CCLXI. ELECTROMETRIC TITRATION OF ZEIN AND IODOZEIN.

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It has been shown by different workers, but especially by Cohn [1925], that the acid and base binding capacity of proteins is connected with their amino-acid content. It is assumed that the free carboxyl groups of the dicarboxylic amino-acids are responsible for the base binding, while the basic groups (the ϵ-amino group of lysine, the guanidino-group of arginine and the iminazole group of histidine) determine the acid binding. The protein molecules must however be regarded as existing in solution predominantly as zwitterions, a view which best explains the values of the dielectric constants [Wyman 1931; Marinesco, 1932] and the calorimetric measurements of Meyerhof [1922]. From this view it follows that the acid binding should depend on the acidic groups and the base binding on the basic groups. Pauli and Valkó [1933] however have already pointed out that the application of the zwitterion theory does not alter the basis of calculation for the acid and base binding capacity of proteins. A protein at its isoelectric point carries an equal number of positive and negative charges. Therefore any excess of acidic or basic groups must exist in an unionised form. If a protein contains A acidic groups and B basic groups and the number of the acidic groups is larger than the number of the basic groups, then at the isoelectric point A^- must equal BH^+. The excess of acidic groups ($A-A^-$) is present in an uncharged form. The carboxyl groups exist at the isoelectric point partly in the form COO^- and partly in the form COOH. If such a protein is titrated from the isoelectric point with acid, the added hydrogen ions will in reality combine with the negatively charged carboxyl groups according to the equation:

$$^+RA^- + H^+ = {}^+RAH.$$

But the number of the titrated carboxyl groups is A^- which is equal to B^+, the total number of basic groups. Thus, in spite of the fact that it is the acidic groups which are titrated, the acid binding is equivalent to the basic groups present.

On the other hand, on addition of alkali, the sum of the excess of acidic groups, *i.e.* the uncharged carboxyl groups and all the basic groups, combines with the hydroxyl ion according to the two reactions:

$$(1)\ {}^-R^+AH + OH^- = {}^-R^+A^- + H_2O.$$
$$(2)\ {}^-RBH^+ + OH^- = {}^-RB + H_2O.$$

Thus the whole amount of titrated groups is $(A-A^-)+B^+$: and since A^- is equal to B^+, the base binding is equal to A, *i.e.* the number of all acidic groups present. If a protein contains more basic than acidic groups then the excess of basic groups is titrated by adding acid. But here also the basis of calculation for acid and base binding remains unaltered, although the interpretation of the actual titration curve has to be changed.

The phenolic group of tyrosine cannot contribute to the zwitterionic character of the majority of proteins at their isoelectric points, because its acid character is too feeble. But it seems to be certain that the phenolic OH group is free in most proteins and binds base at fairly high p_H values.

It is further important to note for the calculation of acid and base binding of proteins, that titration in aqueous solution is only possible within certain p_H ranges. Groups whose dissociation constants are too large or too small cannot be titrated satisfactorily. This applies especially to the guanidino-group of arginine. But if means are taken to lower the dielectric constant of the protein solvent, the range of titration may be enlarged.

Relation between titration curve of a protein and those of its constituent amino-acids.

In order to determine which groups of a protein molecule are free and are able to combine with acid or base, it seems necessary not only to connect the acid and base binding capacity of a protein as a whole with its amino-acid composition but also to compare the titration curve of a particular protein with the curves of its constituent amino-acids. This has been attempted by Simms [1928] for edestin and gelatin. But his interpretation is largely hypothetical, more especially in regard to his "prearginine."

The values of the dissociation constants of the different groups of the free amino-acids are known, but it does not follow that these values remain unchanged when the amino-acids are combined in the protein molecule. The values of the α-carboxyl and the α-amino-groups are altered very considerably by the peptide linkage [Euler, 1907; Simms, 1928; Mitchell and Greenstein, 1930]. But it has been shown by Greenstein [1933] that the values of the second carboxyl group and those of the ε-amino-, the iminazole, the guanidino- and phenolic groups, which are the important groups in regard to the titration curve of proteins, are not greatly changed. The peptide linkage exercises a much stronger influence on the α-amino- and α-carboxyl groups, which are in the immediate neighbourhood of the peptide linkage, than on other more distant groups.

It would be desirable to have more data about the dissociation constants of peptides containing trivalent amino-acids than are available at present. But it may be assumed that when amino-acids are linked to form dipeptides little change occurs in the dissociation constants of those groups peculiar to trivalent amino-acids. From the work of Stiasny and Scotti [1930] it may be concluded that a further increase of peptide linkages will not greatly influence the dissociation constants.

Thus it may be expected that in a protein molecule the dissociation constants of the individual groups will lie within one unit of those of the corresponding amino-acids in the free condition. It must be realised that groups of the same chemical character in the same protein molecule will not have quite the same dissociation constants, for these values depend on the mutual distance of the groups from one another, on their distance from a peptide linkage or from other charged groups. If a protein molecule, *e.g.*, contains a certain number of free carboxyl groups, those groups will not have the same p_K: a carboxyl group which is near to a basic group will have a higher dissociation constant than a carboxyl group which is near to another acidic group; thus the part of the titration curve of this protein which is due to the carboxyl groups will be prolonged. The buffering power which is due to carboxyl groups will be effective over a larger p_H range than with an isolated amino-acid. It is evidently necessary, therefore,

when we attempt to apply our knowledge of the dissociation constants of the free amino-acids to the interpretation of the titration curve of a protein, to make allowance for the effects of structural factors.

Titration of zein.

This paper is concerned with the titration of zein, of which the composition is better known than that of any other protein. The amino-acids which have been recovered from zein account for nearly the whole of the molecule. Cohn *et al.* [1924] have investigated the base binding capacity of zein in water and found values of 28×10^{-5} and 31×10^{-5} mols. per g. protein. No acid binding in water could be found. Later Hoffman and Gortner [1924] reported that acid binding takes place in aqueous-alcoholic solution. During the progress of this work a paper by Cohn *et al.* [1934] appeared in which the authors report that zein in solution in 80 % ethyl alcohol binds $17{\cdot}8$–$21{\cdot}3 \times 10^{-5}$ mols. acid and 12·1 mols. base between the isoelectric point and p_H 9. In the present paper the acid and base binding capacities of four different zein preparations over the whole possible p_H range are reported. An interpretation of the titration curve in connection with the amino-acid composition is given.

In attempting to apportion the different parts of the titration curve of the protein to the various constituent amino-acids, the following methods were adopted.

(1) The temperature coefficients of the dissociation constants of the different groups of amino-acids vary to a large extent. The amino-groups and the guanidino-group of arginine have large temperature coefficients, the phenolic and iminazole groups have fairly large ones, while the dissociation of the carboxyl groups does not alter appreciably with changes of temperature [Schmidt *et al.*, 1930]. Greenstein [1933] has shown that the temperature coefficients peculiar to the dissociation constants of certain groups are not altered significantly when the amino-acids are combined to form peptides. It was shown for glycine [Neuberger, 1934] that in 90 % alcoholic solution also the p_K of the amino-group is greatly influenced by temperature changes, while that of the carboxyl group remains practically unaltered. Thus comparison of the titration curves of a protein at different temperatures must yield some information about the corresponding dissociation groups.

(2) It is known that variation of the dielectric constant of the solvent has a different influence on the acidic and basic groups of amino-acids. Lowering of the dielectric constant decreases predominantly the dissociation of the acid groups, *i.e.* the carboxyl and phenolic groups [Jukes and Schmidt, 1934; Neuberger, 1934]. The same must be expected to apply to these groups in the protein molecule. Change in the dielectric constant of the solvent can be brought about by variation of the alcohol concentration.

(3) Chemical alteration of certain groups has an influence on their dissociation constants. In the present work zein was iodinated under suitable conditions. Analysis of the product indicated that iodine had been introduced exclusively into the benzene ring of tyrosine in the 3 : 5-positions. The p_K of the phenolic group of tyrosine is changed from a value of 10·28 to 6·48 by the introduction of iodine into the benzene ring [Dalton *et al.*, 1930]; (for the interpretation of the different dissociation constants of diiodotyrosine see Cohn [1931]). If the assumptions about the interpretation of the titration curve and the chemical character of the iodination are correct, a shift in the titration curve of the iodinated product in comparison with that of zein ought to be found.

Calculated acid and base binding power of zein.

In Table I a calculation of the acid and base binding capacity of zein is made on the basis that the acid binding is equal to the number of the basic amino-acids (histidine and arginine) and the base binding is equal to the sum of the free carboxyl groups and the phenolic groups. It is difficult to include the free

Table I. *Calculated values of acid and base binding power of zein.*

Acid binding			Base binding			Combining power of histidine *plus* free carboxyl groups	Combining power of arginine *plus* tyrosine
	% of protein	Mols. $\times 10^{-5}$ per g. protein		% of protein	Mols. $\times 10^{-5}$ per g. protein	Mols. $\times 10^{-5}$ per g. protein	Mols. $\times 10^{-5}$ per g. protein
Histidine	0·84* 0·82†	5·4 5·3	Glutamic acid‡	31·3	212·8	32·8–32·9	41·4–43·3
Arginine	1·88* 1·55†	10·8 8·9	Aspartic acid‡	1·8	13·5		
Lysine	—	—	Hydroxy-glutamic acid‡	2·5	15·3		
					241·6		
Total acid binding		14·2*–16·2†	Ammonia†	3·64	214·1		
			Free carboxyl groups (by diff.)		27·5		
			Tyrosine§	5·9	32·5		
			Total base binding		60·0		

* Kossel and Kutscher [1900]. † Osborne and Liddle [1910]. ‡ Dakin [1923]. § Folin and Ciocalteu [1927].

terminal α-amino- and carboxyl groups in the calculation, since the molecular weight of zein is not known. But it must be pointed out that in consideration of the small acid and base binding capacity of zein the correction of the calculated values, caused by these terminal groups, might be considerable. The calculation presented here differs from that given by Cohn *et al.* [1924] in minor points. For the dicarboxylic amino-acids the values of Dakin [1923] have been used, but for the amide-nitrogen the highest value of Osborne and Liddle [1910] has been employed. For the basic amino-acids the analyses of Kossel and Kutscher [1900] and those of Osborne and Liddle [1910] have been recorded. The values of Kossel and Kutscher are corrected for a nitrogen content of 16·13 %. The value for histidine obtained by Hanke [1925] has not been used, since his method seems to give too high results. The latest estimation of tyrosine furnished by Folin and Ciocalteu [1927] indicates a value of 5·9 %. The value obtained by Dakin [1923] is lower by 0·6 %, but may be explained by the unavoidable loss of substance during the process of isolation.

Experimental.

Four different preparations of zein were used. Preparation I was purified from an old laboratory sample by repeated precipitation of the aqueous-alcoholic solution with water, absolute alcohol and ether. Preparation Ch was supplied by Professor Chibnall. Preparation O was prepared from white maize flour by adapting the method of Dill and Alsberg [1925] to zein. The nitrogen content of the different preparations (on a moisture- and ash-free basis) varied between 15·85 and 16·15 %. All preparations gave clear solutions in 90 % alcohol. The general technique of the p_H measurements was as described in a previous paper [Neuberger, 1934]. The titration vessel was fitted with two hydrogen electrodes and the readings could be taken alternatively. The values obtained by the two

electrodes agreed satisfactorily. The electrodes were rapidly poisoned by the protein and had to be replaced after two or three titrations. Hydrogen electrodes gold-plated before platinisation seemed to be very advantageous. The term p_H in connection with alcoholic solutions is used in the conventional sense already described [Neuberger, 1934]. For the calculation of the p_H values from the voltage readings corrections were made for the vapour pressure of the solution according to "International Critical Tables." All p_H measurements were made in a paraffin-bath, which was kept constant within $\pm 0,1°$.

Discussion.

The isoelectric point of zein.

The p_H of the zein solutions, which are almost salt-free, can be regarded as being identical with the isoelectric point. From Table II it follows that the value of the I.P. depends on the temperature and on the alcohol concentration. The

Table II. *The isoelectric point of zein.*

Temp.	25° p_H	45° p_H
90 % alcohol	6·40	6·04
60 % ,,	6·00	5·70
54 % ,,	—	5·61

change of the isoelectric point brought about by the alteration of these physical constants of the solution will be explained later in connection with the interpretation of the titration curve.

Csonka *et al.* [1926] give a value of 6·2 for the I.P. of zein, but the method cannot give clear results in alcoholic solutions.

Table III. *Experimental values of the acid and base binding capacities of different zein preparations.*

All figures in this table are expressed in mols. $\times 10^{-5}$ acid or base per g. protein.

	Acid binding	Base binding	Combining power of groups titrated between acid end-point and p_H 9	Combining power of groups titrated between p_H 9 and p_H 13·2
Prep. I	19·3	54·2	30·4	43·1
Prep. N	16·8	46·2	26·4	36·6
Prep. Ch	20·1	54·6	32·1	42·6
Prep. O	16·6	47·2	28·8	35·0

Acid and base binding capacity of zein.

The values given in Table III for the acid and base binding capacities of the different zein preparations were obtained as the mean values of three or four estimations. Comparing these values with the calculated values in Table I certain points have to be taken into account.

(1) Since the acid and base binding of zein is much smaller than that of other proteins, errors in the estimation and analysis of the constituent amino-acids are magnified. This applies especially to the calculated number of free carboxyl groups, which is given as the small difference of two large magnitudes, namely, the sum of the three dicarboxylic amino-acids and the amide-nitrogen.

(2) In order to obtain the value for the base or acid binding the experimental curve has to be corrected by a blank curve. This method involves the assumption,

which is undoubtedly incorrect, that the activity of the hydrogen is not influenced by the presence of proteins.

(3) The accuracy of p_H measurement in very acid or alkaline ranges is generally smaller than in more neutral ranges, and is still less accurate in the case of alcoholic solutions (liquid junction, traces of CO_2).

(4) Although the range of titration is enlarged by using 90 % alcohol as solvent, a certain number of groups may be still outside the titratable range. This applies to a small number of phenolic or guanidino-groups. This latter point is regarded as the main reason for the small discrepancy which exists between the calculated and the experimental values of the base binding capacity. But as a whole the agreement between the values in Tables I and III is satisfactory, taking into consideration all these sources of error.

The differences in acid binding between the various zein preparations are similar to those differences observed by Cohn *et al.* [1934]. The differences between the base binding capacities of the different zein preparations are mainly due to differences occurring in very alkaline ranges and therefore cannot be regarded as highly significant.

Titration curves.

The titration constants of the free carboxyl groups may be assumed to vary between 3·6 and 4·8 (in aqueous solution). In 90 % alcohol the values of the dissociation constants of the carboxyl groups are increased by about 1·7–1·9 units and in 50–60 % alcohol by roughly about 1·0 unit. This means that nearly all carboxyl groups would be titrated in 90 % alcohol over the range from 4·3 to 7·7 and in 50–60 % alcohol between about 3·7 and 7·0. Since the dissociation of the iminazole groups of histidine is not influenced greatly by the addition of alcohol, the range in which histidine is titrated may be between 4·8 and 7·4 (at 25°). The sum of all groups titrated between the acid end-point and about p_H 9 must be equal to the sum of all carboxyl groups and histidine. The agreement between the calculated values in Table I, column 7, and the experimental values in Table III, column 4, is satisfactory. The p_K of the phenolic group of tyrosine is increased on changing the solvent from water to 90 % alcohol by about 2 units. Making allowance for the influence of other neighbouring groups on the p_K value of the phenolic group, it may be assumed that the range for the titration of this group lies between 10·50 and 13·8 in solution in 90 % alcohol. The p_K of arginine is not greatly influenced by the addition of alcohol and it may be assumed that most of the arginine is titrated between 10·5 and 14·0. The sum of tyrosine and arginine groups therefore must be equal to the number of groups titrated between p_H 9 and the alkaline end-point. But since the titration is only carried as far as p_H 13·2, it is to be expected that the experimental value will be somewhat lower than the calculated value. This is really the case, as the comparison between Table I, column 8, and Table III, column 5 shows.

Influence of temperature variation. Figs. 1 and 2 and Table II show the influence of variation of the temperature of the solution on the titration curve and on the value of the isoelectric point. By raising the temperature from 25° to 45° the titration curve is shifted mainly in two ranges. Between p_H 5·5 and 7·0 the p_H values are lower by 0·30–0·35 unit, while in alkaline p_H ranges the difference is much larger (about one p_H unit). At acidities greater than p_H 5·5 no considerable difference between the titration curves at the two temperatures can be observed. Other zein preparations of which the titration curves are not reproduced here show the same behaviour. These changes can easily be explained. At acidities greater than p_H 5·5 practically only carboxyl groups are titrated, of

which the dissociation is not greatly influenced by temperature changes. Over the range between p_H 5·5 and 7·0 the iminazole group is titrated as well as carboxyl groups. Variation of the temperature therefore has an appreciable effect on the titration curve, but this effect is not so strong as it would be if iminazole groups only were titrated. Over alkaline ranges (p_H 11–13) the phenolic and guanidino-groups are titrated. The effect of temperature variation is greater, as might be expected.

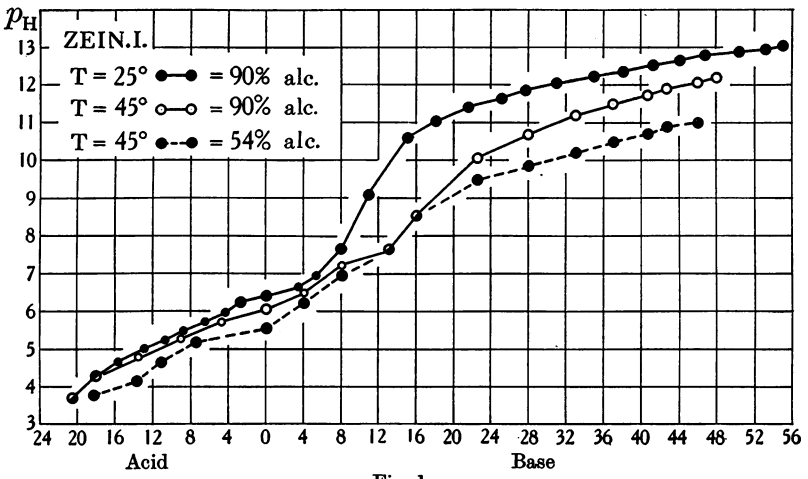

Fig. 1.

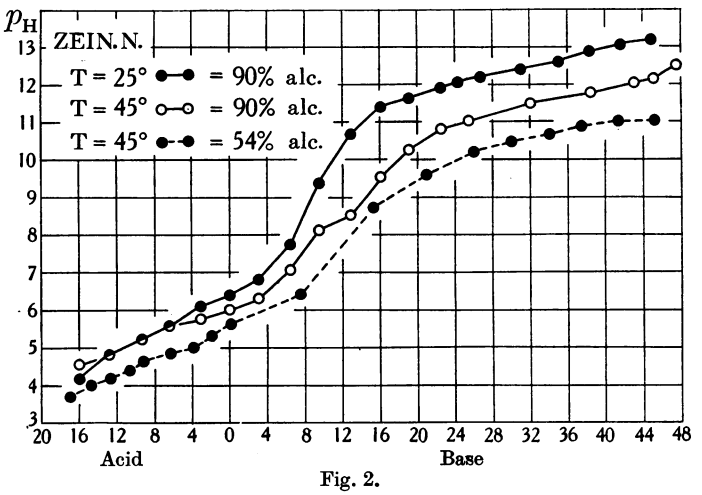

Fig. 2.

It must be mentioned that at 45° all zein preparations investigated bind much more base between p_H 7 and 9 than at 25°. The difference in the two curves is here nearly 1·5 units. It is possible that a reversible dissociation of the large zein molecules takes place at the higher temperature, resulting in the liberation of new terminal amino-groups.

Influence of variation of the alcohol concentration. The effect of lowering the alcohol content of the solvent on the titration curve is shown in Figs. 1, 2 and 3.

In solutions containing 60 % alcohol the maximum shift of the titration curve is about 0·4 p_H in the acid range and about 0·9 in the alkaline range. When the solvent contains 54 % alcohol the maximum shifts are a little larger, *viz.* 0·5 and 1·1 respectively. The effect is smaller in the acid range because the dissociation of the iminazole group coincides to some extent with that of the carboxyl groups. The very large shift of the curve in strongly alkaline ranges shows that here chiefly phenolic groups are titrated.

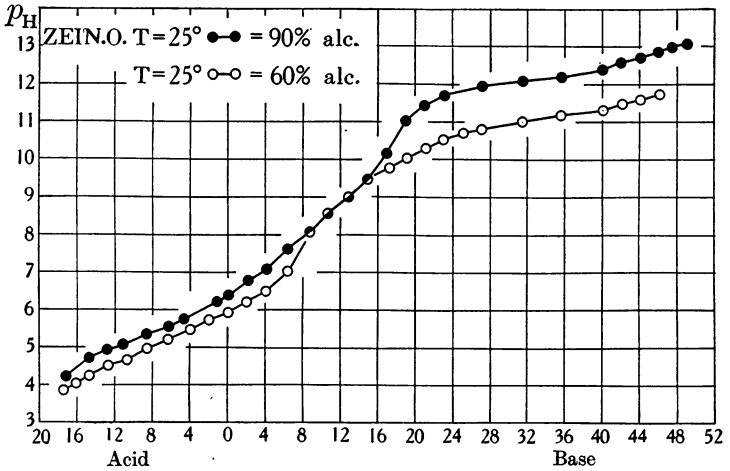

Fig. 3.

Iodozein.

The iodozein required for the titrations was prepared as follows.

Purified zein (3·75 g. calculated on an ash- and moisture-free basis) was dissolved in a mixture of methyl alcohol (210 ml.), water (25 ml.) and aqueous ammonia (15 ml. of sp. gr. 0·880). The solution was cooled in ice and treated gradually with a solution of iodine in potassium iodide (2·8*N*). The addition of iodine was interrupted after 1·84 ml. had been introduced, this amount representing about 5 % excess above that required to iodinate the tyrosine contained in the quantity of zein used. The iodine disappeared rapidly until the last stages of the addition were reached; the final presence of a slight excess was indicated by a persistent faint brown tinge in the solution.

Glacial acetic acid was now added in slight excess and the mixture poured into water. The white precipitate which separated was collected and washed several times with water. For further purification it was dissolved in 90 % ethyl alcohol and reprecipitated by pouring into 5 vols. of water containing 0·5 % lithium chloride, this process being repeated twice. The yield was 3·5 g. of air-dried product. The preparation contained 4·4 % moisture and 0·1 % ash. On a moisture- and ash-free basis it contained 14·78 % N and 7·54 % I.

On the assumptions that the original zein contained 5·9 % of tyrosine and that the iodine was all used up in iodinating this amino-acid the product should contain 7·65 % I; it may therefore be reasonably concluded that the iodine has in fact exclusively entered the tyrosine molecule.

This conclusion is supported by the fact that the iodinated product gives no Millon reaction, but gives on the other hand an intense reaction with nitrous acid and ammonia such as is characteristic for the *o*-diiodophenolic grouping.

Iodozein resembles zein in its general solubilities; it is insoluble in ether, acetone, absolute alcohol and in water at neutral or acid reaction; it is soluble in aqueous alkali at a much lower p_H than is necessary to dissolve zein. Iodozein is soluble in 90 % alcohol but the solution has a tendency to become slightly cloudy. This cloudiness may be increased by the addition of small amounts of base but does not interfere with the accuracy of the electrometric titration; it disappears completely on the addition of larger quantities of base or of acid.

Table IV shows that the acid and base binding power of iodozein is similar to that of zein; this proves that no scission of peptide or amide linkages has occurred. Fig. 4 indicates however that the p_H range over which most of the base binding takes place is shifted about 3·5 p_H units to the acid side.

Table IV. *Acid and base binding of iodozein.*

	Acid binding mols. × 10^{-5} per g. original zein	Base binding mols. × 10^{-5} per g. original zein
Prep. I	16·8	46·2
Prep. II	16·2	48·5

If it is assumed that the p_K of the phenolic group of iodozein is increased by about 2 units on passage from water to 90 % alcohol, then it is to be expected that the titration of this group in iodozein will take place between p_H 6·5 and 10·5, with maximum base binding at about p_H 8·5. That this is actually the case is shown by Fig. 4.

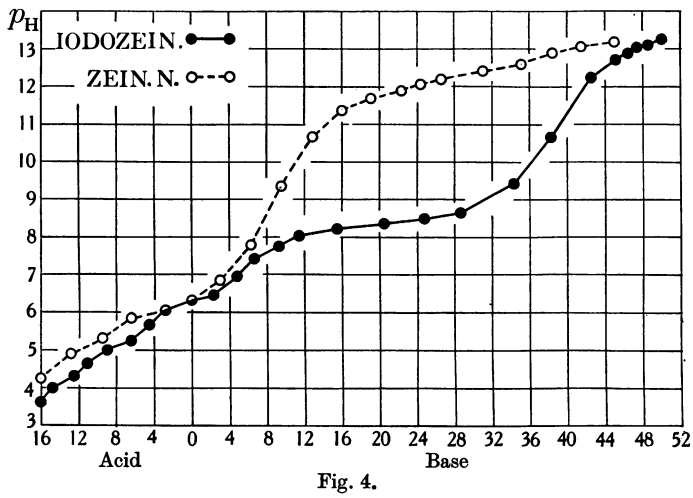

Fig. 4.

For purposes of comparison the figures for the acid and base binding power of iodozein given in Table IV have been calculated as mols. × 10^{-5} per 0·1613 g. N, *i.e.* per g. of original zein. The number of groups in iodozein titrated between the isoelectric point and p_H 10·5 should be equal to the sum of the number of groups in zein titrated between the isoelectric point and p_H 9 and the number of tyrosine groups, *i.e.* 40·5 to 44·7, the figures having the significance explained above. The experimental values vary between 41·5 and 43·0.

It should also be pointed out that the acid portion of the titration curve of iodozein is shifted slightly to the acid side in comparison with that of zein.

The titration of iodozein provides further and conclusive evidence that iodination of zein has really resulted in introduction of iodine into the benzene ring of tyrosine. Comparison of the titration curves of zein and iodozein also shows very clearly that most of the base binding of zein is due to the tyrosine groups.

The solubility of zein can now also be easily understood. If it is assumed that zein has a molecular weight of about 100,000, then at the isoelectric point the molecule carries about 11–17 positive charges and the same number of negative charges. Addition of acid does not bring about any increase in the number of ionised groups but causes the negatively charged groups to lose their charges. Addition of alkali on the other hand causes ionisation of the phenolic and carboxyl groups and the number of negatively charged groups increases to about 60 per molecule. The point of maximum charge [Cohn, 1931] lies at high alkalinity (about p_H 12·0).

It is generally agreed that the solubility of proteins is increased by ionisation and it is thus easy to understand why zein should be insoluble in acid and soluble in alkali. The fact that a lower p_H is required to dissolve iodozein than to dissolve zein is to be ascribed to the lower p_H at which the iodinated phenolic groups become ionised in comparison with the simple phenolic groups of zein.

Summary.

1. The acid and base binding capacities of proteins and their titration curves are discussed on the basis of the zwitterion theory and in connection with the dissociation constants of the constituent trivalent amino-acids.

2. The acid and base binding power of zein has been estimated in solution in 90 % alcohol. The values for the acid binding of different preparations vary between 16·6 and 20·1 and for the base binding between 46·2 and 54·6 mols. $\times 10^{-5}$ per g. protein.

3. Titrations of zein at different temperatures and concentrations of alcohol have been made. It has been shown that the titration curve of zein is determined by the number of free carboxyl groups, phenolic, iminazole and guanidino-groups.

4. Iodozein has been prepared by iodination of zein in solution in aqueous methyl alcohol in presence of ammonia. The product is a white powder. The analysis and the titration curve show that iodine is introduced exclusively into the benzene ring of tyrosine. The titration curve of iodozein is further evidence that most of the base binding power of zein is due to the tyrosine groups.

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