Proton Nuclear Magnetic Resonance Study of Histidine Ionizations in Myoglobins of Various Species. Comparison of Observed and Computed pK Values†

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ABSTRACT: Observed pK values for histidine residues in a series of myoglobins [Botelho, L. H., and Gurd, F. R. N. (1978), *Biochemistry 17* (preceding paper in this issue)] were compared with computed values by an extension of the Tanford–Kirkwood theory based on the modification by Shire et al. [Shire, S. J., Hanania, G. I. H. and Gurd, F. R. N. (1974) *Biochemistry 13,* 2967]. The extended treatment draws on the three-dimensional structure of sperm whale myoglobin to specify which imidazole nitrogen atom is the more exposed to solvent in a given histidine residue. The choice of pK_{int} is then taken as 6.60 if N^{τ} is the more exposed or 6.00 if N^{π} is the more exposed, and the corresponding fractional static solvent accessibility [Lee, B., and Richards, F. M. (1971), *J. Mol. Biol. 55,* 379] is adopted for the given case. The solvent-accessibility factor is employed directly in the treatment to provide for the relative degree of electrostatic communication between point charges through the aqueous medium of high dielectric constant containing moving ions and the internal medium of low dielectric constant. The computed $pK_{1/2}$ values, corresponding to the pK of half titration of the given group, show good agreement with experiments. Cautious adjustment of the sperm whale myoglobin structure to allow for residue substitutions in other myoglobins likewise led to computed $pK_{1/2}$ values in good agreement with experiment.

The preceding paper in this issue reported assignments of histidine C-2 proton resonances observed among a set of myoglobins obtained from 16 animal species (Botelho and Gurd, 1978). The histidine resonances were identified with residues 8, 12, 35, 36, 48, 81, 113, 116, 119, 128, and 152. The present report deals with the interpretation of the observed titration behavior in each case in terms of chemical-shift ranges and pK values. The observed pK values are compared with computed values based on the extension by Shire et al. (1974a,b) of the Tanford–Kirkwood electrostatic theory (Tanford and Kirkwood, 1957; Tanford and Roxby, 1972). The treatment draws on the three-dimensional structure of sperm whale myoglobin (Takano, 1977) to help specify the particular imidazole N^{τ} or N^{π} involved primarily in the proton equilibrium (Botelho, 1975). This choice of proton dissociation site in a given histidine residue is used (Botelho, 1975; Matthew et al., 1978b), in turn, to specify, first, a particular intrinsic pK value, pK_{int}, that reflects the inherent differences in proton-binding behavior between N^{τ} and N^{π} (Reynolds et al., 1973) and, second, the fractional static solvent accessibility that applies in the given case (Lee and Richards, 1971; Matthew et al., 1978a). The computer $pK_{1/2}$ values, corresponding to the pK applying at the stage of half titration of a given group, show good agreement with experiment for all the myoglobins studied.

Experimental Section

Materials. The major myoglobin from each species was isolated and studied by the procedures described (Botelho and Gurd, 1978). The differences in amino acid sequences between these myoglobins are summarized in Table I.

Computation of Chemical Shifts. Chemical-shift values corresponding to the completely protonated form of the given imidazole group, δ_a, were computed (Botelho, 1975) according to the three-dimensional structure of sperm whale myoglobin (Takano, 1977). The starting point for a particular histidine residue was the acid-limit chemical-shift characteristic of the C-2 proton in a fully extended random coil (McDonald and Phillips, 1969). To this were added contributions reflecting the electrostatic environment including hydrogen-bond structures (Robillard and Shulman, 1973) as well as positive and negative charges, ring-current effects due to other aromatic side chains (Johnson and Bovey, 1958) and the porphyrin ring (Shulman et al., 1970), and contact and pseudocontact interactions due to the paramagnetic iron atom (Dwek, 1973; Sheard et al., 1970; Kurland and McGarvey, 1970; Kotani, 1961; Horrocks and Greenberg, 1973). Estimates for histidine residues not found in sperm whale myoglobin were made in terms of the sperm whale myoglobin structure by visual substitution involving the least possible deformation of that structure. A skeletal model of the sperm whale myoglobin structure was used to guide the selection of estimated coordinates for substituted atoms, after which several appropriate projections were computed to check the resulting interatomic distances.

Computation of Electrostatic Interactions. The adaptation (Botelho, 1975; Matthew et al., 1978b) of the procedure of Shire et al. (1974a,b, 1975) was employed as follows. The Debye–Hückel theory is used to determine the electrostatic free energy for a set of discrete point charges on a sphere of radius, b, and ion-exclusion radius, a, in a solvent (here water) with an external dielectric constant, D. The charges are placed on the surface of the equivalent sphere which is assumed to form a continuous medium with a (low) internal dielectric constant, D_i. The value of b for myoglobin was taken as 18.0 Å (Takano, 1977) and of a as 20.0 Å. The dielectric constant of the interior, D_1, was taken as 4, and the external dielectric

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TABLE I: Differences in Amino Acid Sequences in Myoglobins.

species	residue position 1	4	5	8	9	12	13	15	19	21	27	28	34	35	45
sperm whale[a]	Val	Glu	Gly	Gln	Leu	His	Val	Ala	Ala	Val	Asp	Ile	Lys	Ser	Arg
dwarf sperm whale[b]	Val	Glu	Gly	Gln	Leu	His	Val	Ala	Ala	Ile	Asp	Ile	Lys	His	Arg
sei whale[c]	Val	Asp	Ala	Gln	Leu	Asn	Ile	Ala	Ala	Val	Asp	Ile	Lys	Gly	Lys
gray whale[d]	Val	Asp	Ala	Gln	Leu	Asn	Ile	Ala	Ala	Val	Asp	Ile	Lys	Gly	Lys
humpback whale[e]	Val	Asp	Ala	Gln	Leu	Asn	Ile	Ala	Ala	Val	Asp	Ile	Lys	Gly	Lys
minke whale[f]	Val	Asp	Ala	His	Leu	Asn	Ile	Ala	Ala	Val	Asp	Ile	Lys	Gly	Lys
common dolphin[g]	Gly	Asp	Gly	Gln	Leu	Asn	Val	Gly	Ala	Leu	Asp	Val	Lys	Gly	Lys
bottlenosed dolphin[h]	Gly	Asp	Gly	Gln	Leu	Asn	Val	Gly	Ala	Leu	Asp	Val	Lys	Gly	Lys
pilot whale[i]	Gly	Asp	Gly	Gln	Leu	Asn	Val	Gly	Ala	Leu	Asp	Ile	Lys	Gly	Lys
Amazon River dolphin[j]	Gly	Asp	Gly	Gln	Leu	Asn	Ile	Gly	Ala	Leu	Asp	Val	Lys	Gly	Lys
common porpoise[k,l]	Gly	Glu	Gly	Gln	Leu	Asn	Val	Gly	Ala	Leu	Asp	Val	Lys	Gly	Lys
dall porpoise[l]	Gly	Glu	Gly	Gln	Leu	Asn	Val	Gly	Ala	Leu	Asp	Val	Lys	Gly	Lys
harbor seal[m]	Gly	Asp	Gly	His	Leu	Asn	Val	Gly	Thr	Leu	Glu	Val	Lys	Ser	Lys
sea lion[n]	Gly	Asp	Gly	Gln	Leu	Asn	Ile	Gly	Ala	Leu	Glu	Val	Lys	Gly	Lys
horse[o]	Gly	Asp	Gly	Gln	Gln	Asn	Val	Gly	Ala	Ile	Glu	Val	Thr	Gly	Lys
man[p]	Gly	Asp	Gly	Gln	Leu	Asn	Val	Gly	Ala	Ile	Glu	Val	Lys	Gly	Lys

species	residue position 51	53	54	56	57	62	66	67	74	83	86	101	109	110	113
sperm whale[a]	Thr	Ala	Glu	Lys	Ala	Lys	Val	Thr	Ala	Glu	Leu	Ile	Glu	Ala	His
dwarf sperm whale[b]	Ser	Ala	Glu	Lys	Ala	Lys	Val	Thr	Ala	Glu	Leu	Ile	Glu	Ala	His
sei whale[c]	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Thr	Gly	Glu	Leu	Ile	Asp	Ala	His
gray whale[d]	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Thr	Gly	Glu	Leu	Ile	Asp	Ala	His
humpback whale[e]	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Thr	Gly	Glu	Leu	Ile	Asp	Ala	His
minke whale[f]	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Thr	Gly	Glu	Leu	Ile	Asp	Ala	His
common dolphin[g]	Thr	Ala	Asp	Lys	Ala	Lys	Asn	Thr	Ala	Asp	Leu	Ile	Glu	Ala	His
bottlenosed dolphin[h]	Thr	Ala	Asp	Lys	Ala	Lys	Asn	Thr	Ala	Asp	Leu	Ile	Glu	Ala	His
pilot whale[i]	Thr	Ala	Asp	Lys	Ala	Lys	Asn	Thr	Ala	Glu	Leu	Ile	Glu	Ala	His
Amazon River dolphin[j]	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Thr	Gly	Glu	Leu	Ile	Glu	Ala	His
common porpoise[k,l]	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Thr	Gly	Asp	Leu	Ile	Glu	Ala	His
dall porpoise[l]	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Thr	Gly	Asp	Leu	Ile	Glu	Ala	His
harbor seal[m]	Ser	Asp	Asp	Arg	Arg	Arg	Asn	Thr	Gly	Glu	Leu	Ile	Glu	Ala	His
sea lion[n]	Ser	Asp	Glu	Lys	Arg	Lys	Lys	Thr	Gly	Asp	Leu	Ile	Glu	Ala	His
horse[o]	Thr	Ala	Glu	Lys	Ala	Lys	Thr	Val	Gly	Glu	Leu	Ile	Asp	Ala	His
man[p]	Ser	Asp	Glu	Lys	Ala	Lys	Ala	Thr	Gly	Glu	Ile	Val	Glu	Cys	Gln

species	residue position 116	118	121	122	127	128	129	132	140	142	144	146	151	152
sperm whale[a]	His	Arg	Gly	Asp	Ala	Gln	Gly	Asn	Lys	Ile	Ala	Tyr	Tyr	Gln
dwarf sperm whale[b]	His	Arg	Ala	Asp	Ala	Gln	Gly	Ser	Lys	Ile	Ala	Tyr	Tyr	Gln
sei whale[c]	His	Arg	Gly	Asp	Ala	Gln	Ala	Asn	Lys	Ile	Ala	Tyr	Phe	Gln
gray whale[d]	His	Arg	Gly	Asp	Ala	Gln	Ala	Asn	Lys	Ile	Ala	Tyr	Phe	Gln
humpback whale[e]	His	Arg	Ala	Asp	Ala	Gln	Ala	Asn	Lys	Ile	Ala	Tyr	Phe	Gln
minke whale[f]	His	Arg	Ala	Glu	Ala	Gln	Ala	Asn	Lys	Ile	Ala	Tyr	Phe	Gln
common dolphin[g]	His	Arg	Ala	Glu	Ala	Gln	Gly	Asn	Lys	Ile	Ala	Tyr	Phe	His
bottlenosed dolphin[h]	His	Arg	Ala	Glu	Ala	Gln	Gly	Asn	Lys	Ile	Ala	Tyr	Phe	His
pilot whale[i]	His	Arg	Ala	Glu	Ala	Gln	Gly	Asn	Lys	Ile	Ala	Try	Phe	His
Amazon River dolphin[j]	His	Arg	Gly	Asp	Ala	Gln	Gly	Asn	Lys	Ile	Ala	Tyr	Phe	His
common porpoise[k,l]	His	Arg	Ala	Glu	Ala	Gln	Gly	Asn	Lys	Ile	Thr	Tyr	Phe	His
dall porpoise[l]	His	Arg	Ala	Glu	Ala	Gln	Gly	Asn	Lys	Ile	Thr	Tyr	Phe	His
harbor seal[m]	His	Lys	Ala	Glu	Ala	Gln	Ala	Lys	Asn	Ile	Ala	Tyr	Phe	His
sea lion[n]	Gln	Lys	Gly	Asp	Thr	His	Ala	Lys	Asn	Ile	Ala	Arg	Phe	Gln
horse[o]	His	Lys	Gly	Asp	Ala	Gln	Gly	Thr	Asn	Ile	Ala	Tyr	Phe	Gln
man[p]	Gln	Lys	Gly	Asp	Ala	Gln	Gly	Asn	Lys	Met	Ser	Tyr	Phe	Gln

[a] *Physeter catodon*, Edmundson (1965); Romero Herrera and Lehman (1974). [b] *Kogia simus*, Dwulet et al. (1977). [c] *Baleanoptera borealis*, Jones et al. (1979a). [d] *Eschrichtius gibbosus*, Bogardt et al. (1976). [e] *Megaptera novaeangliae*, Lehman et al. (1978). [f] *Baleanoptera acutorostrata*, Lehman et al. (1977). [g] *Delphinus delphis*, Wang et al. (1977). [h] *Tursiops truncatus*, Jones et al. (1976a,b). [i] *Globicaphala melaena*, Jones et al. (1978). [j] *Inia geoffrensis*, Dwulet et al. (1975). [k] *Phocoena phocoena*, Bradshaw and Gurd (1969); Meuth et al. (1978). [l] *Phocoenoides dalli dalli*, Meuth et al. (1978). [m] *Phoca vitulina*, Bradshaw and Gurd (1969). [n] *Zalophus californianus*, Vigna et al. (1974). [o] *Equus caballus*, Dautrevaux et al. (1969). [p] *Homo sapiens*, Romero-Herrera and Lehmann (1971).

constant D as that of water at 298 K was taken as 78.5 (Shire et al., 1974a; Orttung, 1970).

The first stage of the calculation for a given set of parameters (b, a, D. D_i, and T) is to compute a table of W_{ij} values, where W_{ij} is the free energy of interaction of a pair of point charges Z_i and Z_j placed on the sphere of radius, b. An intrinsic pK (pK_{int}) is assigned to every site j, and the charge Z_j of every site is calculated at any particular pH. For a given set of sites, the tabulated W_{ij} values are used to compute by iterative methods a set of effective pK_i values,

$$pK_i = (pK_{int})_i - \frac{1}{2.303kT}\sum_{j\neq i}(W_{ij} - SA_jW_{ij})/Z_i \quad (1)$$

Every site j for which $Z_j \neq 0$ contributes to the effective equilibrium constant of site i under the particular conditions, pK_i. Since the charged groups are exposed to different degrees, the SA value for each group j is included to reflect the degree of exposure of each point charge to the solvent with high dielectric constant. The inclusion of the SA_j term in this way is closely equivalent to adjusting the effective dielectric constant for the computation of W_{ij} for each pair of point charges Z_i and Z_j for the relative contributions of the interaction through the internal medium, D_i, and the external solvent, D (Matthew et al., 1978c). For purposes of computation, the SA values were taken equal to the nearest 0.05 increment (0.05, 0.10, 0.15, etc.) in all cases. Values of SA_j vary between 0.05 and 0.95 (Matthew et al., 1978a).

Following Shire et al. (1974a), the proton binding sites were considered in three categories. The first class comprises all groups with normal intrinsic pK values: terminal carboxyl, 3.60; Asp, 4.00; Glu, 4.50; propionic acid, 4.00; His N^{π}, 6.00; His N^{τ}, 6.60 (Reynolds et al., 1973, Botelho, 1975; Wilbur and Allerhand, 1977); Cys, 9.10 (Tanford, 1962); Tyr, 10.00 (Gurd et al., 1972); Lys, 10.40 (Keim et al., 1974); Arg, 12.00. The choice of the histidine value depends on the preponderant exposure of the given imidazole N according to the SA listing (Botelho, 1975; Matthew et al., 1978a). The second class consists of sites that are also available to hydrogen-ion equilibrium but with abnormal pK values, e.g., those groups influenced by such effects as hydrogen bonding. These intrinsic pK values were adjusted (Szabo and Karplus, 1972) by adding 0.50 (Lys, Tyr) or subtracting 0.50 (Glu, Asp). The charge location was assigned to the appropriate atom, e.g., one or the other oxygen in a carboxyl group, according to the hydrogen-bonding pattern or else the greater degree of SA for that atom. The third class represents the masked sites (Shire et al., 1974a) defined according to specific chemical or structural evidence such as burial or masking of histidine residues.

The treatment was extended to myoglobin species other than sperm whale by careful substitution as described above. As Table I shows, only three of the myoglobin species listed contain more than three substitutions in which a charge type is altered at a given residue position. Furthermore, the immediately neighboring residues of any given substituted residue are almost always conserved. Partly for these reasons, the structural adjustments required to take account of any one particular substitution were rarely influenced by any second locus of substitution. Substantially, all substitutions involved sites falling in the first two classes with respect to pK_{int}.

The environment of histidine residue 36 was adjusted as follows. The patterns of carboxymethylation of histidine residue 36 in solution and in the crystalline state are distinctly different (Banaszak et al., 1963; Hugli and Gurd, 1970a,b; Nigen and Gurd, 1973; Botelho and Gurd, 1978); clearly, the structural arrangement of this side chain in the crystal (Takano, 1977) does not apply in every respect in solution (Gurd, 1970).[1] In view of the high pK value, between 7.7 and 8.0, for residue 36 (Botelho and Gurd, 1978), the local structure was arbitrarily adjusted[2] to bring the carboxyl group of glutamic acid residue 38 into hydrogen-bonding distance of N^{τ}. The arrangement adopted is comparable with that observed in the interaction of β chain histidine-146 with β chain aspartic acid-94 in human deoxyhemoglobin (Perutz, 1970; Kilmartin et al., 1973). The arbitrary change places the carboxyl oxygen of glutamic acid residue 38 at a distance of 2.85 Å from N^{τ} of residue 36 by removal of the intervening water molecule (Takano, 1977). This hypothetical structural alteration involves simple rotation about the C^{α}–C^{β} and C^{β}–C^{γ} bonds without eclipsing the hydrogen atoms and without disturbing residue 37.

For purposes of comparison of computed pK_i values with the observed pK values, pK_{obsd}, the computed values are reported for the condition of half titration and are designated p$K_{1/2}$ (Shire et al., 1974a). The computed p$K_{1/2}$ values reported in this paper apply to an ionic strength of 0.01 and 25 °C (Shire et al., 1974a). The effects of temperature and solvent variations are discussed below.

The experimental methods for the measurement of the pK values have been described (Botelho and Gurd, 1978).

Results

Chemical-Shift Ranges. Table II presents chemical-shift limits for the various observed resonances in which the minke whale myoglobin and common porpoise myoglobin are taken as representatives of the baleen whales and the porpoise and dolphin group, respectively. Each observable histidine C-2 proton resonance is listed with the values of the theoretical acid chemical-shift limit, the observed acid and base limits, and the observed span of chemical-shift limits, $\delta_a - \delta_b$. The theoretical values of δ_a show reasonable agreement with the observed values in most cases, except for residue 81. More informative are the general similarities in observed chemical-shift limits for given residues in the various myoglobin species. These similarities between species for a given histidine residue, like the conservation of pK values (Botelho and Gurd, 1978), are evidence for a broadly conserved three-dimensional structure. The spectral similarities are well illustrated under comparable conditions near the acid-titration limit within the baleen whale group (Figure 1) and the porpoise and dolphin group (Figure 2).

The titration span, $\delta_a - \delta_b$, for all resonances falls within the range 0.93 to 1.07 ppm, with the exception of the resonances of residues 119 and 36. As illustrated in Figure 3 for the gray whale myoglobin, these two resonances deviate at the alkaline extreme. The resonance of residue 119 undergoes a reversal to reduce the magnitude of $\delta_a - \delta_b$ to a range of 0.76 to 0.81 ppm, whereas that of residue 36 experiences a further increment to an overall range of 1.0 to 1.20 ppm. As described previously, these titrations were analyzed to yield the pK values reported below for the primary, lower pH process corresponding to the direct proton dissociation from the imidazole group distinct from the further change related to the hemic acid dissociation (Botelho and Gurd, 1978). The hemic acid p$K_{1/2}$ at ionic strength 0.01 ranges between 8.31 and 8.89 for several of the species studied here (Shire et al., 1975).

[1] Under conditions of very high ionic strength, the dissolved protein recovers some of the reactivity pattern characteristic of the crystalline state (Botelho, 1975).

[2] The alternative of assuming a pK_{int} of 7.8 (Shire et al., 1974a; Matthew et al., 1978b) will provide a fit to the observed results but lacks as straightforward a structural basis as the procedure adopted here.

TABLE II: Computed and Observed Chemical Shifts of Histidine C-2 Resonances.[a]

His res	computed δ_a	observed δ_a	observed δ_b	observed $\delta_a - \delta_b$	observed δ_a	observed δ_b	observed $\delta_a - \delta_b$
		sperm whale			dwarf sperm whale		
12	8.65	8.71	7.71	1.00	8.73	7.69	1.04
35					8.49	7.46	1.03
36	8.35	8.34	7.16	1.18	8.30	7.20	1.10
48	8.56	8.62	7.63	0.99	8.59	7.66	0.93
81	8.52	8.76	7.74	1.02	8.78	7.71	1.07
113	8.60	8.58	7.58	1.00	8.57	7.53	1.04
116	8.58	8.60	7.63	0.97	8.61	7.61	1.00
119	8.65	8.70	7.89	0.81	8.68	7.92	0.76
		minke whale			common dolphin		
8	8.57	8.74	7.73	1.01			
36	8.35	8.28	7.11	1.17	8.31	7.12	1.19
48	8.56	8.63	7.69	0.94	8.62	7.66	0.96
81	8.52	8.78	7.74	1.04	8.72	7.74	0.98
113	8.60	8.61	7.61	1.00	8.60	7.59	1.01
116	8.58	8.62	7.64	0.98	8.61	7.64	0.97
119	8.65	8.67	7.89	0.78	8.67	7.89	0.78
152	8.73				8.53	7.51	1.02
		harbor seal			sea lion		
8	8.57	8.66	7.70	0.96			
36	8.35	8.31	7.16	1.15	8.32	7.12	1.20
48	8.56	8.62	7.62	1.00	8.61	7.66	0.95
81	8.52	8.73	7.73	1.00	8.73	7.71	1.02
113	8.60	8.63	7.57	1.06	8.61	7.61	1.00
116	8.58	8.62	7.59	1.03			
119	8.65	8.69	7.90	0.79	8.66	7.89	0.77
128	8.58				8.65	7.74	0.91
152	8.73	8.45	7.48	0.97			
		horse			man		
36	8.35	8.26	7.12	1.14	8.31	7.11	1.20
48	8.56	8.62	7.66	0.96	8.63	7.69	0.94
81	8.52	8.69	7.74	0.95	8.73	7.76	0.97
113	8.60	8.62	7.63	0.99			
116	8.58	8.60	7.66	0.94			
119	8.65	8.66	7.88	0.78	8.69	7.88	0.81

[a] The acidic and basic chemical-shift limits downfield of Me_4Si are represented by δ_a and δ_b, respectively.

Individual pK Values. The observed and computed pK values for each observable histidine residue are listed in Tables III–V for the 16 species. The species are again grouped according to structural similarity or, in certain cases, identity (Table I). The first columns in the table list the SA values for N^{π} and N^{τ} (Matthew et al., 1978a) and the value of pK_{int} which is chosen as 6.00 when SA is greater for N^{π} and 6.60 when SA is greater for N^{τ}. The pK_{int} value so chosen for each residue is then used to compute for each myoglobin species the value of $pK_{1/2}$ corresponding to the pK_i value at the pH of one-half titration of the particular group, followed in the next column by the observed pK (pK_{obsd}). The magnitude of the electrostatic correction term, the second term on the right in eq 1, is seen directly by comparing $pK_{1/2}$ and pK_{int}. The agreement between theory and observation is shown by comparing $pK_{1/2}$ with pK_{obsd} and is illustrated for each species in Figure 4. In this figure, values of $pK_{1/2}$ based on pK_{int} values of 6.60 and 6.00 are indicated by closed and open circles, respectively. In all cases, the fit of theory with experiment is satisfactory. The correlation coefficients for the plots in Figure 4 range from 0.94 (sperm whale, Figure 4A) to 0.99 (man, Figure 4L), in contrast with the correlation coefficients for $pK_{1/2}$ with pK_{int} that fall near 0.35. Note that the higher and lower pK_{int} values lead in particular instances to the opposite order for $pK_{1/2}$.

The results in Tables III–V and Figure 4 illustrate the value of making the preliminary distinction between N^{τ} and N^{π} as preferred sites of proton dissociation for each histidine residue to guide the choice of pK_{int}. Since the summations of W_{ij} terms involve all groups for which $Z \neq 0$, the values of pK_{int} and SA_j for other classes of groups apart from the imidazole groups enter all computations as well. The results obtained here for the histidine residues that are not present in sperm whale myoglobin (Table III) also indicate the predictive possibilities of the treatment, since $pK_{1/2}$ and pK_{obsd} fall within approximately 0.3 unit for residues 8, 35, 128, and 152 in all cases.

The comparison of theory with experiment in Tables III–V and Figure 4 leaves out of account the differences in temperature (25 and 17 °C, respectively), ionic strength (0.01 and 0.1, respectively), and solvent (H_2O and D_2O, respectively). Accepting an enthalpy of disassociation of an imidazole residue of 7 kcal/mol (Breslow and Gurd, 1962), the computed $pK_{1/2}$ for 25 °C should be increased by 0.14 unit to correspond to the condition of observation of approximately 17 °C. The effect of the difference in ionic strength likewise means that the computed $pK_{1/2}$ for 0.01 should be increased by approximately 0.07 unit to correspond to the observations made at 0.10 (Shire et al., 1974a). The NMR measurements were performed in D_2O without correction of the pH meter readings (Botelho and Gurd, 1978). They may be taken to underestimate pD by approximately 0.4 unit, a correction that is not necessarily offset by the isotope effect proper. Therefore, the correlation between

TABLE III: Computed and Observed pK Values for Histidine Residues in Myoglobins of Sperm Whales and Baleen Whales.

His residue				sperm whales				baleen whales					
				sperm whale		dwarf sperm whale		gray whale		sei whale	humpback whale	minke whale	
sequence no.	N atom	SA	pK_{int}	p$K_{1/2}$	pK_{obsd}	p$K_{1/2}$	pK_{obsd}	p$K_{1/2}$	pK_{obsd}	pK_{obsd}	pK_{obsd}	p$K_{1/2}$	pK_{obsd}
8	π	0.90	6.00									5.93	6.10
	τ	0.80											
12	π	0.85	6.00	5.70	6.28	5.92	6.21						
	τ	0.65											
35	π	0.70	6.00			5.20	5.52						
	τ	0.50											
36	π	0.30											
	τ	0.85	6.60	7.77	7.97	7.76	7.74	7.74	7.50	7.61	7.53	7.53	7.74
48	π	0.30											
	τ	0.70	6.60	6.53	6.73	6.59	6.69	6.51	6.44	6.53	6.56	6.51	6.59
81	π	0.30											
	τ	0.95	6.60	6.55	6.17	6.53	6.12	6.27	6.18	6.23	6.25	6.27	6.23
113	π	0.50											
	τ	0.65	6.60	5.35	5.49	5.28	5.42	5.34	5.48	5.40	5.42	5.33	5.46
116	π	0.05											
	τ	0.95	6.60	6.36	6.55	6.35	6.45	6.39	6.42	6.43	6.42	6.39	6.41
119	π	0.20	6.00	5.42	5.46	5.41	5.41	5.83	5.49	5.38	5.42	5.84	5.44
	τ	0.05											

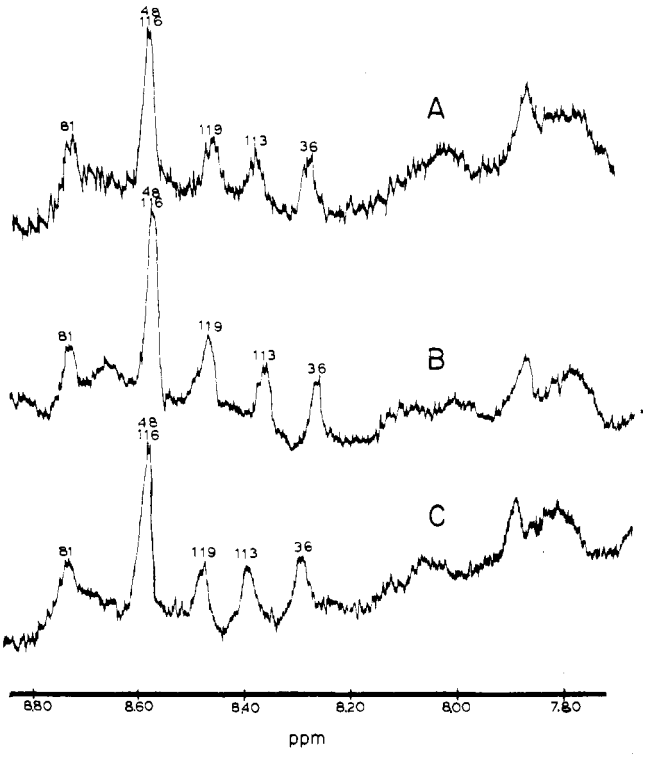

FIGURE 1: Comparison at pH 5.0 of spectra of myoglobins of (A) humpback whale, (B) gray whale, and (C) sei whale. Resonance assignments to histidine residues are indicated.

the NMR and titration values in H_2O may be no more than approximate (Bradbury and Brown, 1973). These various factors are absorbed in the choice of the pK_{int} values (Reynolds et al., 1973) with a minimum of ad hoc assumptions. Because of these uncertainties, the computations have been applied to the conditions used by Shire et al. (1974a,b, 1975), which were chosen for comparison with titration curves before the present pK assignments had been completed (Botelho and Gurd, 1978).

Discussion

As pointed out in the preliminary reports (Matthew et al.,

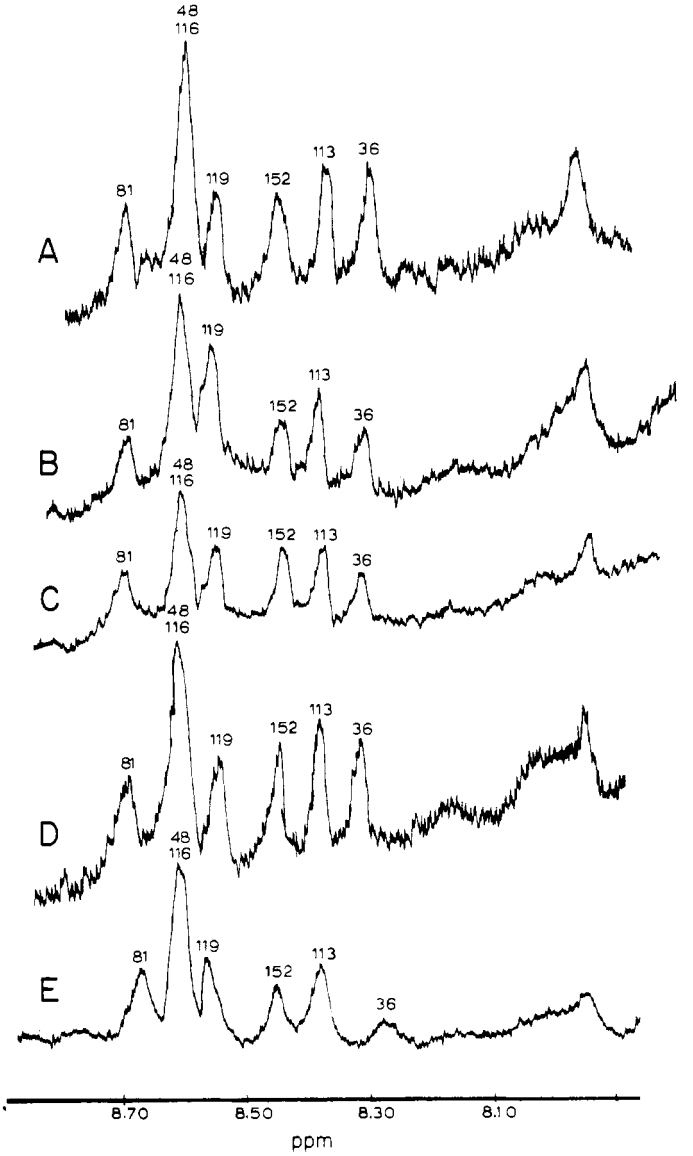

FIGURE 2: Comparison at pH 5.0 of spectra of myoglobins of (A) Amazon River dolphin, (B) common porpoise, (C) pilot whale, (D) bottlenosed dolphin, and (E) common dolphin.

TABLE IV: Computed and Observed pK Values for Histidine Residues in Myoglobins of Dolphins and Porpoises.

His residue sequence no.	N atom	SA	pK_{int}	common dolphin p$K_{1/2}$	common dolphin pK_{obsd}	bottle-nosed dolphin pK_{obsd}	pilot whale pK_{obsd}	Amazon River dolphin p$K_{1/2}$	Amazon River dolphin pK_{obsd}	common porpoise p$K_{1/2}$	common porpoise pK_{obsd}	dall porpoise pK_{obsd}
36	π	0.30										
	τ	0.85	6.60	7.74	7.78	7.79	7.88	7.74	7.79	7.75	7.77	7.87
48	π	0.30										
	τ	0.70	6.60	6.51	6.47	6.47	6.48	6.51	6.45	6.51	6.50	6.47
81	π	0.30										
	τ	0.95	6.60	6.21	6.39	6.32	6.31	6.28	6.31	6.21	6.33	6.31
113	π	0.50										
	τ	0.65	6.60	5.34	5.40	5.52	5.44	5.34	5.54	5.32	5.45	5.46
116	π	0.05										
	τ	0.95	6.60	6.35	6.42	6.29	6.33	6.36	6.32	6.36	6.27	6.37
119	π	0.20	6.00	5.60	5.29	5.46	5.42	5.54	5.46	5.59	5.45	5.46
	τ	0.05										
152	π	0.50	6.00	5.86	6.18	6.12	6.02	5.84	5.95	5.89	5.96	6.18
	τ	0.35										

TABLE V: Computed and Observed pK Values for Histidine Residues in Myoglobins of Miscellaneous Species.

sequence no.	N atom	SA	pK_{int}	harbor seal p$K_{1/2}$	harbor seal pK_{obsd}	sea lion p$K_{1/2}$	sea lion pK_{obsd}	horse p$K_{1/2}$	horse pK_{obsd}	man p$K_{1/2}$	man pK_{obsd}
8	π	0.90	6.00	5.84	6.14						
	τ	0.80									
36	π	0.30									
	τ	0.85	6.60	7.70	7.72	7.65	7.66	7.76	7.62	7.97	7.77
48	π	0.30									
	τ	0.70	6.60	6.74	6.58	6.51	6.61	6.54	6.61	6.80	6.71
81	π	0.30									
	τ	0.95	6.60	6.22	6.22	6.21	6.19	6.48	6.37	6.27	6.27
113	π	0.50									
	τ	0.65	6.60	5.38	5.32	5.30	5.59	5.36	5.70		
116	π	0.05									
	τ	0.95	6.60	6.33	6.37			6.40	6.54		
119	π	0.20	6.00	5.62	5.32	5.59	5.44	5.70	5.41	5.82	5.56
	τ	0.05									
128	π	0.95	6.00			5.48	5.53				
	τ	0.15									
152	π	0.50	6.00	6.32	6.29						
	τ	0.35									

1978a,b), the modification by Shire et al. (1974a,b) of the Tanford–Kirkwood theory (Tanford and Kirkwood, 1957; Tanford and Roxby, 1972) has been simplified by adopting the following formalism. The treatment summarized in eq 1 takes into account (a) the distance of separation of point charges on the equivalent sphere, (b) the degree of exposure to solvent of the given proton-bearing atom in terms of fractional static solvent accessibility, SA, (c) the characteristic dielectric constant of the medium, D, and of the interior of the protein molecule, D_i, and (d) the choice for the imidazole group of N^{τ} or N^{π} as the proton-bearing atom according to exposure to solvent. Following Reynolds et al. (1973), the intrinsic pK_i for N^{τ} is taken as 0.60 unit larger than that for N^{π}. In the present work, the choice between N^{τ} and N^{π} has been confined to the evidence from the computed static solvent accessibility (Lee and Richards, 1971; Matthew et al., 1978a) as to which is the more exposed, and, in turn, these two imidazole nitrogen atoms are associated consistently with pK_{int} values of 6.60 and 6.00, respectively. The substitution of the atomic coordinate set of Takano (1977) for that of Watson (1969) used previously (Shire et al., 1974a,b) has altered some individual estimates of SA, and the more recent, refined structural data have now been exploited at the level of the individual proton-bearing atoms. While making fuller use of the structural information, the present treatment does not circumvent other underlying simplifying approximations of the electrostatic theory. It should be borne in mind that Tanford and Roxby (1972) foresaw the possible advantage of a formalism that recognizes the extent of protrusion of the charged groups into the external medium.

The structure of sperm whale ferrimyoglobin crystals equilibrated with concentrated salt solutions (Kendrew and Parrish, 1956; Watson, 1969; Takano, 1977) prescribes a certain pattern of reactivity of histidine residues toward reagents such as bromoacetate, which is confirmed experimentally (Hugli and Gurd, 1970a). This reactivity pattern is largely preserved in the dissolved state of the protein, except where such residues as histidine-48 are sterically blocked by a neighboring protein molecule in the crystal lattice (Hugli and Gurd, 1970b). The most important exception is in the case of histidine residue 36 which loses its reactivity at pH 6.8 when dissolved in dilute salt solution.[1] The crystallographic information, therefore, does not apply to this residue under the conditions of the present study, and the arbitrary adjustment has been made of rotating certain C–C bonds in the side chain of glutamic acid residue 38 to bring an oxygen into hydrogen-bonding distance of the exposed N^{τ} of residue 36. This is the only such arbitrary adjustment applied by us to the struc-

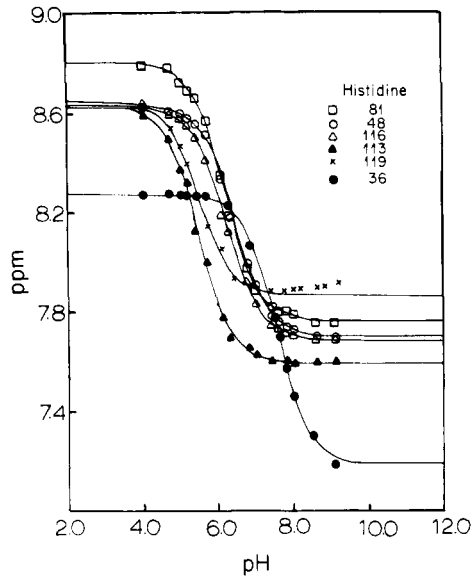

FIGURE 3: Titration curves for gray whale myoglobin with histidine residues identified on the figure.

ture of Takano (1977) to accommodate the electrostatic treatment for the sperm whale protein.

Crystallographic evidence exists for small systematic changes in the sperm whale myoglobin structure near pH 9 in the upper range of titration studied here (Watson and Chance, 1966; Schoenborn, 1971). The main changes in the structure with respect to histidine residues apply to residues 36 and 119, and their small but clear NMR consequences have been discussed already (cf. Figure 3). Some more subtle pH-dependent changes in ^{13}C NMR (Jones et al., 1976), near-ultraviolet circular dichroism (Friend et al., 1977), and enthalpy (Atanosov, 1970) have been observed in the range of the present titration studies. Computed chemical-shift values in the acid pH limit for the C-2 protons fit tolerably well with those computed for the crystalline stucture, with the exception of that for histidine residue 81 (Table II). Furthermore, with the exceptions noted above of the entries for residues 36 and 119, the overall chemical-shift changes in Table II, $\delta_a - \delta_b$, are within the normal range of near 1.00 ppm.

The justification for the extension to other myoglobins of the structural information applying to the sperm whale protein lies in close similarities of several kinds. In the first place, crystallographic evidence early showed certain consistencies (Kendrew and Parrish, 1956), later work has shown the common porpoise myoglobin as isomorphous with the sperm whale protein (Kretsinger et al., 1968), and, finally, the similarities found for the harbor seal myoglobin are gratifyingly numerous (Scouloudi, 1969; H. Scouloudi, personal communications). In the second place, the similarities in chemical shifts of ^1H NMR reported in the present work (Table II) as well as by other workers (Wüthrich et al., 1970; Cohen et al., 1972; Hayes et al., 1975) confirm numerous points of near identity between several of the myoglobin species studied here. Observations by ^{13}C NMR likewise support numerous points of structural similarity (Wilbur and Allerhand, 1977; Nigen et al., 1973). Thirdly, reactivity patterns toward bromoacetate in solutions of sperm whale and harbor seal myoglobins are generally comparable (Nigen and Gurd, 1973). Lastly, the overall patterns of the masking of histidine residues revealed by proton titration curves are consistent for several of the myoglobin species represented here (Breslow and Gurd, 1962; Hartzell et al., 1968; Friend et al., 1977).

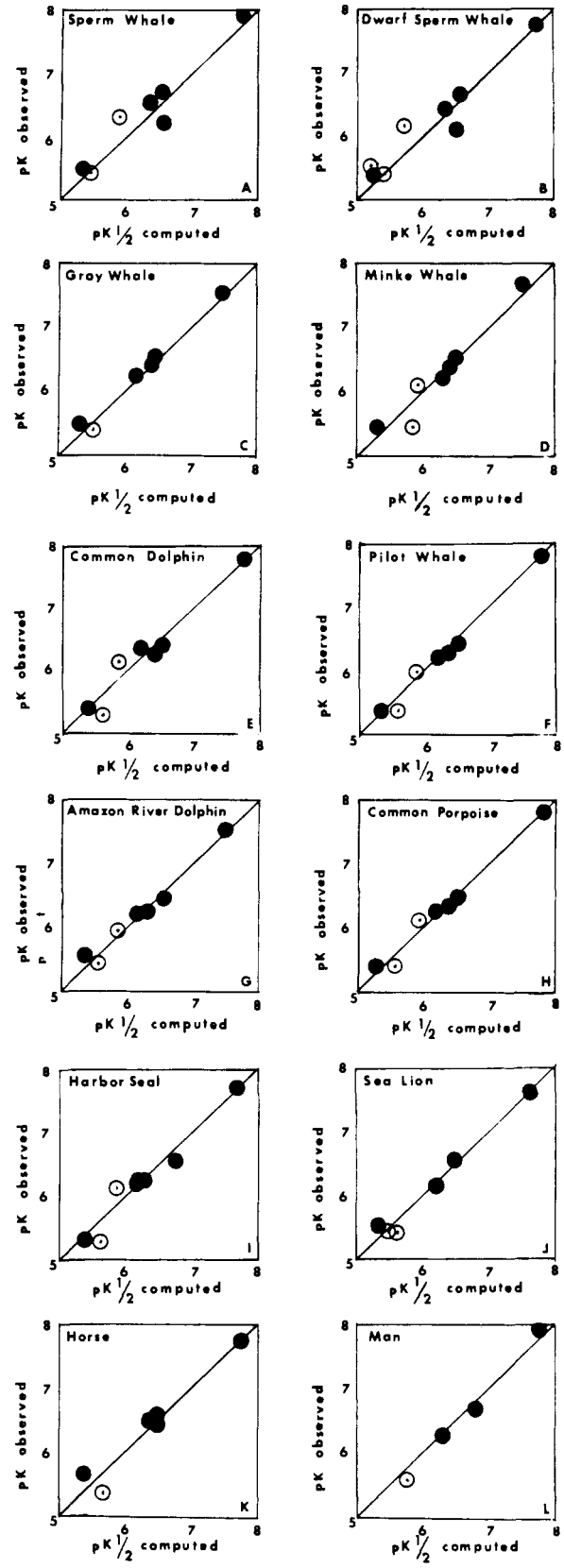

FIGURE 4: Comparison of computed $pK_{1/2}$ values with the corresponding observed pK values for a series of myoglobins indicated in each part, A-L. The histidine pK values were computed according to the assumed pK_{int} values of 6.60 (closed circles) or 6.00 (open circles), according to the static solvent accessibility discrimination described in the text. See Table III.

The results in Tables III-V and in Figure 4 show that for the two sperm whales the computed $pK_{1/2}$ values for residue 12 fit with pK_{obsd} relatively poorly. There is a corresponding

relative discrepancy between $pK_{1/2}$ and pK_{obsd} for residue 81 in these same two cases. These discrepancies may be related to the discrepancy between computed and observed values of δ_a (Table II) mentioned above. Another seemingly systematic discrepancy is in the observed and computed values of the pK for residue 119 in the baleen whale group. On the other hand, it is interesting that the correspondence of $pK_{1/2}$ with pK_{obsd} for residue 36 is good for both of the sperm whale cases, even with the substitution in the dwarf sperm whale myoglobin of the histidine residue for serine at position 35 (Table III). In this comparison, the deprotonation of residue 35 is substantially complete before the range of deprotonation of residue 36 begins. The significant contributions to pK_i involve large numbers of interactions according to eq 1 and, thus, qualitative arguments based on surveys of the relatively closely neighboring groups can be quite misleading.

Besides the procedural adaptations of the present treatment relative to that of Shire et al. (1974a,b, 1975), some important details have been altered here. The first is that the suggestion of Romero-Herrera and Lehmann (1974) that residue 122 in sperm whale myoglobin is aspartic acid rather than asparagine (Edmundson 1965) has been amply confirmed in this laboratory (DiMarchi et al., 1978a).[3] This change has been incorporated by Takano (1977) for the ferrimyoglobin structure. With this introduction of a negatively charged site, it has been possible to abandon the previous assumption of a closely bound anion in the region of residue 45 (Shire et al., 1974a). The second change is that the assignment of histidine-36 as an observable and identifiable residue has been made (Botelho, 1975; Hayes et al., 1975; Botelho and Gurd, 1978), which, in turn, puts aside the tentative assignment of histidine residue 64 as the one with a high pK value (Shire et al., 1974a). We have not been able to observe the resonance of histidine-64 in the present work. However, Wilbur and Allerhand (1977) have observed by ^{13}C NMR a resonance titrating with a pK value of approximately 5 or less that we have tentatively assigned to histidine residue 64. This residue has the SA value for N^{π} of 0.20, and it has been assigned an arbitrary pK_{int} of 5.00, to try to allow for linkage with incipient conformational change (Friend et al., 1977).[4] In this way, residue 64 makes itself felt only at the extreme acid limit of the pH range in question and is effectively treated as masked in the unprotonated form at neutral pH (Breslow and Gurd, 1962). Allowing for such adjustments and the formally more direct procedure for assigning pK_{int} values to the titratable histidine residues, the present treatment encounters little conflict with the implications of the earlier work in which only the assignments of the hemic acid and α-amino group titrations were offered as firm (Shire et al., 1974a,b, 1975).

In addition to histidine residue 64, C-2 proton resonances of residues 24, 82, 93, and 97 have escaped recognition in all cases studied here, either because of the lack of internal mobility or of the proximity to the paramagnetic heme center (Botelho, 1975; Hayes et al., 1975).

The tabulation of Wilbur and Allerhand (1977) and the detailed results of Hayes et al. (1975) show that where comparisons can be drawn between myoglobin species (sperm whale, horse, and man) the observations reported here and in the preceding paper of this issue (Botelho and Gurd, 1978) are in generally satisfactory agreement with those from other laboratories. The scope of the present work and its foundation on earlier chemical-modification studies have complemented the resonance assignments and, hence, provide the basis for an extensive test of the electrostatic interaction analysis. The results of Wilbur and Allerhand (1977) are of particular interest in that the ^{13}C NMR resonances of unprotonated carbons in myoglobin are narrow enough for observation in the absence of internal mobility, so that such restrained side chains as those of histidine residues 24 and 82 are probably observable. Protonations of N^{π} and N^{τ} have opposing chemical-shift effects on the most readily observable ^{13}C resonances of the histidine residues (Reynolds et al., 1973; Deslauriers et al., 1974; Ugurbil et al., 1977; Wilbur and Allerhand, 1977), so that the interpretation of the ^{13}C NMR results is not quite so straightforward as that of the ^{1}H NMR results. However, the combination of the two methods should prove highly informative (Wilbur and Allerhand, 1977).

The present treatment is being applied to the estimation of the summed electrostatic contribution to the stability of many of the myoglobins represented here. The most stable myoglobins toward acid denaturation are those of the two sperm whales, but other contrasts of obvious interest have been drawn between the myoglobin of the minke whale and those of the other baleen whales (Friend et al., 1977). The distinction between the sperm whale and dwarf sperm whale myoglobins, on the one hand, and all the other myoglobins, on the other hand, conflicts with the interesting suggestion of a relationship between the stability of the protein and the weight of the animal (McLendon, 1977). The present work is being extended to the myoglobin of the finback whale whose sequence (DiMarchi et al., 1978b) offers some promising substitutions within the closely related baleen whale group (Jones et al., 1979a). The electrostatic interaction treatment used here has been extended successfully to the significantly different cases of hemoglobin and cytochrome c (Matthew et al., 1978b,c) with the same set of pK_{int} values as are used here.

The results of comparable ^{1}H NMR analyses of various heme complex derivatives of some of these myoglobins and of modification experiments in very concentrated salt solutions (Botelho, 1975) are being prepared for publication.

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[3] A previous report to the contrary was contained in footnote 2 of Shire et al. (1975). The isoionic point of all samples of the main fraction of sperm whale myoglobin studied in this laboratory has been identical (Hardman et al., 1966; Hartzell et al., 1974a) and there is no reason to question the assignment of Romero-Herrera and Lehmann. See also Meuth et al. (1978) and Jones et al. (1979b).

[4] Presumably, histidine-64 must swing outwards from its normal location to become adequately mobile and sufficiently distant from the paramagnetic iron center to be observed by ^{1}H NMR.

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