

AN ANALYSIS OF THE TITRATION DATA OF OXYHEMOGLOBIN OF THE HORSE BY A THERMAL METHOD

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There is a great amount of data on the titration of proteins with acids and bases. Most of this has received some degree of interpretation in terms of the composition of the molecules. In a number of cases the total acid- and base-combining power has been shown to be in very close agreement with that calculated on the basis of the analytical figures for the amino acid content of the proteins in question. In at least one case, moreover, it has been possible to fit the data in a satisfactory way over the whole range by dividing the total number of acid- and base-combining groups into a relatively small number of classes and assigning to each class a suitably chosen acidity constant, and the required choice of classes and constants has not been inconsistent with the known composition of the protein.

There is another method of approach available for the analysis of the acid- and base-combining power of the proteins which so far has not been tried, but which may be expected to shed new light on the problem and to supplement the information derived in other ways. This, which we may call the thermal method, consists in determining the effect of temperature on the titration curve. Since the different acid- and base-binding groups of these polyvalent molecules are for the most part characterized by different heats of dissociation, we should expect, in accordance with the van't Hoff equation, that the effect of temperature would be different in different ranges of the titration curve, and this should make it possible to see directly in which ranges different groups are active. In this paper we describe the application of this method to the case of a well known protein, oxyhemoglobin of the horse. First we shall give the results of the titration of oxyhemoglobin in solution at three temperatures and the apparent heat of

dissociation as a function of pH calculated from these data; we shall then develop the theory underlying the method, and make use of it in the analysis of the data on oxyhemoglobin.¹

The horse hemoglobin was crystallized by the method of Ferry and Green, as described by German and Wyman (2), from concentrated cells kindly furnished by the Massachusetts Antitoxin and Vaccine Laboratory. The final crystals were dissolved in 0.3 M aqueous sodium chloride to provide the stock solution which was used for all the measurements. This solution contained, according to a Kjeldahl analysis, 12.42 gm. of nitrogen per liter, or 73.8 gm. of hemoglobin per liter, on the basis of a nitrogen factor of 5.93 (Vickery and Leavenworth (3)). At the end of the measurements the solution was found to have an oxygen-combining capacity of 9.71 volumes per cent. Assuming the molecular weight of hemoglobin to be 66,800, this indicates that only 1.9 per cent of the protein was inactive. This amount is so small that any corrections based on it are less than the experimental error, and it has been ignored in calculating the results.

Measured amounts of approximately 0.1 N HCl or NaOH were added to aliquots of stock solution, and the resulting mixture diluted with water to give the same final concentration of protein in all cases. This involved a dilution of the stock solution to 132 per cent of its original volume. The titrated aliquots were equilibrated with oxygen at a pressure of about 90 to 100 cm. of mercury in a tonometer before injection into the electrode for measurement.

The pH was determined with a glass electrode in connection with a high resistance vacuum tube bridge (1). The measurements were made at three temperatures, 6.5°, 25°, and 37.7°, controlled by water pumped from a constant temperature bath through a glass jacket surrounding the electrode. The electrode was calibrated constantly throughout the course of the measurements with various standard buffers. The composition of these buffers and the pH assigned to each for each of the three temperatures are given in Table I. The values of the pH are based on interpolations or extrapolations from data given by Clark (4) wherever the values are not given directly by him.

¹ It should be noted that in 1924 Stadie and Martin (1) made an estimate of the heat of ionization of hemoglobin as a base from the shift of the isoelectric point with temperature.

TABLE I
pH of Standard Buffers

					6.5°	25°	37.7°
10	cc. citrate*				4.92	4.98	5.03
9.0	" "	+	1.0	cc. NaOH*	5.07	5.13	5.18
6.0	" "	+	4.0	" " *	5.93	6.00	6.03
0.25	" Na ₂ HPO ₄	+	9.75	" KH ₂ PO ₄ †	5.31	5.28	5.26
1.0	" "	+	9.0	" " †	5.92	5.90	5.87
49.6	" "	+	50.4	" " ‡	6.83	6.80	6.78
84.1	" "	+	15.9	" " ‡	7.52	7.50	7.48
87.0	" "	+	13.0	" " ‡	7.64	7.60	7.58
89.4	" "	+	10.6	" " ‡	7.73	7.70	7.67
94.7	" "	+	5.3	" " ‡	8.05	8.01	7.98
10	" borate§				9.33	9.19	9.10

* Clark (4), Table 42.

† Clark (4), Tables 41 and 43.

‡ Clark (4), Table 43.

§ Clark (4), Table 38.

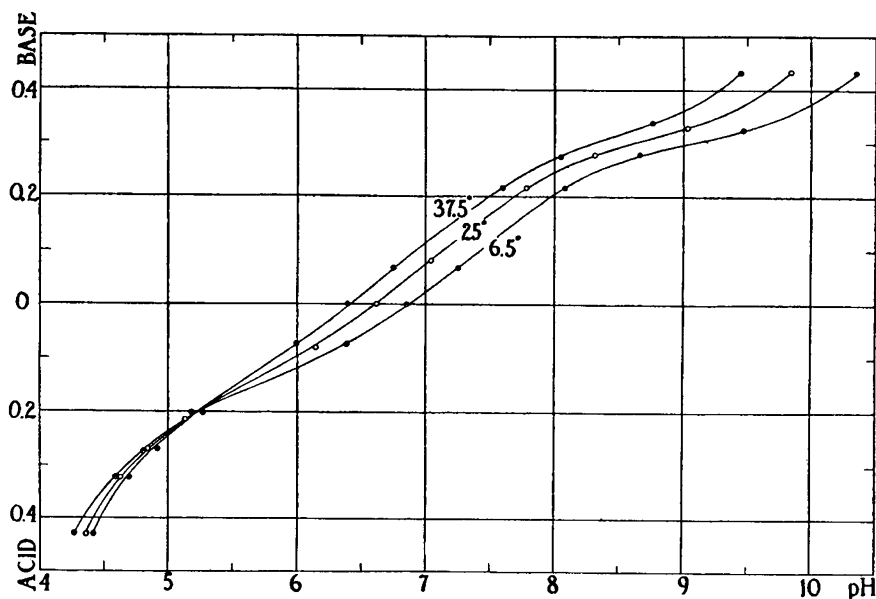


FIG. 1. Titration curves of oxyhemoglobin at three temperatures. Acid and base are expressed in milliequivalents per gm. of hemoglobin.

The titration curves for each of the three temperatures are shown graphically in Fig. 1. In Fig. 1 the ordinates give milliequivalents of acid or base per gm. of hemoglobin, which, if desired, may be converted into equivalents per mole simply by multiplying by 66.8 on the assumption of a molecular weight of 66,800. The results at 25° are in good agreement with other data and it is unnecessary to tabulate them numerically.

The point in which we are primarily interested here is a second order effect; namely, the difference of pH between the three curves for given values of the ordinates. It is this quantity which determines what we shall call the apparent heat of dissociation, Q' , and define, by analogy with the case of a weak monobasic acid, by the equation

$$(1) \quad Q' = -2.303 RT^2 \left(\frac{\partial \text{pH}}{\partial T} \right)_B$$

In this equation and subsequently, R denotes the gas constant, T the absolute temperature, and B the total amount of base present. The justification of this definition is given below. In Fig. 2 we have plotted values of this quantity expressed in calories as a function of pH over the entire range covered by the data. These values were actually calculated from the pH values taken from the smooth curves of Fig. 1 at various convenient values of the ordinate by the expression

$$(2) \quad -4.579 T_1 T_2 \frac{\text{pH}_2 - \text{pH}_1}{T_2 - T_1}$$

in which subscripts 1 and 2 refer to the two different temperatures in question. In Fig. 2 each value so calculated is plotted against the corresponding mean pH; *i.e.*, $(\text{pH}_2 + \text{pH}_1)/2$.

Let us now consider first very briefly the case of a simple acid SH which dissociates to give the products S^- and H^+ in accordance with the equation

$$(3) \quad \frac{a_{\text{S}} a_{\text{H}^+}}{a_{\text{SH}}} = k$$

where a denotes activity and k is the thermodynamic equilibrium constant. Q , the heat of dissociation per mole,² is given by the

² This is the increase of total heat; *i.e.*, the heat absorbed as a result of the dissociation.

van't Hoff equation

$$(4) \quad Q = RT^2 \frac{\partial \ln k}{\partial T}$$

Actually, what is more readily determined from the titration data is not the thermodynamic constant k but the acidity constant k' defined as

$$(5) \quad \frac{C_{S^-}}{C_{SH}} a_{H^+} = k' = k \frac{f_{S^-}}{f_{SH}}$$

in which C denotes concentration, and f activity coefficient. The van't Hoff equation, expressed in terms of k' , is

$$(6) \quad Q = RT^2 \frac{\partial}{\partial T} \left(\ln k' + \ln \frac{f_{S^-}}{f_{SH}} \right)$$

The expression $RT^2(\partial \ln k' / \partial T)$ gives what we call the apparent heat of dissociation Q' . This may be calculated from the titra-

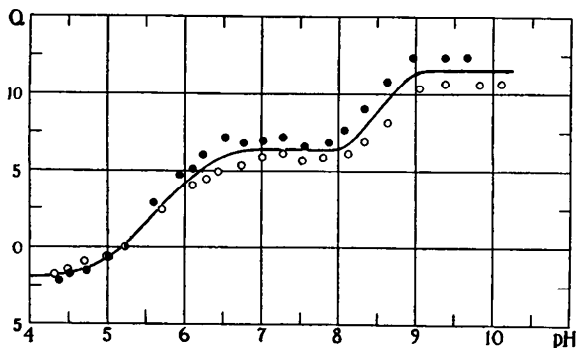


FIG. 2. Apparent heat of dissociation of oxyhemoglobin as a function of pH. ● = values for the 25-37.7° interval; ○ = values for the 6.5-25° interval.

tion data at once without any knowledge of activity coefficients from the equation

$$(7) \quad Q' = RT^2 \frac{\partial \ln k'}{\partial T}$$

Q' is of course not the same as the true heat of dissociation Q , but in general it is very close to it, since $\ln (f_{S^-}/f_{SH})$ varies but little

with temperature regardless of its absolute magnitude. The fact that Q' involves only the temperature derivative of f_S/f_{SH} and not this quantity itself is of considerable importance for the more complex case about to be considered, since it shows that even very great modifications in the residue attached to a given dissociating group can have little or no effect on the apparent heat of dissociation in so far as they lead only to changes in f_{SH} and f_S , however great. Provided the acid is sufficiently weak in relation to its concentration and the pH of the solution to allow us to make the usual approximation of setting C_{S-} equal to the amount of base added,³ the apparent heat of dissociation may be simply reckoned from the change of pH with temperature of a partially titrated solution containing a fixed amount of base, by the following equation, which results from Equations 5 and 7

$$(8) \quad Q' = -RT^2 \left(\frac{\partial \text{pH}}{\partial T} \right)_B$$

This method of reckoning Q' is the only one practicable in the more complex case which we will now consider, that of a polyvalent molecule, like a protein, containing n acid or basic groups. We shall treat all these groups as acid groups and express their dissociation in terms of n acidity constants k . For convenience of notation in what follows we shall omit the prime marks when referring to the acidity constants and apparent heats of dissociation and shall use H to refer to the activity of the hydrogen ions, omitting the symbols a and $+$. We shall assume that the n groups are independent, so that the dissociation of each is unaffected by that of the others. As a result of the independent dissociation of these n groups there will be $n + 1$ classes of ions, to which we shall refer by subscripts 0, 1, . . . n . Each class is characterized by the total number of hydrogen ions dissociated, or, what amounts to the same thing, by the number of equivalents

³ The error involved in this approximation is

$$\frac{C_{S-} - C_B}{C_{S-}} = \frac{C_{H+}(C_{H+} + k')}{k'(C_{SH} + C_{S-})}$$

provided we assume the base to be completely dissociated, neglect the dissociation of water, and take $C_{H+} = a_{H+}$.

of base bound, per mole, and this number is the same as the subscript. These $n + 1$ classes are all in equilibrium with one another, and the concentration C_{S_i} of ions in the i 'th class may be expressed in terms of the concentration C_{S_0} of ions of the 0'th class as follows:

$$(9) \quad \frac{C_{S_i}}{C_{S_0}} = \frac{K_i}{H^i}$$

Each of the n constants K depends on the n individual k 's and K_i is the sum of all the $n!/(n-i)!i!$ different products of i k 's chosen from the total number of n k 's. The total concentration of all the ions of all classes is

$$(10) \quad C_S \left(1 + \frac{K_1}{H} + \cdots \frac{K_n}{H^n} \right) \equiv C_S A$$

and the total amount of base bound is

$$(11) \quad C_S \left(\frac{K_1}{H} + \frac{2K_2}{H^2} + \cdots \frac{nK_n}{H^n} \right) \equiv C_S G$$

We have introduced the symbols A and G for convenience to refer to the two expressions in parentheses. The amount of base bound per mole of material is

$$(12) \quad b = G/A$$

A formal differentiation of b in terms of $\ln H$ and $\ln K_1, \dots \ln K_n$ gives

$$(13) \quad db = \left(\frac{\partial b}{\partial \ln H} \right)_{K_i} d \ln H + \sum_{i=1}^n \left(\frac{\partial b}{\partial \ln K_i} \right)_{H, K_j} d \ln K_i$$

If b is constant and $\ln H$ and $\ln K_i, \dots$ are expressed as functions of the temperature, this becomes, after multiplication by RT^2 ,

$$(14) \quad -RT^2 \left(\frac{\partial \ln H}{\partial T} \right)_b = \sum_{i=1}^n \left(\frac{\partial b}{\partial \ln K_i} \right)_H \frac{Q_i}{(\partial b / \partial \ln H)_{K_i}}$$

in which

$$(15) \quad Q_i = RT^2 \frac{\partial \ln K_i}{\partial T}$$

If we make use of Equations 10, 11, and 12 and remember that $\partial \ln x / \partial x = 1/x$, Equation 14 becomes

$$(16) \quad RT^2 \left(\frac{\partial \ln H}{\partial T} \right)_b = \frac{A \sum_{i=1}^n \frac{iK_i Q_i}{H^i} - G \sum_{i=1}^n \frac{K_i}{H^i} Q_i}{A \sum_{i=1}^n \frac{i^2 K_i}{H^i} - G^2}$$

It follows from the structure of K_i that Q_i consists of $n!/i!(n-i)!$ terms all constructed in the same way, of which the first is

$$k_1 k_2 \cdots k_i (q_1 + q_2 + \cdots q_i) / K_i$$

Each q is the apparent heat of dissociation of an individual group; *e.g.*, $q_1 = RT^2 \partial \ln k_1 / \partial T$. Q_i is the apparent heat of dissociation for the total step from class 0 to class i : $S_0 \rightarrow S_i + i$ hydrogen ions. $Q' = RT^2 (\partial \ln H / \partial T)_b$ is the apparent heat of dissociation of the molecule as a whole and may be determined from the experimental data by Equation 1, as was done in constructing Fig. 2.

When the q 's are the same for all the groups and equal to q , Q' calculated from Equation 16 reduces to q , as we know it must. When the q 's are different, but the k 's are all the same, it follows from the same equation that Q' is independent of pH and equal to the arithmetic mean of all the q 's. If the groups fall into two equal classes, each characterized by a given q and k , then Q' is the same as for a simple dibasic acid with one group having one k and q , the other group having the other k and q . Equation 16 shows how in any given case Q' varies as the pH is changed. The sharpness of the transition of Q' from one level to the next and the separation of the levels depend on the spacing of the successive pK 's as well as on the corresponding q 's and increase as the spacing increases.

The behavior of Q' in a number of special cases is illustrated in Figs. 3 and 4. Fig. 3 is for a molecule containing two groups having the constants pk_1 and $pk_2 > pk_1$ and heats q_1 and $q_2 > q_1$. The ordinate gives $(Q' - q_1)/(q_2 - q_1)$; the abscissa gives $pH - (pk_1 + pk_2)/2$. The curves are all symmetrical and in each case the value of the upper asymptote is given. The positions of the pk 's are shown by the circles.⁴ It may be seen that when the

⁴ *I.e.*, values of the abscissa for which $pH = pk_1$ or $pH = pk_2$.

separation of the pK 's is about 1.5 units or more the case is not very different from that in which the pK 's are infinitely separated.

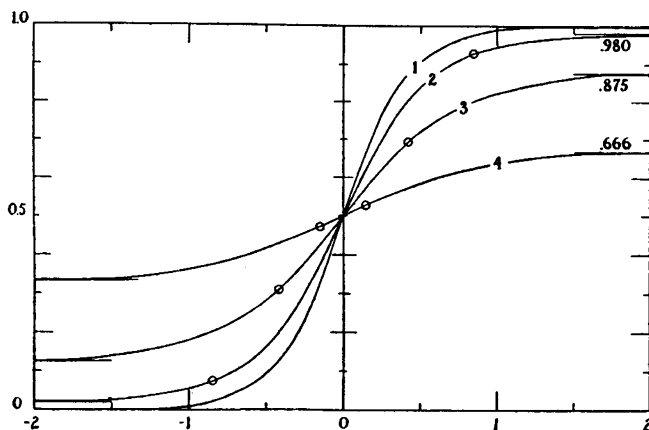


FIG. 3. The ordinates give $(Q' - q_1)/(q_2 - q_1)$; the abscissas $pH - (pk_1 + pk_2)/2$. Curve 1, $pk_2 - pk_1 \rightarrow \infty$; Curve 2, $pk_2 - pk_1 = 1.690$; Curve 3, $pk_2 - pk_1 = 0.845$; Curve 4, $pk_2 - pk_1 = 0.301$.

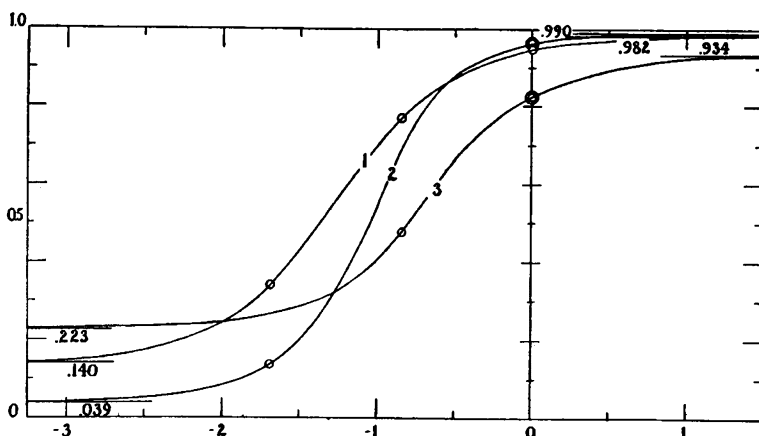


FIG. 4. The ordinates give $(Q' - q_1)/(q_2 - q_1)$; the abscissas $pH - pk_3$. Curve 1, $pk_3 - pk_2 = pk_2 - pk_1 = 0.845$; Curve 2, $pk_3 = pk_2$, $pk_2 - pk_1 = 1.690$; Curve 3, $pk_3 = pk_2$, $pk_2 - pk_1 = 0.845$.

Fig. 4 illustrates three cases of a molecule containing three groups characterized by pk_1 and q_1 , $pk_2 > pk_1$ and $q_2 > q_1$, and $pk_3 \geq pk_2$ and $q_3 = q_2$. The ordinates give $(Q' - q_1)/(q_2 - q_1)$ and

the abscissas $\text{pH} - \text{pk}_3$. Here again the positions of the $\text{pk}'\text{s}$ are indicated with circles, and the values of the upper and lower asymptotes are given.

In the light of this analysis it appears from Fig. 2 that over the range from pH 4 to pH 10 the dissociating groups of oxyhemoglobin fall into three classes on the basis of the heat of dissociation. Members of Class 1 have an average apparent heat of dissociation of -2000 or -3000 calories, and are active in the acid range of the titration. Members of Class 2 have an average apparent heat of dissociation of about $+6200$ calories and are active in the middle range. Members of Class 3 have an average apparent heat of dissociation of $+11,500$ calories and are active in the alkaline range of the titration. These three classes correspond to the three plateaus of Fig. 2, separated by two transition regions whose mid-points lie close to pH 5.5 and 8.5 respectively. The right-hand transition is complete in about 1 pH unit and this rather sudden transition suggests a wide separation, 2 units or more, between the pk of the most alkaline group of Class 2 and that of the most acid group of Class 3, which must be, respectively, not more than about 7.5 and not less than about 9.5. With a separation of the nearest groups of the two classes as great as this the effect of other groups further to the right and left is negligible.

In contrast to this, the left-hand transition in Fig. 2 occupies at least 2 pH units. This suggests that the separation of the pk of the last group of Class 1 and that of the first groups of Class 2 is much less than 2 units and that other groups to the right and left have an effect. This situation when the groups are closely spaced is more complicated. Fig. 4 gives some idea of what may be expected. Curve 1, which represents the case where the three $\text{pk}'\text{s}$ are equally spaced and separated by 0.85 unit, is the one in which the transition is most flattened and prolonged. The addition of other groups to the right and left would serve to extend the transition slightly more and to shift the asymptotes toward $+1$ and -1 , and with this modification Curve 1 is probably fairly representative of the acid transition of Fig. 2. We may conclude, therefore, that the pk of the most alkaline group of Class 1 is about 5 and that of the most acid groups of Class 2 about 6.

Let us consider the amount of base bound by the groups of Class 2, having a heat of dissociation of 6200 calories. If we recall that more than 80 per cent of the titration of any acid group is accomplished within a space of 2 pH units centered about the pK value, we see that there can be very little overlapping as regards the base bound by these groups and those of Class 3. The latter do not begin to bind any considerable amount of base up to about pH 8.5 or more. In the range of the left-hand transition of Fig. 2 we may expect an appreciable amount of overlapping as regards base bound by groups belonging to Classes 1 and 2. Consideration shows, however, that owing to the roughly symmetrical way in which the overlapping occurs we obtain approximately the correct result by attributing all the base bound on the alkaline side of the mid-point of the left-hand transition region of Fig. 2, *i.e.* pH 5.5 or 5.6, to the groups of Class 2 and all the base bound on the acid side of this point to groups of Class 1. The difference between the total amount of base bound per gm. of protein at 25° between pH 8.5 and 5.5 read from the middle curve of Fig. 1 is 0.46 equivalent. This corresponds to 31 equivalents per mole. This is almost the same as the analytical figure for the number of histidine units per mole; namely, 33 (3, 5). Now the characteristic heat of dissociation of the imidazole group of histidine is known to be 6200 or 6900 calories (6, 7). There can be little doubt therefore that Class 2 consists of the imidazole groups of the histidine units. Since the pH range covered by groups of Class 2 includes the range where the base-binding power of hemoglobin is affected by oxygenation (2), it must be certain of these groups which lie close to the oxygen-combining centers and are affected by the introduction of oxygen into the molecule.

On the other hand, the groups of Class 1, the acid class, are certainly carboxyl groups. These are known to have a very small heat of dissociation, sometimes positive, sometimes negative, but never more than ± 2000 or 3000 calories (6) and this agrees well with the observed heats.

The remaining groups to be considered are the guanido groups of the 13 to 16 arginine units (4, 5), the amino groups of the 37 to 44 lysine units (4, 5), the hydroxyl groups of the 12 tyrosine units (8), and the sulfhydryl groups of the somewhat uncertain number of cysteine units, given by chemical analysis of the hemoglobin

molecule. Data are lacking on the heat of dissociation of the sulfhydryl group, but that of the hydroxyl group in dihalogenated tyrosines is 800 to 900 (7), that of the guanido group of arginine is 12,400 (7), and that of the amino group is between 9000 and 12,000 (7). All these groups dissociate at strongly alkaline reactions, but our results indicate that the strongly alkaline groups of Class 3 must be either the amino groups of lysine or the guanido groups of arginine.

This analysis is in good accord with the recent results of Cohn and others (9) involving the titration of carboxyhemoglobin, which is believed in this respect to be like oxyhemoglobin, in the absence of salt. They have shown that between pH 1.5 and 13 the curve involves in all the dissociation of 261×10^{-5} equivalent of hydrogen ions per gm. or 174 equivalents per mole of hemoglobin, and they show that the data may be fitted within the accuracy of the experiments by assuming the presence in each molecule of fourteen groups of $pK' = 11.6$, forty of $pK' 10.8$, twenty of $pK' 7.5$, thirteen of $pK' 5.7$, four of $pK' 4.8$, and 83 of $pK' 3.7$ or 4.0 . They suggest, as our results confirm, that the thirty-three groups of $pK' 5.7$ or 7.5 are the imidazole groups of histidine. They also suggest that the forty groups of $pK' = 10.8$ are the amino groups of lysine, which accords with the heats that we have observed.

SUMMARY

The apparent heat of dissociation of a polyvalent substance like a protein is defined in terms of the effect of temperature on the titration curve. An analysis is given showing how this apparent heat of dissociation is related to the heats of dissociation of the individual groups of the molecule. From the observed variation of the apparent heat of dissociation with pH and a knowledge of the characteristic heats of dissociation of the individual groups it is possible to decide which groups are active in different ranges of the titration curve.

The apparent heat of dissociation of oxyhemoglobin of the horse has been determined between pH 4 and 10. The data indicate that the groups active acid to pH ~ 5.5 are carboxyl groups, that those active between pH ~ 5.5 and pH ~ 8.5 are the imidazole groups of histidine residues, and that those active alkaline

to $\text{pH} \sim 8.5$ are either amino groups or the guanido groups of arginine residues. From this it follows that the groups affected by oxygenation of hemoglobin are the imidazole groups of a certain number of histidine residues.

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