THE REACTION OF CASEIN WITH HYDROCHLORIC ACID.

A THESIS

Submitted to the Committee on Post-Graduate Studies
Of the University of Manitoba.

by

EDWARD GERALD METCALFE, B.S.A.

In Partial Fulfillment of the Requirements for the
Degree of
MASTER OF SCIENCE.

April
1 9 3 7.

ACKNOWLEDGMENT.

The Writer wishes to express his sincere appreciation for the criticisms and valuable advice given him by Dr. A. D. Robinson, under whom this work was done.

E. G. M.

TABLE OF CONTENTS.

INTRODUCTION:

Probably no other class of organic compounds has been the object of greater attention in recent years than the proteins. Many and varied are the studies that have been made of them. This has led to the accumulation of much adverse data, each particular study leading investigators far afield. There has been a great deal of disagreement regarding results and conclusions, and separate investigators studying similar properties have been in disagreement when drawing conclusions from their observations.

Among the properties of proteins which have so far not been satisfactorily explained is the nature of their reactions with acids and bases. Many different studies have been made of this property. The literature dealing with the investigators' results lead one to conclude that there are three schools of thought regarding protein-acid, protein-base systems. One group of workers holds that the reaction is one involving strictly chemical forces. Such men as Loeb, Hitchcock and Schmidt support this theory. Another group maintains that the forces of adsorption alone are responsible. The third group is in favor of chemical binding between pH values of 2.5 and 10.5 while outside this range absorption is the main factor. Hoffman and Gortner defend this last view.

The above controversy has largely arisen out of interpretations of experimental results and in observing

their own methods each group is no doubt justified in their conclusions. Apparently then, agreements must be reached regarding methods of attack and modes of experimentation. Greater precision and accuracy in experimental measurements might also assist in bringing about a better mutual understanding. Studies on whole groups of compounds under varied conditions would be valuable. Hoffman and Gortner (1925) have done this, using the Prolamines, but investigations of this kind are time-consuming and therefore expensive.

Since our time was limited we chose the one compound casein, because of its ease of preparation in a high degree of purity. It was hoped that by a careful study of its behaviour we might be able to enhance the knowledge of casein-acid systems.

HISTORICAL:

The field of protein reactions with acids and bases has interested many workers, with the result that a voluminous literature has accumulated, far beyond the scope of this introduction. However the papers of Hoffman and Gortner, (1925), Gortner (1929) and Robinson, Gortner and Palmer (1932) contain extensive bibliographies.

That proteins are amphoteric has been known for years, That is to say, they react with both acids and bases.

METHODS USED IN INVESTIGATIONS:

Various methods have been employed in an attempt to determine how and to what extent proteins reacted to acids and bases. Assuming that the reaction was a chemical one, Van Slyke and Hart (1905) used alcohol to precipitate "Ca caseinate." This however, did not prove satisfactory. Hoffman, and Gortner (1925) refer to a method used by Cohnheim and Kreiger based on the fact that phosphotungstic acid proteins yield insoluble salts depending on the fact that the protein is in combination with an acid as HCl. Then the reaction is as follows:

$$\underset{(sol)}{\text{Protein HCl}} + \underset{(sol)}{\text{Ca phosphotungstate}} \longrightarrow$$

$$\underset{(insol)}{\text{Protein phosphotungstate}} + \underset{(sol)}{CaCl_2}$$

The $CaCl_2$ in the filtrate furnishes an indication of the amount of HCl bound by the protein.

The work of Sjoquist referred to by Robinson, Gortner and Palmer (1932), and Lacquer and Sockur in Hoffman and Gortner (1932) is based on the fact that the conductivity of protein-base, and protein-acid complexes is considerably lower than that of the free base of acid. Hence the addition of protein to an acid or base is accompanied by a decrease in conductivity. Sjoquist added increasing amounts of albumin to 0.025 N HCl. The molar conductivity decreased till a constant value was obtained. This was first reached at a 4.25 per cent concentration of albumin. It was inferred that at this point all the acid had been bound by the albumin and that the constant conductivity value was due to the compound formed by the HCl on the albumin. Hoffman and Gortner (1925) obtained similar results but failed to add sufficient protein to give them a constant conductivity value. Greenberg and Larson (1935) found that the conductivity of solutions of casein, edestin and gelatin in anhydrous lactic acid and glacial acetic acid was little greater than the acids themselves, but in anhydrous formic acid the conductivity is nearly as good as that of alkali and alkaline earth formates in formic acid. They say that the proteins in formic acid have formed ionisable salts having electrochemical properties that would be expected of formic acid salts of rather high valency.

Robinson, Gortner and Palmer (1932) mention that Bugarsky and Liebermann introduced potentiometric measure-

ments as a means of determining the amount of acid or base bound by a protein. We used this method, the amount bound being determined by the difference between the original concentration and the residual concentration of HCl. Hoffman and Gortner (1925) mention the work of Blasel and Natula who used the following formula:

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

Where n = amount of acid or alkali bound,

N = original normality of acid or alkali,

cH = the measured ($\overset{+}{H}$) of the protein-acid or protein-base solution at equilibrium,

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

Pauli and Spitzer (1922) used the same formula for strong alkalis as for acids:--

$$n = N - \frac{cOH}{q}$$

For weak bases they used the expression including K the dissociation constant.

$$n = \frac{K(N-cOH) - (cOH)^2}{K - cH}$$

Hoffman and Gortner (1925) state that "The degree of ionisation of acid and alkali as determined by conductivity measurements and by potentiometric methods do not agree. The values from conductivity data cannot be used for calculat-

ing the amount of acid or alkali bound by a protein."
They made use of the following formula:--

$$n = N - \frac{cH}{\alpha'} \quad \text{or} \quad n = N - \frac{cH\ (corr)}{\alpha'}$$

where (alpha) is the degree of ionisation determined potentiometrically. They favor the former but it must be noted that both these formulae assume complete ionisation of the protein salt, regardless of the pH. Some discrepancy has arisen as to the effect of binding of Cl ions by the protein. With regard to this, Hoffman and Gortner (1925) say the following:

"The experimental data show that it is only the lower concentrations of acid where considerable ionisation occurs, and at these concentrations the correction for the Cl ion is very small. At the higher concentrations of acid the correction for the Cl is quite large but the ionisation is very small (as a rule less than 50 per cent). The true value for the amount of acid bound appears to be much nearer the value obtained from the formula:-

$$n = N - \frac{\overset{+}{H}}{\alpha'} \quad \text{than from} \quad n = N - \frac{\overset{+}{H}\ (corr)}{\alpha'}$$

where α' is the degree of ionisation as determined by potentiometric methods."

We used the calculation of Cohn and Berggren as mentioned by Robinson, Gortner and Palmer (1932), and this will be discussed later.

Indicators have been used in the determination of

acid and alkali binding but they are objected to for two reasons: (1) Due to their amphoteric character and also to their multiple combining capacity the change of hydrogen or hydroxyl ion concentration when acid or alkali is added is not very great, thus the end points are not sharp. (2) Many of the substances used as indicators are either acids or bases and may combine with the proteins.

Of more recent years different methods of study have appeared. Faneslow (1928) has studied the influence of electrolytes and non-electrolytes upon the optical activity and relative resistance to shear of gelatin systems. Carpenter (1927) used specific rotation studies in determining the influence of salts on the optical rotation of gelatin.

Van Slyke and Bosworth (1913) and (1913_a) dissolved protein in measured amounts of alkali and titrated the excess with standard acid. The end point was marked by the settling out of the casein.

PROTEIN REACTIONS WITH ACIDS AND BASES.

The methods mentioned do not begin to tell a complete story of the work on proteins and acid-base binding. Conflicting conclusions had been drawn from the work done; some investigators favoring a chemical theory of binding; others one of adsorption. Undoubtedly proteins do form chemical compounds with acids and bases, since results have been put forward in proof of this. Then again, there is proof that colloidal forces play a part.

Alkali bound by protein increases with rising pH and the acid bound increases with lowering pH. This suggests that forces of adsorption are being enhanced by rising pH on the one hand and lowering pH on the other.

Loeb (1922) holds that the hydrogen ion concentration must be rigidly controlled. He claims that at the same pH a protein will bind equivalent amounts of different acids or alkalis. He used casein, gelatin and albumin with Hydrochloric, Oxalic, Sulphuric and Phosphoric acid and concluded that acids were bound in equivalent proportions.

Robinson, Gortner and Palmer (1932) mentions the work of Robertson in proof of chemical binding where increasing amounts of casein were dissolved in potassium hydroxide. If the normality of the KOH neutralised by the casein added is plotted as ordinates against the original normality of the KOH in which the casein was dissolved as abscissae, an interesting series of curves result. The line slopes upward, then falls off, tending to become horizontal to the X - axis. For higher concentrations of alkali the original line continues further, but falls off again. That is, the higher the concentration of alkali, the more we can expect casein to act as an ordinary acid.

Weber, in Robinson (1930) studied the swelling of fibrin in HCl. He concluded that the acid is chemically bound, since the binding reaches a well defined end point, beyond which acid binding cannot be increased by further add-

ition of acid. No negative temperature coefficient was observed for the acid binding by insoluble protein. The hydrogen ion binding and increase in free NH_2 groups are not parallel processes in protein cleavage by digestion, but the hydrogen binding follows approximately the same curve as the increase in free carboxyl groups. The acid binding curve of fibrin is not an adsorption curve.

W.M.Sandstrom (1930) studied acid and alkali binding by native and deaminised proteins. He reports that the acid bound by casein, edestin, arachin, fibrin and durumin is roughly proportional to the lysine content. Acid binding was decreased when the proteins were deaminised. At pH 10.5 however, sodium hydroxide bound by deaminised proteins is more than native proteins. In the regions studied, acid and alkali binding is strictly adsorptive.

In some phase rule studies on the proteins casein, arachin, gliadin and edestin, Bancroft and Barnett (1930) dealt with the solid compounds of these proteins with hydrochloric acid and ammonia. They found that casein, zein, arachin, fibrin and gliadin absorb NH_3 without the formation of chemical compounds while casein, arachin, gliadin and edestin form definite compounds with HCl, while zein does not. These investigators placed a weighed amount of powdered base or acid in a vessel of known volume. Then a known amount of HCl gas or ammonia is run in. From the pressure at equilibrium the volume of the gas remaining in the flask is calculated and the

volume removed by the solid found by difference.

Piettre (1931) in his studies on the influence of adsorption on the Physicochemical Properties of Organic Colloids, states that the pH curve of HCl is displaced toward the neutral point by the addition of serum proteins. This can be observed only when acids weaker than 0.002 N are used. It is due to the adsorption of the electrolyte.

Carpenter (1927) studied the influence of salts on the optical rotation of gelatin, using KCl, KBr and KI.

Schulz and Ettisch (1933) maintain that the reactions of acids and bases with proteins are divided into a primary and secondary reaction. (1) This is instantaneous in nature and ionic in character, and follows the mass action law. (2) This one is very much slower and with small amounts of base is reversible, but a large amount destroys the protein molecule. This reaction with acids is very weak.

Hitchcock (1932) studying the combination of certain proteins with HCl states that the E.M.F. measurements of cells without liquid junction, (Ag,AgCl, HCl protein, H_2) with gelatin, edestin, casein in 0.1 N NCl solution furnish data consistent with the assumption of, (1) a constant combining capacity of each protein for (H) ion; (2) no combination with (Cl) ion; (3) Farley's principle of a lineal variation of the log of the mean activity coefficient of the acid with increasing protein concentration. Combining capacities obtained were; edestin 13.4×10^4; gelatin 9.6×10^4; casein 8.0×10^4 in

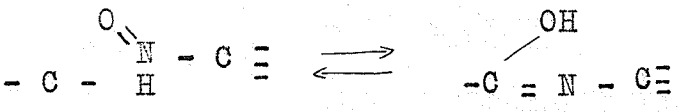
equivalents of combined (H) per gram of protein.

Lloyd (1933) studying the combination of proteins with acids and bases states that while it is true that the adsorption equation fits the acid titration curve of proteins nearly as well as the stepped curves drawn through the experimental points, the divergences from the adsorption curve are real because repeatedly found at the same pH value by different workers. The alkali adsorption curve shows more marked divergence from that of the adsorption equation. This supports the chemical theory of acid and alkali binding by proteins.

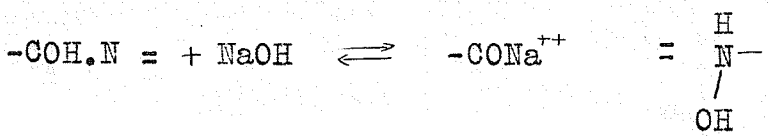
Pauli (1927) concludes that the behavior of soluble proteins as albumins toward acid is explained by the fact that the amino group has a positive ion and the COOH group has a negative ion. If equal in number the molecule is neutral. Mineral acids depress ionisation of the COOH group and increase that of the amino group and the protein residue becomes a multivalent positive ion. This is the generally accepted mode of chemical combination of the protein with acids and carboxyl groups. Whether the amino group adds water to give $R\text{-}NH_3OH$ which ionises like NH_4OH is a matter on which individual opinion will differ.

From the results of potentiometric determinations on proteins plus acid or alkali, it is obvious that some acid is bound by the proteins in some manner or another. Assuming that the combination is chemical, the reaction may have follow-

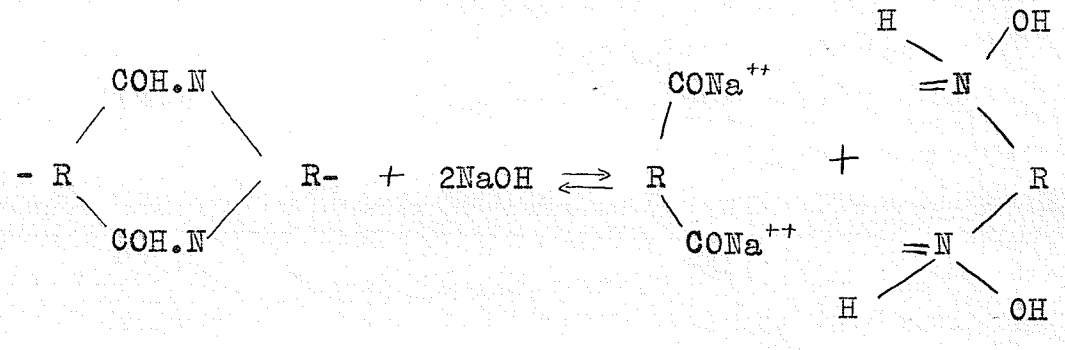
ed one of at least three types. Robertson (1918) suggests combination at the peptide linkage following a keto-enol transformation of the peptide group,



which is followed by:-



That is to say, the protein is ionised by the rupture of the peptide linkage with the formation of two oppositely charged protein fragments. The diamino and bicarboxyl groups also play a leading part in this theory and maybe represented by:-



which merely serves to point out that according to this theory, no free inorganic ions would be formed, but only an equal number of oppositely charged protein ions. However, Hoffman and Gortner (1928), quoting from Pauli, state the following:

"The difference in electrical conductivity between sodium caseinate and ammonium and potassium caseinates correspond to the difference in mobility of the sodium ion compared

with the ammonium and potassium. This behavior is compatible only with the existence of free metal ions."

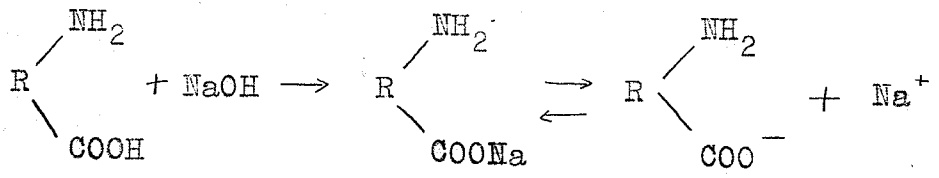
Further, differences are noted in the amounts of hydrogen and chlorine ions bound. If Robertson's theory is correct, these should be the same. In defence of his theory, Robertson says that it shows how the composition of the salt formed is dependent on the concentration of protein and base and is not dependent on total dilution, as no water is involved in the binding.

- - - - - - - -

Hoffman and Gortner (1925) make reference to Hammersten's suggestion in 1877 that the combination is not chemical since he was able to remove all of the acid from a casein-acid solution by repeated washing. However, when we consider the light in which we regard equilibria and balanced reactions we must concede that Hammersten may have been dealing with a case of chemical combination.

Loeb (1922) added a salt to gelatin and then washed it with water. Following the sixth washing, however, it was found that the gelatin would give quantitative tests for certain of the ions. All this favors chemical binding.

Two other methods of protein reactions with acids and bases presuppose a chemical type of combination. In the one the free carboxyl groups react as acid valencies and the free amino groups react as basic valencies, thus:--



The other type is the "Zwitterion" reaction, which suggests an ionisation differing from the above according to Birch and Harrison (1932)

The old view:--

$$Cl^- + {}^{+}NH_3\ R.COOH \quad \xleftarrow{+HCl} \quad NH_2.R.COOH \quad \xrightarrow{+NaOH} \quad NH_2.R.COO^- + Na^+$$

The new view:--

$$Cl^- + NH_3.R.COOH \quad \xleftarrow{+HCl} \quad NH_3.R.COO^- \quad \xrightarrow{+NaOH} \quad NH_2.R.COO^- + Na^+$$

Van Slyke and Hart (1925) and Van Slyke and Van Slyke (1926) make an interesting observation in the study of casein acid reactions. The lower the temperature the more slowly was equilibrium obtained but the more was bound. This would indicate a negative temperature coefficient for binding, which, though not customary with chemical reactions, yet which is often observed in adsorption reactions.

Among those expressing binding studies by the use of adsorption curves are Toleman and Bracewell (1919), and Toleman and Stearn (1918). Hoffman and Gortner (1925) mention the work of Izaquirre, who, by using certain published data on gelatin and hydrochloric acid, showed that a logarithmic relationship exists between the amount of acid bound and the original concentration, thus resembling the usual adsorption reaction.

Studies employing optical methods have been used. These suggest that adsorption is responsible, in part at least,

for protein reactions. Kraemer (1926) studied tyndall effect and specific rotation of gelatin-hydrochloric acid systems of definite pH values. From these values he determined the isoelectric points of the gelatin samples. Since these varied over a considerable range he concluded that gelatin did not consist of definite and uniform chemical molecules as units in aqueous gelatin solutions. He added that this was true of other protein systems such as casein.

However, Almquist and Greenberg (1931), when using changes in rotatory power as an indication of the mode of combination of acid and alkali with proteins, obtained the following results. At a pH of 5.04 $[\alpha]_D^{22}$ of egg albumin was -30.8°. On adding alkali it rose slowly, if at all, to pH 11.0 and then rose sharply to a maximum of -60.6° between pH 11 and 12. The addition of acid casued the value to rise to 35.1° at a pH of 3.15, where it remained to a pH of 1.72. Addition of acid or alkali to either of the above mentioned systems brought the $[\alpha]$ reading back to the original of -30.8°. The authors say that this points to a chemical reaction between proteins and acids and alkalis and not to an adsorption one.

Robinson, Gortner and Palmer (1932) mention the work of Thomas and Mayer who added 0.1004 N hydrochloric acid to 0.881 grams of ash free gelatin and made interferometer readings. These increased in a straight line ratio with the acid added until a "stoicheometric" point was reached, at which the interferometer readings began to increase much more rapidly

than the acid added, this new function also being a straight line one. This appears to be in agreement with the hypothesis of Hoffman and Gortner (1925). That is to say, chemical combination takes place up to the "Stoichiometric point" after which it is followed by adsorption.

Bancroft and Barnett (1930) studied the reactions of proteins with dry acids, with gaseous ammonia and with gaseous hydrogen chloride. Knowing the amount of gas added to the reaction and measuring the partial pressure of the gas at equilibrium they were able to calculate the amount bound by the protein. They maintain that the pressure concentration curves for these gases with solid acids or bases showed clearly whether or not definite chemical compounds were formed. A stoichiometric reaction was shown by the appearance of a flat in the curve during which time the solid acid or base was reacting with the gas to form a chemical compound. With proteins it appeared that casein, arachin, fibrin, gliadin, and zein absorb ammonia readily with no evidence of the formation of a chemical compound. Casein, fibrin, arachin, gliadin and edestin did form definite compounds with hydrogen chloride. The graphs for those proteins which did form chemical compounds indicate that they also absorb more gas than is necessary for the formation of the chemical compound alone.

Hoffman and Gortner (1925) (p.357) conclude their work with the suggestion that there are two types of combination between proteins and acids and alkalis: (1) A chem-

ical type of combination which takes place between a hydrogen ion concentration represented by pH 2.5 and pH 10.5. (2) An adsorption type of combination which takes place when the hydrogen ion concentration is greater than pH 2.5 or the hydroxyl ion concentration is more than pH 10.5. They studied acid- and alkali-binding by various proteins over a wide pH range and sum up as follows:

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

1. The logarithms of the amount of acid or alkali bound 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 more than 10.5.

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

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, <u>regardless of</u>

their chemical composition, bind approximately the same amount of acid or of alkali.

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

3. The logarithms of the amount of acid or alkali bound plotted against the logarithms of the original acid or alkali concentrations 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 possibility.

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

THE BINDING OF ACID BY CASEIN:

We were interested in the amount of acid bound by casein under different conditions and we studied this phenomenon over a relatively wide pH range. Hoffman and Gortner (1925) include some work of this kind done on casein, while Van Slyke (1928) and Palmer and Richardson (1926) have carried out similar

studies. The amounts of acid bound in the above cases are not in strict agreement, Van Slyke, using very dilute acids, concludes that, "One gram of casein, shaken with 100 cc. of 0.001 N HCl for three hours, takes up from solution nearly 50 per cent of the acid. The amount of acid thus taken up is no definite and fixed amount, but varies (a) with the concentration of the acid, (b) with the duration of the contact until equilibrium is reached, which requires some hours, (c) with the degree of agitation until equilibrium is reached, (d) with the temperature, and (e) with the kind of acid used. Some of the acid is always taken up, however small the amount of acid used, but the acid is never completely removed from the solution, however large the proportion of casein present."

The following statement is also to be found, "Therefore, the result of treating casein with an acid, when no solution of casein occurs, is not a chemical compound of casein and acid, but a case of adsorption." However the publication lacks tabled data and graphs which would seem necessary in support of a definite statement of this kind.

Hoffman and Gortner (1925) and Palmer and Richardson (1926), on the other hand, include several tables in support of their work, although, as mentioned previously, these data are not entirely in agreement. However, since these two works, along with that of Robinson, Gortner and Palmer (1932) come among the most detailed studies on the reactions of casein and para-casein with acids and alkalis, it is of interest to include a few of their figures.

PALMER & RICHARDSON.

c.c. N. 1.033 HCl	pH.	Gm. equivs. bound per gm. Casein.
0.75	2.495	51
0.80	2.456	54
0.85	2.396	56
0.90	2.377	56
0.95	2.364	59
1.00	2.311	58

HOFFMANN & GORTNER (1) pp. 304 and 306.

at 22.0° C.

c.c. Normal HCl	pH	Gm equivs. bound per gm Protein.
2.5	2.392	83
3.0	2.249	92
3.5	2.155	104
4.0	2.096	118
5.0	1.978	143
6.0	1.843	155
	- - - - - - -	
	At 35.0° C	
2.5	2.434	90
3.0	2.299	101
3.5	2.164	108
4.0	2.105	123
5.0	1.970	145
6.0	1.843	159

It will be noted that the figures chosen fall below pH 2.5 which is the point at which chemical combination ceases to be of importance, and where adsorption is the main factor, according to Hoffmann and Wortner (1925). However, it will also be noted that while these investigators claim a negative temperature coefficient at the higher concentrations of acid, this statement is not borne out by the data they present, since what they claim to be an adsorption reaction should indicate less binding at the higher temperature and this it does not do. These discrepancies are not only to be noted with regard to casein, but also with other proteins, such as fibrin, and not only are they evident in the protein-acid mixtures, but also in the protein-alkali mixtures at pH values above 10.5

Robinson (1930) refers to the work of Kolthoff and Bosch on the influence of neutral salts on acid-base equilibria. Since the effect of neutral salts entered into our work it is necessary that some of the above investigators be included at this point. In this work, the dissociation constants of the acids were calculated from dilution data. Salt solutions were added to the system, and the resultant effect on the activities and dissociation constants of the components was noted. They used a mixture of 0.25 N acetic acid and 0.25 N sodium acetate in successive dilutions. They determined the ratio $\frac{f_1}{f_0}$, where (f_1) represents the activity coefficient of the acetate and (f_0) the same of the undissociated acid.

By the Debye-Huckel equation:--

$$-\log f_1 = .5\sqrt{\mu}$$

where ( ) represents the molarity of the acetate.

Now, if a neutral salt is added to a mixture of an acid and its salt, quite generally the pH can be calculated from the equation:--

$$pH = \log \frac{salt}{acid} + pK + \log f_1 - \log f_o$$

or

$$-\log \frac{f_1}{f_o} = pK - pH,$$ if the acid and its salts are present in the same concentration. Using a series of neutral salts, the following results were obtained:--

Influence of neutral salts on the pH of .005 N acetic acid and .005 N sodium acetate at 18° C.

Salt	Concentration	pH	$-\log \frac{f_1}{f_o}$
KCl	.1 molar	4.610	.125
"	.25 "	4.567	.168
"	.5 "	4.544	.191
KBr.	.5 "	4.570	.165
K I	.5 "	4.577	.158
K_2SO_4	.25 "	4.591	.144
$BaCl_2$	.25 "	4.346	.389
No salt	-----	4.697	-----

Investigating the ratio $-\log \frac{f_1}{f_o}$ further, Kolthoff calculated figures for (f_o) representing the activity of the undissociated acid molecules. The following shows these results which are general and concern no special acid:

Salt	Concentration	μ	log f_1 calculated	log f_o	f_o
KCl	.1	.106	.116	.014	1.03
"	.25	.256	.155	.015	1.04
"	.5	.506	.189	.021	1.05
NaCl	.1	.106	.130	.040	1.09
"	.25	.256	.182	.048	1.11
"	.5	.506	.230	.050	1.12

From this and other similar data Kolthoff and Bosch assumed that the effect of salts was to increase the activity coefficient of the undissociated acid.

This point was of importance to us since we were interested in determining the extent to which neutral salts influenced the binding of acid by casein, if at all.

Robinson, Gortner and Palmer (1932) carried out extensive studies on the effect of neutral salts on casein- and paracasein-sodium hydroxide systems and conclude as follows:--

"The amount of sodium hydroxide 'bound' by casein and paracasein is increased by the presence of potassium chloride, potassium bromide, or potassium iodide".

"The addition of neutral salts causes a shift in the equilibrium pH of a casein-NaOH or a paracasein-NaOH system toward a lower pH value. The 'salt-effect' is much more pronounced in these protein base systems than has been reported to be the case for simpler acid-base systems. (This refers to the work of Kolthoff and Bosch, mentioned above) (parentheses ours). Concentrations of neutral salts, as low as 0.001 N in some instances, produced a measurable pH shift."

"The magnitude of the salt effect is essentially the same for KCl, KBr and KI. This 'salt effect' exhibits no marked lyotropic series for these aminos. These protein-NaOH systems are more sensitive to the addition of salts at a pH closer to their isoelectric points than at one farther away."

This salt effect must be taken into consideration in the interpretation of the electrometric titration curve of proteins. If one titrates proteins electrometrically with either acid or alkali, and then back-titrates the resulting system, the back-titration curve, due to the salt effect, will not coincide with the original titration curve. If the protein solution contains even small quantities of neutral inorganic salts, the equilibrium pH which is attained on the addition of a definite quantity of acid or alkali will be different from that which would be attained in the absence of the neutral inorganic salts.

Noting this last it is of interest to know that

Hoffman and Gortner (1925) experienced this difficulty when comparing the 'titration curve' and 'back-titration curve' of a protein-NaOH-HCl system.

The work of Robinson, Gortner and Palmer (1932) offered a new method of attack on the problem of the extent to which the reaction of proteins into alkalis is an adsorbtion one and the extent to which it is a strictly chemical one. They agreed with Hoffman and Gortner (1925) that it was an adsorption one above pH 10.5. Below that point they were led to believe that the protein reacted into the added alkali in both ways. The closer the pH of the medium to the isoelectric point of the protein, the greater was the tendency toward chemical binding. It appeared desirable to make the same sort of investigation of the reaction of proteins with acids. This study then, is designed to investigate the reaction between casein and hydrochloric acid, using insofar as possible, similar methods to those employed by Robinson, Gortner and Palmer (1932).

EXPERIMENTAL:

PREPARATION OF CASEIN:

Casein was prepared according to the methods used by Van Slyke and Baker (1918) and Van Slyke and Bosworth (1913).

Five gallons (approximately 20 litres) of fresh skimmed milk were placed in a large insulated crock and cooled to about 4°C, when normal acetic acid was added in order to precipitate the casein. The acid was added by means of an apparatus similar to the one employed by Van Slyke and Baker (1918). This consists of a capillary tube dipping below the surface of the milk with the end of the tube bent at right angles so that the acid emerges in a horizontal plane, and is swept away by the stirring action of a paddle operated by electric motor which makes several hundred revolutions per minute. The acid enters the capillary tube from an aspirator jar. Use of an apparatus of this sort insures an even distribution of acidity throughout the body of the milk at all times. It is also used for the addition of the alkali mentioned below.

Normal acetic acid was added till pH of 4.6 - the isoelectric point of casein - was reached. The precipitated casein was allowed to settle and the supernatant whey was siphoned off. The casein was separated from the remainder of the whey by centrifuging.

The casein was suspended in about 10 litres of distilled water and 0.1 N NH_4OH added slowly, to dissolve the casein, until a pH of approximately 7.2 was reached. This was done at room temperature. The solution was then cooled to 4° C, and the casein precipitated at the isoelectric point by the addition of normal acetic acid. The above procedure of siphoning, centrifuging and suspending in water was repeated. The casein was again dissolved by means of 0.1 N NH_4OH and again cooled to 4° C and precipitated with normal acetic acid. This third precipitation was considered sufficient to give a product of the required degree of purity. The casein was centrifuged and washed, in succession, in the centrifuge cups with water, hot neutral 70 per cent alcohol, absolute alcohol, absolute ether and petroleum ether. The substance was finally dried by exposure to the atmosphere.

The casein was ground in an agate mortar to a fine powder and placed in a well stoppered bottle.

THE ANALYSIS OF CASEIN:

The casein prepared was analysed for moisture, ash, nitrogen, phosphorus and sulphur. For the first three mentioned the A.O.A.C. (1930) methods of analysis were used.

Two-gram samples were analysed for moisture by drying in aluminium moisture dishes in a vacuum oven at 100° C. The per cent of ash was determined by igniting 10 gram-samples in platinum ash dishes at a dull red heat until a white ash was obtained. 0.1-gram samples were analysed for nitrogen

by the Kjeldahl-Gunning method.

For the determination of phosphorus the Briggs (1924) method was used. This is a modification of the Bell-Doisy (1920) method. 1.0-gram samples of casein were mixed with 10 per cent calcium acetate solution in platinum ash dishes, and ignited at a dull red heat until a white ash remained. The calcium phosphate so formed was extracted with dilute HCl, and the extract diluted to 100 c.c. A 10 c.c. aliquot of this phosphoric acid solution was treated in a 100 c.c. volumetric flask with 10 c.c. of 5 per cent ammonium molybdate solution, 10 c.c. of 10 per cent sodium sulphite, and 10 c.c. of 0.25 per cent amino-naphthol sulphonic acid in place of the hydroquinone. This method is used by Morris, H.P; Nelson, J.W. and Palmer, L.S. (1931), Ind. and Eng. Chem. 3: 164-7 (1931) The whole was diluted to volume and the blue color formed was compared in a Bausch & Lomb colorimeter, with a standard made by treating 10 c.c. of KH_2PO_4 solution (of such strength that 10 c.c. contained 1 milligram of phosphorus) in a manner similar to that of the 10 c.c. aliquot. The blue color was formed by the reduction of phosphomolybdic acid due to the amino naphthol sulphonic acid and the sulphur dioxide. The solutions were prepared according to Hawk and Bergeim (1927)--Hawk, D.B; and Bergeim, Olaf. Practical Physiological Chemistry, p. 403 (1927). P. Blakiston's Son & Co. (Philadelphia).

Sulphur was determined according to Dennis (1910). 2.0-gram samples were evaporated twice with concentrated HNO_3

in porcelain dishes on a water bath. To the residue was added 10 c.c. of oxidising solution (25 gms. $Cu(NO_3)_2$, 25 gms. NaCl and 10 gms. NH_4NO_3 in 100 c.c. distilled water), and it was again evaporated to dryness. The residue left after this treatment was ignited at redness and dissolved in 10 c.c. of 10 per cent HCl. The solution was transferred to a beaker, diluted to 150 c.c., heated to boiling, and sulphur precipitated as $BaSO_4$ by the addition of 10 c.c. of 5 per cent $BaCl_2$ solution. After standing overnight the precipitated $BaSO_4$ was filtered off on quantitative filter paper, and ignited in tared platinum crucibles at a dull red heat to constant weight.

The results of the analysis are given in Table No.1

TABLE No.1.

ANALYSIS OF CASEIN (Per Cent).

	I	II	Average	Moisture Free
Moisture	9.06	9.03	9.055	
Ash	0.072	0.080	0.076	0.084
Nitrogen	14.2	14.2	14.2	15.62
Phosphorus	1.026	0.999	1.01	1.10
Sulphur	0.481	0.490	0.485	0.529

A comparison with the analyses obtained by other investigators has a place at this time:--

Reference	Sulphur	Nitrogen	Phosphorus
Bosworth (1914)	0.72	15.80	0.71
Van Slyke and Bosworth (1913)	0.72	15.80	0.71
Robinson, Gortner & Palmer (1932)	0.66	15.73	0.91
Our sample	0.529	15.62	1.10

These values are close enough to the average to suggest a high degree of purity. The unusually high content of phosphorus could not have been an impurity, otherwise the per cent of ash would have been much higher than 0.084. It must have been an integral part of the casein molecule. We assumed that the preparation was satisfactory.

THE PREPARATION OF REAGENTS:

Reagents used were Casein, Hydrochloric acid, Sodium hydroxide, water and neutral salts.

The preparation of casein has been described. C.P. Hydrochloric acid was used and diluted to the desired strength of 0.1 Normal. Carbonate free sodium hydroxide was used and this too was diluted to 0.1 Normal, which solution was standardised against pure, oven-dry succinic acid using phenolphthalein as indicator. The 0.1 N. HCl was standardised against the 0.1 N. NaOH solution.

All water was redistilled before use, and kept in well stoppered Pyrex flasks, to avoid contamination due to carbon dioxide.

The neutral salts used were pure chemicals, ground and dried in the oven at 100° C before use.

APPARATUS AND METHODS.

The amount of hydrochloric acid bound by the protein was determined potentiometrically and is expressed as gram equivalents of HCl bound per gram of casein. Measurements were made with an L & N type of potentiometer, using a high resistance galvanometer, both manufactured by the Leeds and Northrup Company. Readings were made to a tenth of a millivolt.

The set up was tested by the use of sodium hydroxide-hydrochloric acid mixtures. A set of these is reproduced in Table III, and indicates the mode of chemical combination.

One gram samples of the casein were used at all times and since these would not dissolve readily in the acid, measurements were made on the suspension. This same method was employed by Palmer and Richardson (1926).

For protein-acid systems, the sample of casein was first wetted with 100 c.c. of pure, double distilled water in a beaker and placed in a water-bath at constant temperature. Hydrogen was bubbled through the mixture using a bubbling electrode of the Hildebfand type, see Clark (1928), which permitted the hydrogen to escape through the tube enclosing

the electrode. E.M.F. measurements were made following the addition of measured amounts of 0.1 normal hydrochloric acid. Ten to fifteen minutes were allowed for the system to reach equilibrium. In this way, the casein was never in contact with the acid for more than a few hours. We anticipated no hydrolysis in this time.

The amounts of acid bound were calculated according to the equations of Cohn and Berggren as used by Robinson, Gortner and Palmer (1932). From the E.M.F. of the cell was subtracted the value for the calomel as given by Clark (1928) (p.672). The difference was divided by the value for 0.0001983T where T is the absolute temperature. This given the pH value.

$$pH = \frac{\text{E.M.F. of cell - C.M.F. of calomel}}{0.0001983\ T}$$

The concentration of the free acid was determined as follows:-

$$\frac{(H\ )}{(HCl)} = \gamma$$

$$\text{then} \quad pH = \text{log of } \frac{1}{HCl}$$

$$= pHCl + p\gamma$$

$$\text{or } pHCl = pH - p\gamma$$

Values for $p\gamma$ were obtained from the values of Lewis and Randall repreduced in Gortner (1929). The values of

pHCl was subtracted from the logarithm of 1 which is 0.000, thus giving the logarithm of the concentration of free HCl. The concentration of free HCl was determined from logarithm tables. The above is based on the assumption that

$$pH = \log \frac{1}{(H^+)}$$

Subtracting the amount of free HCl from the original concentration gave the amount bound by the protein from which was calculated the acid bound per gram of protein.

Values for ν were obtained from Gortner (1929) after Lewis and Randall. As pointed out by Robinson, Gortner and Palmer (1932) we are making certain assumptions when we use a calculation of this kind. The values for ν were determined from electromotive force measurements, in which the activity of the HCl was affected by the presence of sodium chloride. There was no sodium chloride present in our study, but we did have "protein salts" present. Our assumptions for purposes of calculation are:--

1. That protein and Hydrochloric acid react in stoichiometric proportions.

$$\text{Protein} + HCl \longrightarrow \text{Protein chloride} + H_2O$$

2. That the protein chloride is completely dissociated,

$$\text{Protein } Cl \rightleftharpoons \text{Protein} \quad Cl$$

3. ν for Cl^- of $Protein^+ + Cl^-$ =

ν Cl^- of $Na^+ + Cl^-$

4. Therefore γ Protein^{+} = γ Na^{+}

5. There is no adsorption of Cl^{-} or of HCl on the micelles, and no effect of the ionic micelles on the γ of Cl^{-}.

The quantitative effect of the protein and the protein salts present on the activity of the Cl^{-} and HCl present is not known, thus forcing us to make the above assumptions. It has been pointed out by Robinson, Gortner and Palmer (1932) that the results obtained by this means agree with those obtained by other methods of calculation using the same data.

THE AMOUNT OF HYDROCHLORIC ACID BOUND BY CASEIN.

We wished to determine how the amount of acid bound by casein varied with pH. Consequently, suspensions were prepared of casein in water to which additions of hydrochloric acid were made at regular intervals and E.M.F. readings taken following each addition of acid. This was described above. The readings were calculated to pH and equivalents of acid bound per gram of protein, also described above. These results are described in Table II.

A similar determination was made of the binding of hydrochloric acid by sodium hydroxide. These results are found in Table III. These data will serve as a comparison between the binding of acid by a protein and the binding of acid by sodium hydroxide, a reaction which is known to be of a strictly chemical nature.

Data from Table II is recorded graphically in Figure 1. Data from Table III is recorded in Figure 2.

TABLE II.

AMOUNT OF HYDROCHLORIC ACID BOUND BY CASEIN.

Conc. = 1 gm. Casein in 100 c.c. water. Temperature = 25° C.

C.C. HCl added	Normality HCl	pH	Equivalents acid bound per gm. protein x 10⁵
0.0	0.00000	6.379	0.000
0.5	0.00050	3.323	0.200
1.0	0.00099	3.254	4.2
1.5	0.0015	3.162	8.0
2.0	0.00196	3.188	13.0
2.5	0.00243	3.130	17.0
3.0	0.00291	3.041	20.0
3.5	0.00338	2.917	22.0
4.0	0.00385	2.873	25.0
4.5	0.00431	2.834	29.0
5.0	0.00476	2.794	32.0
5.5	0.00521	2.768	36.0
6.0	0.00566	2.758	40.0
6.5	0.00610	2.716	43.0
7.5	0.00698	2.736	54.0
8.0	0.00741	2.592	51.0
8.5	0.00783	2.545	52.0
9.0	0.00826	2.533	56.0
9.5	0.00868	2.447	53.0
10.0	0.00910	2.433	56.0
10.5	0.00950	2.362	53.0
11.0	0.00991	2.305	50.0
11.5	0.01031	2.277	51.0
12.0	0.01071	2.240	50.0
12.5	0.01111	2.210	49.0
13.0	0.01150	2.181	49.0
13.5	0.01189	2.152	48.0
14.0	0.01236	2.125	48.00
15.0	0.0130	2.115	53.0
20.0	0.0167	1.942	49.0
25.0	0.0200	1.827	43.0
30.0	0.0231	1.741	38.0
35.0	0.0259	1.675	31.0
40.0	0.0286	1.626	29.0
45.0	0.0310	1.579	20.0
50.0	0.0333	1.552	26.0
55.0	0.0355	1.506	5.0

(Continued)

TABLE II (Cont'd).

AMOUNT OF HYDROCHLORIC ACID BOUND BY CASEIN.

C.C. HCl added	Normaltiy HCl	pH	Equivalents acid bound per gm. protein x 10^5
60.0	0.0375	1.481	5.0
65.0	0.0394	1.459	2.0
70.0	0.0412	1.438	---
75.0	0.0429	1.411	---
80.0	0.0444	1.394	---

TABLE III.

AMOUNT OF HYDROCHLORIC ACID BOUND BY SODIUM HYDROXIDE.

Temperature = 25° C.

Normality HCl	NaOH in grams per 1000 cc	pH	Equivalents of HCl bound per gram NaOH *
0.0530	1.90	2.064	0.023
0.0523	1.91	2.245	0.024
0.0520	1.92	2.318	0.024
0.0518	1.93	2.386	0.024
0.0515	1.94	2.467	0.025
0.0512	1.95	2.563	0.025
0.0510	1.96	2.695	0.025
0.0507	1.97	2.883	0.025
0.0505	1.98	3.184	0.025
0.0502	1.99	5.941	0.025
0.0500	2.00	9.601	0.025

* Theory 1 gm. NaOH should bind 0.025 gms. HCl.

FIGURE I.

AMOUNT OF HYDROCHLORIDE ACID BOUND BY CASEIN.

Conc. = 1 gm. Casein in 100 c.c. water. Temperature = 25° C.

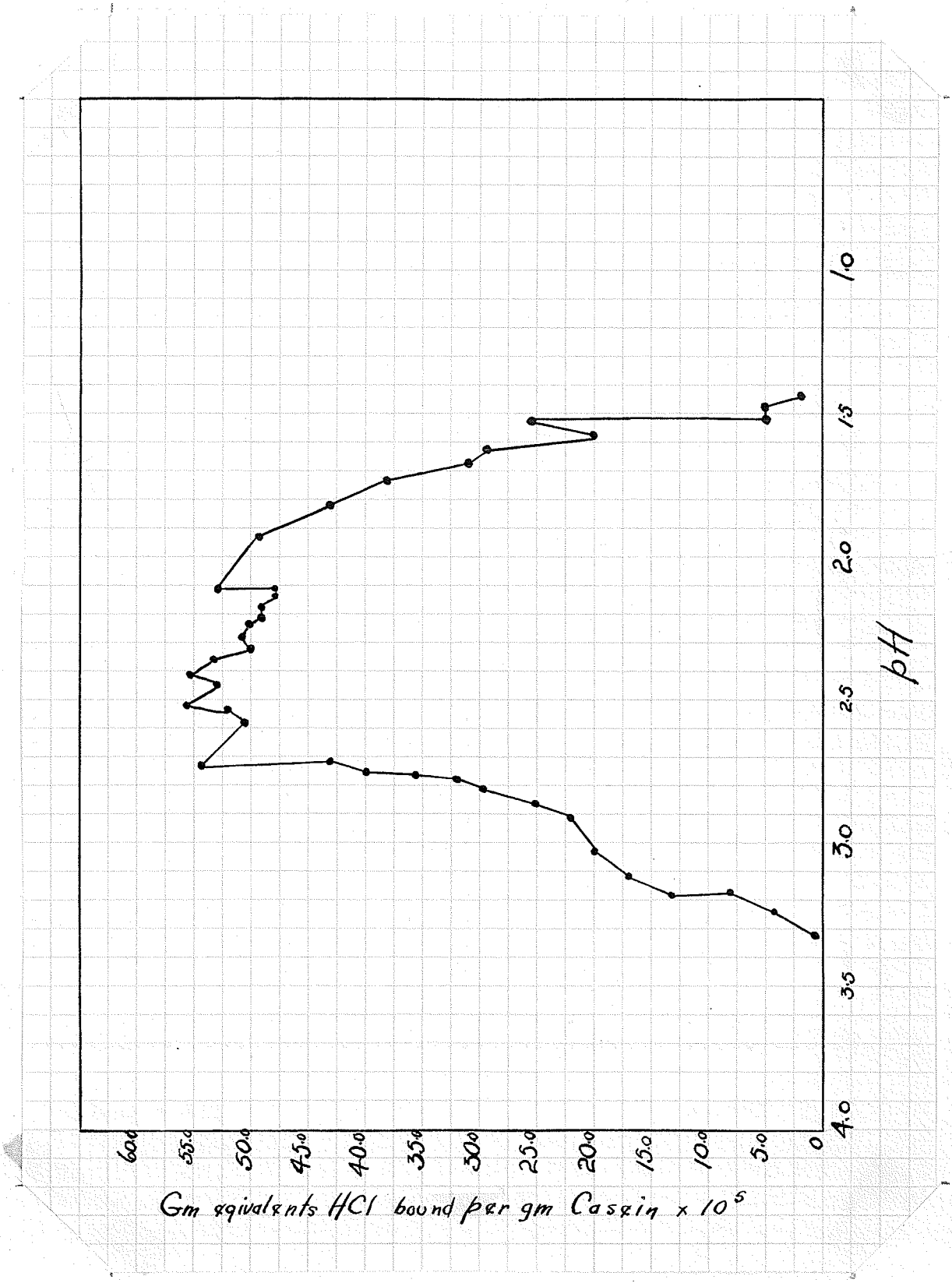
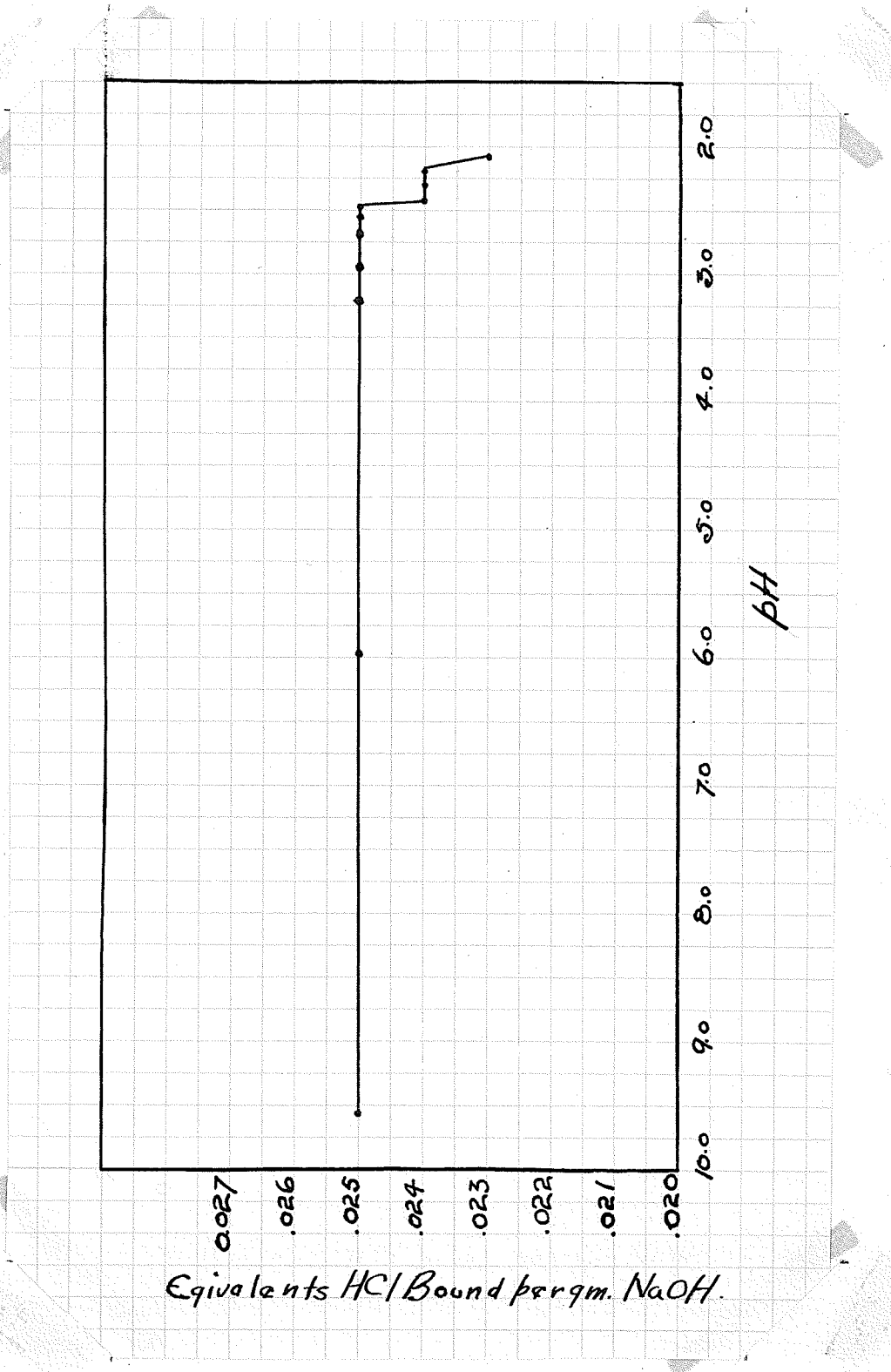


FIGURE II.

AMOUNT OF HYDROCHLORIC ACID BOUND BY SODIUM HYDROXIDE.

Temp. = 25° C.



INFLUENCE OF NEUTRAL SALTS ON THE BINDING OF HYDROCHLORIC ACID BY CASEIN.

The material presented above, while embodying some singular results is largely a repetition of work done by previous investigators.

We decided to study the effect of neutral salts and acid binding by casein at both high and low acidities. Work of this kind was carried out by Robinson, Gortner and Palmer (1932) on the alkaline side of neutrality, using casein and paracasein.

At the high acidity we used sodium bromide, sodium chloride and potassium bromide. The data are presented in Table IV.

TABLE IV.

EFFECT OF SALTS ON THE AMOUNT OF HYDROCHLORIC ACID BOUND BY CASEIN AT HIGH DEGREE OF ACIDITY.

Conc.= 1 gm.Casein in 100 c.c. N/10 Hydrochloric Acid.
Temp. = 25° C.

Normality of Salt Solution	NaBr pH	NaCl pH	KBr pH
0.0000	1.056	1.051	1.052
0.0005	1.059	1.064	1.076
0.001	1.056	1.064	1.054
0.0025	1.054	1.051	1.052
0.005	1.052	1.074	1.054
0.01	1.054	1.049	1.052
0.025	1.051	1.049	1.056
0.05	1.044	1.047	1.049
0.1	1.039	1.037	1.051
0.25	1.019	1.019	1.046
0.5	0.980	0.986	1.024
1.0	---	0.912	[illegible]
2.5	---	0.624	

In the experiments at low acidity we used potassium chloride, sodium chloride and potassium bromide. The results will be found in Table V. They are reproduced graphically in Figure 3.

TABLE V.

EFFECT OF SALTS ON THE AMOUNT OF HYDROCHLORIC ACID BOUND BY CASEIN AT LOW DEGREES OF ACIDITY.

Conc. of Casein = 1 gm. in 100 c.c. 0.003 N HCl.

Temp.= 25°C.

Normality of salt solution	KCl pH	KCl Equivalents bound x 10^5	NaCl pH	NaCl Equivalents bound x 10^5	KBr pH	KBr Equivalents bound x 10^5
0.000	3.112	22.0	3.083	21.6	3.176	23.1
0.0005	3.404	26.0	3.044	20.6	3.347	25.3
0.001	3.306	25.0	3.174	23.1	3.403	25.9
0.0025	3.430	26.0	3.387	25.7	3.435	26.2
0.005	3.438	26.0	3.475	26.5	3.420	26.1
0.01	3.496	26.6	3.452	26.3	3.574	27.2
0.025	3.658	27.7	3.597	27.4	3.636	27.6
0.05	3.734	28.1	3.699	27.9	3.805	28.4
0.1	3.812	28.4	3.853	28.5	3.971	28.9
0.25	4.042	29.0	4.056	29.1	4.173	29.3
0.5	4.156	29.0	4.125	29.2	4.284	29.5

These particular salts were chosen to provide a variety of monovalent cations and anions, so that any variation in salt effect due to the nature of the individual ion would be revealed.

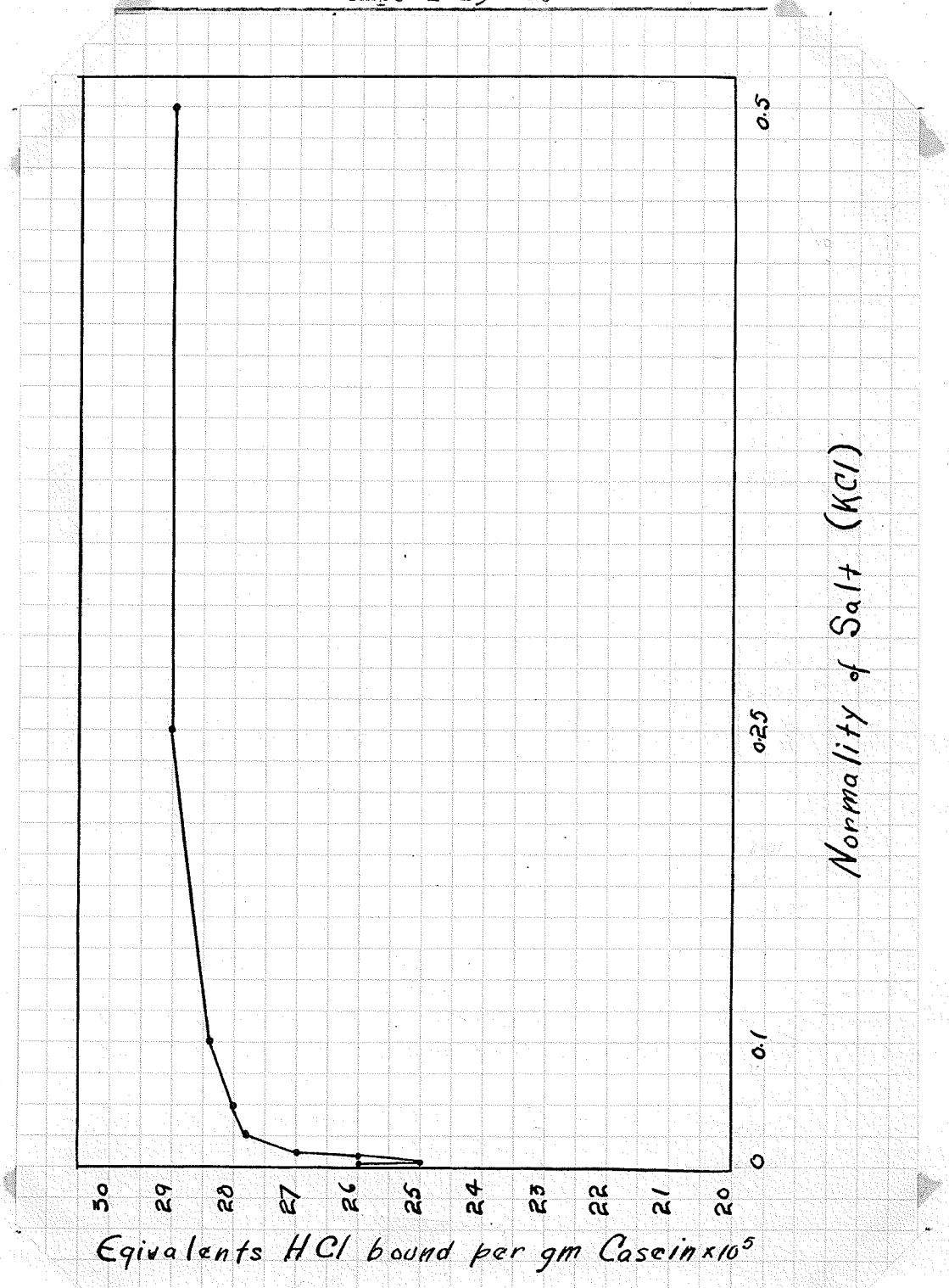
The difference in acidity was obtained by suspending casein in 0.1 N HCl solution for the study whose results are

recorded in Table IV, and suspending it in 0.003 N HCl solution for the study whose results are recorded in Table V.

FIGURE 3.

EFFECT OF SALTS ON THE AMOUNT OF HYDROCHLORIC ACID BOUND BY CASEIN AT LOW DEGREES OF ACIDITY.

Conc. of Casein = 1 gm. in 100 c.c. 0.003 N. HCl.
Temp. = 25° C.



EFFECT OF TEMPERATURE ON THE INCREASE IN AMOUNTS OF HYDROCHLORIC ACID BOUND BY CASEIN, DUE TO THE PRESENCE OF POTASSIUM CHLORIDE, AT LOW DEGREES OF ACIDITY.

Following the work on casein-hydrochloric acid alone and casein-hydrochloric acid plus various salts, we decided to experiment with the "salt-effect" at various temperatures. The salt used was potassium chloride. The results appear in Table VI.

TABLE VI.

EFFECT OF TEMPERATURE ON THE INCREASE IN AMOUNTS OF HYDROCHLORIC ACID BOUND BY CASEIN DUE TO THE PRESENCE OF KCL AT LOW DEGREE OF ACIDITY.

Conc. of Casein = 1 gm. in 100 c.c. of 0.003 N HCl.

Normality of salt Solution	Temp. = 25° C		Temp. = 30°C		Temp. = 35°C		Temp. = 40°C	
	pH	Equivalents bound x 10^5	pH	Equivalents bound x 10^5	pH	Equivalents bound x 10^5	pH	Equivalents bound x 10^5
0.0000	3.112	22.0	3.085	21.5	3.074	21.2	3.114	22.0
0.0005	3.404	26.0	3.190	23.3	3.178	23.1	3.217	23.7
0.001	3.306	25.0	3.265	24.4	3.280	24.5	3.261	24.3
0.0025	3.430	26.0	3.326	25.1	3.345	25.3	3.357	25.4
0.005	3.438	26.0	3.406	25.9	3.401	25.9	3.436	26.2
0.01	3.496	26.6	3.486	26.6	3.478	26.5	3.488	26.6
0.025	3.658	27.7	3.614	27.5	3.607	27.4	3.604	27.4
0.05	3.734	28.1	3.734	28.1	3.732	28.1	3.738	28.1
0.1	3.812	28.4	3.884	28.6	3.846	28.5	3.833	28.5
0.25	4.042	29.0	4.040	29.1	3.989	28.9	4.003	29.0
0.5	4.156	29.0	4.146	29.3	4.077	29.1	4.069	29.1

EFFECT OF TEMPERATURE ON THE AMOUNTS OF HYDROCHLORIC ACID BOUND BY CASEIN AT VARIOUS DEGREES OF ACIDITY.

Following the work on the salt effect at various temperatures we decided to investigate the influence of temperature alone in order to determine whether or not there is a temperature effect. The results of this work are presented in Table VII.

TABLE VII

EFFECT OF TEMPERATURE ON THE AMOUNTS OF HYDROCHLORIC ACID BOUND BY CASEIN AT VARIOUS DEGREES OF ACIDITY.

Initial Conc. of Casein = 1 gm. in 100 c.c. Water.

c.c. of HCl added	Normality of HCl	Temp. = 25° C pH	Equivalents bound x 10^5	Temp. = 30° C pH	Equivalents bound x 10^5	Temp. = 35° C pH	Equivalents bound x 10^5	Temp = 40° C pH	Equivalents bound x 10^5
0.0	0.0000	6.379		5.549		5.339		5.380	
2.0	0.0020	3.049	11.0	3.180	13.4	3.170	13.2	3.428	16.0
4.0	0.0038	2.865	25.0	2.962	28.0	2.993	28.0	3.184	32.0
6.0	0.0057	2.748	41.0	2.769	41.0	2.833	44.0	2.907	47.0
8.0	0.0074	2.584	50.0	2.579	50.0	2.604	51.0	2.601	51.0
10.0	0.0091	2.409	54.0	2.401	53.0	2.396	52.0	2.388	51.0
15.0	0.0130	2.115	53.0	2.098	49.0	2.083	46.0	2.074	43.0
20.0	0.0167	1.942	49.0	1.930	44.0	1.902	35.0	1.903	31.0
25.0	0.0200	1.827	43.0	1.810	35.0	1.792	15.0	1.792	26.0
30.0	0.0231	1.741	38.0	1.724	27.0	1.707	16.0	1.705	14.0
35.0	0.0259	1.675	31.0	1.657	18.0	1.628	---	1.636	1.0
40.0	0.0286	1.626	29.0	1.599	6.0	1.584	---	1.581	---
45.0	0.0310	1.579	20.0	1.561	3.0	1.552	---	1.536	---
50.0	0.0333	1.552	26.0	1.519	---	1.502	---	1.501	---
55.0	0.0355	1.506	5.0	1.484	---	1.470	---	1.467	---
60.0	0.0375	1.481	5.0	1.464	---	1.444	---	1.441	---
65.0	0.0394	1.459	2.0			1.420	---	1.415	---
70.0	0.0412	1.438	---			1.396	---	1.367	---
75.0	0.0429	1.411	---			1.376	---	1.361	---
80.0	0.0444	1.394	---			1.355	---	1.351	---

DISCUSSION:

It is noted at once in Figure II that the hydrochloric acid-sodium hydroxide curve is almost a straight line parallel to the X axis. This shows, that no matter what the degree of acidity may be, sodium hydroxide "binds" the same amount of hydrochloric acid. In view of the laws of stoichiometric proportions, this would be expected. The second outstanding feature of the figure is that the protein-binding curve rises with decreasing pH to a more or less well defined peak and then proceeds to fall off. Finally, at a pH value of 1.438, binding has ceased. This indicates, for casein, a different type of binding from that of sodium hydroxide, and which seems to be in agreement with the conclusions of Gortner, (1930) that the reaction is, in part at least, an adsorption one. The casein-hydrochloric acid curve agrees with the curve of Palmer and Richardson (1926). However, these workers only carried the experiment to an acidity corresponding to pH 2.300, whereas we obtained readings as low as pH 1.394, at which point no binding of acid was made evident by the method of calculation which we used. At the same time, we notice, in the curve for casein of Palmer and Richardson, a definite flattening out at their final reading. This suggests that, had they carried the acidity still lower, their curve would have fallen off as did ours.

Hoffman and Gortner (1935) however show an increase in binding for casein as low as pH 1.843 with nothing to indicate that the binding ceased or fell off at that point. This

is in strict disagreement with our work and with the work of Palmer and Richardson (1926), not only as regards the limits of binding, but also as to amounts bound.

Obviously, there is something more than chemical combination influencing the reaction of casein with hydrochloric acid. Presumably the forces of adsorption are playing a noticeable part in the process. It is difficult to say to what factors we can attribute the falling off of the curve following a maximum of binding between pH 2.5 and pH 2.3. This of course, makes evident the fact that, after a point has been reached, binding is decreased by a lowering pH.

In our work a maximum binding was reached around pH 2.5, shown by the flattening out of the casein curve in Figure I as noted previously. This same phenomenon was apparently due to take place in the work of Palmer and Richardson (1926) since their casein-acid curve flattens out around pH 2.4 and shows a tendency to fall off. As mentioned before, the work of Hoffman and Gortner (1925) indicates no such contingency even though they reached pH values well below 2.0.

The falling off of, and eventual disappearance of binding in the case of our results may be due to hydrolysis induced by the relatively high concentration of acid. The splitting off of phosphoric acid from the casein molecule would tend to lower the pH value, thus suggesting a decrease in binding. Ultimately binding would apparently disappear. This theory is strengthened by the fact that at high temperatures the bind-

ing seems to fall off more rapidly than at lower temperatures. This is explained by the fact that the higher the temperature, the more rapidly would the casein be hydrolysed and the more phosphoric acid set free in a unit time, thus completely masking the binding process earlier in the reaction. Of course, if adsorption is involved it would explain the more rapid decrease in binding noted at higher temperatures. The high phosphorus content of the casein already noted suggests that there would be ample phosphoric acid available to bring this about in the event of hydrolysis.

The effect of neutral salts on the reactions between casein and hydrochloric acid was studied. Tables IV, V and VI contain the data and a representative curve for potassium chloride is to be found in Figure III. There are definite indications that the presence of these neutral salts at low concentrations of acid increase the amount of acid bound by a unit weight of protein.

Table IV shows the effect of the addition of neutral salts to casein suspended in tenth normal acid. It is noticed that the pH of the systems before any salt was added, was 1.05. This is substantially lower than the final reading in Table II, which is pH 1.394 and no binding is evident at either value. Table IV also shows that there was no evident binding at any time throughout the course of the experiment. This suggests that the same forces that were responsible for a decrease in the work embodied in Table II were acting in the Table IV ex-

periment too. What these forces are remains a problem.

Table V contains the data obtained in studying the salt effect at a lower degree of acidity. (It will be noted that the initial pH of the systems, previous to the addition of the salts is around pH 3.1 and corresponds to gram equivalents bound of about 22.0×10^5. In Table II, the pH reading closest to the above is 3.13. This corresponds to gram equivalents bound of 17.0×10^5.) From the data in Table V it is evident that at low acidities, as the amount of neutral salt present increases, so does the amount of hydrochloric acid bound by the casein increase.

Robinson, Gortner and Palmer (1932) found the above to be true for the binding of alkalis by casein and paracasein, i.e., the amount of alkali bound by casein and paracasein is increased due to the presence of neutral salts.

In Table V the lower concentrations of salts do not correspond to so much increased binding as do the higher concentrations. There seems to be no difference between the effects of potassium chloride, sodium chloride, or potassium bromide as regards increased binding by the casein.

It seems, then, that just as neutral salts increase the binding of sodium hydroxide by casein so do they increase the binding of hydrochloric acid by casein, especially in regions of low acidity. The different neutral salts seem to exert identical effects regardless of the anion and cation present, i.e., potassium bromide exerts the same effect as sodium chloride.

Extremely low concentrations of the salts exert varied effects, if they exert any effect at all. This can be seen in Table V. However, at the higher concentrations, the "salt effect" is quite noticeable. The data for the salt effect of potassium chloride from Table V are represented graphically in Figure III. One outstanding feature of the curve is that it is not one indicative of adsorption.

In Table VI are reproduced figures obtained in a study of the salt effect at different degrees of temperature. They indicate clearly that while the salt does increase the amount of acid bound by the casein, as would be expected from earlier observations, the changes in temperature are practically without effect. Then again, these results are in agreement with the theory that chemical binding is supposed to take place between pH 2.5 and pH 10.5. The amount of acid bound by the protein increases with increasing salt concentration. This may be explained as being due to the increased activity of the undissociated casein molecule due to the presence of the neutral salt. Whether this result is increased chemical activity due to increased dissociation or whether it results in increased activity due to increased adsorption should be shown by the effect of temperature. In adsorption the amounts bound are less at higher temperatures. In chemical combination the amounts are the same although the reaction proceeds more slowly at lower temperatures. The data of Table VI indicates that the salt effect is the same regardless of the temperature. Therefore

it would appear that the increased binding of casein is due to the greater dissociation of the casein molecule. We might logically expect these same groups to be involved in the reaction if more acid were added to the mixture. This would suggest that the binding of acid by casein at immediately lower pH's than the range in Table VI is strictly chemical in nature.

We felt justified in using potassium chloride here in preference to any other of the salts since there was no reason for suspecting that any other of the salts would have produced an effect different from that of potassium chloride at the different temperatures.

It will be noted, that at low concentrations of acid, where a definite salt effect was observed, the different anions produced the same effect. That is to say there is no lyotropic series for the anions Cl, Br, and I. in these systems. Neither did the cations Na and K produce any marked difference; suggesting no lyotropic series for these, either.

The results of the study of the binding of hydrochloric acid by casein at different temperatures are recorded in Table VII. Hoffman and Gortner (1925) conducted numerous experiments of this kind, using the prolamines and casein at 22°C and 35° C, and reported a negative temperature coefficient.

From the data in Table VII it is significant that the binding reaches maximum for all temperatures at about pH 2.4. Up to this point, at similar pH values, the binding increases with increasing temperature. Following this point, however,

the binding begins to fall off and the higher the temperature in general, the more rapidly does the binding drop, although there is very little difference between 35°C and 40°C. The falling off of binding at 25°C is substantiated by the similar results obtained at 30°, 35° and 40°C, again indicating that some other factor or factors in addition to adsorption and chemical binding are affecting the reaction of casein with hydrochloric acid. The higher the temperature, the more rapidly do these other factors make themselves evident. Strangely enough their effect begins to appear at about pH 2.5 regardless of the temperature, but the higher the temperature the sooner binding ceases.

Obviously, the temperature effect cannot be referred to as negative, in view of the above results.

SUMMARY AND CONCLUSIONS:

1. Down to a pH of 2.4 the amount of hydrochloric acid bound by casein increased. However, after this point, further additions of acid decreased the binding which finally became zero. This suggests that after a certain acidity has been reached, hydrolysis sets in, resulting in the production of free phosphoric acid, and a decreased pH. This is partly upheld in view of the high phosphorus content of the casein and by the fact that at higher temperatures the binding falls off more rapidly, indicating a speedier hydrolysis at increased temperatures.

2. Addition of neutral salts had no noticeable effect on binding at very low pH's indicating once more that hydrolysis masks the binding process. In a less acid medium, however, neutral salts increased the binding of acid by casein. The higher the concentration of salt, the greater was the effect on binding. No lyotropic series was evident for either anions or cations. The salts used were potassium chloride, potassium bromide, sodium chloride and sodium bromide. This effect was noted with concentrations of salts as low as 0.0005 Normal.

3. The salt effect at pH 3.0 seems to be the same regardless of temperature. This supports the theory that only chemical binding takes place between pH 2.5 and pH 10.5 since the amount of adsorption would be less at higher temperatures.

4. The reaction of casein with hydrochloric acid appears to be principally a chemical reaction rather than adsorption. This may be because the casein is hydrolysed at acidities at which the adsorption type of reaction might be expected to be dominant.

BIBLIOGRAPHY.

1930 Association of Official Agricultural Chemists Official and Tentative Methods of Analysis.

Almquist, H.J., and Greenberg, D.M.

1931 "Changes in the Rotary Power of Egg Albumin as Evidence of the Mode of Combination of Acid and Alkali with the Proteins."

Journal of Biological Chemistry 93: 167-170

Bancroft, W.D., and Barnett, C.E.

1930 "Phase Rule Studies on Proteins I. Determination of Solid Compounds with Hydrochloric Acid or Ammonia."

J. Phys. Chem. 34: 449-98

Bell, R.D., and Doisy, D.A.

1920 "Rapid Colorimetric Methods for the Determination of Phosphorus in Urine and Blood."

J.B.C. 44: 55-67

Birch, T.W., and Harris, L.J.

1930 "Zwitterions II. Amino Acids, Polypeptides, etc., and Proteins as Zwitterions with Instances of Non-Zitterion Ampholytes."

Biochem, J. 24: 1086-1097

Bosworth, A.W.

1914 "The Action of Rennin on Casein."

J.B.C. 19: 397-8

Briggs, A.P.

1924 "Some Applications of the Colorimetric Phosphate Method."

J.B.C. 59: 255-64

Carpenter, D.C.

1927 "The Influence of Salts on the Optical Rotation of Gelatin."

J. Phys. Chem. 31: 1873-79

Clark, W. M.

1928 "The Determination of Hydrogen Ions."

The Williams and Wilkins Co., Baltimore,Md.

Dennis, W.

1910 "The Determination of Total Sulphur in Urine."

Jour. Biol, Chem. 8: 401-3.

Faneslow, J.R.

1928 "The Influence of Electrolytes and Non-Electrolytes upon the Optical Activity and Relative Resistance to Shear of Gelatin Systems."

Colloid Symposium Monograph 6: 237-52

Gortner, R.A.

1929 "Outlines of Biochemistry" pp. 420-421

John Wiley & Sons. New York.

Greenberg, D.M., and Larson, C.E.

1935 "The Physical Chemistry of the Proteins in Non Aqueous and Mixed Solvents. II. Electorchemical Properties of Protein Solutions in Certain Glacial Organic Acids."

J. Phys. Chem. 39: p. 665

Harris, L.J.

1930 "Zwitterions I. Proof of the Zwitterion Constitution of the Amino-Acid Molecule."

Biochem. J. 24: 1080-1085

Hawk, D.B. and Bergeim, Olaf

1927 "Practical Physiological Chemistry." p. 403

P. Blakiston's Son & Co.

Hitchcock, D.I.

1932 "Combination of Certain Proteins with HCl."

J. Gen. Physiol. 16: 357-366

Hoffman, W.F., and Gortner, R.A.

1925 "Physico Chemical Studies on Proteins. I. The Prolamines - Their Chemical Composition in Relation to Acid and Alkali Binding."

Colloid Symposium Monograph 2: 209-368

Kraemer, E.O.

1926 "Observations of the Colloidal Behavior of Aqueous Gelatin Systems."

Colloid Symposium Monograph 4: 102-121

Lloyd, Dorothy J.

1933 "Combination of Proteins with Acids and Bases."

J. Intern. Soc. Leather Trades Chem. 17: 1 245-258.

Loeb, J.

1922 "Proteins and the Theory of Colloidal Behavior".

McGraw-Hill Co. New York.

Morris, H.P., Nelson, J.W., and Palmer, L.S.

1931 "Quantitative Determination of Calcium, Magnesium and Phosphorus in Feedstuffs and Cattle Excreta."

Ind. and Eng. Chem. Vol.3, No.2 pp 164-167

Palmer, L.S., and Richardson, G.A.

1926 "The Colloid Chemistry of Rennet Coagulation".

Colloid Symposium Monograph 3: 113-134.

Pauli,W, and Spitzer,

1922 Quoted by Pauli in "Colloid Chemistry of the Proteins." Part I.

J.A.Churchill, London.

Piettre, M.

1931 "Influence of Adsorption on Physico-Chemical Properties of Organic Colloids."

Compt. Rend. 192: 894-897

Robertson, T.B.

1918 The Physical Chemistry of the Proteins."

Longmans, Green and Co.,New York.

Robinson, A.D.

1930 "Thesis for Degree of Doctor of Philosophy at University of Minnesota. "

Privately loaned.

Robinson, A.D., Gortner, R.A., and Palmer, L.S.

1932 "Physico-Chemistry Studies on Proteins". "VI. The Effect of Salts on the Casein and Paracasein Sodium Hydroxide Equilibria."

J. Phys. Chem. 36: 1857-1881

Sandstrom, W. M.

1930 "Physico-Chemical Studies on Proteins."

J. Phys. Chem. 34: 1071-1101

Schulz, G.V., and Ettisch, G.

1933 "Primary and Secondary Reactions of Proteins with Acids and Bases."

Z. Physik Chem. A. 164: 97-120. Abstracted in Chem.Abstracts p. 2862 (1933)

Toleman, R.C., and Bracewell, R.S.

1919 "The Molecular Mechanism of Colloidal Behavior. II. The Swelling of Fibrin in Alkalis."

J.A.C.S. 41: 1503-1510

Toleman, R.C., and Stearn, A.E.

1918 "The Molecular Mechanism of Colloidal Behavior. I. The Swelling of Fibrin in Acids."

J.A.C.S. 40: 264-272

Van Slyke, L.L.

1928 "A Summary of Research Studies Relating to Casein and Some of the Applications."

New York State Agri. Exp. Sta. Tech. Bull. No. 139. (1928)

Van Slyke, L.L., and Baker, T.C.

1918 "The Preparation of Pure Casein."

Jour. Biol. Chem. 35: 127-136.

Van Slyke, L.L, and Bosworth, A.W.

1913 "Method of Preparing Ash-Free Casein and Paracasein."

Jour. Biol. Chem. 14: 203-206

Van Slyke, L.L., and Bosworth, A.W.

1913_a "Preparation and Composition of Basic Calcium, Caseinate and Paracaseinate."

Jour. Biol. Chem. 14: 207-209

Van Slyke, L.L., and Bosworth, A.W.

1913_b "Preparation and Composition of Unsaturated or Acid Caseinates and Paracaseinates."

Journ. Biol. Chem. 14: 211-225

Van Slyke, L.L., and Hart, E.B.

1905 "Casein and Paracasein in Some of Their Relations to Bases and Acids."

Am. Chem. J. 33: 461-496.