Effects of Heme–Globin and Chain–Chain Interactions on the Conformation of Human Hemoglobin. A Kinetic Study†

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ABSTRACT: The reactivity of the β-93 sulfhydryl groups with *p*-hydroxymercuribenzoate has been used as a kinetic probe to investigate conformational changes in globin, deoxyhemoglobin, and various liganded forms of hemoglobin using stopped-flow devices. The pH dependence and the energy of activation of the various reactions have been determined. Addition of heme to the α chains of globin causes only a very small change in the rate of reaction of the β-93 SH group. Further addition of heme which fills up the β-heme sites to form hemoglobin results in a tenfold decrease in the *p*-hydroxymercuribenzoate reaction rate, suggesting that the β-93 SH is less exposed in hemoglobin than in globin. The reaction of the β-93 SH group in isolated β-O_2 chains is slightly faster than that of HbO_2. This finding is consistent with the previous circular dichroic (CD) spectra and ligand-binding kinetic studies which indicated a difference between the heme environment in isolated β chains and in the β chains of liganded hemoglobin. The *p*-hydroxymercuribenzoate reaction rate is decreased nearly 70-fold at pH 7 when oxyhemoglobin is converted to deoxyhemoglobin. In the ligand-bound conformation of hemoglobin, the *p*-hydroxymercuribenzoate reaction was not sensitive to the nature of the ligand for the five forms studied (HbO_2, HbCO, HbCN, HbN_3, and methemoglobin). These results are consistent with a masking of the β-93 SH group when the hemoglobin molecule undergoes conformational changes from the oxy- to deoxyhemoglobin form, as indicated by crystallographic studies. The reaction of hemoglobin has been studied in dilute solution and in salt solutions thought to produce α–β dimers. In salt solution the kinetics remain essentially unchanged, indicating that if dimers are formed, they react with a rate similar to tetrameric hemoglobin. The studies in dilute solution indicate that at a heme concentration of 1.75 μM the fraction of the oxyhemoglobin dissociated into chains cannot be greater than 4–7%.

The reactivity of the sulfhydryl groups of human deoxyhemoglobin has been shown to be different from that of the ligand-bound form toward a number of SH reagents (Riggs, 1961; Benesch and Benesch, 1962; Guidotti, 1965; Antonini and Brunori, 1969; Maeda and Ohnishi, 1971). The different reactivity is considered to derive from conformational differences between oxy- and deoxyhemoglobin revealed by X-ray crystallographic determinations (Perutz, 1970). The conformational change accompanying the deoxygenation of human hemoglobin appears to involve interaction of the imidazole moiety of the C-terminal histidine of β chains with aspartate-94 resulting in a decrease in the accessibility of the adjacent β-93 SH group for various reagents. Antonini and Brunori (1969) and Brunori *et al.* (1970) have presented evidence that the alteration of the rate constant for the combination of *p*-hydroxymercuribenzoate with the SH groups of hemoglobin depends primarily on the presence of the ligand on the β chains, and that the presence or absence of the ligand on α chains has no effect on the observed kinetics. On the other hand, Maeda and Ohnishi (1971), using half-methemoglobins, have found that the reactivity of the β-93 SH shows dependence on the ligand state of the α-chain heme.

It appeared to us that the reactivity of the β-93 SH groups could be used as a sensitive probe to elucidate factors determining the geometry of the β-chain heme sites in hemoglobin in the synthetic sequence globin → ICII[1] → hemoglobin and in the reaction α chains + β chains → hemoglobin. To characterize further the reactivities of the SH groups of the different molecules, we have determined the activation energy and the pH dependence of all the relevant reactions.

The studies on isolated chains showed a large difference both in rate and absorbance change on a heme basis with respect to hemoglobin. Since the *p*-hydroxymercuribenzoate reaction can be followed at micromolar concentrations, it provided a sensitive and rapid assay for the presence of chains in dilute hemoglobin solutions where unusual ligand-binding kinetics have been observed (Antonini and Gibson, 1967). The reaction of β chains with *p*-hydroxymercuribenzoate is kinetically complex and will be treated in a further paper.

Experimental Section

Hemoglobin was prepared from pooled erythrocytes of multiple donors as described by Geraci *et al.* (1969) and then crystallized from Drakbin's buffer (1946). For control experiments, hemoglobin was prepared from the blood of a single donor both with and without the use of toluene and was used in the same day. Hemoglobin was also reconstituted from isolated chains (Geraci *et al.*, 1969), and from ICII by addition of hemin–dicyanide. Globin was prepared by the method of Winterhalter and Huehns (1964). ICII was prepared according to Winterhalter (1966). Protohemin was purchased from Fluka. *p*-Hydroxymercuribenzoate samples from Fluka, Mann, and Sigma were purified according to Boyer (1954) before use. Hemoglobin concentrations at pH

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[1] Abbreviations used are: ICII, a synthetic hemoglobin containing heme only in the α chains and with cyanide or CO bound to the Fe; HbO_2, oxyhemoglobin; Hb, deoxyhemoglobin; HbCO, carbon monoxide–hemoglobin.

TABLE I: Rate Constants for the Combination of *p*-Hydroxymercuribenzoate with Various Hemoglobin Derivatives.[a]

pH	Derivative					$k(HbO_2)/k(Hb)$
	Globin	ICII	HbO_2	MetHb	Hb	
6	3300	2700	470	480	10	47
7	3000	2500	510	480	8	64
8	1700	2000	460	480	14	33
9	1500	1800	180	200		

[a] Temperature 20°; buffers were 50 mM phosphate for pH 6–8 and 50 mM sodium borate at pH 9. Rate constants are in M^{-1} $sec^{-1} \times 10^4$.

7.0 in 0.05 M potassium phosphate buffer were determined spectrophotometrically using ϵ_{nm} 15.6 (Antonini, 1965) at 576 nm for oxyhemoglobin or ϵ_{nm} 11 (Cameron and George, 1962) at 541 nm for the cyanomet form. The concentration of *p*-hydroxymercuribenzoate solutions was determined using ϵ_{nm} 16.9 at 232 nm in 100 mM phosphate, pH 7.0, and was confirmed by titrating known amounts of β-mercaptoethanol according to the procedure of Boyer (1954).

Deoxyhemoglobin was prepared by passing water-saturated argon (S10 Ultrapure, Italy; High Purity, Matheson) over the oxyhemoglobin solution. The progress of the deoxygenation process was followed by monitoring the absorption spectrum in a Cary 14R recording spectrophotometer. Methemoglobin was prepared by addition of a threefold excess of ferricyanide to oxyhemoglobin solutions and removing excess reagent on a Sephadex (Pharmacia) G-25 fine column equilibrated with the buffer used for the reaction. The homogeneity of ICII and of isolated α and β chains and the ability of the latter to recombine to form hemoglobin were determined by electrophoresis on cellulose acetate strips (Millipore) in barbital buffer, pH 8.6, and by starch–gel electrophoresis, using the discontinuous buffer of Poulik (1957). The protein bands were stained with Amido Black and with benzidine.

The reaction of *p*-hydroxymercuribenzoate with the SH groups of the different molecules was measured statically at 254 nm in a Beckman DU-2 spectrophotometer. The reaction rates were measured at 254 nm in a Gibson-Durrum stopped-flow apparatus and in a stopped-flow device designed by one of us (L. P.) and described elsewere (Boelts and Parkhurst, 1971). The light path was 2 cm and the reaction temperature was measured to better than 0.1° at the outflow of the reaction block as well as in a hole 1 mm from the reaction cuvet in the reaction block. During the later stages of the study, the stopped-flow devices were interfaced to a Supernova computer (Data General Corp.) following a design of De Sa (R. J. De Sa, personal communication) and thereby provided us with online data reduction and kinetic analyses. The values of absorbance change measured in the Beckman spectrophotometer were used to calibrate those observed in the stopped-flow spectrophotometers.

Results

The second-order rate constants for the combination of *p*-hydroxymercuribenzoate with globin, ICII, oxy-, met-, and deoxyhemoglobin as a function of pH are shown in Table I and in Figures 1 and 2. The reaction rates for globin and ICII

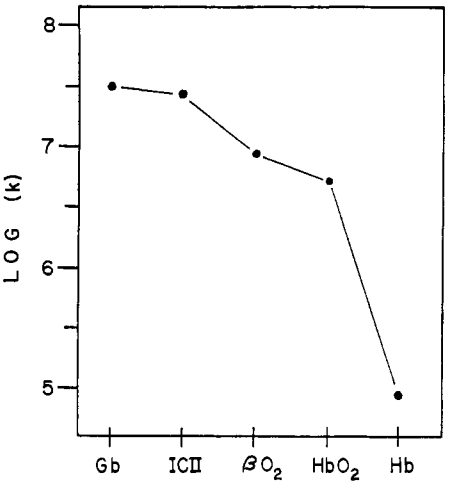

FIGURE 1: A plot of the log of the second-order rate constant for the reaction of *p*-hydroxymercuribenzoate with the β-93 SH of various human hemoglobins and intermediates at pH 7, 20°: Gb = globin; ICII is globin with hemes only in the α-chain heme sites; βO_2 = isolated oxy-β chains (the fast rate is reported); HbO_2 = oxyhemoglobin; Hb = deoxyhemoglobin.

were so rapid that it was necessary to carry out the study on these compounds using equal concentrations of globin SH groups and *p*-hydroxymercuribenzoate at 1 μM. In all experiments with ICII, either cyanide or CO was bound to the Fe in the functional α chains. There were no significant differences in reaction rates for these two liganded forms. For the hemoglobin reactions, the usual concentrations were: *p*-hydroxymercuribenzoate 15 μM; hemoglobin, 5 μM in heme, 2.5 μM in β-93 SH. Owing to the reaction of *p*-hydroxymercuribenzoate with dithionite, deoxyhemoglobin was prepared by flushing the hemoglobin with wet argon in a tonometer and was then studied in a stopped-flow apparatus which had previously been extensively flushed with argon-saturated buffers. At pH 6 and 7, the deoxyhemoglobin reactions were monophasic and the expected absorbance change was observed. At pH 8, the increased affinity of the hemoglobin for oxygen resulted in a biphasic reaction, approximately half of which reacted with a slow rate attributed to deoxyhemoglobin. At pH 9 we were unable to maintain the protein as deoxyhemoglobin in the stopped-flow apparatus, and the reaction rate was that characteristic of oxyhemoglobin at pH 9. With the exception of deoxyhemoglobin, the various proteins show a falling off of the rate with an increase in pH, whereas a

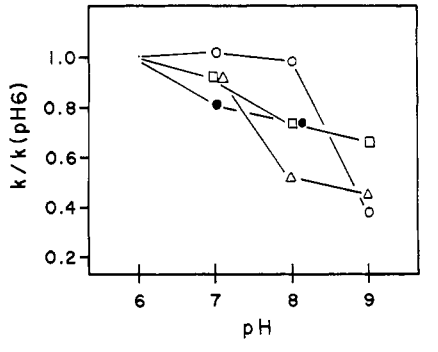

FIGURE 2: A plot of the ratio, k/k(pH 6), of the second-order rate constant at various pH values to the value at pH 6 for the *p*-hydroxymercuribenzoate reaction with the β-93 SH of various human hemoglobins and intermediates, 20°: (△) globin; (□) ICII; (○) HbO_2; (●) Hb.

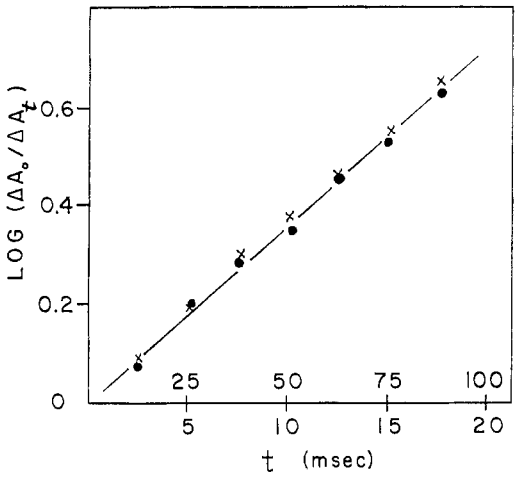

FIGURE 3: The time course for the reaction of *p*-hydroxymercuribenzoate with human hemoglobin at two different hemoglobin concentrations. The reaction was run at 20°, 0.1 M potassium phosphate buffer, pH 7.0. The upper time scale is for hemoglobin 0.75 μM in heme (X), *p*-hydroxymercuribenzoate concentration = 3 μM. The lower time scale is for hemoglobin 5 μM in heme (●), *p*-hydroxymercuribenzoate concentration = 15 μM. ΔA_0 is the total absorbance change during the reaction; ΔA_t is the absorbance change remaining at time *t*.

model compound, β-mercaptoethanol, shows a progressive increase in rate. The reactions of globin and ICII were essentially an order of magnitude greater than those of liganded hemoglobin. Deoxyhemoglobin reacted 64 times more slowly with *p*-hydroxymercuribenzoate than did oxyhemoglobin at pH 7.

Carbon monoxide-hemoglobin (HbCO), cyanomet, and azidomet forms of hemoglobin all reacted with rates which differed by no more than 20% from that of oxyhemoglobin. For cyanomet hemoglobin and azidomethemoglobin, it was necessary to have no excess ligand in solution, for reaction of these ligands with Hg greatly modifies the observed rate of the SH reaction with *p*-hydroxymercuribenzoate. The reaction rate of methemoglobin is also shown in Table I as a function of pH and is seen to be very similar to HbO_2.

The rate constant for the reaction of HbO_2 with *p*-hydroxymercuribenzoate was constant over a *p*-hydroxymercuribenzoate concentration range of 7.5–60 μM when the hemoglobin was 2.5 μM in SH. All three sources of *p*-hydroxymercuribenzoate gave identical rates for the reaction, as did fresh hemoglobin, crystallized hemoglobin, hemoglobin prepared with and without toluene, and hemoglobin prepared from ICII by the addition of hemin as the dicyanide complex. The same rate could also be obtained from recombined oxygenated α and β chains.

Activation energies for the various proteins were determined over the temperature range 8–31° at pH 7.0. For globin and ICII, no variation in the rate of the reaction was seen over the temperature range 10–26°, indicating that the activation energy for the reaction must be very small and in any case no greater than 3 kcal/mol. A value of 3 ± 1 kcal/mol was obtained for deoxyhemoglobin, whereas the activation energy for oxyhemoglobin was 5.3 ± 0.5 kcal/mol.

Figure 3 shows the time course for the *p*-hydroxymercuribenzoate reaction for a sevenfold change in hemoglobin concentration. At the lower concentration, extensive dissociation into chains would have altered the time course considerably, as well as the observed absorbance change. Oxyhemoglobin was also studied in 2 M NaCl under conditions thought to promote dissociation of the tetramer into dimers (Rossi-Fanelli *et al.*, 1961). Under these conditions the reaction is slower by a factor of about 2. It is not clear whether or not this is merely a salt effect on the reaction of *p*-hydroxymercuribenzoate since both the isolated α and β chains show kinetic salt effects in 2 M NaCl.

Discussion

Antonini and Brunori (1969) have reported rate constants for the reaction of *p*-hydroxymercuribenzoate with oxy- and deoxyhemoglobin in an investigation on the time course of a conformational change in hemoglobin. Their rate constants differ from those which we have determined by about a factor of ten. The ratio of their rate constants for oxy- and deoxyhemoglobin, however, is in excellent agreement with our values. A recent study of Maeda and Ohnishi (1971) is based on hemoglobins reconstituted from chains. The rate constants quoted in that work are lower than ours by a factor of more than 3 for oxyhemoglobin and by a factor of 2 for deoxyhemoglobin. As described in the Experimental Section, we have used *p*-hydroxymercuribenzoate from three different sources and have purified it before use. In addition, we have studied hemoglobin prepared in different ways and stored for different periods of time, crystallized and not. Furthermore, the reaction was studied on two quite different stopped-flow devices. We find identical values for the rate constants in all instances, ruling out a difference arising from our preparative procedures for the hemoglobin, the origin of the *p*-hydroxymercuribenzoate, or our apparatus. The half-times of the reactions were found to be inversely dependent on the *p*-hydroxymercuribenzoate concentration when experiments were run under pseudo-first-order conditions.

The results for globin and ICII show that introduction of the heme group into the α-heme site in globin scarcely alters the reactivity of the β-93 SH group. Addition of heme to the β site to form hemoglobin slows the reaction by a factor of 10 as shown in Table I. It appears that the environment of the β-93 SH group is only slightly influenced, if any, by the introduction of the heme group into the cavity of the associated α chains and that a major alteration arises only after the introduction of heme into the β chain.

The effect of α chain–β chain interaction on the β-93 SH reactivity can be obtained from a comparison of the kinetic properties of that group in liganded hemoglobin and in isolated liganded β chains. The combination of *p*-hydroxymercuribenzoate with the SH groups of isolated β chains is kinetically complex and will be described in detail elsewhere (Parkhurst, L. J., and Geraci, G., manuscript in preparation). It is sufficient for the analysis here to compare the rate constants at pH 7.0 as reported in Figure 1. The reaction of oxy-β chains is strongly biphasic. The rapid phase is about 1.7 times more rapid than the rate for HbO_2 and is almost certainly due to the reaction of the β-93 SH group. (Owing to the very high affinity of β chains for O_2, it was not possible for us to study reliably the reaction of *p*-hydroxymercuribenzoate with deoxy-β chains.) It thus appears that α–β interaction produces a smaller kinetic effect on the β-93 SH than entry of heme into the β chain. These results are in accord with previous findings from circular dichroic (CD) spectra (Geraci and Li, 1969) which showed that α chain–β chain interaction affected the environments of the hemes and are consistent with the results of ligand-binding kinetics on hybrid heme hemoglobins (Parkhurst *et al.*, 1970). The conclusions from *p*-hydroxymercuribenzoate kinetics appear to be that the major contribution to

the β-93 SH group reactivity in hemoglobin derives from the introduction of heme into the β-heme site and that a secondary but important role is derived from α–β interactions. Only small kinetic effects arise from the binding of heme to the α sites in globin. This latter conclusion is in accord with the work of Javaherian and Beychok (1968), who studied the CD spectra of horse globin, globin 50% saturated with heme (probably mainly ICII), and hemoglobin, and concluded that no measurable effect on the conformation of β chains was apparent upon binding of heme to α chains.

The rate constants for the globin and ICII reactions with *p*-hydroxymercuribenzoate and the low activation energies are nearly those expected for a diffusion-limited reaction in which the reaction site in the protein is somewhat inaccessible to the small molecule reactant (Alberty and Hammes, 1958). In the case of liganded hemoglobin, the activation energy is about 5 kcal/mol. This value is much lower than the activation energy for the reaction of *p*-hydroxymercuribenzoate with the SH group in isolated α chains. We observe no differences, within experimental error, between HbO_2, HbCO, methemoglobin, cyanomet-, and azidomethemoglobin in the rates of the *p*-hydroxymercuribenzoate reactions, indicating that the reactivity of the β-93 SH is not sensitive to small conformational changes in the ligand-bound conformation which might arise from these different ligands. Furthermore, the fact that oxy- and methemoglobin show similar rates as a function of pH, as shown in Table I, implies that the reactivity of the β-93 SH is not sensitive to small conformational changes such as are thought to occur in the high-spin to low-spin transformation in methemoglobin (Hoard, 1971). The very large difference in rate between deoxy- and oxyhemoglobin already reported by other investigators (Maeda and Ohnishi, 1971, and references cited therein) is in accord with the crystallographic work (Perutz, 1970) which reports that the β-93 SH group is less accessible in the deoxyhemoglobin molecule. The interpretation that the reactivity is altered primarily by a steric block of the SH group appears substantiated here by the finding that the activation energy for the reaction of the SH group in deoxyhemoglobin is even lower than that for oxyhemoglobin. The anomalous pH dependence of the reaction for both oxy- and deoxyhemoglobin, however, indicates that the effect of the protein in modifying the SH reactivity cannot be explained solely in terms of steric factors.

In a number of studies, the β-93 SH region has been studied in several liganded forms of hemoglobin using nitroxide spin labels (Ogawa and McConnell, 1967; Ogawa *et al.*, 1968; McConnell *et al.*, 1968; Ho *et al.*, 1970; Baldassare *et al.*, 1970). These studies are complementary to *p*-hydroxymercuribenzoate kinetic studies since they give information on the immobilization of the label after covalent attachment at the β-93 SH site, whereas the kinetic results appear to reflect the relative accessibility of the site to *p*-hydroxymercuribenzoate attack. In several instances results from the two techniques appear to allow similar conclusions to be drawn. The immobilization of the spin label is a function of pH, ionic strength, and temperature, and varies with the liganded state of the hemoglobin and with the particular spin label used. The studies of McConnell *et al.* (1968) show differences between oxyhemoglobin and methemoglobin which are not apparent in our kinetic work. In that study, the immobilization of the spin label in the deoxy form was regarded as "intermediate," whereas in methemoglobin there was apparently an equilibrium between "weakly" and "strongly" immobilized states. If this equilibrium were rapidly established, then perhaps intermediate and weak immobilization of spin labels would correlate with the slow and rapid *p*-hydroxymercuribenzoate binding rates observed for deoxyhemoglobin and liganded hemoglobin, respectively. Spin-label studies (Ho *et al.*, 1970; Baldassare *et al.*, 1970) have shown that there must be more than two conformations of hemoglobin for fully cooperative hemoglobins. Similar conclusions were reached from kinetic *p*-hydroxymercuribenzoate binding studies (Maeda and Ohnishi, 1971) on valence hybrids. Spin-label studies (Ogawa *et al.*, 1968) have also shown that the signal from a spin label at β-93 is sensitive to changes at the α-heme site. *p*-Hydroxymercuribenzoate kinetic studies (Maeda and Ohnishi, 1971) also give evidence for the propagation of such conformational changes across the α–β interface.

The reaction of *p*-hydroxymercuribenzoate with the SH groups of hemoglobin provides a rapid and accurate assay for the presence of isolated chains. On a heme basis, isolated chains have, as an average, 1.5 SH groups, whereas hemoglobin has only 0.5 available for reaction with *p*-hydroxymercuribenzoate. This results in a threefold amplification of the signal to be measured, increasing the sensitivity of the assay. Moreover, one of the two SH groups of β chains and the SH group of α chains have rate constants very much smaller than that of hemoglobin. The presence of free chains in hemoglobin would change the kinetics from monophasic to strongly biphasic, with an increase in the total absorbance change. The sensitivity of the method allows one to measure micromolar concentrations of SH groups in a 1-cm light path. Antonini and Gibson (1967) reported the appearance of a quickly reacting hemoglobin when HbCO was diluted in a flow-flash apparatus. We examined the *p*-hydroxymercuribenzoate reaction with HbO_2 in 0.1 M potassium phosphate, pH 7, and 1.75 μM in heme (*before* mixing in the stopped flow) and found that there could be no more than 4–7% free α and β chains in the solution. Our estimate of the maximum amount of chains present is based entirely on the bandwidth of the noise in the oscilloscope traces, since we did not actually see any slow phase in our reaction. On the basis of our experiments, we must conclude that free α and β chains could not have given rise to the rapidly reacting material seen in the experiments of Antonini and Gibson (1967). Although the work in 2 M NaCl is somewhat ambiguous owing to kinetic salt effects, the work at 1.75 μM in heme is in the concentration range where the hemoglobin should be largely in the dimeric state (Schachman and Edelstein, 1966). It appears, therefore, that hemoglobin reacts with *p*-hydroxymercuribenzoate at the same rate in the dimeric or tetrameric form, and that dimers, rather than free chains, were responsible for the appearance of the rapidly reacting hemoglobin reported by Antonini and Gibson (1967).

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Natural Abundance ^{13}C Nuclear Magnetic Resonance Study of Gelatin†

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ABSTRACT: Natural abundance Fourier transform ^{13}C nuclear magnetic resonance (nmr) spectra were obtained for calf skin gelatin. Most resonances can be assigned to individual carbon atoms. Change of pH affects only the C^{γ} and C^{δ} resonances of the glutamic acid residues. Spin–lattice relaxation times were measured by the partially relaxed Fourier transform technique. The values are to be expected for a random coil molecule. The ^{13}C nmr spectra for poly(hydroxy-L-proline) are also reported.

Since the first ^{13}C nuclear magnetic resonance (nmr) study of amino acids by Horsley and Sternlicht (1968), the technique has been developed into a powerful new tool for elucidating the structures of biological molecules. A partial list includes studies of peptides (Horsley *et al.*, 1970), gramicidin S-A (Gibbons *et al.*, 1970), lysozyme (Chien and Brandts, 1971), chloresteryl chloride and AMP (Allerhand *et al.*, 1971), ribonuclease (Allerhand *et al.*, 1970; Glushko *et al.*, 1972), oligopeptides (Gurd *et al.*, 1971; Christl and Roberts, 1972), oligosaccharides (Dorman and Roberts, 1971), lecithin vesicles and erythrocyte membranes (Metcalfe *et al.*, 1971), carboxyhemoproteins (Conti and Paci, 1971; Moon and Richards, 1971), polynucleotides (Mantsch and Smith, 1972), and yeast transfer RNA (Komoroski and Allerhand, 1972). We have used ^{13}C nmr to study the helix–coil transition of collagen. In the course of this work, it was found necessary to thoroughly characterize the ^{13}C nmr of gelatin. In this paper, the nmr spectrum is reported along with the assignment of the individual carbon resonances, the effect of the pH, and the determination of spin–lattice relaxation times for the carbon nuclei in various residues.

Materials and Methods

Materials. Calf skin gelatin (NJ 869 0-69-792) was obtained from Kind and Knox Gelatin Co. The amino acid composition is taken to be that given by Veis (1964) for bovine corium collagen. It was dissolved at a concentration of 15 w/vol % in 0.2 M KCl, 0.2 M KH_2PO_4–K_2HPO_4 buffer, 2 M KCNS, 2 M $CaCl_2$, and 0.5 M $(NH_4)_2SO_4$. These solutions were then adjusted to the desired pH with either 1 N HCl or 1 N KOH using Radiometer type PHM 26 pH meter standardized at 25° for the measurement. Poly(hydroxy-L-proline) (mol wt 8120) was obtained from Sigma Chemical Co. and was dissolved in 6 M LiBr at a concentration of 0.28 M.

Methods. High-resolution ^{1}H broad band decoupled, natural abundance Fourier transform ^{13}C nmr spectra were were obtained at 22.63 MHz with a Brucker HFX-90 spectrometer. The ^{13}C analytical channel was operated with a 10-mm insert maintained at a temperature of 31° in the single coil mode where a 12-μsec pulse resulted in a $\pi/2$ spin nutation. Hexafluorobenzene contained in a 5-mm coaxial tube was used to secure field-frequency stabilization. Free induction decays were accumulated in a Nicolet 1080 time-averaging computer using 8192 channels internally swept at a rate of 100 μsec per channel (5 kHz frequency domain). Frequency components greater than 5 kHz were removed by a low-pass filter built into the Nicolet SD80 signal digitizer. A delay of 100 μsec was introduced between the time of the

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