The Formaldehyde-Hemoglobin Reaction

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It is now generally accepted that formaldehyde reacts initially with amino groups of proteins to form methylolamines, which then react more slowly to form methylene bridges between the amino groups and such other groups as the amide group and the phenolic, indole, and imidazole rings (Fraenkel-Conrat and Olcott (1); Alexander et al. (2)). Some of these bridges are intermolecular. The present potentiometric study shows that pH changes underlying the formal titration are consistent with the accepted mechanism and suggest that methylolamine formation has nearly the same formation constant in proteins as in the amino acid reactions reviewed by French and Edsall (3). The reaction of formaldehyde with amide groups is important in acid solution (4) but not under the conditions of the present study.

Hemoglobin was chosen as the protein for this study for several reasons: it is readily available in quantity, its oxygen equilibrium is altered by formaldehyde (Guthe (5)), and it has a high histidine content. Although histidine reacts with formaldehyde, imidazole compounds without amino groups do not (Levy (6)), and it has been uncertain whether protein imidazole groups react. The present data suggest that the initially reactive groups of hemoglobin are the ε-amino and the terminal α-amino groups, and that reactions of imidazole are secondary.

The formaldehyde-hemoglobin reaction has also been studied by spectroscopic methods, with special attention to the effect of formaldehyde on the ε-amino methionemoglobin reaction (Schelor et al. (7); Tzushima (8)). The spectroscopic studies establish that formaldehyde reacts with heme-linked groups, a finding directly relevant to the effect of formaldehyde on oxygenation. The present potentiometric method detects any changes in equilibrium constants of amino and imidazole groups of hemoglobin, whether they are heme-linked or not.

EXPERIMENTAL

Materials and Methods

Hemoglobin—Human red blood cells from which the serum had been separated by centrifugation were kindly furnished by the Division of Biologic Laboratories of the Massachusetts Department of Public Health. Human hemoglobin was prepared, recrystallized by ammonium sulfate precipitation (9), and then dialyzed salt-free. All steps were carried out at 2 to 5°. The hemoglobin content of the final dialyzed solution was calculated from the Kjeldahl nitrogen content, assuming that hemoglobin contains 16.9% nitrogen.

* Many of the experiments reported were performed at the Biological Laboratories, Harvard University, Cambridge, Massachusetts.

The dialyzed solution was either used directly as oxyhemoglobin or was first oxidized to methemoglobin by addition of a slight excess of potassium ferricyanide. At high formaldehyde concentrations (1.0 M or more), the solutions turn brown, probably due to denaturation, and they gel at alkaline pH. Formaldehyde alone did not react with ferricyanide in several days, at room temperature.

Formal Titration—Commercial formaldehyde was carefully neutralized. The concentration of the stock solution, determined by iodometry (10), was always close to 12.5 M. Known amounts of hemoglobin and formaldehyde solutions, salt-free except for added ferricyanide, were pipetted into the reaction vessel, which was kept under an atmosphere of commercial "Dry Nitrogen." Base or acid was added by burette, and the solution stirred for about 2 minutes. About 20 titration points were thus measured on each hemoglobin aliquot in 1 hour.

Measurements of pH were made with commercial glass and calomel electrodes. After a glass electrode had been used for several weeks, the observed value dE/dpH fell from the theoretical 59 to 60 mvolts per pH to a value near 50. This low value was reached after several months use, and remained constant thereafter. The response was still linear over the pH range 1.0 to 9.6, but dE/dpH had to be determined by the use of two standard buffers in each experiment.

Most experiments were made at 23-26°. No effect of temperature was apparent, and the heats of the formaldehyde-amine reactions are small (French and Edsall (3)).

Protein titration curves were plotted in terms of h, the equivalents of acid bound per 100 grams of protein. The raw data were corrected for uncombined acid or base present at the measured pH and for the sodium error of the glass electrode. The corrections were determined by titrating a blank in which sodium chloride replaced the sodium proteinate. The true pH of the blank for a given value of free base was calculated from $K_w$ (International Critical Tables) and the activity coefficients of the hydroxyl ion (Lewis and Randall (11)). The acid bound was arbitrarily set as zero at pH 7.1, the isoionic point for human oxyhemoglobin (12).

RESULTS

Titration Curves—When neutral formaldehyde is added to a solution, no base or acid is added, and, if free hydrogen and hydroxyl ion concentrations are negligible, the acid or base bound is unchanged. The pH was therefore determined before addition of formaldehyde, and h was read from a previously determined formaldehyde-free titration curve. This value of h was the starting point for the formaldehyde titration. As thus determined, h depended on a single pH measurement, and might
beyond D may include 11 phenolic hydroxyl groups and six confirmed by other, unplotted titrations. Other groups titrated and four titratable charges on the four heme irons. Between C to the 36 imidazole groups, four terminal cr-amino groups (17), and D, 43 equivalents are titrated, corresponding to 44 e-amino and eight propionyl carboxyls from the hemes, and two free carboxyl data show 37 to 52 free side-chain carboxyl groups (from aspartic h values A and B, titration shows 106 equivalents per 106 grams of various formaldehyde (F) concentrations. The ordinate is acid equivalents per 100,000 g of protein.

be in error by 1 to 2 equivalents per 10^5 grams. It was assumed that formaldehyde did not change the sodium error of the glass electrode from its value in water at the same measured pH, since the mole fraction of formaldehyde was always small. This error was negligible in solutions more acid than pH 10. At high formaldehyde concentration, the necessary correction for base bound by formaldehyde was determined by including formaldehyde in the blank.

A few representative titrations at different formaldehyde concentrations are shown in Fig. 1. They are comparable to the 30-minute curve of Zaiser and Steinhardt (13), and are also fairly consistent with the analytical data (Hanos (14)). Between $h$ values A and B, titration shows 106 equivalents per 10^2 grams protein, or 71 groups per molecule (mol. wt. 67,000). Analytical data show 37 to 52 free side-chain carboxyl groups (from aspartic and glutamic acid residues after correction for amide content), eight propionyl carboxyls from the hemes, and two free carboxyl end groups (15), a total of 47 to 62 groups. Discrepancies between analytical and acid titration data in hemoglobin have been discussed in detail by Steinhardt and Zaiser (16). Between B and C, titration shows 44 groups per molecule, corresponding to the 36 imidazole groups, four terminal a-amino groups (17), and four titratable charges on the four heme irons. Between C and D, 43 equivalents are titrated, corresponding to 44 e-amino groups of lysine residues. The existence of plateau D is confirmed by other, unplotted titrations. Other groups titrated beyond D may include 11 phenolic hydroxyl groups and six sulphydryl groups (Cole et al. (18)). The guanidyl groups of arginine are not titrated below pH 11 in unmodified proteins (Cohn and Edsall (19)) or below pH 9 in the presence of formaldehyde (Levy (6)).

Reversibility—A hemoglobin solution was brought to a formaldehyde concentration of 1.1 M and left for 1 hour at room temperature. It was then dialyzed against distilled water for 3 days in the cold, and its titration curve determined. Because the true isoinionic point of the dialyzed protein was unknown, the titration curve was arbitrarily shifted vertically to agree with the formaldehyde-free curve of untreated protein near pH 8. Fig. 2 shows the results. The points for the dialyzed solution after reaction at pH 6.37 coincide very well with the original curve in the alkaline region. The curve for the solution after reaction at pH 10.30 agrees less well; perhaps all the formaldehyde had not been removed or the relatively high pH caused additional irreversible changes. Near neutrality the points for the two dialyzed solutions agree well with each other but not with the native curve.

In the alkaline region, the original curve is restored at least in large part by dialysis, suggesting that methylolamines are formed reversibly and that any methylene bridges formed can be broken at the $-\text{CH}_2-\text{NH}-$ bond to free the amino groups. Near neutrality complete restoration of the curve does not occur; some of the imidazoles may have been irreversibly modified as they are in the histidine-formaldehyde reaction (Neuberger (20)). Formaldehyde irreversibly bound to nontitratable groups would not be detected.

Nature of Reactive Groups—Which protein groups react with formaldehyde is indicated by the pH dependency of the vertical difference (i.e. the difference in $h$ at constant pH) between the curves obtained in the absence and presence of formaldehyde (Fig. 3). If formaldehyde shifts the pK of a number of acid groups, the shape of the curve will be unchanged and the vertical difference between the original and shifted curves will be bell-shaped when plotted against pH. The observed difference is well fitted by the sum of two such curves. Most if not all of the titration shift in hemoglobin can therefore be explained by the reaction of only two kinds of groups. Such curves permit only a minimum estimate of the number of reacting groups, unless the pH shift is large. In each hemoglobin molecule, at least 45 alkaline groups and at least 12 more acid groups react. The first figure is close to the total number of lysine residues (analytically 44), which suggests that the epsilon amino groups of all three residues react with formaldehyde. The second figure is considerably larger than the number of alpha amino groups (four), and might result from a small shift in apparent pK of the 36 imidazole groups. Part of the difference in the neutral region may also be due to unmasking of groups as in acid titrations (Steinhardt and Zaiser (16)), to secondary changes resulting from cross-linkage between amino and imidazole groups, or to changes in the hydration shell of the protein (21).

Formaldehyde is not expected to react with most of the other titratable groups. Carbonyl groups and ferric iron do not react. Although phenolic hydroxyl and guanidyl groups react slowly and secondarily to receive bridges from methylolamines, they are not titrated in any numbers at the pH's studied. Sulphydryl groups are few in number (six per molecule) and should not be titratable in the neutral range.

Dependence of pH on Formaldehyde Concentration—The reaction was followed by measuring the decrease in pH when formal-
FIG. 2. Reversibility of formaldehyde (F) shift by dialysis after reaction at two different initial pH (pH₀) values. Ordinate is acid equivalents per 100,000 g of protein.

FIG. 3. Additional base bound in formaldehyde, in equivalents per 100,000 g of protein. The points are vertical differences between the titration curve in the absence of formaldehyde (Fig. 1) and a curve at an initial formaldehyde (F) concentration of 0.4 M (intermediate between the 0.2 M and 2.0 M curves of Fig. 1). O-O is the sum of the two indicated bell curves.

dehyde is added, as described in the review of formaldehyde-amino acid reactions by French and Edsall (3). When pH is plotted against the logarithm of the formaldehyde concentration (log F), the shapes of the curves vary with pH₀, the initial pH. One kind of curve is obtained near pH₀ 6.5, another near pH₀ 10, and intermediate curves at intermediate pH₀. This again suggests that the pH shift in formaldehyde may be referred to only two kinds of polar groups in the hemoglobin molecule. At pH₃ above 10, the shapes of the curves are distorted by effects of glass electrode errors and changes in free hydroxyl ion concentration, but no third reaction need be postulated even at pH₃ as high as 11.

The potentiometric data for pH₃ near 10 (9.90 to 10.23) have been plotted (Fig. 4) in terms of (pH₃ - pH). The initial hemoglobin concentration was about 3 g/100 ml in each case. The agreement among experiments with different pH₃ confirms the
constancy of \((pH_o - pH)\) in this region. A few preliminary experiments on serum albumin showed similar results.

Experiments near neutral \(pH_o\) (6.2 to 7.8) are shown in Fig. 5. Initial hemoglobin concentrations ranged from 2 to 10%, but the results did not depend on the concentration.

Since commercial formaldehyde contains about 15% methanol, formaldehyde was also prepared by steam distillation of paraformaldehyde (22). Several experiments at \(pH\) 6 and 10 showed that freshly prepared formaldehyde produced the same results as commercial formaldehyde. Addition of methanol to such solutions caused no change.

At low \(F\), the \(pH\) drifted toward lower \(pH\) in the alkaline experiments. Measurements were made as rapidly as possible (about 2 minutes between points) in an attempt to determine the \(pH\) change in the initial reaction. In the neutral experiments, there was a less pronounced drift toward higher \(pH\) at low \(F\). At formaldehyde concentrations above 0.2 \(M\), no drift was apparent in either neutral or alkaline experiments and the \(pH\) change was reversible by dilution. This reversibility makes it possible to apply Levy's equilibrium theory (6) to the data.

DISCUSSION

The theory developed by Levy (6) for his experiments on the reaction of formaldehyde with amino acids serves for the present results. Formaldehyde reacts only with the uncharged form of the amino group, and the following equilibria are present:

\[
\begin{align*}
RNH_2 + H^+ & \rightleftharpoons RNH_3^+ \\
RNH_2 + CH_2O & \rightleftharpoons RNCH_2OH \\
RNH_2 + 2 CH_2O & \rightleftharpoons RN(CH_2OH)_2
\end{align*}
\]

If the \(pH\) in the absence of formaldehyde is adjusted to \(pH_o\) and formaldehyde is added to reach a concentration \(F\), the \(pH\) becomes

\[
\text{pH} = pH_o - \log (1 + L_1F + L_2F^2),
\]

or

\[
\text{pH} = pH_o - \log (1 + L_1F + L_2F^2).
\]

At high formaldehyde concentrations, the last term within the parentheses dominates and the slope of \((pH_o - pH): \log F\) curve approaches two. Since an imine combines with only one molecule of formaldehyde \((RN/CH_2OH)\), the plot for an imine reaction will have a limiting slope of unity. This theory fitted Levy's potentiometric data and the polarimetric data of Frieden et al. (23).

The curves of Fig. 4 are amine and imine curves. Neither fits all the points, but the amine curve fits the points at low \(F\) and the imine at high \(F\). Initial methylolamine formation will explain the curve at low \(F\). These methylolamines may then form methylene bridges to amides, or to phenolic, indole, or imidazole rings:

\[
\begin{align*}
RNHCH_2OH & \rightleftharpoons RNH + CH_2 + H_2O \\
R'CONH_2 & \rightleftharpoons R'CONH + CH_2 + H_2O
\end{align*}
\]

Analogous bridges are formed when formaldehyde reacts with asparagine (Levy and Silberman (24)) and histidine (Neuberger (20)). The product may have a changed \(pK\) and may in turn form \(N\)-methylolamines and produce the imine curve at high \(F\). The acid drift in \(pH\) observed at low formaldehyde concentrations and the steadiness of \(pH\) at high concentrations strengthens the hypothesis that the initial rapid reaction forms methylolamines which more slowly form bridges. The observed points move from the amine curve toward the imine curve.

The curves of Fig. 4 were calculated from constants that seem reasonable on other grounds (3). The amine curve was based on the constants for the formaldehyde reaction of lysine (\(L_1\) 240, \(L_2\) 300). The imine constant \((L_2)\) was estimated as 100 to agree with the observed linearity of the curve down to \(\log F = 0.2\). An \(L_2\) of 100 is not unreasonable for an imine, since the constants for proline and hydroxyproline are of this order. A similar estimate can be made from the final curve (Levy and Silberman (24)) for the imine group formed in the asparagine reaction. It also appears that the \(pK\) of the imine is more acid than that of the original amine, and the curve is drawn for a shift of 1.06 units.
The curves of Fig. 5 represent an attempt to fit the observations near pH 7 in the same way. The amine curve was drawn with the constants for leucylvalcyclidine (L4 25, L3 38), as a reasonable estimate for the reaction of terminal α-amino groups. The imine curve was arbitrarily drawn with L4 4 and with no shift in pK.

The generally accepted reaction scheme of Fraenkel-Conrat and Olcott (1) fits the data at alkaline pH. Near pH 7, the initial reaction may be methylolamine formation by the α-amino and Olcott (1) fits the data at alkaline pH. Near pH 7, the shift in pK.

The generally accepted reaction scheme of Fraenkel-Conrat and Olcott (1) fits the data at alkaline pH. Near pH 7, the initial reaction may be methylolamine formation by the α-amino groups. Secondary pH changes resulting from bridge formation to imidazoles (1), from unmasking as in acid titrations (16), or from substitution of formaldehyde in the hydration shell (21) are hard to predict quantitatively. Reaction of the four α-amino groups alone would not yield the observed vertical shift (Fig. 3), which corresponds to 12 groups per molecule.

The rapid formation of methylolamines and the slower formation of bridges are probably followed by other still slower reactions. The spectroscopic and azide interaction studies (7, 8) were carried out several hours after formaldehyde addition to allow completion of the reactions observed. Similar delay was found in the establishment of the oxygen equilibrium in formaldehyde (5). The compound observed in those studies is therefore not simply an N-hydroxymethylated hemoglobin, but a hemoglobin with bridges that may have undergone further rearrangement. Both papers (7, 8) present strong evidence that a hemin-linked group, probably histidine, reacts 1 to 1 with formaldehyde over a period of several hours.

Although other explanations of the observations are possible, the foregoing interpretation is consistent with present knowledge of the reaction. Further analysis would require special investigations of the kinetics of the reaction and detailed experimental analysis of the effects of chemical modification of polar groups of the protein.

**SUMMARY**

1. The reaction of formaldehyde with two kinds of acid-base groups of human hemoglobin, probably the α- and ε-amino groups, can explain the initial shift in pH of a hemoglobin solution when formaldehyde is added. Presumably methylolamines are formed.

2. The present data do not unambiguously determine formation constants for the methylolamines, but they are satisfactorily fitted by constants for formaldehyde-amino acid reactions.

3. In the alkaline region, near pH 10, a further reaction proceeds rapidly at high formaldehyde concentration. The pH change is consistent with condensation of the methylolamines with other protein groups to form methylene bridges.

4. In the neutral region, near pH 7, further reaction of the protein with formaldehyde at high concentration is shown; possible further reactions are discussed.

5. Dialysis restores the original titration curve near pH 10. This suggests that the methylene bridge break at their amino ends. Near pH 7, the titration curve is not completely restored by dialysis, suggesting that irreversible reaction has occurred.

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