Spectrophotometric Titration Curves of Human Hemoglobin and Its Carboxypeptidase Digests*

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Carboxypeptidase A and B digests of human hemoglobin have been made, and the spectrophotometric titration curves of the modified proteins isolated from the digests have been measured to determine the state of the tyrosine side-chains near the carboxyl ends of the peptide chains of hemoglobin (*i.e.*, α_{141} and β_{145}). Of the six tyrosine side-chains in each half molecule of carbon monoxide hemoglobin, two ionize with abnormally high pK (>12) and four with a normal pK (10.6). When tyrosine residues α_{141}, β_{145}, or both together are removed, three new hemoglobins result which have correspondingly lower numbers of only normally ionizing tyrosine side-chains. From this observation it is concluded that tyrosines α_{141} and β_{145} ionize normally in carbon monoxide hemoglobin. The spectrophotometric titration curve of cyanide-methemoglobin has also been determined. This complex is found to have three normal and three abnormal tyrosine side-chains, and it is concluded that its globin has a somewhat different conformation from the globin of carbon monoxide hemoglobin. The carboxypeptidase digests of carbon monoxide hemoglobin have been converted to the corresponding cyanide-methemoglobins. The spectrophotometric titration curves of these three proteins are, however, not affected by the different state of oxidation and complexing of the heme iron ion. The results on the cyanide-methemoglobins can be explained in two ways. For one, it is possible that only one of the tyrosines removed is abnormal in the native protein but that this abnormal tyrosine becomes normal upon removal of the other, normal one (and the adjacent carboxyl terminal amino acid). This is possible if this abnormal tyrosine was bonded to the removed chain segment *or* if removal of the chain segment causes an unfolding of the protein structure in the neighborhood of the abnormal tyrosine. However, it is also possible that the conformation of the globin of the digested cyanide-methemoglobins is not the same as that of cyanide-methemoglobin but is, rather, the same as that of carbon monoxide hemoglobin. In that case, the experiments presented cannot lead to a conclusion regarding the normality or abnormality of side-chains α_{141} and β_{145} in cyanide-methemoglobin. The relevance of the data to the results on the acid denaturation of hemoglobin and to the heme-heme interaction is also discussed.

A recent paper (Hermans, 1962) on the ionization of the tyrosine groups of hemoglobins and myoglobins (in the carbon monoxide form) was concluded with a postulate regarding which particular side-chains in human hemoglobin have an abnormally high pK. This postulate rests on the assumption that if the chain folding of two or more proteins is largely the same then the backbone and side-chain interactions holding these protein molecules together are also largely the same. This is supported by the finding in the case of hemoglobin and myoglobin not only that the tertiary structure of these proteins is similar (Perutz *et al.*, 1960) but that their amino acid sequence is also closely related (Watson and Kendrew, 1961). From this it follows that if a given amino acid occurs in the same position along the peptide chain of such related proteins and has been shown to be involved in a hydrogen bond (or other tertiary interaction) in one of these proteins, then in all likelihood this hydrogen bond occurs in the related proteins as well.[1]

Following these rules, we postulated that in human hemoglobin the tyrosine groups near the carboxyl ends of both α and β chain (residues α_{141} and β_{145}; Braunitzer *et al.*, 1961; Konigsberg *et al.*, 1961) are abnormal, in analogy with sperm-whale myoglobin, in which the tyrosine residue occupying the equivalent position (H_{22}, Watson and Kendrew, 1961) in the chain is involved in some kind of intramolecular interaction in crystals of this protein (Kendrew, 1961). Furthermore, the abnormalities noted in the tyrosine titration curve of sperm-whale myoglobin (Hermans, 1962) indeed correspond (in principle) with the structural features observed by Kendrew (1961).

A method apparently suitable for investigating our postulate is to remove the particular tyrosine residues in question and to redetermine the tyrosine ionization curve. Loss of a normal or of an abnormal tyrosine residue might then be taken as evidence of the state of the tyrosine which has been removed. Removal of tyrosines α_{141} and β_{145} has recently been performed by Antonini *et al.* (1961). With carboxypeptidase A these authors selectively removed histidine β_{146} and tyrosine β_{145} and with carboxypeptidase B arginine α_{142} and tyrosine α_{141}. In both cases removal of a third amino acid is a very much slower process, so that nearly homogeneous derivatives can be prepared. While both the experimental approach and the method of interpretation of the results appear straightforward, it is necessary to investigate the latter a little more closely.

For the interpretation of data on proteolytically modified proteins it is first necessary to analyze what happens to the side-chains of a globular protein when a part of the polypeptide chain is removed, and what effect this has on the number of normal and abnormal side-chains after the removal. Side-chains are normal when they are surrounded by the solvent, abnormal when they are linked to other parts of the protein

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[1] Ingram (1961) suggests that different proteins evolve through random stepwise changes in the amino acid sequence. Since mutations leading to inactive or unstable proteins are not viable, it is to be expected that certain key amino acids are much less subject to changes than are the others, and it is among the former that one will look for the side-chains which are responsible for holding the molecule in its specific conformation.

molecule, for instance through hydrogen bonds or hydrophobic bonds. Removal of a part of the protein will disrupt some of these tertiary bonds: First, those involving side-chains of the chain segment removed and, second, those involving side-chains which in the native protein interacted with the removed segment. Hence it is possible to find the number of normal groups of a certain kind to be unaffected by the shortening of the peptide chain when a normal group is part of the removed segment, if at the same time an abnormal group was bonded to the removed segment.

We have been supposing that the conformation of the new and somewhat smaller protein molecule is the same as that of the native protein molecule, but this is not generally justifiable; a (partial) unfolding of the molecule is a possibility we may not neglect. Since most tertiary bonds have a stabilizing effect on neighboring ones, there is a third possibility, that removal of some side-chains and the accompanying tertiary bonds could easily lead to the disruption of a few more bonds, while the protein chain would take up a random conformation.

The formation of new side-chain interactions by a reverse process appears unlikely because the composition and relative positions of the side-chains which might form such new bonds will not generally meet the specific requirements to do so.

We may, therefore, conclude that the removal of amino acids from a protein may be accompanied by the transformation of abnormal groups to normal ones. Thus, even though the number of abnormal side-chains may diminish in the process, this does not necessarily mean that the removed amino acids had abnormal side-chains; however a decrease in normal groups does not allow of more than one interpretation.

A conformational difference between hemoglobin and oxygen hemoglobin has been suggested to explain the S-shape of the oxygen binding curve (Haurowitz and Hardin, 1954). The conformation of carbon monoxide hemoglobin should be like that of oxygen hemoglobin, since the carbon monoxide binding curve is equally S-shaped. Recent unpublished x-ray studies by Perutz confirm the existence of the conformational difference unequivocally. Further, from the results presented below it follows that another small change in the chemical composition of the hemoglobin molecule can bring about a conformational change. One should, therefore, also consider the possibility that the removal with carboxypeptidase of even two amino acid residues from a hemoglobin complex causes the conformation of the protein to change and become like that of another complex. In that case, one cannot get information about the normality or abnormality of the side-chains of the removed amino acids by comparing data on the native and the modified protein, since these data reflect the properties of two structures in which different sets of side-chains are abnormal. Antonini *et al.* (1961) have found that digestion with carboxypeptidases A and B leads to considerable changes in the oxygen binding curve (the S-shape even being absent with the product of carboxypeptidase A digestion). While this evidence is circumstantial, recent work from the same laboratory (Antonini *et al.*, 1963) confirms that there is a conformational difference between hemoglobin and oxygen hemoglobin, but not between hemoglobin carboxypeptidase A and oxygen hemoglobin carboxypeptidase A.

It is, therefore, clear that caution is needed in the interpretation of the results reported below. The analysis presented above is fully taken into account in the discussion.

Experimental

A. Materials.—Materials not mentioned were the same as used before. Carboxypeptidases A and B (CPA and CPB) were procured from Worthington Biochemicals, Freehold, N. J.

B. Methods.—Carboxypeptidase digests of human hemoglobin (Antonini *et al.*, 1961) were prepared by allowing a solution of carbon monoxide hemoglobin to which a certain amount of carboxypeptidase solution had been added (carboxypeptidase A in 10% LiCl, carboxypeptidase B in water) to react overnight at 30° and *p*H 8 in a carbon monoxide atmosphere. The resulting solution was passed through a column of Sephadex G-25, medium grade, and the effluent containing the proteins and that containing the amino acids were collected in separate vessels. The concentration of hemoglobin in the one was measured by fully converting a suitable dilution to the carbon monoxide form (with $Na_2S_2O_4$ and CO), measuring the visible spectrum against a solvent blank, and making use of the finding by Antonini *et al.* (1961) that carboxypeptidase digestion does not alter the spectrum. The concentration of tyrosine in the other solution was obtained by measuring the difference spectrum *p*H 13 *vs.* *p*H 7 at a suitable dilution and using the calibration spectrum reported earlier to obtain the concentration. The number of tyrosines removed per molecule can then easily be computed. Results are given in Table I, experiments 1 to 4. Clearly these data agree with the results of Antonini *et al.* (1961). We have not performed any additional check on our product, but have relied on their results. The resulting proteins shall be designated below as hemoglobin CPA and hemoglobin CPB.

Table I

Results of Digestion of Human Hemoglobin with Carboxypeptidases A and B

Total volume in each digestion about 20 ml.

Expt. No.	Hemoglobin (mg)	Carboxypeptidase A (mg)	Carboxypeptidase B (mg)	Time (hr.)	Moles Tyrosine Lost / Mole Hemoglobin
1	100	0.4	—	15	2.0
2	100	0.2	—	15	2.3
3	100	—	0.4	15	2.3
4	100	—	0.5	15	2.3
5	150	0.1	0.5	43	6.0
6	100	0.4	0.5	6	5.7
7a	150	0.2	—	37	2.1
7b	Sample 7a	—	0.2	38	2.0

We have also digested human hemoglobin with both carboxypeptidases. When a digestion is performed with both enzymes present simultaneously (experiments 5 and 6 of Table I), proteolytic action exceeds the sum of the action of the separate enzymes, as was noted by Antonini *et al.* (1961). In fact, the number of tyrosine residues removed approaches a limit of *six* residues per molecule of hemoglobin. If the proteolytic action proceeds beyond lysines α_{140} and β_{144}, this is as expected, since aside from tyrosine residues α_{141} and β_{145} only tyrosine β_{130} has a position near the carboxyl ends of the peptide chains (Braunitzer *et al.*, 1961; Konigsberg *et al.*, 1961). Therefore, assuming a constant rate of removal of amino acids, the number of removed tyrosines is at a constant value of three per half molecule for a considerable period of time. The resulting sam-

ples will be designated below as hemoglobin (CPA + CPB).

To obtain a sample for which we could be reasonably certain that tyrosine β_{130} had not been removed in any of the molecules, we resorted to stepwise digestion (experiment 7 of Table I). First, hemoglobin CPA was prepared in the usual manner. To the reaction mixture a few milligrams of citric acid were added in order to inhibit carboxypeptidase A action (Smith and Hanson, 1949). After standing for some time, the solution was deionized, upon which it became cloudy. The precipitate was collected by centrifugation, and its light color suggests that it consisted of inactivated (by citrate) and denatured (by deionization) carboxypeptidase A. The dissolved protein was separated from tyrosine on a Sephadex column and then subjected to digestion with carboxypeptidase B in the usual manner. In this second digestion the number of tyrosine residues removed leveled off at two. We therefore are confident that this sample consists of hemoglobin molecules which all lack residues α_{141}, α_{142}, β_{145}, and β_{146}, but very few others. We shall call this sample hemoglobin (CPA, CPB).

Solutions of the corresponding cyanide-methemoglobins were obtained by adding some $K_3Fe(CN)_6$ to the solutions of carbon monoxide hemoglobin, waiting for 15 minutes, then deionizing the solution by passing it through a short column of mixed-bed deionizing resin. KCN was added to the stock solutions in the amount to make the final solutions 0.01 M with respect to this compound. While no ferricyanide could be detected in the deionized solutions by precipitation with ferrous ion, small amounts might be present, bound very firmly to the protein. However, the presence of a trace of $K_3Fe(CN)_6$ is not expected to affect the spectrophotometric titration curve appreciably.

The concentration of hemoglobin in the cyanide-methemoglobin solutions was determined by converting the protein to carbon monoxide hemoglobin (with $Na_2S_2O_4$ and CO) and measuring the visible spectrum. In a control experiment this procedure gave satisfactory results.

Results

A. *Hemoglobin CPA and Hemoglobin CPB*

Carbon Monoxide Hemoglobins.—The spectrophotometric titration curve (*i.e.*, values of $\Delta\epsilon_{245}$ *vs.* *p*H) of carbon monoxide hemoglobin as presented in a previous communication (Hermans, 1962) has been reproduced in Figure 1 (curve A). Analysis of this curve showed that there are four normally and two abnormally ionizing tyrosine residues per half molecule. In Figure 1 circles represent the experimental data on carbon monoxide hemoglobin CPA, squares those on carbon monoxide hemoglobin CPB. As before, data at high *p*H could be extrapolated to zero time. These points are indicated by symbols with arrows. Clearly, both derivatives have the same titration curve. Curve B, which fits the points fairly well, was drawn to have ordinates equal to three fourths of those of the lower part of curve A and to go to a level of five sixths of the higher segment of curve A. There are, therefore, three normal and two abnormal tyrosines per half molecule of these proteins. Normal tyrosines were lost in each case, and, as is seen from the introduction, this is in contradiction to our earlier postulate about the residues removed (α_{141} and β_{145}).

Cyanide-Methemoglobins.—Difficulties are encountered when one tries to measure tyrosine ionization difference spectra on solutions of hemoglobin or of methemoglobin. With the former it is difficult to avoid the presence of oxygen or else eliminate oxygen in a short time (high *p*H). With solution of the latter an important spectral change (Haurowitz, 1924) accompanies the ionization $(>Fe \leftarrow OH_2)^+ \rightarrow (>Fe \leftarrow OH)$ of the heme ferric ion water complex (Keilin, 1953), and the ultraviolet difference spectrum is sufficiently perturbed to make interpretation in terms of tyrosine ionization impossible. This effect can, however, be circumvented by complexing the ferric ion in its sixth coordination position with a cyanide ion rather than a water molecule or hydroxyl ion, since this ligand is about 100 times more firmly bound than the latter ion (Coryell *et al.*, 1937). Thus in 10^{-2} M KCN the fraction of methemoglobin hydroxide is small below *p*H 13, and tyrosine titration curves are easily obtained.

Data for cyanide-methemoglobin (open circles) and for cyanide-methemoglobin CPA (filled circles) and cyanide-methemoglobin CPB (filled squares) are shown in Figure 2. Comparison with Figure 1 at once shows that there are three normal and three abnormal tyrosines per half molecule in cyanide-methemoglobin[2] and

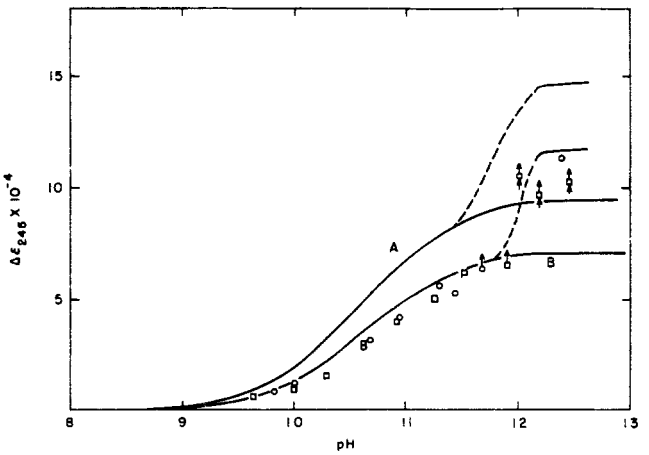

FIG. 1.—Spectrophotometric titration data of carbon monoxide hemoglobin and carbon monoxide hemoglobins CPA and CPB. Top pair of curves: earlier data on carbon monoxide hemoglobin (Hermans, 1962). ○, experimental data on hemoglobin CPA; □, data on hemoglobin CPB. Bottom set of curves drawn to be three quarters of bottom part of upper curves, five sixths of top part of upper curves. Arrows indicate extrapolations.

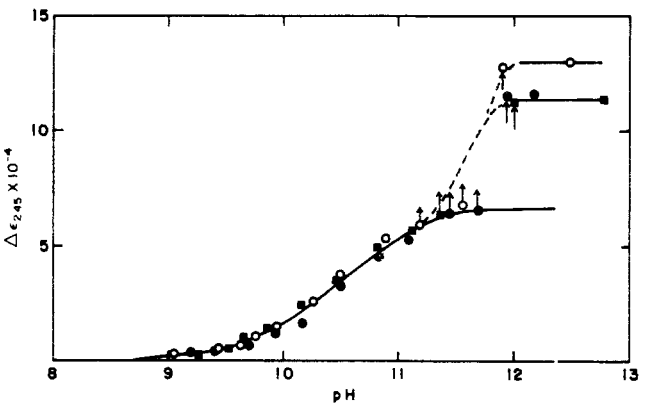

FIG. 2.—Spectrophotometric titration curves of cyanide-methemoglobin and cyanide-methemoglobins CPA and CPB. Experimental data, ○, on hemoglobin; ●, on hemoglobin CPA; ■, on hemoglobin CPB. Curves drawn to fit the points.

[2] The tyrosine titration curves of cyanide-methemoglobin (horse), carbon monoxide myoglobin (horse), and sperm-whale myoglobin are the same.

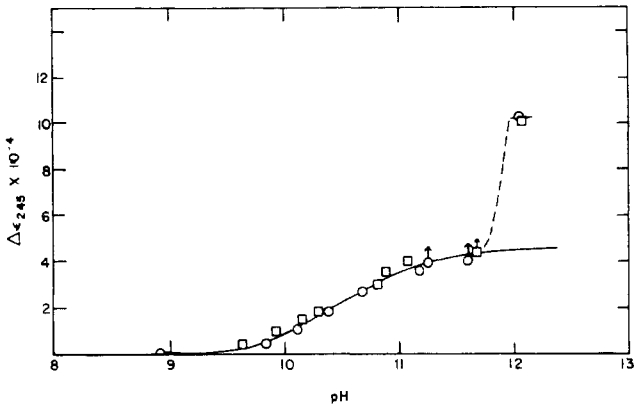

FIG. 3.—Spectrophotometric titration of the hemoglobin digest, which has four tyrosines less per molecule than hemoglobin. ○, data on carbon monoxide hemoglobin (CPA, CPB); □, data on cyanide-methemoglobin (CPA, CPB).

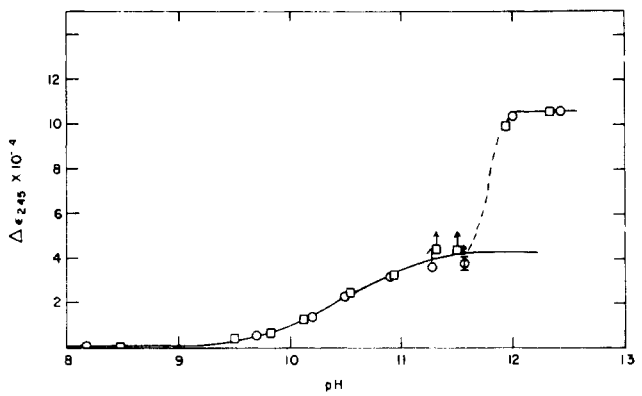

FIG. 4.—Data on the hemoglobin which has six tyrosines less than hemoglobin. ○, data on carbon monoxide hemoglobin (CPA + CPB); □, data on cyanide-methemoglobin (CPA + CPB).

that there are also three normal tyrosines in the two cyanide-methemoglobins which do not have tyrosines α_{141} or β_{145}.

Thus we have found conclusive evidence that a true conformational difference (however slight) exists between carbon monoxide hemoglobin and cyanide-methemoglobin. Yet neither form can be termed "denatured," since both have abnormal tyrosine residues, the met-form the greater number. Also, the optical rotatory dispersion curves of hemoglobin, oxygen hemoglobin, and methemoglobin are practically identical (Briehl, 1962). There are, therefore, at least two different conformations of the hemoglobin molecule, both of which are stable and possess well-organized tertiary structures. Some information regarding the differences between these structures may come from the data on the carboxypeptidase digests. Let us examine these.

Removal of tyrosine α_{141} *or* β_{145} from carbon monoxide hemoglobin yields proteins which have one normal tyrosine less. On the other hand, the cyanide-methemoglobins which can be made from these two proteins have one abnormal tyrosine less than unaltered cyanide-methemoglobin. It is tempting to deduce the state of these tyrosines in native human hemoglobin from these experiments: normal in carbon monoxide hemoglobin and abnormal in cyanide-methemoglobin. It is a simple matter to verify this reasoning by studying the titration curve of a hemoglobin lacking *both* tyrosines.

B. Hemoglobin (CPA, CPB) and Hemoglobin (CPA + CPB)

The tyrosine titration curves of hemoglobin (CPA, CPB), the sample which has lost two tyrosines per half molecule, are shown in Figure 3 (circles: carbon monoxide complex; squares: cyanide-met complex). A single curve represents both sets of data, and four normal tyrosines are present in both complexes. In other words, a normal tyrosine residue is lost in the progression from carbon monoxide hemoglobin CPA to carbon monoxide hemoglobin (CPA, CPB) and also from cyanide-methemoglobin CPA to cyanide-methemoglobin (CPA, CPB). This is consistent with the view that α_{141} and β_{145} are normal in carbon monoxide hemoglobin, but not with the conclusion that both are abnormal in cyanide-methemoglobin. The answer to this contradiction lies in the fact that we have in this paragraph ignored the possibility of conformational changes indicated in the introduction. This will be discussed.

Since a sample lacking three tyrosines per half molecule has also been prepared, its spectrophotometric titration curves have been measured. These are shown in Figure 4 (circles: carbon monoxide complex; squares: cyanide-met complex). There are four normal (and therefore two abnormal) tyrosines in each complex, which suggests that tyrosine β_{130} is abnormal in hemoglobin (CPA, CPB), the protein which has four tyrosines missing. It has been seen in the introduction that generally care must be exercised with such conclusions, and the final answer concerning the state of tyrosine β_{130} cannot be given on the basis of these data.

In Figures 3 and 4 the high *p*H value of $\Delta\epsilon_{245}$ is slightly over 10^4. This is, of course, higher than can be accounted for on the basis of the complete ionization of eight or six tyrosine residues per molecule (the value of $\Delta\epsilon_{245}$ at high *p*H per tyrosine residue varies between 10^4 and 1.2×10^4). It seems likely that the high value is caused by changes in the heme spectrum, but a satisfactory explanation has not been found. Although these results throw some doubt on the validity of the interpretation of data under conditions under which hemoglobin is denatured, we still feel confident of our interpretation of the results at lower *p*H (*p*H < 11.7).

DISCUSSION

Before discussing the results obtained on the digested hemoglobins, we wish to point out that our results apparently show that carbon monoxide hemoglobin and cyanide-methemoglobin differ somewhat in conformation. We are not aware of any existing evidence pointing to a difference between these particular complexes of hemoglobin. (Some of the evidence for a difference between hemoglobin and oxygen hemoglobin has been discussed in the introduction.) It has been established that the rate of acid denaturation of methemoglobin is much faster than that of oxygen hemoglobin, carbon monoxide hemoglobin, and cyanide-methemoglobin (Keilin, 1960; Beychok and Steinhardt, 1959). One possible explanation of these findings put forward by these authors is to assume that methemoglobin has one conformation and oxygen hemoglobin, carbon monoxide hemoglobin, and cyanate-methemoglobin another, and that the protein conformation determines the rate of denaturation. However, our findings indicate that the conformation of two of the complexes which denature slowly is not the same. While this is insufficient proof of the incorrectness of the explanation referred to above, we would like to consider another

explanation of the denaturation data, namely, to attribute a stabilization of the heme-protein bond to the presence of the strongly bound ligands, carbon monoxide and cyanide ion. In particular, one would expect an effect of the ligand on the histidine-iron linkage (Keilin, 1960). That a strengthening of this linkage would affect the rate of denaturation is to be expected, in view of the fact that the groups which "trigger" the acid denaturation are histidine side-chains (Steinhardt *et al.*, 1962).

We shall now turn to our results on the carboxypeptidase digests and review them in the light of the analysis given in the introduction. It was shown above that only normal groups are lost when α_{141} and β_{145} are removed separately or together from carbon monoxide hemoglobin, and *we conclude that these tyrosine residues have normal side-chains in carbon monoxide hemoglobin.*

Removal of tyrosine α_{141} or β_{145} from cyanide-methemoglobin results in the loss of one abnormal group.[3] We have seen that such a loss can occur for three reasons: (1) removal of an abnormal group, (2) removal of a normal group plus the site to which an abnormal group was bonded; or (3) removal of a normal group by the proteolysis with consequent (limited) unfolding of the remaining protein, in which an abnormal group becomes normal. Any one of these three circumstances may hold in the case of the removal of tyrosines α_{141} or β_{145}.

The choice between these possibilities is strongly limited if we consider at the same time the results of removal of tyrosine α_{141} from cyanide-methemoglobin CPA. A normal group is now lost. As we have seen, this may generally be interpreted to mean that α_{141} is normal in cyanide-methemoglobin CPA. As a result, the sum total of findings on cyanide-methemoglobin can be explained by assuming that tyrosine β_{145} is normal and α_{141} is abnormal in the starting material, but that removal of amino acids β_{145} and β_{146} causes the side-chain of α_{141} to become normal, either because α_{141} was bonded to this chain segment or because a conformational change in which α_{141} becomes normal occurs upon removal of the chain segment. In this statement residues α_{141} and β_{145} can be interchanged to obtain another possible explanation of our data. It is easily verified that these models and no others behave according to our findings on cyanide-methemoglobin. The assumption of either of these models places restraints on the tertiary structure of the hemoglobin molecule. However, it is pointless to discuss these in the light of the alternate possibility that the digestion of cyanide-methemoglobin with carboxypeptidase causes a conformational change. The likelihood of this possibility has been discussed in the introduction.

Since we have found that the spectrophotometric titration curves of all the digested carbon monoxide hemoglobins are the same as those of the cyanide-methemoglobins prepared from them, it is for instance possible that the conformation of the digested cyanide-methemoglobins is that of the corresponding carbon monoxide hemoglobins. As stated, we may, in that case, draw no conclusions regarding the state of tyrosines α_{141} and β_{145} in cyanide-methemoglobin from our data.[4]

[3] It will be clear from the Results section that the modified cyanide-methemoglobins are prepared from the modified carbon monoxide hemoglobins. Thus, when we say that tyrosine residues are removed from cyanide-methemoglobin, we are assuming that the order (1) digestion with carboxypeptidase (2) conversion to cyanide-methemoglobin can be reversed to give the same end-product.

Returning, finally, to the initial postulate, it is seen that it is not upheld, since our findings show that tyrosines α_{141} and β_{145} are normal in carbon monoxide hemoglobin. The evidence presented is certainly more compelling than the reasoning which led to the postulate. The possibility remains that α_{141} and β_{145} are abnormal in another complex of hemoglobin. If they are abnormal in reduced hemoglobin, these side-chains play a role in the conformation change which accompanies oxygenation (heme-heme interaction, Haurowitz and Hardin, 1954), which is indeed a possibility suggested by the results of Antonini *et al.* (1961). We have no data on reduced hemoglobin, and, as was explained above, our data give us no conclusive information about side-chains α_{141} and β_{145} in cyanide-methemoglobin because a conformational change may accompany the removal of these residues.

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[4] If digestion of *carbon monoxide hemoglobin* with carboxypeptidase A and carboxypeptidase B were to change the conformation of the protein we would no longer be able to conclude that tyrosines α_{141} and β_{145} are normal in this protein. We may, however, exclude this possibility by the following consideration. The affinity of hemoglobin to oxygen increases in the course of the binding of oxygen because the conformation of hemoglobin and oxygen hemoglobin is different. Hence, the data of Antonini *et al.* (1961), which show that the oxygen affinity of the carboxypeptidase digests is at all degrees of saturation the same as or greater than that of native hemoglobin, indicate that the conformation of hemoglobin is affected by the digestions, but that the conformation of oxygen hemoglobin probably is not.