THE DETERMINATION OF THE EQUIVALENT WEIGHT OF PROTEINS.*

By ALLEN E. STEARN.

(From the Department of Chemistry, University of Missouri, Columbia.)

(Accepted for publication, November 25, 1927.)

According to Hoffman and Gortner (1) proteins combine stoichiometrically with acids and bases only within a limited pH range, which he places approximately between the limits 2.5 and 10.5. At pH values below 2.5 acid is still increasingly bound, but the binding in this region follows adsorption laws. Bases, above a pH of 10.5, behave in an analogous manner. This conclusion is based on the facts that (*a*) at no pH does the amount of acid or alkali bound seem to become constant and independent of further pH change, and (*b*) the temperature coefficient of acid and alkali bound begins to manifest itself to a significant degree at pH values below 2.5 and above 10.5 respectively.

Naturally it is difficult to obtain accuracy in electrometric titration at low or high pH values, where a small change in the ratio of hydrogen ion concentration means a large actual change. Regarding the conclusions drawn from temperature coefficient, no account is taken of the possible temperature effect on the difference in the activity of hydrogen ion in pure water and in protein solution, an effect which should at least be considered before too definite conclusions are drawn.

Jordan-Lloyd and Mayes (2) are in general agreement with the above conclusion from work done on the titration of gelatin with HCl. They state that up to a concentration of 0.04 normal acid a typical titration curve is obtained, but that above this concentration more acid begins to be bound.

Other workers find, at least in the case of the binding of HCl by

* This work was carried out in the chemical laboratory of Stanford University, and the author wishes to acknowledge his appreciation of the courtesy of the department extended to him as visitor.

gelatin, a typical titration curve showing no discontinuous section even in solutions considerably more acid than 0.04 normal (3, 4).

Volumetric conductivity titrations have been suggested from time to time as a means of obtaining the equivalent weight of proteins (5, 6, 7), but in only one case does the method seem to have been directly applied. Hitchcock (8) titrated gelatin with various acids and obtained concordant values for the equivalent weight of gelatin which agreed substantially with values obtained by electrometric titration.

The question arises as to whether any light can be thrown on the generality of Gortner's claim of a type of binding, easily observable at high or low pH values, different from that which one finds through the intermediate pH range. Doubtless a certain amount of "peptid-linkage" binding will take place, but does it occur to the extent assumed by Gortner?

Theoretical Considerations.

If we are interested in the increase in conductance of a titration mixture to which quantities of acid are being added after the stoichiometrical end-point has been reached we have the following rough relationships in the case of protein titrations.

Starting from a point where hydrolysis has been largely repressed (*n*, Fig. 1), we have for the change in λ, the conductance corrected for volume change during titration, with x, the number of equivalents of acid added,

$$\frac{d\lambda}{dx} = k\,\Lambda_0 \tag{1}$$

in which the constant k depends on cell constant and degree of ionization, and Λ_0 is the equivalent conductance of the acid in ionic form. This relation holds only in case no appreciable "adsorption" takes place. In the latter case we have

$$\lambda = \lambda_0 + k\,\Lambda_0\,(x - n) + k'\,p\,\Lambda_0\,nu \tag{2}$$

where k has the same significance as above, k' has analogous signifi-

cance for the protein salt, n is the number of equivalents of acid bound by adsorption, u is the transference number of the protein salt anion, and λ_0, the integration constant of equation (1) (and also of equation (5)), is the conductance of the mixture at the stoichiometrical end-point, corrected for hydrolysis. p is defined by the ratio $\frac{\Lambda_0 \text{ of protein salt}}{\Lambda_0 \text{ of acid}}$ so that

$$\Lambda_{0\,\text{protein salt}} = p\ \Lambda_{0\,\text{acid}}$$

But, though n is not, strictly speaking, a function of x, if we confine ourselves to conditions such that the increase in hydrogen ion concentration is nearly proportional to the amount of acid added, then, if n_0 is a constant and b the adsorption exponent,

$$n = n_0\, x^b \tag{3}$$

Substituting (3) in (2) we have

$$\lambda = \lambda_0 + k\ \Lambda_0\, x - n_0\, \Lambda_0\, (k - k'\, pu)\, x^b \tag{4}$$

Both u and p are fractions, the latter being rather small in general, and therefore, since k will not differ greatly from k',

$$k > k'\, p\, u$$

so that

$$\lambda = \lambda_0 + k\ \Lambda_0\, x - \text{const.} \times x^b$$

and

$$\frac{d\lambda}{dx} = k\ \Lambda_0 - b\ \text{const.} \times x^{b-1} \tag{5}$$

The two slopes differ therefore by the quantity $-$ const. x^{b-1}, where the constant is in all cases positive. In practically all cases reported by Hoffman and Gortner b is less than 1, or $(b-1)$ is negative. Thus for very high values of x, assuming no disturbing complications, the two slopes become identical.

If we are titrating a given amount of acid with a protein solution,

and if we study the same portion of the titration curve as above, *i.e.* with acid still in goodly excess, we have, if no adsorption takes place,

$$\lambda = \lambda'_0 - k\ (\Lambda_{0_{\text{acid}}} - \Lambda_{0_{\text{protein salt}}})\ x'$$

where λ'_0 is the conductance of the acid solution before any protein is added.

Putting in

$$\Lambda_{0_{\text{protein salt}}} = p\ \Lambda_{0_{\text{acid}}}$$

we have

$$\lambda = \lambda'_0 - k\ \Lambda_0\ (1 - p)\ x' \tag{6}$$

or

$$\frac{d\lambda}{dx'} = -\ k\ \Lambda_0\ (1 - p) \tag{7}$$

where x' is the number of equivalents of protein added in titration.

In case of appreciable adsorption we have

$$\lambda = \lambda'_0 - k\ \Lambda_0\ (1 - p)\ (x' + nu) \tag{8}$$

Under such conditions, if c be the number of equivalents of acid originally present, we cannot represent the concentration of free acid by the quantity $(c-x')$ as we could if there were no adsorption. The free acid will be $(c-x'^a)$ where the exponent a takes account of adsorbed as well as neutralized acid.

Thus,

$$n = n_0\ (c - x'^a)^b \tag{9}$$

and

$$\lambda = \lambda'_0 - k\ \Lambda_0\ (1 - p)\ x' - k\ \Lambda_0\ (1 - p)\ un_0\ (c - x'^a)^b \tag{10}$$

so that

$$\frac{d\lambda}{dx'} = -\ k\ \Lambda_0\ (1 - p) + bk\ \Lambda_0\ (1 - p)\ un_0\ (c - x'^a)^{b-1} \cdot ax'^{a-1} \tag{11}$$

In this case the two slopes differ by the quantity const. x'^{a-1} $(c-x'^a)^{b-1}$, where the constant is in all cases positive. As x' increases

$(c-x'_a)$ diminishes and, since $a>1$ and $b<1$, these two curves will tend to diverge in place of becoming parallel, as was the case with increase of x in titrating protein with acid.

These tendencies are shown by the diagrammatic graphs in Fig. 1. Curve A represents titration of a sample of protein with acid and Curve B the titration of a sample of acid with protein. Let the dotted lines represent the experimental curves. If we assume that appre-

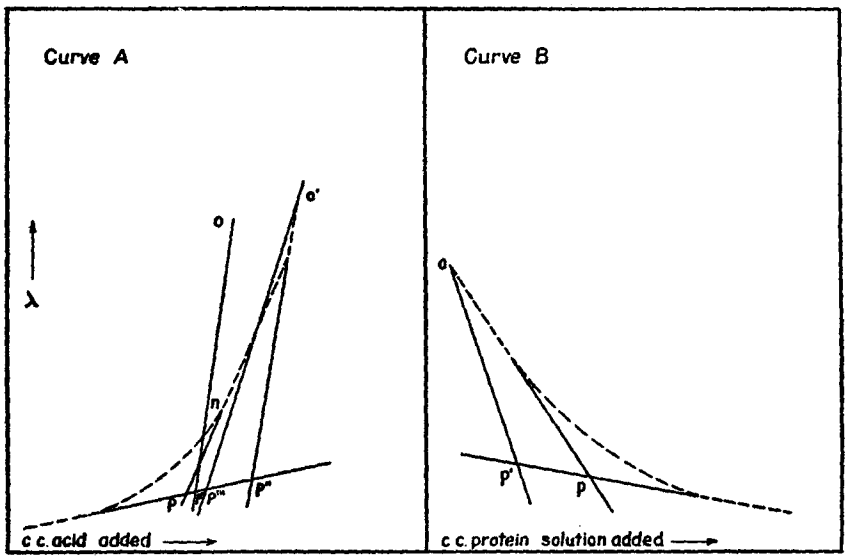

FIG. 1. Curve A represents the change in conductance when protein is titrated with acid, while Curve B gives the corresponding change when acid is titrated with protein. Analogous curves might be drawn to represent behavior toward bases. The dotted line represents a hypothetical experimental curve. For explanation of the various end-points, P, P', P'' and P''', see body of text.

ciable adsorption has taken place we may, on the basis of the above equations, draw in hypothetical arms, OP' in both curves, representing the curve one would get if no adsorption took place, *i.e.* if chemical neutralization were the only type of binding. Thus the point P is the observed end-point, P' is the hypothetical end-point which would give the stoichiometrical equivalent weight of the protein, and in case the titration were carried to the point where the two arms become parallel, the observed end-point would shift to P''.

It will be seen that if one calculates the number of cc. of standard alkali, or in this case acid, which is equivalent to 1 gm. of protein, one will not, in general, get the same result when titrating protein with acid as when titrating acid with protein unless adsorption is negligible. When titrating protein with acid (Curve A), points P and P' are rather close together since they are bound to occur at or near the

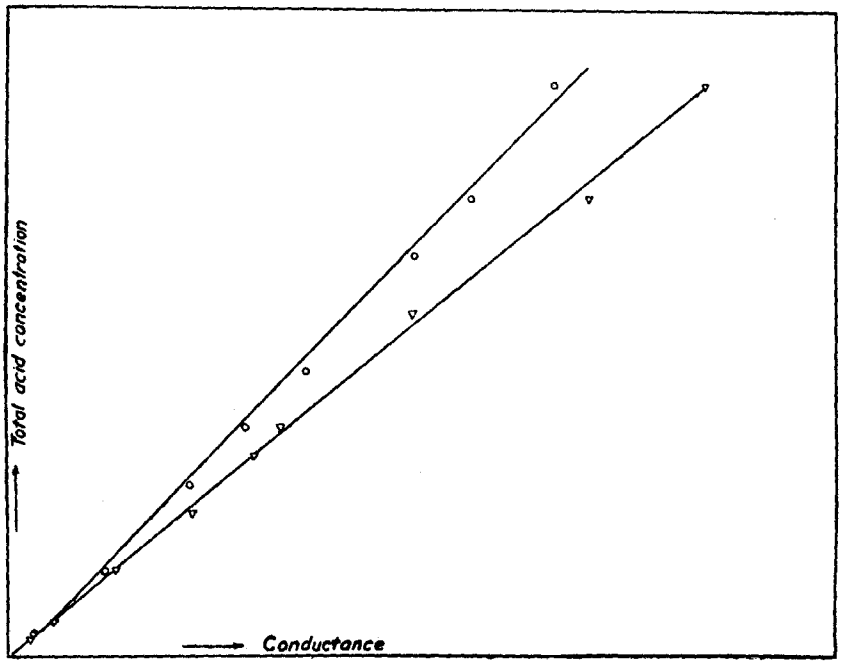

FIG. 2. Showing the difference in slope of conductance titration curves *after the stoichiometrical end-point has been passed* (I) when no adsorption takes place, and (II) when appreciable adsorption takes place. The curves are plotted from data calculated from results by Hoffman and Gortner (1) on the addition of HCl to water and to 1 per cent teozein solutions at 15°C. respectively.

point of convergence of the real and hypothetical arms. Thus, unless one goes far enough in one's titration to reach P″ as end-point, which is quite unlikely,[1] the observed end-point and the point which would give

[1] If one did get into this region one would probably imagine he should be getting a straight line and either disregard the particular experiment as untrustworthy or else arbitrarily take some average slope OP'''.

the stoichiometrical equivalent weight of the portein will lie rather close together.

Such is not the case (Curve B) when titrating acid with protein. In such a case the points P and P' will be read far from the point of convergence of the two arms. The estimated equivalent weight from the observed end-point P should therefore differ appreciably from that estimated from P' if appreciable adsorption takes place. Thus the observed value obtained by titrating protein with acid should be lower than that obtained by titrating acid with protein.

The same reasoning and predictions would apply also to the binding of bases.

The question whether the difference due to adsorption, on the basis of the results of Hoffman and Gortner, is large enough to warrant the above consideration may be easily answered affirmatively since they indicate, if their conclusions are valid, that over 90 per cent of the acid-binding and over 95 per cent of the alkali-binding is due, in the case of their prolamines, to adsorption. From their electrometric titration data on teozein at 15 degrees the conductance curves in Fig. 2 are plotted. These show the actual difference in slope between the curve obtained when HCl is added to water and that when it is added to completely "neutralized" teozein in 1 per cent solution.[2] The electrometric titrations were carried down nearly to a pH of 0.5.

The present paper presents data indicating that, in the case of gelatin and HCl, the same value is obtained for the equivalent weight of gelatin whether the gelatin is titrated with acid or the acid with gelatin. In case of gelatin and NaOH, contrary to obtaining a higher equivalent weight for the gelatin when titrating the base with the protein, a slightly lower value was obtained, due probably to carbon dioxide absorption.

[2] Plotted conductances were obtained by multiplying the various ion concentrations by their respective ion conductances at 15 degrees and adding. The value 25 was assumed for the protein ion conductance. The hydrogen ion concentration was obtained from pH values, the chloride ion concentration from the sum of hydrogen and total bound acid, the latter being calculated from data on page 336 (1), and the protein ion concentration was assumed equal to the second named component of the chloride ion concentration. The significant fact is that there is a real difference in slope through a pH range below that at which all of the protein has been "neutralized."

EXPERIMENTAL.

Since it is impracticable to titrate acid or base with gelatin without considerable volume change, all four titrations reported were made with about the same volume change, and the various conductances were corrected to the original volumes of the corresponding solutions. A preliminary pair of titrations first with a fairly concentrated, though unstandardized, acid and then with the same acid diluted to one-tenth of its original concentration gave the same end-point, when volume corrections were made, in equivalents of acid bound per gm. of gelatin.

TABLE I.

Acid added	$k \times 10^5$ measured	$k \times 10^5$ corrected to 75 cc.	Gelatin added	$k \times 10^6$ measured	$k \times 10^6$ corrected to 77 cc.
cc.			*cc.*		
0	105.0	105.0	0	3366.0	3366.0
1	154.0	156.0	1	3001.0	3040.0
3	213.1	221.6	2	2632.0	2700.0
5	282.7	301.6	3	2302.0	2392.0
7	388.0	424.0	4	2003.0	2107.0
9	562.0	629.7	5	1762.0	1877.0
11	785.0	900.0	6	1569.0	1691.0
13	1027.0	1205.0	7	1427.0	1557.0
15	1266.0	1519.0	8	1336.0	1475.0
17	1501.5	1842.0	10	1249.0	1411.0
20	1835.5	2325.0	12	1233.0	1425.0
			15	1256.0	1501.0
			17	1286.0	1570.0
			19	1311.5	1635.0
			20	1323.0	1667.0

The first three columns give the data obtained when different amounts of 0.1175 normal HCl were added to 1 gm. of gelatin in an original volume of 75 cc. The last three columns give corresponding data obtained when a solution of gelatin containing 0.0385 gm. per cc. was added in varying amounts to a solution containing 2 cc. of 0.1175 normal HCl in an original volume of 77 cc.

The titrations were made in a constant temperature bath thermally regulated. No adjustment to a particular temperature was made but the value 25.65° ± 0.05°C. was maintained.

A 1 per cent solution of the gelatin gave to water of specific conductance 3×10^{-6} (at room temperature) a pH of 4.90 and a specific conductance of about 100×10^{-6}. A sufficient quantity of gelatin solution was made up for all four titrations so that the magnitude of the correction necessary to bring it to its isoelectric point would be the same in all cases and thus the results of titrating

gelatin with acid and acid with gelatin could be compared regardless of the uncertainty of any correction. The magnitude of the correction was read from an independent electrometric titration curve (4). (See also (8)).

Data are given in Tables I and II and the results in Table III. The data for

TABLE II.

Base added	$k \times 10^6$ measured	$k \times 10^6$ corrected to 77 cc.	Gelatin added	$k \times 10^6$ measured	$k \times 10^6$ corrected to 77 cc.
cc.			*cc.*		
0	105.0	105.0	0	2140.0	2140.0
1	127.3	129.0	1	1989.0	2015.0
3	163.5	170.0	2	1847.0	1895.0
5	241.7	257.4	4	1580.0	1662.0
7	368.0	401.5	6	1364.0	1470.0
9	523.0	584.0	8	1208.5	1334.0
11	683.0	780.5	10	1109.0	1253.0
13	839.0	980.4	12	1064.0	1229.5
15	989.0	1182.0	14	1054.0	1246.0
17	1134.0	1384.0	16	1068.0	1290.0
20	1341.0	1689.0	18	1098.0	1355.0
			20	1140.0	1436.0
			23	1199.0	1557.0
			26	1260.0	1685.0
			30	1324.0	1840.0

The first three columns give the data obtained when different amounts of 0.1219 normal NaOH were added to 1 gm. of gelatin in an original volume of 77 cc. The last three columns give corresponding data obtained when a solution of gelatin containing 0.0385 gm. per cc. was added in varying amounts to a solution containing 2 cc. of 0.1219 normal NaOH in an original volume of 77 cc.

TABLE III.

Experiment	End-point (cc. titrating sol. obtained from curves)	0.1 N acid or base per gm. gelatin (uncorrected)	pH original gelatin sol.	Correction 0.1 N acid or base to isoelectric point	Corrected value of cc. 0.1 N acid or base per gm. gelatin
		cc.		*cc.*	
Titration of gelatin with acid.....	8.15	9.58	4.90	−0.65	8.93
" " acid with gelatin.....	6.40	9.54	4.90	−0.65	8.89
" " gelatin with base.....	5.50	6.70	4.90	0.65	7.35
" " base with gelatin.....	9.15	6.93	4.90	0.65	7.58

the acid titrations are plotted in the graphs of Fig. 3, and those for the alkali titrations are plotted in those of Fig. 4.

A solution of 7.70 gm. dry gelatin in 200 cc. was prepared by dissolving the gelatin in warm water, cooling and making up to volume. At such a concentra-

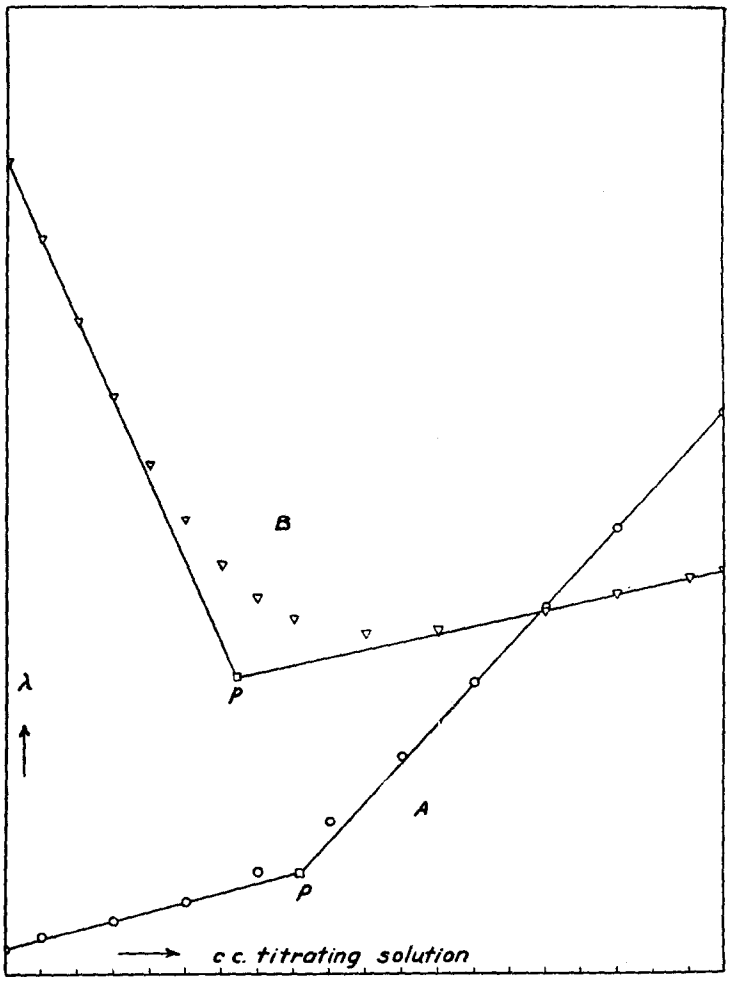

FIG. 3. Conductance titration curves of gelatin and HCl. Curve *A* is for titration of gelatin with acid and Curve *B* is for the titration of acid with gelatin solution. Conductances, corrected to original volumes are in both cases ordinates, while cc. of acid or of gelatin solution added are respective abscissæ.

tion the solution, upon standing for considerable time, would set to a gel at room temperature, but when freshly prepared it could be added from a burette for some time, even at room temperature. In making the titrations with the gelatin solutions, care was taken to obtain proper draining of the rather viscous liquid so as not to introduce appreciable volume error into the titration. For titration of the gelatin with acid or base, 26 cc. of this gelatin solution, which contained almost exactly 1 gm. of the dry gelatin, were used as samples. The standard acid was an HCl solution of normality 0.1175, and the base, prepared by diluting a 50 per cent NaOH solution from which the carbonate had settled with CO_2-free water, had a normality of 0.1219.

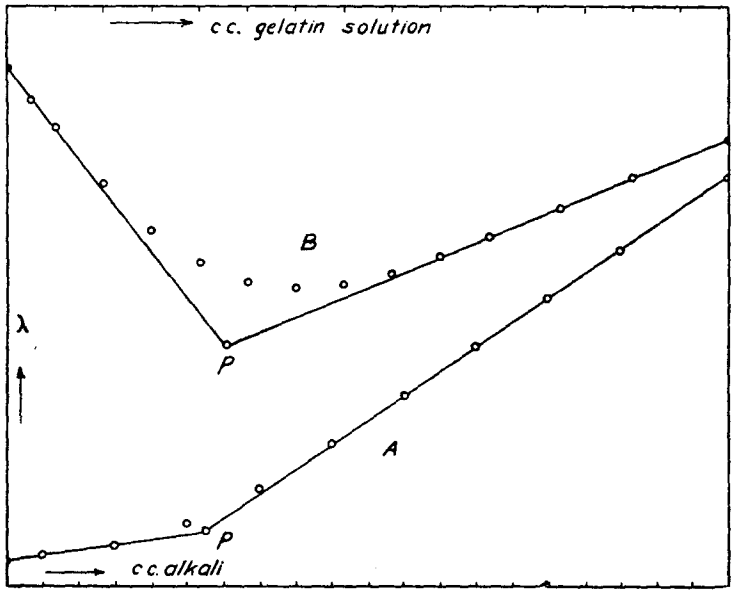

FIG. 4. Conductance titration curves of gelatin and NaOH. Curve *A* is for titration of gelatin with base, and Curve *B* is for the titration of base with gelatin solution. Conductances, corrected to original volumes are in both cases ordinates, while cc. of base or of gelatin solution added are respective abscissæ. (In this figure the abscissæ scale for Curve *B* is two-thirds that for Curve *A*.)

DISCUSSION AND SUMMARY.

The magnitude of the correction in the fifth column of Table III may be open to some doubt, as are all corrections of such a character, and the significance of the above experiment in the author's mind lies

not so much in the actual magnitude of the values given in the last column of this table as in their comparative magnitudes. For this reason the entire experiment reported was performed in a single session[3] using the same gelatin solution, so that, whatever the magnitude of the correction, it would be the same in all cases.

Actually the results in the case of the acid titrations are in fair agreement with those of Hitchcock (8). In the present experiment it is seen that, within the limits of experimental error, one gets the same value for the number of cc. of tenth normal acid bound by 1 gm. of gelatin whether one titrates with the acid or with the gelatin. In the case of the base there is a small difference, due probably to carbon dioxide, but this effect is in a direction opposite to that which one would expect on the assumption that it is due to appreciable adsorption.

From this it is concluded that the binding due to adsorption in the case of gelatin is not significant compared to that due to chemical neutralization. The author realizes that gelatin is a poor choice for a basis of generalizations, and similar work is at present in progress on various other proteins. He does feel, however, that the conclusions of Hoffman and Gortner from their work on the prolamines may also be too widely generalized, and that, on the whole, the acid or alkali bound by adsorption in the case of proteins will not constitute the large majority of the total amounts bound, though certainly one will expect a certain amount of such binding in all cases. It also seems that before placing undue emphasis on the conclusions of these workers the possibilities of equivocal results due to specific technique should be considered. This technique consisted in introducing weighed amounts of dry protein into a definite volume of standard acid or base at the equilibrium temperature, in general, and, "after about 15 minutes, during which time the flask was shaken several times," determining the pH of the equilibrium solution. Is it possible that the actual speed of solution of the protein is such that, even though reproducible results are obtained using identical technique, actual equi-

[3] The experiment reported is one of four performed. It may be stated that the last three gave substantially the same results, the first experiment being the only one yielding peculiar results. These peculiarities were found to be due in the main to insufficient care in titrating with the viscous gelatin solution.

librium conditions are approached only when comparatively high concentrations of acid or alkali are employed, in which cases the solution velocity of the protein may be expected to be greater, other factors remaining constant?

BIBLIOGRAPHY.

1. Hoffman, W. F., and Gortner, R. A., Colloid Symposium Monograph, New York, 1925, ii, 209.
2. Lloyd, D. J., and Mayes, C., *Proc. Roy. Soc. London, Series B*, 1922, xciii, 69.
3. Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 733.
4. Stearn, A. E., Paper in preparation.
5. Harris, L. J., *Proc. Roy. Soc. London, Series B*, 1924–25, xcvii, 364.
6. Wintgen, R., and Vogel, H., *Kolloid-Z.*, 1922, xxx, 45.
7. Hirsch, P., *Z. anal. Chem.*, 1926, lxviii, 160.
8. Hitchcock, D. I., *J. Gen. Physiol.*, 1923–24, vi, 201.