added. This point was confirmed in curve C where no excess nitrite was present.

The generation of $H_2O_2$ during the oxidation of hemoglobin by nitrite explains the loss in GSH noted by Harley and Robin (1962) for glucose-6-P dehydrogenase-deficient cells incubated with sodium nitrite. The erythrocyte enzyme GSH peroxidase catalyzes the oxidation of GSH by $H_2O_2$ (Mills, 1959); the loss in GSH is not readily reversed in glucose-6-P dehydrogenase-deficient cells (Cohen and Hochstein, 1961) due to insufficient generation of NADPH required for GSSG reductase activity. The increased sensitivity of glucose-6-P dehydrogenase-deficient individuals to methemoglobin formation during administration of sodium nitrite (Brewer *et al.*, 1962) may be related to the instability of GSH: After the GSH level has declined, the $H_2O_2$ can contribute to the oxidation of hemoglobin (Cohen and Hochstein, 1963).

The failure of nitrite to produce hemolysis *in vivo* despite its ability to generate $H_2O_2$ and to oxidize hemoglobin might appear to be contradictory to the concept that $H_2O_2$ generation is an important factor in the drug-induced hemolytic anemias. However, it must be emphasized that nitrite possesses only a limited capacity to generate $H_2O_2$ since generation must cease after the hemoglobin has been oxidized. Furthermore, nitrite, by functioning as a substrate for complex I, can act *in vivo* to detoxify intracellular $H_2O_2$. In this manner, nitrite may compensate for the diminished GSH peroxidase pathway of glucose-6-P dehydrogenase-deficient individuals. It should be noted that decomposition of $H_2O_2$ by catalatic activity does not appear to be an important pathway in erythrocytes (Cohen and Hochstein, 1963). The failure of nitrite to act as an hemolytic agent is therefore not at all in conflict with the concept that hemolytic agents act by generating $H_2O_2$. Nitrite merely represents an example of an agent whose toxicity is self-limited because the necessary cofactor (oxyhemoglobin) is removed during the process of $H_2O_2$ generation, and because nitrite can act to detoxify $H_2O_2$ as well as to generate it.

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# Effect of Ligands and Oxidation State upon the Reaction of Myoglobin and Hemoglobin with Zinc*


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Additional supporting evidence is presented for the previous proposal that the *rate-controlling* step in the Zn-mediated suppression of the Soret band of myoglobin and hemoglobin involves macromolecular conformational changes concomitant with rupture of the otherwise inaccessible iron-imidazole linkage and occupancy of the imidazole group by Zn. Thus, carbonmonoxymyoglobin and carbonmonoxyhemoglobin are very much less reactive toward Zn than their corresponding ferriproteins, and the Zn-reacted ferroproteins are very sensitive to oxidation to the ferri state. Also, ferricytochrome c with its covalent iron-ligand bonds is spectrally refractory to Zn. The greater reactivity of ferrimyoglobin and ferrihemoglobin as compared with their corresponding ferro- forms is conceivably related to charge transfer from the ligands to the iron in the former.


Recently (Cann, 1963, 1964) it was shown that reaction of Zn ions with sperm whale ferrimyoglobin at *p*H 6.4 causes major changes in the ultraviolet and visible absorption spectra of the protein as well as loss of solubility at salt concentrations as low as 0.25 M and increased sensitivity to tryptic digestion. It seems clear, therefore, that Zn-reacted myoglobin is conformationally quite different from the unreacted protein. Reaction of the protein with Zn can be reversed to yield renatured ferrimyoglobin by one of the following three methods: (a) lowering the Zn concentration by dilution; (b) sequestering the Zn ions at *p*H 6.4 with ethylenediaminetetraacetate (EDTA) or citrate; or (c) lowering the *p*H of the reaction mixture to a value of 5.2. The renatured protein can be readily crystallized in the same crystal habit as ferrimyoglobin never exposed to Zn.

The most characteristic spectral change is a marked reduction in the Soret-band intensity accompanied by

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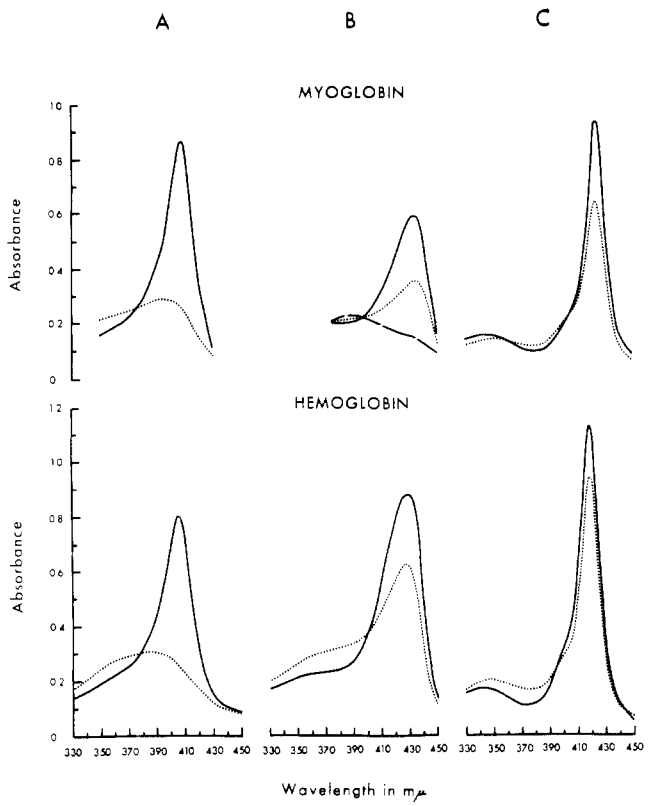


FIG. 1.—Comparative rates of Zn-mediated suppression of the Soret band of: A, ferri-; B, ferro-; and C, carbonmonoxymyoglobin or hemoglobin; ——, control;- - - -, Zn-reacted for standard length of time given. Myoglobins: protein concentration, 0.01%; standard reaction time of 10 minutes at 46°; (spectrum of ferromyoglobin after 1 hour at 46° is also shown, — —); reaction of ferromyoglobin carried out under argon and in presence of excess sodium dithionite, $2.5 \times 10^{-4}$ M; reaction of carbonmonoxymyoglobin under about 1.3 atm of CO; 1-cm light path. Hemoglobins: 0.088% ferri-, 0.10% ferro-, and 0.096% carbonmonoxy-; standard reaction time of 20 minutes at 46°; reaction of ferrohemoglobin carried out under argon; 1-mm light path.

a shift of the band from an absorption maximum of 408 mμ to one at 390 mμ (Fig. 1A). Kinetic measurements (Cann, 1964) on suppression of Soret absorption and its reversal indicate a three-step process: (a) labilization of the protein structure by binding of Zn ions to sites on the surface of the macromolecule; (b) unfolding of the weakened structure, at least to a limited extent, concomitant with binding of a single Zn ion to a critical and otherwise inaccessible site, as the *rate-controlling* step; (c) rapid polymerization of the Zn-reacted myoglobin such that interaction between the ferriheme moieties of the polymer suppresses and broadens the Soret band. It was proposed that in the *rate-controlling* step the Zn ion ruptures the $Fe^{3+}$-F8 imidazole linkage and occupies the F8 imidazole group.

Ferrihemoglobin is also spectrally alterated by reaction with Zn (Figs. 1A and 2B); and, as with ferrimyoglobin, suppression and broadening of the Soret band and the spectral changes in the region 475–650 mμ are reversed by sequestering the Zn ions with EDTA (Fig. 2). These observations led, in turn, to the study reported herein on various derivatives of myoglobin and hemoglobin. The results of these experiments furnish further evidence in support of the above proposal as to the mechanism of action of Zn.

## EXPERIMENTAL

Solutions of Mann's sperm whale ferri(met)myoglobin were dialyzed against distilled water in the cold and

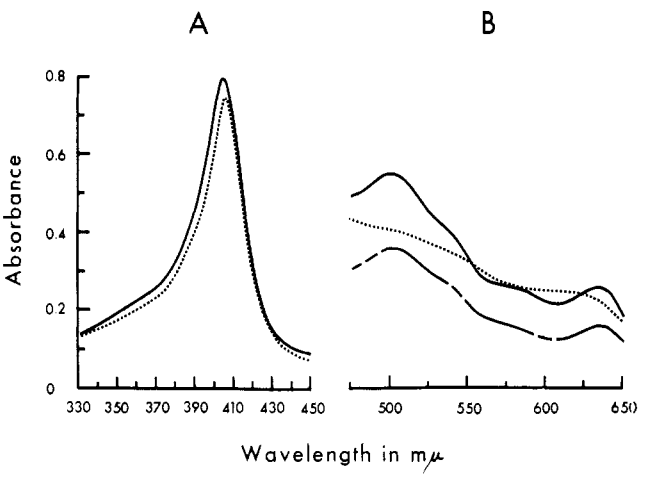


FIG. 2.—Spectra of ferrihemoglobins. A, comparison of Soret absorption of EDTA-renatured ferrihemoglobin,- - - - with that of control, ——. Renaturation was accomplished as follows: 1 ml of the Zn-reacted protein of Fig. 1A diluted with 4 ml of $10^{-2}$ M sodium acetate followed by addition of 5 ml of 0.1 M EDTA, *p*H 6.4, and 20 minutes at 46°; 1-cm light path. B, comparison of control, ——, Zn-reacted, - - -, and EDTA-renatured protein, — — —. The control and Zn-reacted protein are those of Fig. 1A. Renaturation was accomplished by dilution of the latter with one volume of 0.1 M EDTA, pH 6.4, followed by 20 minutes at 46°. About 35% of the protein precipitated an addition of EDTA; the solution was subsequently clarified by centrifugation. Absorbance measurement, 1-cm light path, have been multiplied by 2 for sake of comparison.

lyophilized. Solutions of ferromyoglobin were prepared by reducing 0.01% ferrimyoglobin with $2.5 \times 10^{-4}$ M sodium dithionite at *p*H 6.4; carbonmonoxymyoglobin by reduction of 0.1% ferrimyoglobin with $2 \times 10^{-3}$ M dithionite followed by saturation with CO.

Preparation of carbonmonoxy- and ferrihemoglobin has been described previously (Cann, 1964). Oxyhemoglobin was prepared in a manner analogous to the carbonmonoxy derivative; ferrohemoglobin was prepared from its oxy- derivative by flushing out the $O_2$ with argon.

The cytochrome c was Mann's amorphous powder (horse heart) which spectral analyses revealed to be 84% ferri- and 16% ferrocytochrome.

The wavelengths of maximum absorption required for analysis of the various spectra are as follows: (a) Soret band of ferrihemoglobin, 405 mμ; carbonmonoxyhemoglobin, 418 mμ; oxyhemoglobin, 415 mμ; (b) α- and β- bands of carbonmonoxyhemoglobin, 569.5 and 538.9 mμ; oxyhemoglobin, 579.3 and 544 mμ.

Reaction mixtures contained $9 \times 10^{-3}$ M $ZnAc_2$ and 0.01 M KCl, *p*H 6.4. The low ionic strength was necessary in order to avoid precipitation of the Zn-reacted hemoglobin. Control solutions contained NaAc in place of $ZnAc_2$. Except when otherwise stated, reactions were carried out under air. In order to avoid photodissociation of the carbonmonoxy derivatives (Gibson, 1956), the reaction vessels were wrapped in Al foil except, of course, for the light reaction, in which case the reaction mixture was illuminated with light from a 500-w projection lamp. Reaction mixtures and controls were incubated in a thermostated bath for the desired length of time and then rapidly cooled to 0° to stop the reaction (Cann, 1964). The spectra were recorded on a Beckman DK-2 spectrophotometer thermostated at about 10° using stoppered cuvets previously flushed with the appropriate gas.

The *p*-mercuribenzoate was obtained from Nutritional Biochemicals Corp. and *n*-ethylmaleimide from Schwarz Laboratories, Inc.

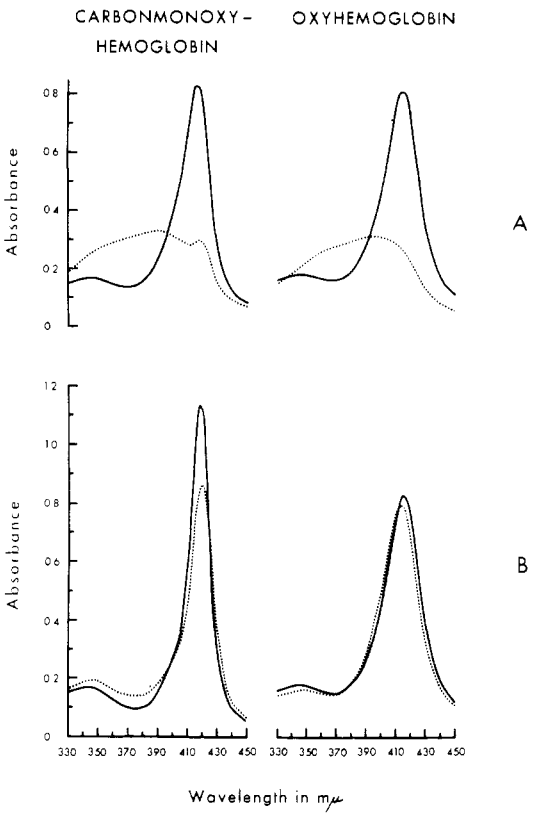


FIG. 3.—A demonstration of the stability of carbonmonoxy- and oxyhemoglobin toward the action of Zn. Inhibition of reaction with Zn at low protein concentrations, 0.0091% carbonmonoxyhemoglobin and 0.010% oxyhemoglobin, by CO or $O_2$: A, reacted for 20 minutes at 46° under air (compare results on carbonmonoxyhemoglobin with that for the higher protein concentration shown in Fig. 1C); B, under about 1.3 atm of CO or $O_2$. Control, ——; Zn-reacted, - - -.

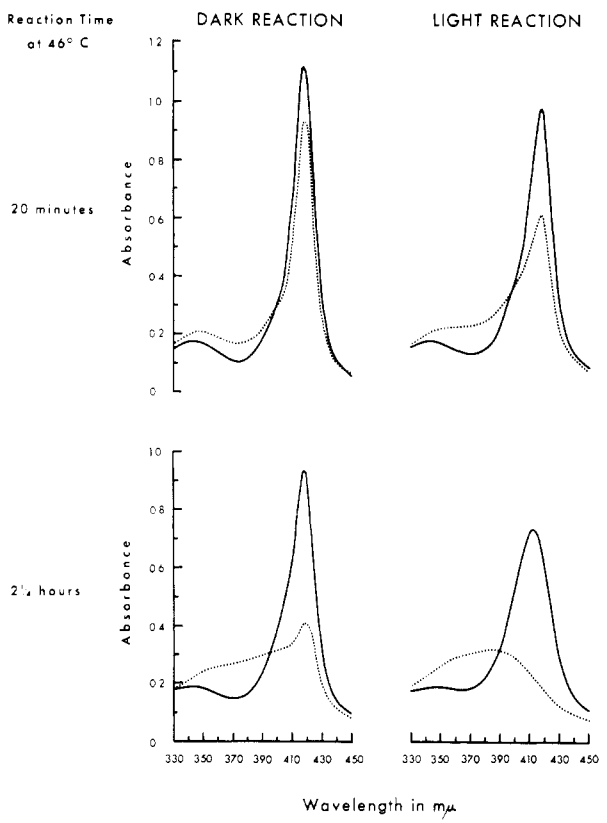


FIG. 4.—Another demonstration of the stability of carbonmonoxyhemoglobin towards the action of Zn. Acceleration of reaction at high protein concentration, 0.096% by light from a 500-w projection lamp. After 20 minutes of reaction the spectra were recorded with a 1-mm light path; after 2.25 hours the cooled protein solutions were diluted 1:10 with Zn solution and a 1-cm light path was used.

## FORWARD REACTION

The relative rates of reaction of the ferri-, ferro-, and carbonmonoxy- forms of myoglobin or hemoglobin with $9 \times 10^{-3}$ M Zn at *p*H 6.4 are illustrated in Figure 1. Under the conditions of these experiments the spectral changes are complete in the case of the ferri- forms but not nearly so for the other forms. Rather surprising is the stabilization toward Zn acquired merely by reduction of the ferriheme moiety to the ferrous state—only 44 ± 3 and 29% decrease in absorbance for ferromyoglobin and ferrohemoglobin, respectively. As anticipated, a further stabilization is conferred on the two proteins by formation of their carbonmonoxy- derivatives, 31 and 17% decrease in absorbance for the myoglobin and hemoglobin derivatives.

Whereas both carbonmonoxy- and oxyhemoglobin are quite stable toward the action of Zn at a protein concentration of about 0.1% (Fig. 1), they are very reactive at about 0.01% (Fig. 3A). This result suggests that in order for reaction to occur these derivatives must first dissociate into CO or $O_2$ and ferrohemoglobin, the latter and any ferrihemoglobin formed therefrom being the reactive forms. That this is in fact the case is demonstrated by two different kinds of experiments. In the first kind, reaction at the lower concentration was greatly inhibited by carrying out the process under about 1.3 atm pressure of CO or $O_2$ (Fig. 2B).

In the second kind of experiment, reaction of carbonmonoxyhemoglobin at the higher protein concentration under an atmosphere of air was accelerated by shining light upon the reaction mixture (Figs. 4 and 5A), which treatment alone is known to dissociate carbonmonoxyhemoglobin into CO and ferrohemoglobin (Gibson, 1956). In contrast to the dark reaction, the light reaction proceeded to completion in 2.25 hours, and during this time the carbonmonoxyhemoglobin in the control mixture containing no Zn was largely converted to oxyhemoglobin with very little oxidation to ferrihemoglobin. (The dark control survived largely as carbonmonoxyhemoglobin). Both the Soret band and the spectrum in the region 475–650 mμ of the Zn-reacted protein thus obtained were identical to those shown in Figures 1B and 2B for the reaction product of ferrihemoglobin with Zn.

In contrast to the ferri- and ferro forms of myoglobin and hemoglobin, we have been unsuccessful in altering significantly (wavelength of maximum absorption and maximum absorbance remaining unchanged) the Soret band of cytochrome c by incubation with Zn even in 10% ethanol, which agent cooperates synergistically with Zn or Cd in suppressing the Soret band of ferrimyoglobin (Cann, 1964).

## REVERSE REACTION

Suppression and broadening of the Soret band of carbonmonoxyhemoglobin and oxyhemoglobin by Zn can be reversed by sequestering the metal ions with 0.05 M EDTA at *p*H 6.4; but the renatured protein, except under special conditions, is entirely ferri- instead of ferrohemoglobin. Consider the case of the partial reaction of about 0.09% carbonmonoxyhemoglobin during 2.25 hours' incubation with Zn in the dark (Figs. 4 and 5A). Upon addition of EDTA to the Zn-reacted solution the spectra changed to those shown in

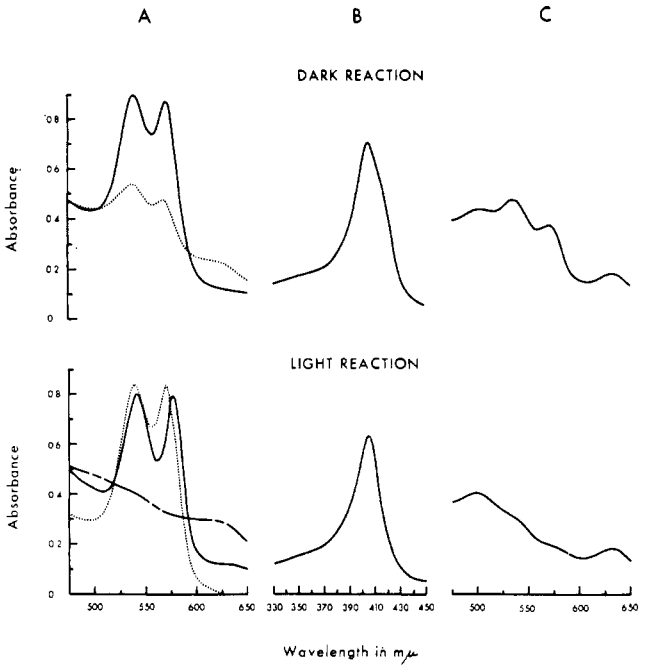


FIG. 5.—Spectra of Zn-reacted and EDTA-renatured carbonmonoxyhemoglobin. A, dark reaction, comparison of control, ——, with partially Zn-reacted protein, - - - - -; light reaction, comparison of control, ——, freshly prepared carbonmonoxyhemoglobin, - - -, and completely Zn-reacted protein, — — —. Reaction of 0.096% carbonmonoxyhemoglobin for 2.25 hours at 46°; solutions same as those whose Soret bands are shown in Fig. 4 but undiluted; 1-cm light path. B and C, spectra of EDTA-renatured protein obtained by dilution of above solutions of Zn-reacted protein with one volume of 0.1 M EDTA, *p*H 6.4, followed by incubation for 30 minutes at 46° and clarification by centrifugation. B, renatured protein diluted 1:10; 1-cm light path. C, 1-cm light path and absorbance measurements multiplied by 2.

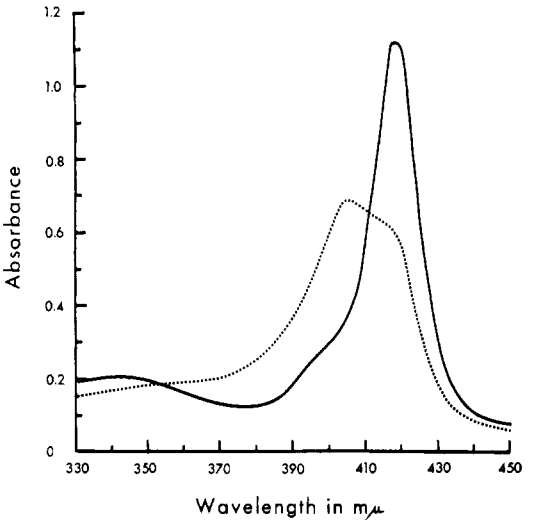


FIG. 6.—Reaction of 0.02% carbonmonoxyhemoglobin with Zn for 25 minutes at 46° under $N_2$ and renaturation with 0.05 M EDTA under CO; 30 minutes at 46°. Comparison of Soret absorption of the control, ——, and the renatured protein, - - -; 1-cm light path. Analysis of these data shows that the renatured protein consists of 67% ferrihemoglobin, the remainder being carbonmonoxyhemoglobin. In three other experiments of this kind the proportion of ferrihemoglobin was found to be 66, 62, and 51%.

Fig. 5B, C. These latter spectra clearly reveal a mixture of unreacted carbonmonoxyhemoglobin and of ferrihemoglobin derived from the Zn-reacted protein by EDTA treatment. When reaction with Zn is complete as after 2.25 hours of light reaction (Figs. 4 and 5A), the spectra of the subsequently derived EDTA-renatured protein (Fig. 5B, C) identify the latter as ferrihemoglobin. Ferrihemoglobin was also obtained by EDTA-renaturation following virtually complete reaction of 0.02% protein with Zn in the dark; resaturation of the EDTA-renatured material with CO did not alter the Soret band and about 80% of the control protein survived the entire process as carbonmonoxyhemoglobin. Quite similar observations have been made with oxyhemoglobin. These results are not surprising when one considers that the spectra of completely Zn-reacted carbonmonoxyhemoglobin are identical with those of Zn-reacted ferrihemoglobin.

Experiments were also made in which the dark reaction of about 0.02% carbonmonoxyhemoglobin with Zn and the subsequent renaturation of the Zn-reacted protein with EDTA were carried out in the absence of oxygen. Salt solutions were prepared in triple-distilled water, degassed by boiling, and cooled under nitrogen. Reaction with Zn was carried to 80% completion under an atmosphere of nitrogen, and EDTA-renaturation under 1.3 atm of CO. Even with these precautions the renatured protein consisted of 61 ± 6% ferrihemoglobin, the remainder being carbonmonoxyhemoglobin (Fig. 6). Obviously, the solutions were contaminated with traces of oxidants; but it is important to note that the control survived the entire procedure as carbonmonoxyhemoglobin. Finally, when reaction with Zn was carried out under air but EDTA-renaturation was carried out under CO, the renatured material was 86% ferrihemoglobin.

These results reveal that reaction of hemoglobin with Zn greatly increases the ease of oxidation of the heme iron.

## DISCUSSION

Kendrew's 2A model of ferrimyoglobin (Kendrew *et al.*, 1961; Kendrew, 1961) reveals that the iron of the ferriheme is bonded to the imidazole group of the F8 histidyl residue. It has long been supposed that the heme-linked groups in hemoglobin are also imidazole and, according to Perutz (1962), amino acid sequence together with X-ray results on hemoglobin leave little doubt that the irons are actually bonded to imidazoles, probably those of the 87$\alpha$ and 92$\beta$ histidyl residues. Previously (Cann, 1964) it was proposed that the *rate-controlling* step in the Zn-mediated suppression of the Soret band of ferrimyoglobin involves macromolecular conformational changes (presumably an unfolding of tertiary and secondary structure into a less tightly packed configuration since the Zn-reacted protein shows increased susceptibility to tryptic digestion) concomitant with rupture of the otherwise inaccessible $Fe^{3+}$-F8 imidazole linkage and occupancy of the F8 imidazole group by Zn. By analogy, a similar mechanism is evoked for hemoglobin. (Unlike myoglobin, hemoglobin contains cysteine residues. Native human hemoglobin possesses two reactive –SH groups and four unreactive ones [Ingbar and Kass, 1951; Hughes, 1949; Perutz, 1962]. Stoichiometric reaction of the two available groups with the –SH specific reagent, *p*-mercuribenzoate [Ingbar and Kass, 1951], drastically reduces heme-heme interaction [Riggs, 1952]. Possible direct involvement of the two available –SH in the reaction of hemoglobin with Zn seems to be eliminated by the results of experiments in which these groups were blocked prior to exposure of the protein to Zn: 0.1% ferrihemoglobin was reacted with $6.5 \times 10^{-5}$ M *p*-mercuribenzoate at *p*H 7 for

30 minutes at room temperature or 36°. The rate of reaction of 0.01% of the resulting modified protein with $9 \times 10^{-3}$ M Zn at 36° was only about 15% less than for unmodified ferrihemoglobin. In another set of experiments, prior treatment with $6.5 \times 10^{-5}$ M *N*-ethylmaleimide at *p*H 7.4 and room temperature [Ingram, 1955] caused no significant alteration in the rate of reaction of the protein with Zn.)

The proposed mechanism correctly predicts that the rate of suppression of Soret absorption by Zn will depend upon the strength of the iron-ligand bonds: the weaker the bonds the greater the rate. Thus, ferrihemoglobin with its weaker bonds reacts much faster (*vide supra*) than ferrimyoglobin (Cann, 1964). Conversely, carbonmonoxymyoglobin and carbonmonoxy- and oxyhemoglobin with their covalent bonds react much more slowly (indeed, if they react at all) than their corresponding ferri forms which have ioniclike bonds. (On the basis of paramagnetic-susceptibility measurements the bonding of the iron in hemoglobin and myoglobin derivatives has long been classified as either "essentially ionic" or "essentially covalent" [Haurowitz and Hardin, 1954]. In the case of the diamagnetic CO and $O_2$ derivatives there can be no doubt as to the covalent nature of the iron-ligand bonds but paramagnetic-resonance-absorption measurements reveal [Ingram and Bennett, 1955; George, 1955] that the bonding in ferrihemoglobin and ferrimyoglobin is not essentially ionic in the same sense as it is in the hydration shell of $F3^{3+}$ aquo-ion. The same may also be true of the ferroproteins. It appears that there are probably fewer than five unpaired electrons in the ferriproteins). This result parallels the difference in stability of carbonmonoxyhemoglobin and ferrihemoglobin toward acid (Steinhardt *et al.*, 1962). Likewise, ferricytochrome c in which the iron-ligand bonds are covalent (Boeri *et al.*, 1953) is spectrally inert to Zn, which is consistent with the lower *p*H required to modify its Soret band (Boeri *et al.*, 1953) than in the case of ferrimyoglobin (Breslow and Gurd, 1962) and ferrihemoglobin (Steinhardt *et al.*, 1963). (Rupture of the covalent bonds between the iron atom and the two hemichrome-forming groups, presumably imidazoles, of the protein moiety in ferricytochrome c by protonation of the groups causes a blue shift of the Soret band but intensifies rather than suppresses absorptivity [Boeri *et al.*, 1953]. In contrast to myoglobin, the prosthetic group of cytochrome c is joined firmly to the protein moiety by two thioether linkages [Theorell, 1938; Paul, 1950]. Thus the heme can not be released from the protein simply by protonation of the two donor groups supplied to the iron by the protein; nor would heme-mediated macromolecular aggregation be expected. This may account for the fact that the Soret band of cytochrome c is not suppressed by acid. Sedimentation analyses of acid-denatured myoglobin [Cann, 1964] reveal a large proportion of globin and a relatively small amount of aggregated protein. The latter fraction contains most, if not all, of the ferriheme. Suppression and broadening of the Soret band has been attributed to interaction between the ferriheme moieties of the aggregate.)

In order for carbonomonoxy- or oxyhemoglobin to react appreciably, the CO or $O_2$ must dissociate from the iron with accompanying change in iron-ligand bond type from covalent to ioniclike (*vide supra*). The reactive form is therefore ferrohemoglobin[1]; but the resulting Zn-reacted ferrohemoglobin is very sensitive to oxidation to the ferri state. Previously (Cann, 1963) it was observed that oxygenation of dithionite-reduced Zn-reacted ferrimyoglobin does not result in formation of a stable oxygen-to-iron bond; rather, the oxygen simply oxidizes the iron to its ferric state. The increased ease of oxidation of the iron in the Zn-reacted proteins is in complete accord with the proposed mechanism of the *rate-controlling* step. Comparison of the oxidation-reduction potentials (Clark, 1960) of hemoglobin, myoglobin, and heme indicates that rupture of the iron-imidazole linkage should increase the relative stability of the higher valence state of the iron and thus the ease of oxidation of ferrous to ferric state.

The finding that ferromyoglobin and ferrohemoglobin, while more reactive than their carbonmonoxy- (and, in the one case tested, oxy-) derivatives, are less reactive than the corresponding ferriproteins was unexpected but consistent with the recent preliminary report of Steinhardt and his co-workers (1963) that, contrary to prevailing views, ferrohemoglobin is very stable toward acid. In seeking an explanation it may be pertinent to note that the absorption spectra of the ferriproteins have been interpreted (Williams, 1956) in terms of charge transfer from the ligands to the iron. Such charge transfer apparently labilizes the protoporphyrin of ferrihemoglobin to attack by molecular oxygen, which oxidizes the ring to a linear tetrapyrrole (Lemberg and Legge, 1949). Conceivably the charge-transfer process, which also involves an imidazole group of the protein, could indirectly labilize those portions of the protein adjacent to the ferriheme moiety to the action of Zn or $H^+$, or even cause macromolecular structural rearrangements. The latter may underlie the crystallographic difference between ferri- and ferrohemoglobin. Thus combination of hemoglobin with CO or $O_2$ is accompanied by a major structural change reflected in a striking difference between crystals of oxy-, carbonmonoxy-, and ferrihemoglobin on the one hand, and those of ferrohemoglobin on the other (Perutz, 1962).

Finally, these results of themselves focus attention upon the structural complex involving the heme and adjacent portions of the protein moiety as the site of attack by Zn. Also, along with previous results (Cann, 1964), they emphasize once again the cooperative action of the heme moiety and protein part of hemeproteins in determining macromolecular structure. Recently, Reichlin and his co-workers (Reichlin *et al.*, 1963) also pointed this out in the context of immunological specificity of globins and hemeproteins.

[1] The same is undoubtedly true of carbonmonoxymyoglobin; but the definitive experiments were done with hemoglobin.

## Acknowledgment

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# Helix Formation by Single- and Double-Chain Gelatins from Rat Skin Collagen

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The formation of a polyproline-type helix by single-chain ($\alpha$1 and $\alpha$2) and double-chain ($\beta_{12}$) gelatins was followed by optical rotation, viscometry, and light scattering. It was found that $\alpha$1 and $\alpha$2 formed helices by a concentration-dependent reaction which was accompanied by an increase in viscosity and molecular weight. This was interpreted as evidence for stabilization of a helix by interchain association. $\beta_{12}$ formed a helix by a concentration-independent reaction and, at concentrations below 0.2 mg/ml, there was no change in molecular weight. At low concentrations there was also a large concentration-independent increase in reduced viscosity to about 2.0 dl/g. These data were interpreted as evidence for an intramolecular configurational change associated with helix formation in double-chain molecules. Melting curves demonstrated a close similarity of the gelatin helix, at the local level, whether formed by inter- or intramolecular association. The hydrodynamic properties of the $\beta_{12}$ helix formed at low concentration indicated a relatively compact molecule with high asymmetry. The kinetics of helix formation by $\beta_{12}$ were consistent with an initial first-order reaction associated with the intramolecular change, followed by a concentration dependent second-order reaction. The results were interpreted in terms of a polyproline helix stabilized by interchain association to form a double-chain helix. There was no evidence for significant amounts of single-chain helices under the conditions employed. Triple-chain helices also appeared not to contribute to gelatin structure in these studies.

When solutions of gelatin are cooled, the polypeptide chains assume a configuration closely related to the poly-L-proline II helix characteristic of native collagen. The accompanying mutarotation provides a convenient means of following the process. The various factors involved have been reviewed in detail by Harrington and Von Hippel (1961a). It is clear that the pyrrolidine ring of proline and hydroxyproline plays a central role in the process (Harrington, 1958; Burge and Hynes, 1959; Piez, 1960; Von Hippel and Wong, 1963a; Josse and Harrington, 1964) and the available evidence has generally been interpreted as demonstrating that helices are initially propagated along single chains without a need for interchain stabilization (Harrington and Von Hippel, 1961b). However, it is not evident how such helices are stabilized since the content of pyrrolidine rings in gelatin from vertebrate collagens is less than one residue in four and may be as low as one residue in six (Piez and Gross, 1960). Flory and Weaver (1960) have suggested that triple-chain helices must be formed, citing the parallel to collagen structure, even though the kinetics are not readily compatible with this mechanism.

One of the difficulties in the interpretation of the studies which have so far been reported may be that denatured collagen is a heterogeneous system in two respects. First, vertebrate collagens consist of three chains, one of which, the $\alpha$2 chain, differs in amino acid composition from the other two, the $\alpha$1 chains (Piez *et al.*, 1961, 1963; Schleyer, 1962). Since the differences include proline and hydroxyproline content, it would be expected that the chains would have different rates of helix formation. Second, denatured collagen contains covalently linked double chains, $\beta_{12}$ ($\alpha$1–$\alpha$2) and $\beta_{11}$ ($\alpha$1–$\alpha$1),[1] the amount varying from 18 to 66%, depending on the source and method of preparation (Piez *et al.*, 1961, 1963). It is not known what effect this type of cross-linking has on helix formation and stabilization. Since $\alpha$1, $\alpha$2, and $\beta_{12}$ can now be prepared in pure form (Piez *et al.*, 1961, 1963; Lewis and Piez, 1964), it should be possible to gain further insight into the problem of helix formation by studies on these single molecular species. This was the object of the study reported here.

## METHODS

*Samples.*—The single-chain gelatins $\alpha$1 and $\alpha$2 and the double-chain gelatin $\beta_{12}$ were isolated from salt- or acid-extracted rat skin collagen by chromatography as previously described (Piez *et al.*, 1961, 1963). Samples prepared in this way have been shown to be homogeneous by the criteria of chromatography, sedimentation velocity, amino acid composition (Piez *et al.*, 1961, 1963), gel electrophoresis (Nagai *et al.*, 1964), and molecular weight (Lewis and Piez, 1964). The only exception is that samples of $\alpha$2 may contain a small amount of $\beta_{12}$, estimated to be less than 5%.

[1] The designations of the covalently linked double chains were previously $\beta$1 and $\beta$2. These have been changed to $\beta_{12}$ and $\beta_{11}$ for clarity. See Bornstein *et al.* (1964) for further discussion of these components and their significance.