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Normal and Abnormal Tyrosine Side-Chains in Various Heme Proteins

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This investigation is concerned with the determination of the presence or absence of abnormally ionizing tyrosine groups in myoglobins and hemoglobins. The tyrosine ionization curves of horse and sperm-whale myoglobin and of horse and human hemoglobin were determined by measuring *p*H difference spectra, using the optical density difference at 245 mμ as a measure of the extent of ionization of the tyrosine groups. The following approximate *pK* values can be assigned to the tyrosine groups: in both myoglobins *pK* 10.3 (normal), one group; *pK* 11.5, one group; *pK* > 12.8, one group. In both hemoglobins: *pK* 10.6 (normal), eight groups; *pK* > 12, four groups per molecule. It is suggested that the same structural feature (a hydrogen bond to a peptide CO group) is responsible for the high *pK* (>12) of the same tyrosine side-chain in the peptide chain of myoglobin and in each of the four peptide chains of the hemoglobin molecule.

Now that data are available on the amino acid sequence of hemoglobin (Braunitzer *et al.*, 1961; Konigsberg *et al.*, 1961) and of myoglobin (Edmundson and Hirs, 1961; Kendrew *et al.*, 1961) and on the folding of the chains of the hemoglobin (Perutz *et al.*, 1960) and myoglobin (Kendrew *et al.*, 1960, 1961) molecules, it is of interest to attempt to detect the presence of specific side-chain interactions in these molecules in aqueous solution in order to correlate the results with these structural data.

We have directed our attention to the ionization of the tyrosine side-chains. This problem has not been studied, except recently for human hemoglobin (Vodrážka and Čejka, 1961), presumably because the absorption by heme and by the tryptophan side-chains dwarfs that by tyrosine. We have, therefore, studied the tyrosine ionization by measuring *p*H difference spectra to obtain greater accuracy.

EXPERIMENTAL

Materials.—The horse myoglobin and horse and human hemoglobin used were twice-crystallized commercial products (Mann Laboratories). Crystalline sperm-whale myoglobin was a gift from Prof. J. C. Kendrew. Tyrosine was a Mann product.

Apparatus.—Difference spectra were measured with a Beckman DU spectrophotometer equipped with photomultiplier and thermospacers through which water at 25° was circulated. *p*H was measured in a thermostated vessel (25°) with a Beckman model G *p*H meter standardized at *p*H 6.85 and 9.18 with standard buffers.

Solutions.—Stock solutions of the CO-heme proteins (concentration approximately 1.5 mg/ml) were prepared by adding some $Na_2S_2O_4$ to the aqueous solution, bubbling carbon monoxide through, then deionizing by passing the solution through a short column of Amberlite MB3* resin. One ml of these solutions was then added to 2 ml of buffer solution of adjusted *p*H (with 1 N KOH) to give a final solution containing approximately 0.5 mg/ml protein, 0.33 M potassium phosphate, and 0.33 M potassium carbonate. Concentrations were obtained by dry-weight determinations.

RESULTS

Tyrosine.—The difference spectrum for the ionization of tyrosine has two maxima, one at 295 mμ, the other at 242 mμ. The extinction coefficient of the latter is 5.1 times as large as that of the former (Fig. 1). For the native protein, ribonuclease (Worthington), these extinctions are in the ratio 1:4.2 (not shown), the maxima being at 295 and 244 mμ.

Myoglobins.—The ultraviolet absorption spectrum and the high *p*H difference spectrum of sperm-

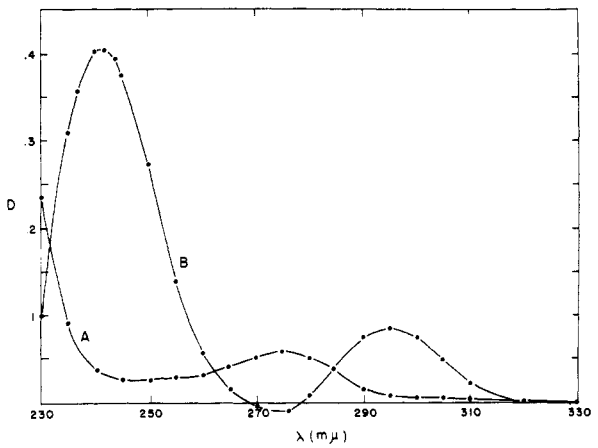

FIG. 1.—Ultraviolet spectrum at *p*H 7 (*A*) and high *p*H difference spectrum for *p*H 12.8 *versus* *p*H 7 (*B*) of tyrosine (3.76×10^{-5}M).

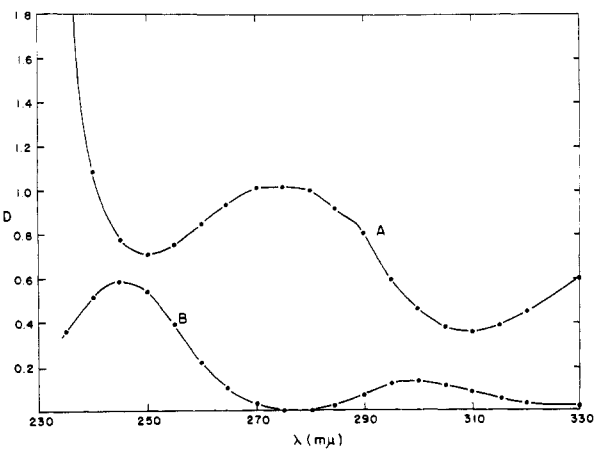

FIG. 2.—Ultraviolet spectrum at *p*H 8.8 (*A*) and high *p*H difference spectrum for *p*H 12.5 *versus* *p*H 8.8 (*B*) of sperm-whale CO-myoglobin (3.09×10^{-5}M).

whale myoglobin are shown in Figure 2. The close similarity between the difference spectrum of this figure and that of Figure 1 assures us that we are dealing in Figure 2 with a tyrosine ionization difference spectrum. The ratio between the maxima is 4.5. Curves for horse myoglobin are very similar.

When these experiments are done at various *p*H values and the values of the optical density difference, which have been converted to values of the molar extinction with use of a molecular weight of 17,800 (Edmundson and Hirs, 1961), are plotted *versus* *p*H, the curve shown in Figure 3 is obtained. It is to be noted that the values obtained for the myoglobins are independent of time at all *p*H levels studied.

Hemoglobins.—Spectra and difference spectra for the two hemoglobins studied are essentially similar to the curves shown in Figure 2. The spectrophotometric titration curves for horse (circles) and human hemoglobin (squares) are shown in Figure 4. Molar extinctions were calculated on the basis of a molecular weight of 64,500, determined on the basis of the amino acid composition (Braunitzer

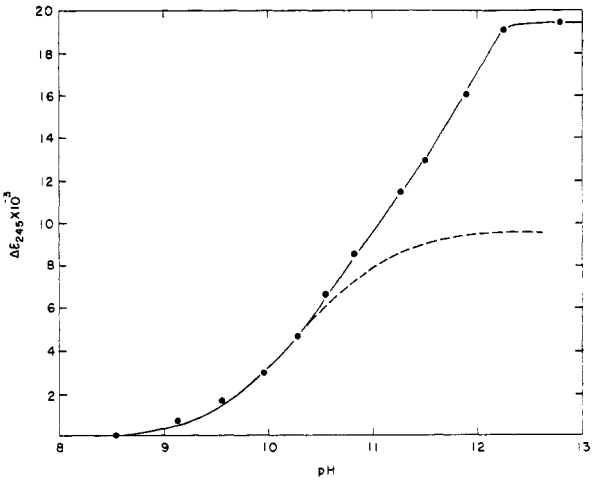

FIG. 3.—Ultraviolet titration curve at 245 mμ of sperm-whale CO-myoglobin. Unbroken curve follows experimental points; the continuous curve terminating in the dashed line is based on equation (1) for ionization of a single group.

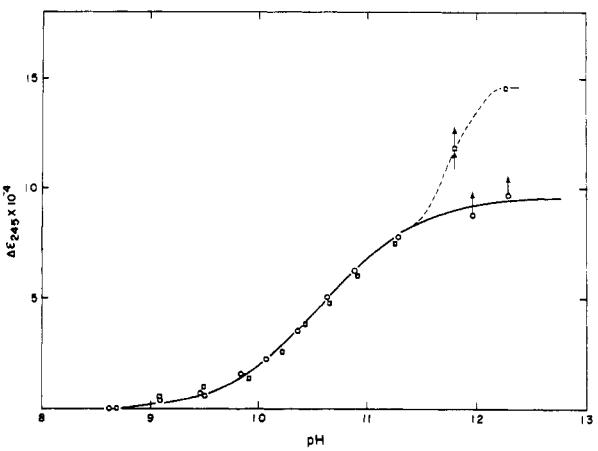

FIG. 4.—Ultraviolet titration curve at 245 mμ of horse CO-hemoglobin (○) and human CO-hemoglobin (□). Unbroken curve is based on equation (1). Dashed part indicates time-dependent measurements. Points with one arrow were obtained by extrapolation to zero time, point with two arrows represents measurements made while the values were changing with time.

et al., 1961). The values of the optical density difference, ΔD, obtained at low *p*H are independent of time. Above *p*H 11.5, however, the values of ΔD increase with time. In the case of horse hemoglobin, the change in ΔD_{245} is so slow that reliable values for zero time can be obtained by extrapolation from a series of rapid measurements started as soon as possible after mixing of the protein stock solution and the buffer. Measurements after *long* periods cannot be interpreted because the ultraviolet spectrum is affected by a concomitant slow change in the heme spectrum from red to green.

In the case of *human* hemoglobin, the increase in ΔD_{245} is much more rapid but the change in the heme spectrum is about as slow as in horse hemoglobin. Thus, it was impossible to obtain values for zero time. On the other hand, the value of

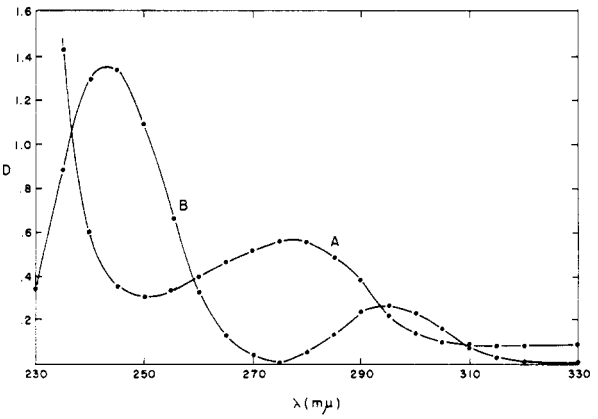

FIG. 5.—Ultraviolet spectrum at *p*H 1.5 (*A*) and ultraviolet difference spectrum, *p*H 12.8 *versus* *p*H 1.3 (*B*) of fully denatured horse globin (1.02×10^{-5} M).

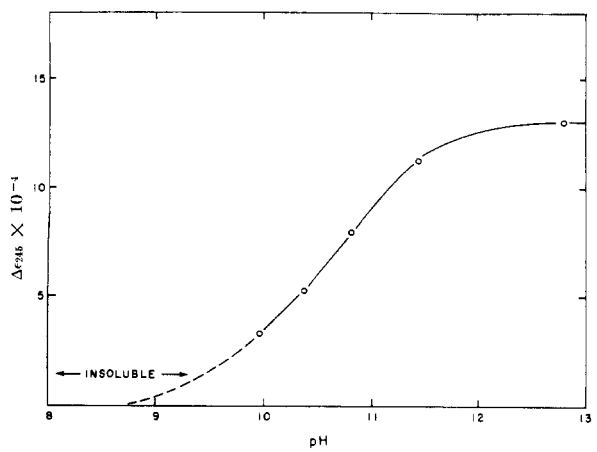

FIG. 6.—Ultraviolet titration curve at 245 mμ of horse globin. Reference solution at *p*H 1.5.

ΔD_{245} at *p*H 12.3 is independent of time as long as the heme spectrum remains unchanged.

Finally, solutions of denatured globin were prepared by precipitating a solution of hemoglobin at *p*H 2 by the addition of acetone and redissolving the precipitate in 0.1 N HCl. Figure 5 shows the spectrum at *p*H 1.3 and the difference spectrum for *p*H 12.8 *versus* *p*H 1.3. In Figure 6 we have shown the data at 245 mμ as a function of *p*H.

DISCUSSION

Tyrosine is a constituent of the heme proteins in the following amounts: human hemoglobin, twelve residues per molecule (Braunitzer *et al.*, 1961); horse hemoglobin, probably twelve (Tristram, 1949); whale myoglobin three (Edmundson and Hirs, 1961). In human hemoglobin, each of the four constituent peptide chains contains three tyrosines.

Globin.—It is reasonable to assume that in denatured globin all twelve tyrosines ionize normally. This gives us a value $(\Delta\epsilon_{245})_{max} = 1.31 \times 10^5/12 = 1.09 \times 10^4$ for horse globin and of 1.19×10^4 for human globin for the change in molar extinction with complete ionization of a single tyrosine group. The value for tyrosine from our own measurements is 1.00×10^4 at 245 mμ, and 2260 at 295 mμ (compared with 2390 in Martin *et al.*, 1958). The values of $\Delta\epsilon_{max}$ per tyrosine group have been listed in Table I.

Figure 6 shows how $\Delta\epsilon_{245}$ varies with *p*H. A smooth titration curve is obtained, and the values of $\Delta\epsilon$ appear to approach zero in the neutral *p*H range, where measurments are impossible because of the insolubility of the denatured globin.

Myoglobin.—In myoglobin, $(\Delta\epsilon_{245})_{max} = 1.94 \times 10^4$. Clearly, only the assumption that two tyrosines are ionizing in the *p*H range studied can give us a value of $\Delta\epsilon_{max}$ per tyrosine (namely 0.97×10^4) in reasonable agreement with the one derived

TABLE I

VALUES OF THE CHANGE IN MOLAR EXTINCTION MEASURED AT 245 MΜ FOR THE COMPLETE IONIZATION OF ONE TYROSINE SIDE-CHAIN PER MOLECULE IN VARIOUS HEME PROTEINS AND FOR TYROSINE[a]

Protein	No. of Tyrosines Assumed Ionizing per Molecule	$(\Delta\epsilon_{245})_{max}$ per Tyrosine
Horse globin	12	10,900
Human globin	12	11,900
Sperm-whale myoglobin	2	9,700
Horse myoglobin	2	10,100
Human hemoglobin	12	12,100
Horse hemoglobin	8	12,100
Tyrosine	1	10,000

[a] We do not know why these values differ by 20% from protein to protein. However, differences in $(\Delta\epsilon_{295})_{max}$ also have been noted from protein to protein (Tanford *et al.*, 1955).

for tyrosine. Since there are three tyrosine groups per molecule of myoglobin, this leads to the conclusion that one tyrosine group in myoglobin has $pK > 12.8$.

In order to evaluate the data at $pH < 12$, we have drawn a curve in Figure 3 (shown as a dashed line where it differs from the observed data), according to the following equation (Hermans *et al.*, 1960):

$$pH - \log [\Delta\epsilon/(\Delta\epsilon_{max} - \Delta\epsilon)] = pK \quad (1)$$

Here $\Delta\epsilon_{max}$ and pK are adjustable parameters, chosen so as to provide the best fit with the experimental data. We have, however, restricted our choice of $\Delta\epsilon_{max}$ to values which are equal to the observed $\Delta\epsilon_{max}$ or to fractions thereof corresponding to an integral number of ionizing tyrosine groups. With this restriction, the experimental data of Figure 3 can best be fitted to a curve based on equation (1) with $pK = 10.3$ and with $\Delta\epsilon_{max}$ equal to one half of that observed (at *p*H 12.8). The fit extends over the *p*H range below 10.5. Apparently, only one tyrosine group has a normal pK value (namely, 10.3). Subtraction of the dashed curve in Figure 3 from the experimental points provides data which *cannot* be fitted with a curve based on equation (1). The second tyrosine group is one half ionized at a *p*H level of about 11.6 and is, therefore, somewhat abnormal. The third

tyrosine group was seen not to ionize below *p*H 12.8, and is highly abnormal.

Horse Hemoglobin.—The data for horse hemoglobin (Fig. 4) attain a value of $\Delta\epsilon_{245}$ at high *p*H, which corresponds to the complete ionization of eight tyrosine groups (see Table I; for the hemoglobins, odd numbers of tyrosine groups have not been considered).

We have noted an increase in the values of ΔD of horse hemoglobin solutions above *p*H 11.5. In our opinion, this means that four tyrosine side-chains are abnormal in each horse hemoglobin molecule, but that at high *p*H the molecule is slowly unfolding with a concurrent liberation of abnormal tyrosine groups, which then also ionize.

A curve according to equation (1) with $\Delta\epsilon_{max}$ equal to that observed fits the data for horse hemoglobin quite well (Fig. 4). The eight readily ionizable tyrosines thus appear normal, with a pK_{obs} of about 10.6 (this value is high, presumably because of the appreciable net negative charge carried by the molecule at this *p*H).

Human Hemoglobin.—Turning to the data for human hemoglobin, we see that at *p*H <11.3, the data coincide with those for horse hemoglobin but that, above *p*H 11.3, $\Delta\epsilon_{245}$ attains much higher values. However, this means only that the unfolding which releases the abnormal tyrosines is much more rapid than in horse hemoglobin—so rapid that at *p*H 11.8 extrapolation to zero time is impossible and at *p*H 12.3 the readings are constant over the first few minutes of our measurements (changes in the heme spectrum being negligible in this period). Therefore, human hemoglobin has eight normal and four abnormal tyrosine side-chains, just as does horse hemoglobin.[1] We would, in fact, go so far as to postulate that a tyrosine side-chain with abnormally high pK is present in each peptide chain, be it of myoglobin or of hemoglobin, and reflects a structural feature which is identical in each chain. According to Kendrew's x-ray data (Kendrew, private communication), this would be a hydrogen bond, with the tyrosine hydroxyl group serving as donor perhaps to a peptide C = O group in an α-helical part of the polypeptide chain several amino acid residues removed but close in space because of the specific folding. Since the chain folding is common to the four chains of hemoglobin and that of myoglobin (Perutz *et al.*, 1960), and since the particular tyrosine residue discussed by Kendrew occupies the same position in each chain (Watson and Kendrew, 1961),[2] our postulate has a firm basis.

Finally, it will be interesting to see if the x-ray studies of whale myoglobin show that a tyrosine residue other than the one discussed above is also involved in some kind of specific interaction which could alter its ionization constant to a value of 11.6.

[1] Vodrážka and Čejka (1961) concluded that there are no abnormal tyrosine residues in human hemoglobin. Since these authors measured not difference spectra but direct spectra and since they studied the optical density not at 245 mμ but at 295 mμ, their data show much more scatter. In fact, if their data of Figure 6 are plotted in our Figure 4 (using an adequate conversion factor), the points are as far removed from our curve as could be expected by allowing for the large scatter of their data. However, our data show less scatter and cannot conceivably fit their proposed curve. Therefore, we feel confident in our interpretation, especially in the light of the results with horse hemoglobin.

NOTE ADDED IN PROOF: Experiments recently performed by the author (unpublished) indicate that in human CO-hemoglobin the tyrosine groups proposed as the abnormal ones[2] are normal. These experiments are still incomplete, and their results will be submitted for publication as soon as possible.

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[2] This position is: one amino acid residue before the carboxyl terminus in both the α- and β-chains of human hemoglobin, and eight residues from the carboxyl terminus in whale myoglobin, the latter molecule having six "extra" amino acids at this end of the chain (Watson and Kendrew, 1961).