensen's scheme to the gluten proteins, Blish and Sandstedt consider their gliadin, glutenin, and mesonin groups, as "the three main dissociable components, each system in turn consisting of a group of lesser dissociable components

or complexes." The only item in which Blish and Sandstedt's views differ from that of Sorensen's is the question of the reversibility of the systems, but they explain that the glutenin components are the least readily reversible due to their susceptibility to denaturation when in contact with the precipitating reagents. The gliadin and mesonin groups are less susceptible to this denaturation.

McCalla and Rose (1935) obtained a series of fractions from a dispersed solution of wheat gluten in sodium salicylate by the addition of varying amounts of magnesium sulfate. They therefore conclude there is no evidence for the existence of "gliadin" or "glutenin" as independent proteins, and that such terms referring to these supposed proteins should be discarded. "Gluten is a single protein complex which may be separated into a great many fractions which differ progressively and systematically in both physical and chemical properties."

Rich (1936) arrived at a similar conclusion stating that by altering the methods of preparation "there is no established limit as to the number, quantities, and character of the protein fractions that can be isolated." Block (1933) is of the opinion that the prepared proteins are merely artifacts produced by laboratory manipulation, and do not exist as such in biological material.

The importance of reviewing these developments in the study of protein characterization, in its application to the reactions of acids and bases with proteins can be appreciated in the understanding of Gortner's (1938) statement:

"The research worker should recognize that solubility is nothing more nor less than peptization and that the proteins

must be considered not alone as complex organic compounds but likewise as colloid micelles, subject to all the varied reactions of a lyophilic system. Only under such conditions will the study of protein classification and reactions characteristic of protein systems be advanced."

EXPERIMENTAL.

EXPERIMENTAL

A. Preparation and Analysis of Glutenin

The glutenin was prepared according to the method of Blish and Sandstedt (1929) with certain modifications suggested by Larmour and Sallans (1932).

Standard bleached flour used for baking purposes was chosen as the source of the protein. The gluten was prepared by mixing 1000 grams of the flour with 600 ccs. of tap water, kneading the mixture until a stiff dough was obtained. The mass was then allowed to stand under water for two hours, after which time the gluten was washed thoroughly under a steady stream of tap water. During the washing, the gluten was submitted to a second and complete process of kneading in an effort to wash away the contained starch. This treatment was continued until the water, passing over the gluten, became clear, at which time it was assumed that all the available starch had been removed. The starch-free gluten was then allowed to remain in water for 16 hours, and finally ground in a meat-chopper.

The ground wet gluten was then submitted to the process of dispersion by treating it with 0.007 normal acetic acid, the concentration of which was suggested by Larmour and Sallans, who found that the most satisfactory results were obtained by using acid of this normality. The acid-gluten mixture was stirred continuously for five hours by means of a motor-driven glass-rod stirrer, at the end of which time a rather viscous milky dispersion resulted which became clearer on standing overnight, leaving however, a certain amount of residue at the bottom of the vessel. The supernatant liquid was decanted and passed through a Sharples

supercentrifuge, which removed any remaining starch. The acetic acid-protein solution was made up to 70% alcohol by the addition of the necessary amount of 95% ethyl alcohol, which served to separate the alcohol-soluble gliadin from the alcohol-insoluble glutenin.

Precipitation of the glutenin was brought about by the careful addition of N/14 sodium hydroxide solution (this particular normality being suggested by Larmour and Sallans), until a point of almost complete neutralization was reached, which was determined by the point of maximum flocculation of the protein, leaving a practically water-clear filtrate. The precipitated glutenin was filtered through cheese-cloth, and submitted to a process of purification.

In purifying the glutenin, it was ground in the chopper and extracted with 70% alcohol several times to insure complete removal of the gliadin, washing with water after each extraction. The protein was then dried with 95% alcohol followed by anhydrous ether. The glutenin was dried *in vacuo* for 12-15 hours, ground in an agate mortar, re-dried *in vacuo* as a powder, then placed in a container and kept well-stoppered.

The residue that remained at the bottom of the vessel after dispersion with acetic acid, was treated with two litres of the acid and submitted to the same process as described above. Only a small fraction of the protein was obtained from this remaining material.

The yield was 22 grams.

o o o

The glutenin was analyzed for nitrogen using the Kjeldahl method, and the following results were obtained:

Sample I - 14.67%

Sample II - 14.96%

Average - 14.82%

As the history of the preparation of glutenin shows, the values for nitrogen content as determined by the Kjeldahl method vary according to the method employed in its preparation. Larmour and Sallans prepared glutenin according to five different methods, including that of Osborne's, which varied in the type and concentration of dispersion medium employed. The nitrogen contents of these five methods are listed below and are given in values of percent:

1. 15.83
2. 11.86
3. 16.07
4. 16.08 and 15.97
5. 15.29

The fourth preparation represents that employed by Larmour and Sallans in their modification of the Blish and Sandstedt (1929) method. Since this was the procedure we employed, we compare our results with those of Larmour and Sallans'.

	Nitrogen content in %		
	I	II	Average
Theirs	16.08	15.97	16.03
Ours	14.67	14.96	14.82

Blish and Sandstedt (1929)without specifying as to the concentration of acetic acid used, give 17.50% for the nitrogen content of their glutenin preparation, which shows the discrepancy between theirs and the results obtained by Larmour and

Sallans. Our results are about 1% lower than those of the latter. This discrepancy in nitrogen content may be due in part to possible contact with alkali in the preparation. Larmour and Sallans state that a sample of glutenin, during its preparation, was left in contact with 0.025 normal sodium hydroxide for a week, after which time analysis of its nitrogen content showed a loss of 4.81% nitrogen.

We concluded that due to the lack of uniformity in results of nitrogen content by different workers, due to the sensitivity of glutenin to slight modifications in its preparation, and due to the existing doubts of the "purity" and "individuality" of glutenin, our preparation, whose nitrogen content is of the same order with that of the preparation of Larmour and Sallans, can be considered pure as far as the meaning of "purity", in the case of glutenin, is concerned. With this belief we felt that our preparation of glutenin was satisfactory for our purposes.

2.

Preparation of Reagents

The reagents employed in this study were glutenin, hydrochloric acid, sodium hydroxide, water, and neutral salts.

The preparation of glutenin has already been described in the preceding section. The hydrochloric acid used, was the C. P. product, and was diluted to 0.1 normal. This was standardized against 0.100 normal sodium carbonate solution using methyl orange as indicator. The sodium carbonate was dried previous to use. 0.100 normal sodium hydroxide solution was prepared from the U. S. P. product, and was standardized against the hydrochloric acid, phenolphthalein acting as the indicator.

The neutral salts employed, were sodium chloride, sodium bromide, potassium bromide, potassium iodide, and lithium bromide. All the salts except lithium bromide were obtained in the dry form, and were the pure variety used in laboratory work. The lithium bromide was obtained in a solution form of unknown concentration, and was analyzed for the lithium content by precipitating the bromide as lithium sulfate by the addition of dilute sulfuric acid. The lithium bromide solution contained a small amount of lithium chloride. All the salts were made up to 1 normal solutions, and stored in well-stoppered Winchester bottles.

No redistilled water was kept on hand, since all water was redistilled immediately before use. This afforded protection against contamination by atmospheric carbon dioxide.

C.

Apparatus and Methods

The amount of acid and alkali bound by the protein was determined potentiometrically and is expressed as gram equivalents bound per gram of protein. The glass electrode was employed in making the measurements. The entire potentiometric set-up, including the potentiometer, galvanometer, working battery, calomel half-cell, and glass electrode, was incorporated as a single unit in the "Beckman pH Meter" manufactured by the National Technical Laboratories at Pasadena, California. In this way, pH readings were taken directly from the instrument, after making adjustments for temperature, calomel half-cell, and amplifier. The function of the radio amplifier is to permit the use of relatively thick and rugged glass instead of the thin glass membrane employed in the original glass electrodes.

The pH readings were accurate to the first decimal place, and approximate to the second. This accuracy was found to be sufficient for our purposes. The glass electrode is subject to errors when used in alkaline solutions containing alkali metal ions, the most serious errors occurring in sodium solutions, and appreciable only at pH values over 9.50. However, since these alkali ion errors can be calculated, a correction sheet had been supplied with the instrument, and all pH readings taken over 9.50 in this study have been subjected to correction. Most of the corrections necessary were very small, since only dilute solutions of alkali were employed.

In all determinations, 0.1 gram of glutenin was used, and was suspended in 100 ccs. of water, this suspension being necessary owing to the insolubility of the protein in neutral media.

The acid or alkali was added in successive measurable quantities from a burette, stirring the protein suspension (which failed to dissolve in either the acid or alkali) thoroughly after each addition. The glass electrode and calomel half-cell were kept immersed in the protein suspension, and readings were taken from the pH electrometer after each addition of acid or alkali.

All working temperatures were kept constant by means of carrying out the measurements in a constant temperature bath, supplied with a thermostatic-controlled electrical heater.

The calculation of the amount of acid or alkali bound by the protein was made by employing the equations of Cohn and Berggren as used by Robinson, Gortner, and Palmer (1932). The fundamental equation for deriving pH is

$$\frac{\text{e.m.f. (observed)} - \text{E (calomel electrode)}}{0.00019837\ T} = \log \frac{I}{K'} = \text{pH}$$

According to the law of Mass Action

$$(H^+) \times (OH') = K_w$$

Cohn converts this equation to the logarithmic form

$$\text{pH} + \text{pOH} = \log \frac{I}{K_w}$$

Now since

$$\frac{(OH')}{(NaOH)} = \gamma$$

where γ is the activity coefficient of sodium hydroxide

then

$$\text{pOH} = \log \frac{I}{\gamma\,(NaOH)}$$

i.e. $$pOH = p\gamma + pNaOH$$

or

$$pNaOH = pOH - p\gamma$$

Thus in determining the value of pOH, and knowing the value of $p\gamma$, the concentration of free base was found. Values for $p\gamma$ were obtained from the values of Lewis and Randall (1923). Certain values not given in this table were obtained by interpolation.

Since

$$pNaOH = \log \frac{1}{NaOH} = \log 1 - \log NaOH$$

Then $$\log NaOH = \log 1 - pNaOH$$

Therefore the values for pNaOH were subtracted from 0.000 giving the logarithm of the amount of free NaOH. The amount of free NaOH was obtained from logarithm tables, and subtracting this amount from the original concentration gave the amount bound by the protein. From this was calculated the gram equivalents bound per gram of protein.

In the case of the acid, amount of free acid was determined from the formula

$$pHCl = pH - p\gamma$$

In using this method of calculation certain assumptions had to be made. In the case of the sodium hydroxide it was neccessary to assume:

1. That the protein and sodium hydroxide react in stoichiometric proportions.

Protein + NaOH = Na proteinate + H_2O

2. That the so formed protein salt is completely dissociated.

Na proteinate = Na + proteinate'

3. That the sodium ions from the sodium proteinate compound have the same activity as sodium ions in a sodium hydroxide solution.

4. That the influence of the protein ion on the activity of the sodium ion is the same as is the influence of an hydroxyl ion.

5. That there is no adsorption of sodium ions or of sodium hydroxide molecules on the protein micelles.

In the case of the hydrochloric acid we similarly assumed:

1. That the protein and hydrochloric acid react in stoichiometric proportions.

Protein + HCl = Protein chloride

2. That the so formed protein chloride is completely dissociated with the same activity coefficient for the chloride ions of the protein chloride as for the chloride ions in an equivalent concentration of hydrochloric acid.

3. That there is no adsorption of the acid in the molecular state which can give rise to hydrogen ions in the solution.

Such assumptions, as complete dissociation of the protein salts or of the metal proteinates, have been completely rejected by various workers, and Robinson, Gortner, and Palmer (1932) have shown that the amount of sodium hydroxide which is bound by casein or paracasein increases with increasing equilibrium hydrogen ion concentration, despite attempts to apply assumptions of a strictly stoichiometric combination. Their results also

appeared to show evidence of adsorption in the higher alkaline regions, an observation which is contrary to the assumption necessary for the calculation that there is no adsorption on the protein micelles.

However, in comparing with other methods of calculation, Robinson, Gortner, and Hoffman have shown that a fair agreement exists between the Cohn and Berggren calculation and the others, except at the higher concentrations of alkali where the discrepancies appear to become more pronounced.

Since no improved method of calculation has been arrived at and since the older methods of calculating the amount of acid or alkali bound by protein have their inadequacies, we decided to retain the Cohn and Berggren method of calculation.

D.

The Amount of Hydrochloric Acid Bound by Alkali

Before any attempt was made to calculate the amount of acid or alkali bound by protein, it was desired to calculate the amount of hydrochloric acid bound by sodium hydroxide using the Cohn and Berggren formula, in order to compare this type of reaction, which is known to be stoichiometrical, with that of acid or alkali with protein. This determination also serves as a check on the apparatus, and of the method of study employed.

Equal quantities of 0.100 normal hydrochloric acid and 0.100 normal sodium hydroxide were mixed together to make up a solution of 50 ccs. The pH of this mixture was determined, and subsequent additions of alkali were carried out until 26 ccs. of sodium hydroxide were present in the solution, pH readings being taken after each addition. At this point, hydrochloric acid was added in varying amounts, and in this manner a representation of the binding of acid by alkali was obtained over a wide range on the pH scale.

These results are listed in Table I, and are recorded graphically in Figure I. It is noticed that a constant value of amount bound, exists at each pH measured.

The figures in the column marked "Bound" refer to gram equivalents of hydrochloric acid bound per gram alkali.

TABLE I

AMOUNT OF HYDROCHLORIC ACID BOUND BY SODIUM HYDROXIDE

Temp. 25°C

Normality of HCl	NaOH in gms/litre	pH	Bound
0.05332	0.1040	2.14	0.025
0.05247	0.1040	2.25	0.025
0.05000	0.1000	3.38	0.025
0.04992	0.1002	3.54	0.025
0.04986	0.1004	3.79	0.025
0.04977	0.1008	5.45	0.025
0.04973	0.1010	6.08	0.025
0.04967	0.1012	6.90	0.025
0.04971	0.1040	7.46	0.025
0.04967	0.1040	8.50	0.025
0.04938	0.1040	10.11	0.025
0.04932	0.1040	10.43	0.025
0.04899	0.1040	10.60	0.025

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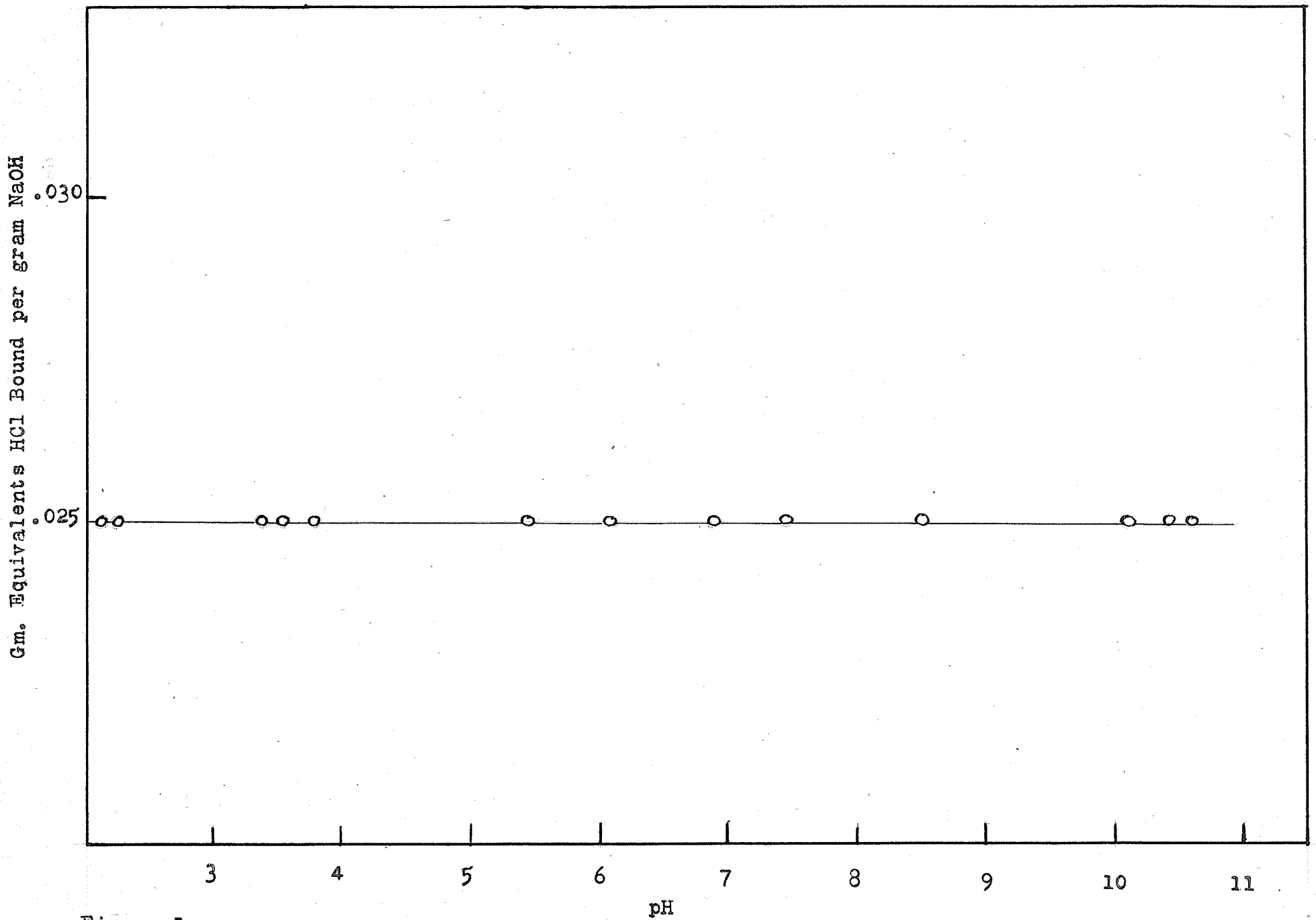

Figure I. Showing The Relation Between Amounts of Acid Bound Alkali with Change in pH.

B.

The Amount of Hydrochloric Acid Bound by Glutenin

We wished to determine how the amount of acid bound by glutenin varied with pH. 0.1 gram of glutenin was suspended in 100 ccs. of water and varying amounts of 0.100 normal hydrochloric acid were added to the suspension. pH readings were taken after each addition. Calculation of the amount bound was made by using the Cohn and Berggren formula.

The results of this determination are listed in Table II, and the plotted points of amount of acid bound against pH are found in Figure 2. In contrast to the NaOH-HCl system, where the amount bound remained the same, in the case of glutenin and acid, the binding shows a maximum with rising pH.

The figures in the column marked "Bound" in Table II, refer to gram equivalents hydrochloric acid bound per gram glutenin x 10^5.

TABLE II

AMOUNT OF HYDROCHLORIC ACID BOUND BY GLUTENIN

Temp. 25°C

ccm. HCl added	Normality of HCl	pH	Bound
0.00	0.00000	6.93	0.0
0.05	0.00005	6.49	5.0
0.10	0.00010	6.02	9.0
0.15	0.00015	5.48	14.6
0.20	0.00020	4.67	17.8
0.23	0.00023	4.43	19.2
0.28	0.00028	4.16	21.0
0.38	0.00038	3.88	24.7
0.61	0.00061	3.57	33.8
0.68	0.00068	3.50	36.1
0.86	0.00086	3.36	41.3
0.98	0.00098	3.28	44.7
1.06	0.00110	3.21	47.5
1.38	0.00140	3.07	53.4
1.59	0.00160	2.99	66.0
1.80	0.00180	2.89	59.0
2.00	0.00196	2.85	51.0
2.40	0.00234	2.74	47.1
2.51	0.00243	2.72	47.2
2.66	0.00259	2.69	62.0
2.80	0.00273	2.66	47.3
3.00	0.00291	2.62	43.3

TABLE II (Cont'd)

ccs. HCl added	Normality of HCl	pH	Bound
3.20	0.00310	2.59	44.4
3.40	0.00329	2.56	44.5
4.00	0.00385	2.48	41.6
4.52	2.00433	2.42	36.6
5.02	0.00478	2.38	42.0
5.25	0.00499	2.34	19.0
5.75	0.00544	2.30	15.9
6.50	0.00610	2.24	- - -
7.00	0.00654	2.20	- - -
7.50	0.00698	2.17	- - -

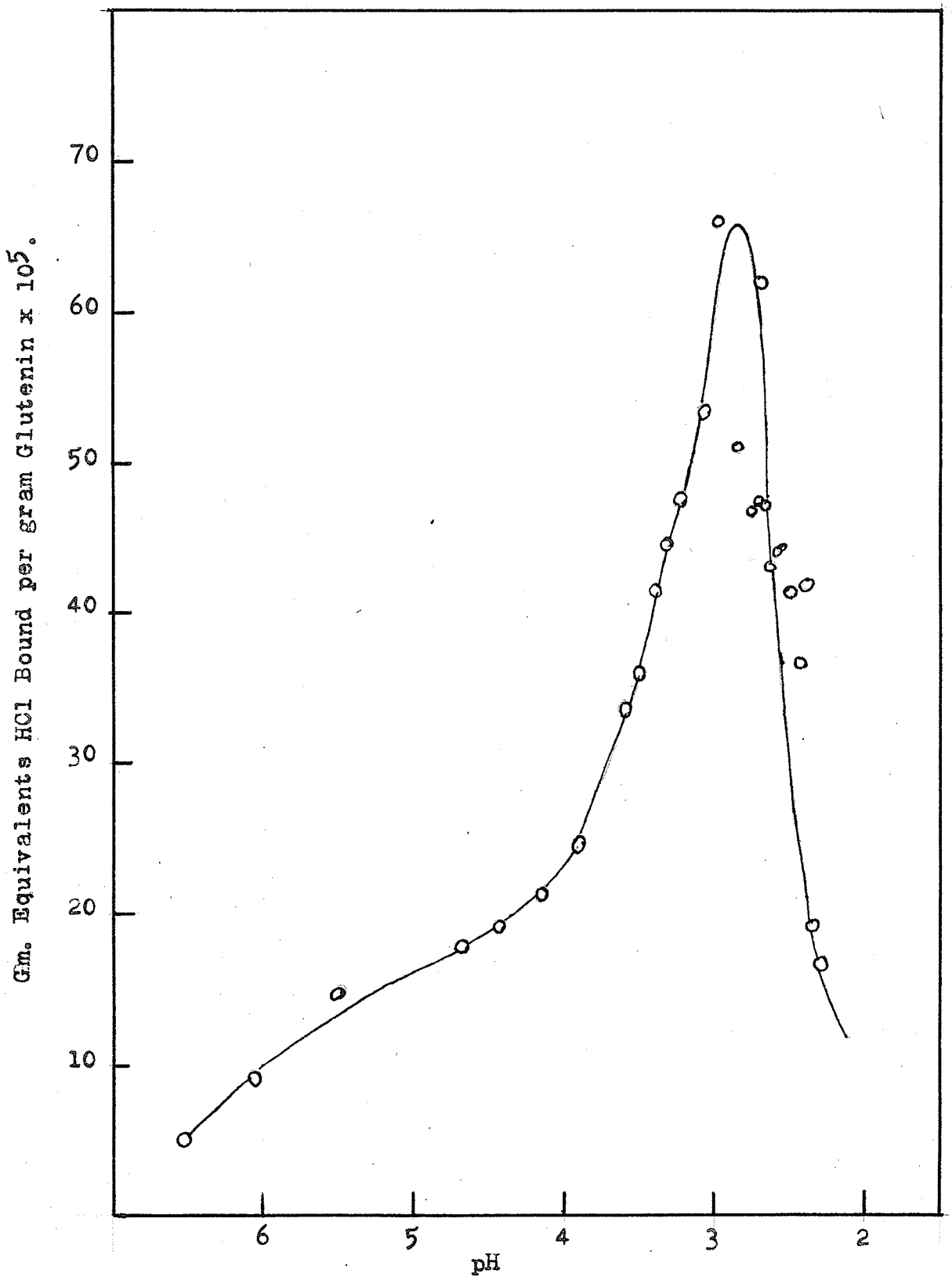

Figure 2. Showing the Relation of Amount of Acid Bound with pH.

F.

The Amount of Sodium Hydroxide Bound by Glutenin.

In the same manner, we wished to determine how the amount of alkali bound by glutenin varied with pH. The procedure used was identical with that of determining the amount acid bound by protein. In this case, however, all pH values over 9.50 have been corrected for the "alkali ion error" effect to which glass electrodes are subjected when used in alkaline solutions containing alkali metal ions.

The results of this determination can be found in Table III, and the graphic representation of the plotted points, in Figure 3. Binding is given in gram equivalents alkali bound per gram glutenin x 10^4. As is the case with glutenin and acid, the binding of alkali by glutenin shows an increase with rising pH. The logarithmic curve of amount of alkali bound against normality is shown in Figure 4.

TABLE III

AMOUNT OF SODIUM HYDROXIDE BOUND BY GLUTENIN

Temp. 25°C.

cc. HCl added	Normality of HCl	pH	Bound
0.00	0.00000	6.81	0.0
0.05	0.00005	8.62	0.2
0.10	0.00010	9.06	0.4
0.14	0.00014	9.24	1.2
0.18	0.00018	9.37	1.5
0.25	0.00025	9.57	2.1
0.30	0.00030	9.62	2.5
0.35	0.00035	9.71	2.9
0.41	0.00041	9.77	3.3
0.46	0.00046	9.84	3.7
0.50	0.00050	9.92	4.1
0.57	0.00057	9.96	4.5
0.62	0.00062	9.99	4.9
0.70	0.00070	10.07	5.5
0.75	0.00075	10.10	6.3
0.90	0.00090	10.20	7.1
0.96	0.00096	10.23	7.5
1.20	0.00119	10.31	9.4
1.34	0.00132	10.36	10.3
1.52	0.00150	10.42	11.8
1.66	0.00163	10.45	12.8
1.82	0.00179	10.49	14.2
2.02	0.00198	10.54	15.6

TABLE III (Cont'd)

cc. HCl added	Normality of HCl	pH	Bound
2.14	0.00205	10.57	16.0
2.27	0.00222	10.60	17.4
2.42	0.00236	10.65	18.2
2.60	0.00253	10.68	19.6
2.90	0.00282	10.72	22.0
3.22	0.00312	10.80	23.7
3.53	0.00341	10.85	25.7
3.80	0.00366	10.87	27.9
4.20	0.00403	10.94	30.0
4.62	0.00442	10.96	33.7
5.00	0.00476	11.01	35.7
5.60	0.00530	11.07	39.5
6.20	0.00584	11.11	43.8
6.80	0.00636	11.14	48.2
7.50	0.00698	11.22	51.1
8.50	0.00783	11.27	57.6
9.52	0.00869	11.33	63.4
10.00	0.00910	11.35	66.6
11.00	0.00991	11.39	72.7
12.00	0.01072	11.43	78.6
13.00	0.01150	11.46	85.1
14.50	0.01267	11.48	97.2
16.00	0.01379	11.49	110.1
18.00	0.01525	11.55	121.4
21.00	0.01736	11.58	158.6

TABLE III (Cont'd)

ccs. HCl added	Normality of HCl	pH	Bound
25.00	0.02000	11.62	176.2
30.00	0.02308	11.69	209.4
60.00	0.03751	11.75	468.9
100.00	0.05000	11.81	808.5

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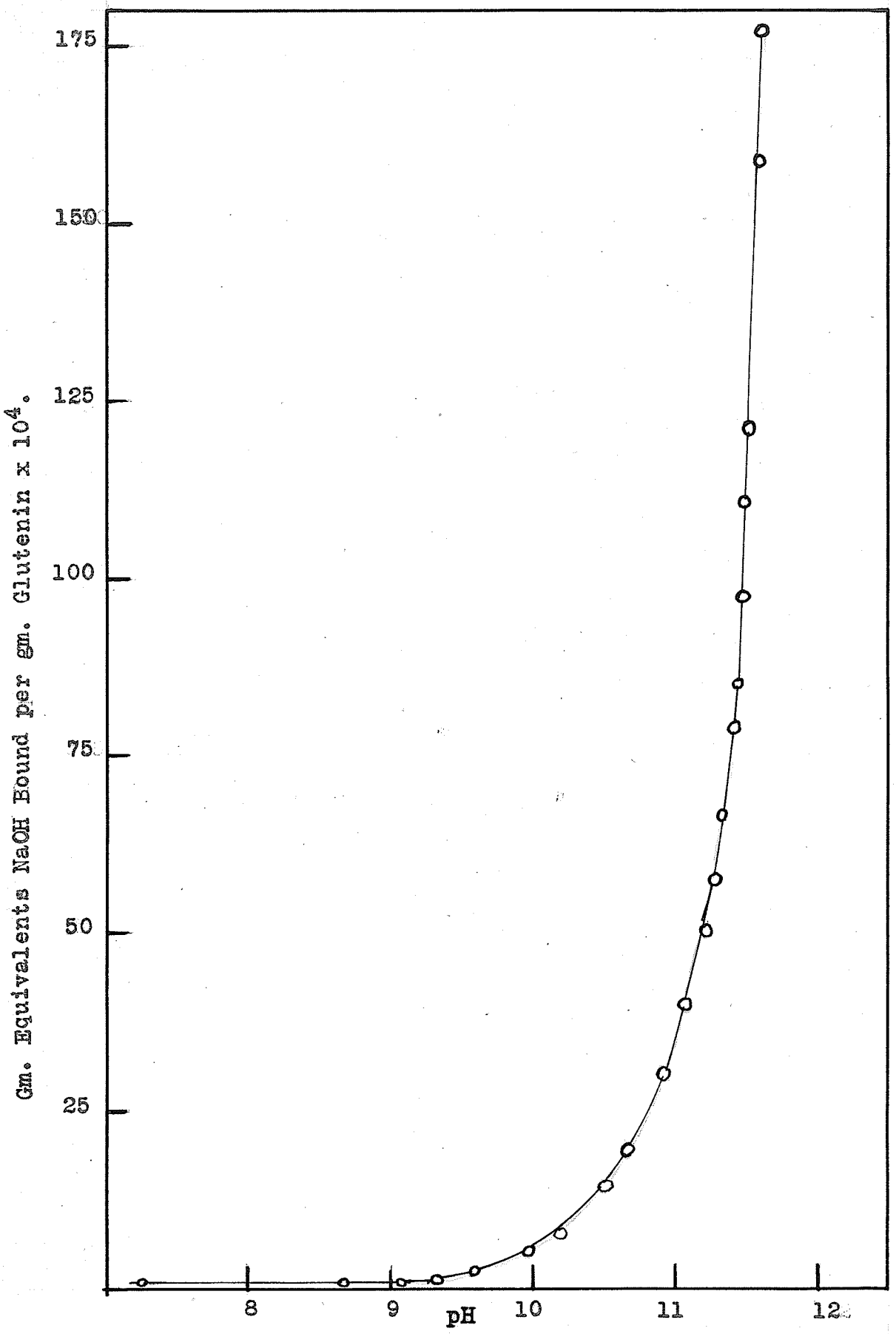

Figure 3. Showing the Relation of Amount of Alkali Bound with pH.

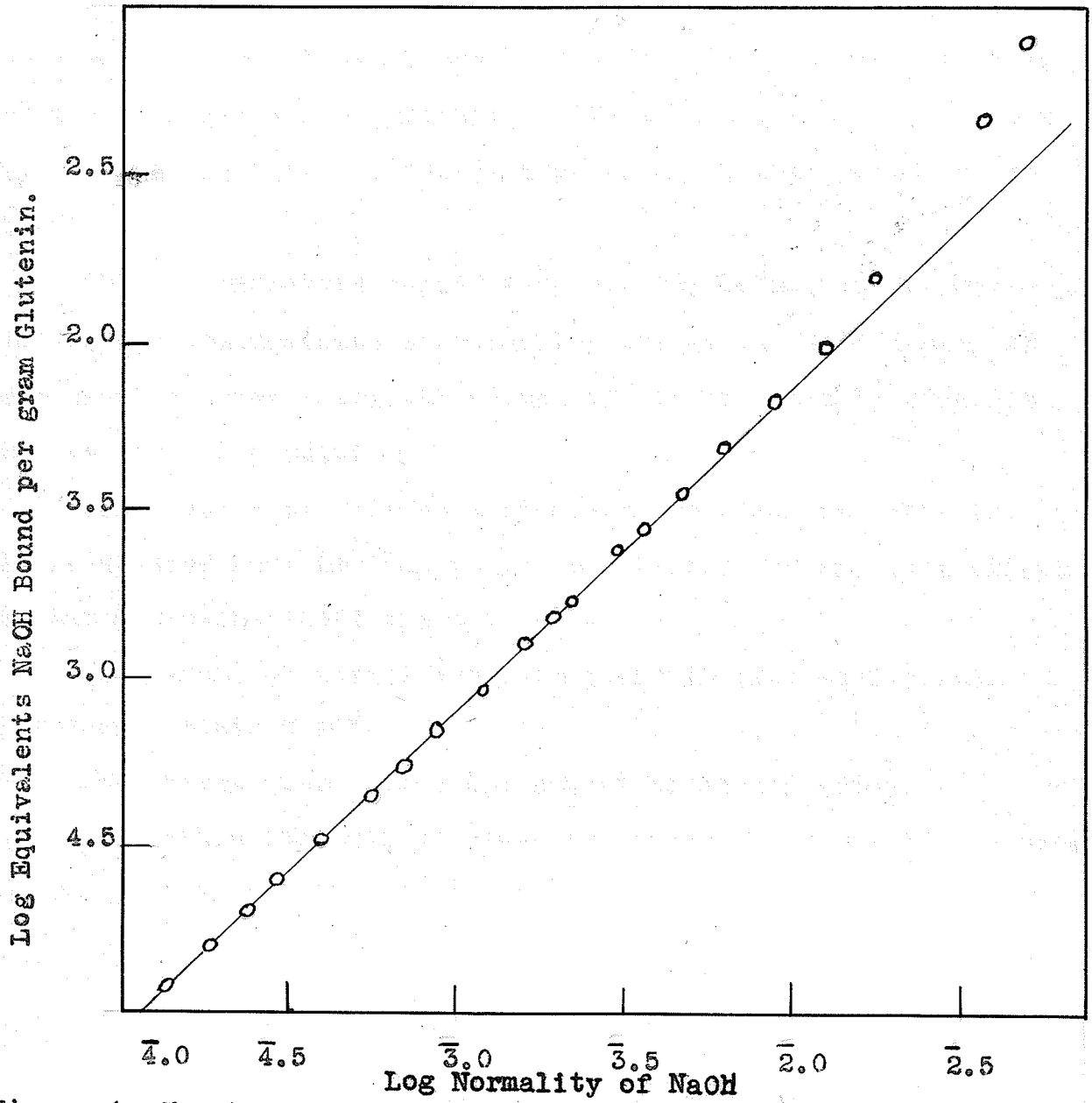

Figure 4. Showing the logarithmic relation between amount of alkali bound with increasing addition of alkali.

C.

The Effect of Temperature on the Amount of Alkali Bound by Glutenin.

It was desired to investigate the influence of temperature on the amount of alkali bound by glutenin in order to ascertain whether a negative temperature coefficient existed as was found by Hoffman and Gortner (1925) at pH values below 2.5 and above 10.5.

Three temperature ranges were chosen, 20^0C., 25^0C., and 30^0C., and measurements were carried out in the same manner as was done in determining the amount of alkali bound by glutenin in the preceding section.

The results of this determination are given in Table IV. It is noticed that no temperature coefficient of any sort exists in the glutenin-alkali system.

The amount of alkali bound is given in gram equivalents per gram protein x 10^4.

The column "ccs" gives the amount of alkali added.

The column "Normality" gives the normality of alkali present in the protein-alkali solution.

TABLE IV

COMPARISON OF AMOUNT OF ALKALI BOUND BY GLUTENIN AT DIFFERENT TEMPERATURES

cc's.	Normality	20°C		25°C		30°C	
		pH	Bound	pH	Bound	pH	Bound
0.0	0.00000	6.86	0.0	6.81	0.0	6.50	0.0
0.1	0.00010	8.28	1.0	9.06	0.4	8.55	0.9
0.3	0.00030	9.25	2.8	9.62	2.5	9.42	2.5
0.5	0.00050	9.71	4.6	9.92	4.1	9.73	4.0
0.7	0.00070	9.96	6.2	10.07	5.5	9.92	5.4
1.2	0.00119	10.35	10.0	10.31	9.4	10.21	8.9
2.6	0.00253	10.76	20.7	10.68	19.6	10.54	19.1
3.8	0.00366	10.95	29.8	10.87	27.9	10.68	28.3
5.0	0.00476	11.06	39.3	11.01	35.7	10.78	37.6
7.5	0.00698	11.26	57.2	11.22	51.1	10.90	58.1
10.0	0.00910	11.34	78.2	11.35	66.6	11.00	80.0
12.0	0.01072	11.40	94.0	11.43	78.6	11.06	94.0
16.0	0.01379	11.44	129.9	11.49	110.1	11.13	127.8
21.0	0.01736	11.50	173.7	11.58	158.6	11.16	173.6

TABLE IV (Cont'd)

cos.	Normality	20°C		25°C		30°C	
		pH	Bound	pH	Bound	pH	Bound
30.0	0.02308	11.55	215.7	11.69	209.4	11.18	218.6
60.0	0.03751	11.61	535.8	11.75	468.9	11.22	542.9
100.0	0.05000	11.63	914.3	11.81	836.0	11.25	921.9

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E.

The Effect of Neutral Salts on the pH-Normality Relationship of Alkali.

It was desired to study what effect neutral salts would have on the alkali binding capacity of glutenin. Work of this type was carried out by Robinson, Gortner, and Palmer (1932), and Metcalfe (1937), the former studying the effect of salts on the alkali binding capacity of casein and paracasein, while the latter studied the salt effect on the casein-acid system.

Before determining what effect neutral salts had on the alkali binding of glutenin, blank measurements were carried out in absence of protein, to notice what effect, if any, the salts had on the hydroxyl ion concentration of the sodium hydroxide. This was deemed necessary, since if the salts altered the pH of the alkali, this alteration would be taken as an increase or decrease in binding of alkali in the presence of glutenin, if such a salt effect on the alkali was unsuspected.

Three concentrations of salts were employed: 0.5 N, 0.05 N, and 0.005 N, diluting each salt from the original 1 Normal salt. The effect of the salts on the pH of the alkaline solution are noted in Table IV, for the 0.5 N salts, and Table V, for the 0.05 N salts. The 0.005 N salts were found to have such a negligible effect that it was impossible to determine whether a true salt effect existed, or whether the effect was due to experimental error only.

The magnitude of the salt effect was determined by taking pH measurements at the same concentrations of alkali as were taken with the salt-alkali system, but this time in the absence of salt. These results are listed also in Tables V and VI, including

the changes of pH, designated as Δ pH, due to the salt effect. The values for Δ pH have been supplied with opposite signs so as to act as correction values for pH in the salt-alkali-protein system. Graphic representations, showing the effect of the salts on the pH-Normality curve of alkali, is demonstrated in Figure 5.

TABLE V

EFFECT OF NEUTRAL SALTS ON THE pH-NORMALITY RELATIONSHIP OF ALKALI

For 0.5 N Salt

Temp. 25°C

Norm.	None pH	NaCl pH	NaCl ΔpH	NaBr pH	NaBr ΔpH	KBr pH	KBr ΔpH	KI pH	KI ΔpH	LiBr pH	LiBr ΔpH
0.00025	9.77	9.38	+0.39	9.15	+0.62	9.52	+0.25	8.95	+0.82	8.33	+1.44
0.00035	9.95	9.61	+0.34	9.42	+0.53	9.80	+0.15	9.15	+0.80	8.55	+1.40
0.00050	10.14	9.85	+0.29	9.70	+0.44	10.01	+0.13	9.45	+0.69	8.81	+1.33
0.00074	10.34	10.17	+0.17	10.00	+0.34	10.34	0.00	9.80	+0.54	9.15	+1.19
0.00098	10.46	10.36	+0.10	10.18	+0.28	10.46	0.00	10.10	+0.36	9.45	+1.01
0.00146	10.64	10.55	+0.09	10.40	+0.24	10.68	-0.04	10.45	+0.19	9.86	+0.78
0.00238	10.86	10.85	+0.01	10.69	+0.17	10.98	-0.12	10.78	+0.08	10.25	+0.61
0.00327	11.02	11.00	+0.02	10.84	+0.18	11.13	-0.11	10.98	+0.04	10.47	+0.55
0.00455	11.16	11.13	+0.03	11.02	+0.14	11.34	-0.18	11.18	-0.02	10.66	+0.50
0.00833	11.40	11.37	+0.03	11.25	+0.15	11.78	-0.38	11.57	-0.17	10.98	+0.42
0.01429	11.59	11.59	0.00	11.42	+0.17	12.17	-0.58	11.93	-0.34	11.21	+0.38

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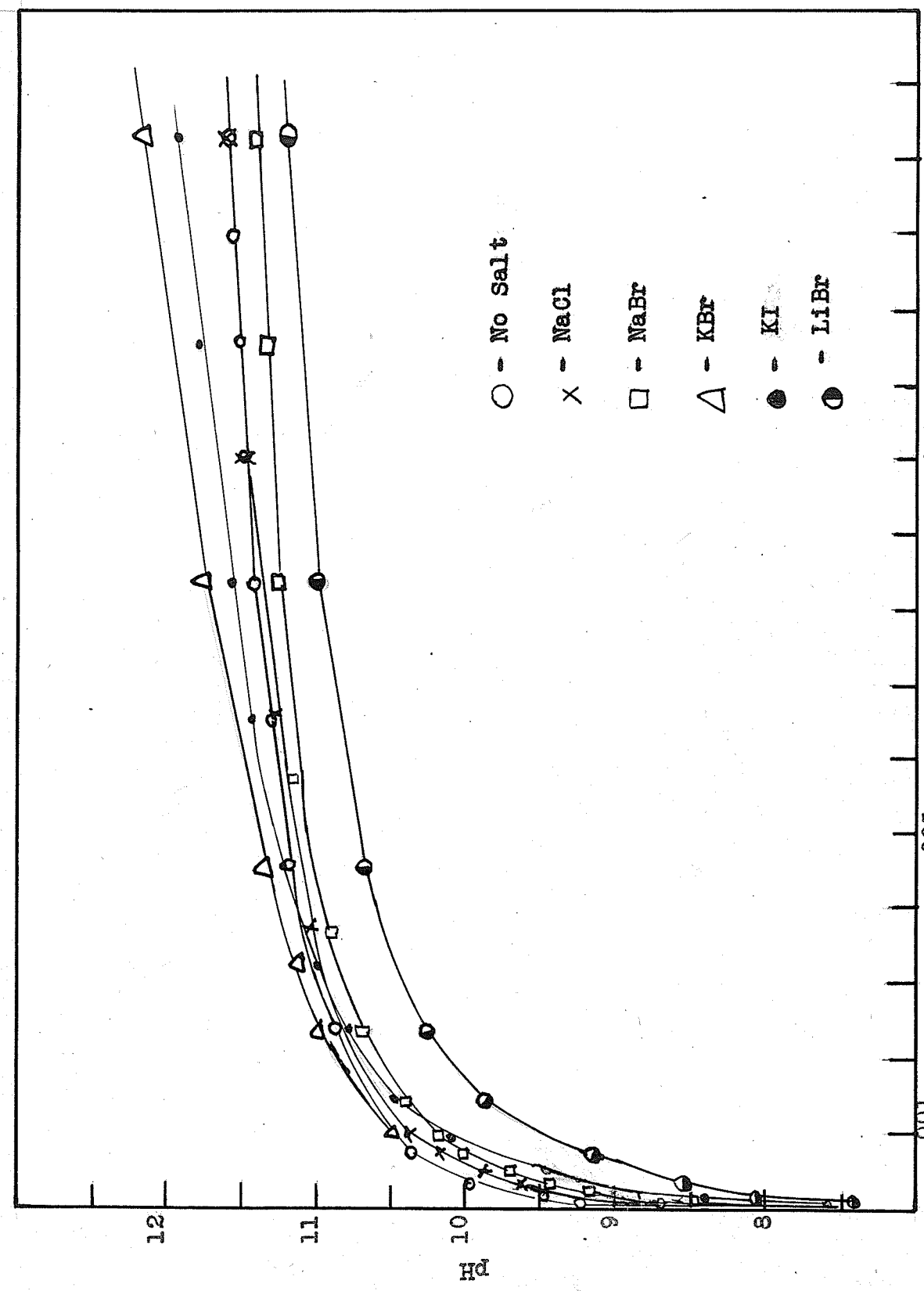

Figure 5. Showing the Effect of 0.5 N Salts on the pH-Normality Curve of Alkali.

TABLE VI

EFFECT OF SALTS ON THE pH-NORMALITY RELATIONSHIP OF ALKALI

For 0.05 N Salt

Temp. 25°C

Norm.	None pH	NaCl pH	NaCl Δ pH	NaBr pH	NaBr Δ pH	KBr pH	KBr Δ pH	KI pH	KI Δ pH	LiBr pH	LiBr Δ pH
0.00029	9.75	9.60	±0.15	9.61	±0.14	9.37	±0.38	9.64	±0.11	9.22	±0.53
0.00038	9.93	9.80	±0.13	9.83	±0.10	9.62	±0.31	9.83	±0.10	9.46	±0.47
0.00047	10.05	9.95	±0.10	9.97	±0.08	9.78	±0.27	9.96	±0.09	9.64	±0.41
0.00067	10.25	10.19	±0.06	10.18	±0.07	10.08	±0.17	10.19	±0.06	9.94	±0.31
0.00094	10.44	10.41	±0.03	10.41	±0.03	10.35	±0.09	10.40	±0.04	10.23	±0.21
0.00140	10.64	10.62	±0.02	10.61	±0.03	10.61	±0.03	10.62	±0.02	10.49	±0.15
0.00186	10.76	10.74	±0.02	10.74	±0.02	10.76	0.00	10.78	-0.02	10.65	±0.11
0.00277	10.97	10.94	±0.03	10.94	±0.03	11.00	-0.03	11.00	-0.03	10.88	±0.09
0.00452	11.20	11.17	±0.03	11.17	±0.03	11.25	-0.05	11.22	-0.02	11.12	±0.08
0.00621	11.33	11.29	±0.04	11.29	±0.04	11.41	-0.08	11.38	-0.05	11.27	±0.06
0.00864	11.46	11.42	±0.04	11.40	±0.06	11.58	-0.12	11.55	-0.09	11.42	±0.04
0.01583	11.65	11.62	±0.03	11.61	±0.04	11.84	-0.19	11.82	-0.17	11.57	±0.08
0.02715	11.77	11.76	±0.01	11.75	±0.02	12.04	-0.27	12.05	-0.28	11.69	±0.08

I.

The Effect of Neutral Salts on the Amount of Alkali Bound by Glutenin

In addition to studying the alkali binding capacity of glutenin, it was desired to know how the amount bound was effected by the presence of neutral salts. As mentioned previously, similar studies on the effect of salts on the acid or alkali binding of other proteins were carried out by Robinson, Gortner, and Palmer, and by Metcalfe. The former studied the salt effect at four distinct ranges of pH, while the latter obtained values at the high and low degrees of acidity. In our research we took measurements of the salt effect through the entire alkaline range, or rather from the neutral point to a pH of about 12, thus obtaining a study through a continuous series of pH values between the limits mentioned.

This was accomplished by making up a protein-salt solution of desired normality, and adding increasing amounts of a salt-alkali solution of the same normality in respect to the salt present. In this way, no matter how much alkali was added to the protein system, the salt concentration remained constant. Salt solutions of the following concentrations were prepared for each salt: 0.5, 0.05, 0.005 normal. In order to obtain a variety of anions and cations, the following salts were employed: sodium chloride, sodium bromide, potassium bromide, potassium iodide, and lithium bromide. 0.1 gram glutenin in 100 cc. of salt solution was used.

The values of Δ pH were applied where necessary so as to make certain that the changes produced in hydroxyl ion concentration were due to the salt effect on the protein only, and not

due in part to that on the alkali. That is, the application of these corrections would convert the pH to its original value that would have existed had there been no salt effect on the alkali itself. In applying this latter method, it was necessary to assume that the salt effect on the alkali in the absence of protein was the same as that when the protein was present.

These results are tabulated in Tables VII, VIII, and IX. A comparison of these results apparently shows that there is no increased binding due to the presence of neutral salts.

The column marked "ccs" gives the amount of alkali-salt solution added.

The column marked "norm" gives the normality of the alkali present.

The column marked "bound" gives the gram equivalents of alkali bound per gram protein x 10^4.

TABLE VII

EFFECT OF 0.5 N SALTS ON AMOUNT OF ALKALI BOUND BY GLUTENIN.

Temp. 25°C

Norm.	No Salt		NaCl		NaBr		KBr		KI		LiBr	
	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound
0.00000	6.81	0.0	6.14	0.0	6.72	0.0	6.63	0.0	6.76	0.0	6.81	0.0
0.00005	8.62	0.2	6.91	0.5	8.06	0.5	7.73	0.5	7.32	0.5	7.48	0.5
0.00015	9.30	1.2	8.60	1.5	8.83	1.4	8.98	1.4	8.34	1.5	9.52	1.1
0.00025	9.57	2.1	8.99	2.4	9.72	1.8	9.37	2.2	9.58	2.0	9.89	1.5
0.00035	9.71	2.9	9.20	3.3	9.93	2.4	9.63	2.9	9.79	2.7	10.06	2.0
0.00050	9.92	4.1	9.55	4.5	10.07	3.5	9.87	4.0	9.95	3.9	10.25	2.7
0.00074	10.09	5.9	9.82	6.6	10.24	5.3	10.16	5.6	10.17	5.6	10.44	4.0
0.00098	10.24	7.8	10.03	8.6	10.37	6.9	10.34	7.1	10.21	7.9	10.53	5.5
0.00146	10.40	11.7	10.29	12.5	10.58	10.0	10.55	10.3	10.39	11.7	10.67	8.9
0.00238	10.66	18.8	10.57	20.0	10.78	16.8	10.74	17.4	10.70	18.2	10.90	14.2
0.00327	10.82	25.7	10.75	27.1	10.95	22.4	10.96	22.3	10.88	24.4	11.08	18.2
0.00455	10.98	36.2	10.88	39.0	11.06	33.4	11.09	32.1	11.06	33.4	11.23	25.4
0.00833	11.29	68.1	11.12	78.5	11.32	66.0	11.32	66.0	11.33	65.2	11.48	50.9
0.01429	11.50	138.3	11.26	164.5	11.55	130.9	11.45	145.0	11.55	130.9	11.70	102.3

TABLE VIII

EFFECT OF 0.05 M SALTS ON AMOUNT OF ALKALI BOUND BY GLUTENIN

Temp. 25°C

Norm.	No Salt		NaCl		NaBr		KBr		KI		LiBr	
	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound
0.00000	6.81	0.0	6.41	0.0	6.79	0.0	7.13	0.0	6.74	0.0	6.90	0.0
0.00010	9.06	0.4	8.54	1.0	8.74	0.9	8.52	1.0	8.47	1.0	8.36	1.0
0.00019	9.39	1.6	9.33	1.6	9.22	1.7	9.08	1.7	9.15	1.7	8.72	1.8
0.00029	9.59	2.4	9.60	2.4	9.53	2.5	9.38	2.6	9.36	2.6	9.48	2.5
0.00038	9.75	3.1	9.78	3.1	9.75	3.1	9.59	3.3	9.56	3.3	9.66	3.2
0.00047	9.86	3.8	10.02	3.4	9.87	3.7	9.72	4.0	9.72	4.0	9.73	4.0
0.00067	10.04	5.3	10.25	4.4	10.11	5.0	10.02	5.4	9.97	5.5	9.98	5.5
0.00094	10.22	7.4	10.42	6.1	10.37	6.5	10.27	7.1	10.25	7.2	10.21	7.4
0.00140	10.38	11.1	10.60	9.0	10.60	9.0	10.55	9.5	10.49	10.2	10.47	10.4
0.00186	10.51	14.7	10.73	11.8	10.72	12.0	10.74	11.7	10.69	12.5	10.58	14.0
0.00277	10.71	21.6	10.92	17.4	10.95	16.6	10.98	15.7	10.94	16.8	10.80	20.0
0.00452	10.99	33.9	11.14	27.8	11.17	26.9	11.21	25.0	11.17	27.0	11.07	31.2
0.00621	11.13	47.1	11.26	40.3	11.28	39.2	11.32	36.5	11.28	39.2	11.22	42.7
0.00864	11.32	63.8	11.37	60.1	11.42	55.8	11.47	50.9	11.44	53.9	11.33	63.1

TABLE VIII (Cont'd)

Norm.	No Salt		NaCl		NaBr		KBr		KI		LiBr	
	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound
0.01583	11.55	130.3	11.56	130.0	11.58	126.1	11.71	113.2	11.65	115.0	11.57	127.6
0.02715	11.69	303.6	11.64	292.3	11.72	274.7	11.89	224.2	11.77	244.6	11.64	292.3

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TABLE IX (Cont'd)

EFFECT OF 0.005 N SALTS ON AMOUNT OF ALKALI BOUND BY GLUTENIN

Temp. 25°C

Norm.	No Salt		NaCl		NaBr		KBr		KI		LiBr	
	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound
0.00000	6.81	0.0	6.42	0.0	6.90	0.0	6.81	0.0	7.25	0.0	6.96	0.0
0.00010	9.06	0.4	9.06	0.8	8.84	0.9	8.90	0.9	8.83	0.9	8.75	0.9
0.00020	9.40	1.7	9.62	1.6	9.46	1.6	9.41	1.7	9.29	1.7	9.45	1.6
0.00030	9.62	2.5	9.85	2.1	9.78	2.2	9.75	2.3	9.50	2.6	9.82	2.1
0.00040	9.77	3.3	10.00	2.7	10.00	2.7	9.94	2.9	9.80	3.2	9.98	2.8
0.00050	9.92	4.1	10.12	3.3	10.14	3.2	10.08	3.5	9.98	3.8	10.12	3.3
0.00070	10.07	5.5	10.32	4.3	10.32	4.3	10.28	4.5	10.22	4.9	10.34	4.2
0.00099	10.24	7.8	10.51	5.8	10.53	5.6	10.48	6.1	10.44	6.4	10.55	5.4
0.00147	10.40	11.7	10.68	8.6	10.62	9.4	10.71	8.2	10.67	8.8	10.77	7.2
0.00195	10.53	15.4	10.81	11.3	10.81	11.3	10.86	10.3	10.83	10.9	10.91	9.2
0.00289	10.75	22.1	10.98	16.4	11.03	15.4	11.05	14.6	11.05	14.6	11.10	12.9
0.00474	11.01	35.7	11.18	28.6	11.26	24.5	11.31	21.4	11.30	22.0	11.30	22.0
0.00651	11.17	48.4	11.29	41.7	11.36	36.7	11.42	32.0	11.45	29.1	11.41	32.7
0.00904	11.34	66.7	11.41	60.9	11.46	56.2	11.58	42.5	11.62	37.0	11.52	49.8

TABLE IX (Cont'd)

Norm.	No Salt		NaCl		NaBr		KBr		KI		LiBr	
	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound	pH	Bound
0.01658	11.57	136.4	11.50	145.6	11.62	128.7	11.74	94.4	11.84	82.5	11.64	125.5
0.02843	11.70	326.7	11.63	312.0	11.72	292.3	11.96	223.0	12.04	177.1	11.69	299.3

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IV

DISCUSSION

DISCUSSION

It is evident from Figures 1, 2, and 3, that reactions of a purely chemical nature cannot be considered identical with those where proteins are involved. In Table I, are tabulated the amounts of acid in gram equivalents bound per gram alkali throughout a pH range of 2.14 to 10.60, a range which represents widely varying degrees of acidity and alkalinity. It is noticed that regardless of the pH, a constant amount of hydrochloric acid is bound per gram of sodium hydroxide. This is to be expected, for it is known that acids and bases combine in purely stoichiometric proportions. Thus from the chemical equation representing the reaction between HCl and NaOH, the theoretical amount of acid bound per gram alkali can be found from the molecular weights. Therefore, in the present case of HCl and NaOH it can be calculated that 0.625 gram equivalents of HCl are bound per gram NaOH. Since this same value was arrived at experimentally employing the Cohn and Berggren method of calculation, the use of the latter, as far as purely chemical reactions are involved, is justified.

In Figures 2 and 3 are shown the graphical representations of the data given in Tables II and III respectively. It can be seen that in cases where proteins are involved, the amounts of acid or alkali bound per gram protein do not lie on a straight line of constant values, as in Figure 1, but increase with change in pH. This would indicate the existence of influencing factors other than those of purely chemical force. This observation appears to be in agreement with that of Hoffman and Gortner (1925) who are of the opinion that forces of adsorption

exist in reactions between proteins and acids or bases.

In the case of glutenin and hydrochloric acid, as is evident from Figure 2, a maximum of acid binding is shown to occur at pH of about 2.9, which corresponds to 66 x 10^{-5} gram equivalents HCl bound per gram protein. Robinson, Gortner, and Palmer (1932) mention that if the increase of amount of alkali bound per gram protein was due to increased ionization of the protein at higher pH values, then a point would be reached where no more alkali would be bound. That is, all the carboxyl groups would have reacted, and there would be no further salt formation between the protein and the alkali. The curve would then show a constancy irrespective of pH.

In our work no such constancy was found, for the maximum of binding was due to a fall of the curve. No explanation can be offered to explain this apparent decrease in acid binding of glutenin, but an actual decrease in binding after a certain point does not seem reasonable. Metcalfe (1937) obtained a similar curve with the system of casein-HCl, and offered an explanation to the apparent decrease in acid-binding. He attributed the fall in the curve to the hydrolysis of the casein induced by the high concentration of acid. Phosphoric acid was therefore set free, which tended to lower the pH, thus suggesting a decrease in binding. Since glutenin is a simple protein there is no possibility of the splitting-off of acid due to hydrolysis, and therefore such a explanation offered by Metcalfe appears to be false.

In the case of alkali (Figure 3), no such maximum exists, as the binding increases continuously with rising pH. This is in line

with the work of Robinson, Gortner and Palmer who found a similar type of curve in the case of the alkali-binding of casein and paracasein. Both the alkali and -acid binding curves of glutenin show the weakness in the theory of strictly stoichiometrical reactions in the case of proteins.

It is noticed that the alkali binding curve of glutenin resembles that of an adsorption isotherm. Since however, many chemical reactions give this same parabolic representation, this cannot be considered evidence for an adsorption type of combination between protein and alkali. According to the Freundlich adsorption isotherm

$$\frac{x}{m} = K \cdot c^{n}$$

where x is the amount of substance adsorbed by m grams of adsorbent, c is the concentration of the dissolved substance in the solution at equilibrium, and K and n are constants.

When one takes the logarithms of the quantities in the equation of the adsorption isotherm, one obtains the expression

$$\log \frac{x}{m} = n \cdot \log c + \log K.$$

That is, log (x/m) is a linear function of log c, so that if one plots the values of log (x/m) against the values of log c, a straight line is obtained. This has been done in Figure 4. Thus, log (x/m) is represented by the logarithm of the amount of alkali bound (expressed in gram equivalents) per gram of glutenin, the adsorbent; log c, the log of concentration of the dissolved substance in the solution at equilibrium, is represented by the logarithm of the original alkali concentration.

The plotted experimental points are observed to follow

very closely the trace of a straight line. The only discrepancy from the straight line relationship occurs at values of greatest alkalinity. The reason for this may be due to the inaccuracy of the glass electrode, for at such extreme ranges of pH, the "alkali ion error effect" is too great for satisfactory correction. Hoffman and Gortner (1925), in plotting their experimental results by the logarithmic method, obtained straight lines only in regions greater than pH of 10.5, and smaller than pH of 2.5. From this evidence, they concluded that adsorption forces exist in these regions. Robinson, Gortner, and Palmer (1932) plotted the logarithms of salt normality against the logarithms of alkali bound per gram of protein. They found that at a pH of 12, the curve approximated a straight line, while at pH 9 and pH 6, sigmoid curves were obtained with a greater curvature at pH 6 than at pH 9. They concluded that there was less of an adsorption effect at pH 6 than at pH 9, and less at pH 9 than at pH 12.

According to our findings, however, in the case of glutenin the adsorption effect appears to exist through the entire alkaline range of the pH scale. The plotted experimental points in Figure 4 show this quite definitely. We therefore cannot substantiate the findings of Hoffman and Gortner (1925) in the case of the prolamines, or those of Robinson, Gortner, and Palmer, in the case of the phosphoproteins, casein and paracasein. These workers find an adsorption effect only in regions of high alkalinity or high acidity, whereas our results appear to increase the pH range of adsorptive activity.

Hoffman and Gortner (1925) present further evidence for an adsorption type of reaction in extreme ranges of pH by show-

ing the existence of a negative temperature coefficient in these ranges. We made a similar study of alkali binding by protein at three different temperature ranges, 20^0, 25^0, and 30^0, the results of which are compiled in Table IV. In comparing the two temperature ranges of 20^0 and 25^0 a definite negative temperature coefficient was found. However, when similar determinations were made at 30^0, a greater, and lesser binding in some cases, existed than at lower temperatures. From these results, therefore, we were unable to establish the existence of a negative temperature coefficient as was found by Hoffman and Gortner. The absence of a negative temperature coefficient would appear to discount the existence of an adsorption type of reaction between glutenin and sodium hydroxide. The findings of such controversial factors show the complexity of the systems in which we are dealing. That is, we cannot state that protein reactions take place according to strict chemical rules, nor can we conclude that proteins adsorb bases in the same manner as charcoal adsorbs gases. We can only say that adsorption forces do act, but may be modified due to the presence of chemical changes.

Robinson, Gortner, and Palmer (1932), in their study of the effect of neutral salts on the alkali binding of casein and paracasein, neglected to consider whether these neutral salts affected the hydroxyl ion concentration of the alkali itself. These workers employed the Cohn and Berggren method of calculating the amount of alkali bound, and since the results depended upon the concentration of free alkali, any change in hydroxyl concentration would be attributed to the action of the

protein alone. Thus if neutral salts decreased the pH of an alkaline solution, this decrease would be taken as an increase in binding by the protein, and it would therefore be concluded that neutral salts increased the acid binding of the protein, when in reality the decrease of pH would be due entirely, or at least in part, to the action of the salt on the alkali, and not to the type of binding by the protein.

For this reason we therefore concluded that it was first necessary to study the alkali in the presence of the neutral salt alone. pH readings of increasing normalities of alkali were taken. The effect of the various salts on these readings were then noted, and are tabulated in Tables V & VI. In the case of the 0.5 N salts, it is noted that there is a decided effect upon the hydroxyl ion concentration. The magnitude of this effect varies according to the salt employed. The salt effect on sodium hydroxide is rendered more obvious in Figure 5.

A study of this graph will show that the difference in salt effect varies, in the main, according to the cation. Thus NaCl and NaBr decrease the pH of the alkali to an appreciably equal degree, while the potassium salts, KBr & KI, show a similarity in their increase of the pH. The lithium salt causes a great decrease in pH of alkali, much greater than that of the sodium salts.

The effect of 0.05 N salts upon the hydroxyl ion concentration of the alkali is much less noticeable. This is further a demonstration of the salt effect upon alkali, since the effect varies directly as the concentration of the salt, which is to be expected.

From these determinations it is evident that especially in the case of 0.5 N salts, the salt effect upon the alkali itself cannot be neglected. If the salt effect was neglected, calculations of alkali binding by the Cohn and Berggren method, would show that sodium and lithium salts greatly increased the binding, while potassium salts decreased the binding, since in the former case, a lower hydroxyl ion concentration was obtained at equilibrium, while a higher hydroxyl ion concentration resulted in the latter.

We therefore decided that before we could determine the true binding of alkali by glutenin in the presence of salts, a correction for the salt effect on the alkali alone must first be applied. This correction was obtained by comparing the determined pH of alkali alone, and the pH of the same alkali concentration but in the presence of a neutral salt. We denoted the difference in pH as Δ pH, and preceded each value with a negative or positive sign depending on whether the difference had to be added or subtracted to obtain the pH reading which would have been obtained if there had been no salt effect on the alkali alone.

In application of this procedure we had to assume that the effect of the individual salts upon the alkali in the presence of the protein was identical with that when the protein is absent.

In Tables VII, VIII, and IX, are tabulated the amounts of alkali bound in the presence of the salts NaCl, NaBr, KI, KBr, and LiBr, whose normalities are 0.5 (in Table VII), 0.05 (in Table VIII) and 0.005 (in Table IX). The pH readings in Tables

VII and VIII have been corrected by applying the values of Δ pH, found in Tables V and VI, each correction being applied at the corresponding normality of alkali.

From these results we were unable to find any definite salt effect upon the alkali binding of glutenin, and consequently there was no question of the existence of a lyotropic series. Our findings of a negative salt effect upon the alkali binding of protein tends to substantiate the results of Cohn, Green, and Blanchard (1937).

Since neutral salts did not appear to show any positive effect upon the alkali binding capacity of glutenin, our study of the type of combination between this protein and sodium hydroxide could not be enhanced by studying such a salt effect.

V

SUMMARY AND CONCLUSIONS

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1. Sodium hydroxide binds a constant amount of hydrochloric acid with increasing pH from 2.14 to 10.60.
2. The acid-binding capacity of glutenin increases with falling pH until a value of 2.9 is reached, after which the binding shows an apparent decrease which may be due to the failure of application of the Cohn and Berggren method of calculating the amount of acid bound by protein in highly acid concentrations.
3. The alkali-binding capacity of glutenin increases indefinitely with rising pH. This increase appears to be due to forces of adsorption, since the logarithms of the amount of alkali bound plotted against the logarithms of the original alkali concentration form a straight line.
4. The presence of a marked negative temperature coefficient was not established as is the case with purely adsorption reactions.
5. Neutral salts of 0.5 normality have a definite effect upon the hydroxyl ion concentration of sodium hydroxide, and this effect must be considered in employing the Cohn and Berggren method of calculation of alkali binding by proteins.

   0.05 N salts also show a definite effect but to a lesser degree than that of 0.5 N salts. The effect of 0.005 N salts is negligible.
6. Neutral salts of the following normalities, 0.5, 0.05, 0.005, have a negative effect upon the alkali binding capacity of glutenin.

VI

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