977. *Reactivity Differences between Hæmoglobins. Part IV.*[1] *The Thermodynamics of Ionization of Some Vertebrate Hæmoglobins.*

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The ionization of eleven vertebrate methæmoglobins to their alkaline forms has been measured at various ionic strengths and temperatures. Large variations are observed in the enthalpy and entropy of ionization, but the free energy of ionization varies only slightly, owing to compensating changes in ΔH and $T\Delta S$. It is concluded that the variations in ΔH and $T\Delta S$ are due to variations in electrostatic interactions originating from different charge configurations in each of the hæmoglobins. This conclusion is supported by the effect of ionic strength on the pK of ionization.

As early as 1928 Barcroft [2] drew attention to the fact that different vertebrate hæmoglobins have different affinities for oxygen and ascribed this to the differences in protein composition. The possible mechanisms by which changes in globin structure can alter the reactivity of a hæmoglobin at its iron atom have been discussed by us in Part I.[3] However, the problem remains of finding criteria by which the importance of each mechanism can be assessed. One such criterion is suggested from the analysis of the thermodynamics of ionization of the human methæmoglobins A, S, and C to their alkaline forms,[3,4] and it is the application of this criterion to the ionization of twelve other vertebrate hæmoglobins that concerns us in this Paper.

In Part I we concluded that the different entropies and enthalpies of ionization of human methæmoglobins A, S, and C may be ascribed to different electrostatic interactions in each hæmoglobin. In Part II [4] we employed Kirkwood's [5] and Tanford and Kirkwood's [6] theory of electrostatic interactions in globular proteins to show that the observed linearity of the plot of $T\Delta S°$ against $\Delta H°$ is consistent with this conclusion. In Part III [1] we extended the theoretical treatment given in Part II to include hæmoglobins which differ considerably in their charged amino-acids, and we showed that if for a series of methæmoglobins the plot of $T\Delta S$ against ΔH of ionization is linear, and the points for human methæmoglobins A, S, and C fall on the line, then: (i) the differences between the entropies and enthalpies of ionization are entirely electrostatic in origin; and (ii) the charge changes which differentiate the hæmoglobins do not occur at positions closer than 10 Å to the iron atoms.

In the present study we have measured the ionization of eleven vertebrate hæmoglobins at different temperatures and ionic strengths. We shall discuss these data in relation to the ideas developed in Parts II and III.

The Variation of pK_3' *with Temperature at Low Ionic Strength.*—Adopting the same nomenclature as in Part I, the ionization of a methæmoglobin to its alkaline form is represented by the equation

$$Pr^{2-}Fe^{+}(H_2O) \rightleftharpoons Pr^{2-}Fe^{+}\cdot OH^{-} + H^{+} \qquad K_3'$$

where $Pr^{2-}Fe^{+}(H_2O)$ and $Pr^{2-}Fe^{+}\cdot OH^{-}$ are the acidic and alkaline forms respectively of methæmoglobin, and K_3' is the ionization constant at finite ionic strength.

The measurement of pK_3' for the methæmoglobins was made at different temperatures at ionic strength, $I = 0{\cdot}05$. This value of I was chosen for two reasons: (1) The desirability of working at ionic strengths low enough to make valid the simplification to the

[1] Part III, Beetlestone and Irvine, preceding Paper.
[2] Barcroft, "The Respiratory Function of the Blood. Part II. Hæmoglobin," Cambridge University Press, London, 1928, ch. 5.
[3] Beetlestone and Irvine, *Proc. Roy. Soc.*, 1964, *A*, **277**, 401.
[4] Beetlestone and Irvine, *Proc. Roy. Soc.*, 1964, *A*, **277**, 414.
[5] Kirkwood, *J. Chem. Phys.*, 1934, **2**, 351.
[6] Tanford and Kirkwood, *J. Amer. Chem. Soc.*, 1957, **79**, 5333.

Kirkwood theory used in Part III. (2) The accuracy of the determination of pK_3' decreases below $I = 0{\cdot}05$ owing to small amounts of the ionizations characterized by pK_1' and pK_2' (see Part I).

The lowest ionic strength at which the effects of pK_1' and pK_2' on the spectrum are negligible was therefore chosen, namely $I = 0{\cdot}05$. The data are summarized in Table 1. We determined ΔH in the usual way from the plot of pK_3' against $1/T$. For all the hæmoglobins we obtained good linear plots, some of which are shown as examples in Fig. 1.

TABLE 1.

Values of pK_3' at various temperatures and $I = 0{\cdot}0506$.

Species	pK_3' ($I = 0{\cdot}0506$)			ΔG	ΔH	$T\Delta S$
Mouse (1)	8·273	8·148	8·000	10,726	7140	3590
	(5·7)	(12·1)	(20·0)			
Rat (2)	8·174	8·095	8·036	10,766	3340	7430
	(4·2)	(12·2)	(20·0)			
Baboon (3)	8·290	8·220	8·112	10,877	4660	6220
	(6·0)	(12·0)	(20·0)			
Patas monkey (4)	8·335	8·236	8·130	10,901	5180	5720
	(5·2)	(12·2)	(20·0)			
Mona monkey (5)	8·362	8·268	8·163	10,945	4930	6025
	(5·2)	(11·7)	(20·0)			
Pig (6)	8·484	8·396	8·211	11,009	7870	3140
	(6·7)	(11·8)	(19·7)			
Dog (7)	8·508	8·403	8·266	11,083	6090	4990
	(5·3)	(12·4)	(20·0)			
Hyaena (8)	8·490	8·415	8·295	11,122	5500	5620
	(6·8)	(12·1)	(20·0)			
Tantalus monkey (9) ...	8·535	8·420	8·300	11,129	5650	5480
	(4·5)	(11·4)	(20·0)			
Cat (10)	8·468	8·388	8·301	11,130	4350	6780
	(5·5)	(11·4)	(20·0)			
Pigeon (11)	8·602	8·494	8·290	11,115	9160	1960
	(7·0)	(11·9)	(20·0)			
Horse [7] (12)				11,276	3660	7620
Human A[3] (13)				10,914	3400	7510
Human S[3] (14)				10,986	4090	6900
Human C[3] (15)				10,928	4880	6050

The temperature at which each pK_3' was measured is indicated in parentheses beneath the value of the pK'_3. The values of ΔG, ΔH, and $T\Delta S$ are at 20·0°. The number in parentheses after each animal name gives the key to the points on the graphs in all the Figures. All values of pK_3' are subject to an error of between $\pm 0{\cdot}01$ and $\pm 0{\cdot}02$.

$T\Delta S$ was calculated from the equation $\Delta G = \Delta H - T\Delta S$, using the values of ΔG and ΔH at $I = 0{\cdot}05$. We did not attempt to calculate $T\Delta S°$, the value of $T\Delta S$ at $I = 0$, as is the usual practice. In the latter procedure $\Delta G°$ is obtained from $pK°$, the value of pK extrapolated to zero ionic strength. ΔH measured at some finite ionic strength is then assumed equal to $\Delta H°$, the value of ΔH at $I = 0$, and then $T\Delta S° = \Delta H° - \Delta G°$. However, the assumption that ΔH ($I = 0{\cdot}05$) is equal to $\Delta H°$ is not valid in these systems, since D_E and $\partial(1/D_E)/\partial T$, and consequently ΔH, are functions of ionic strength. This is borne out by data at high ionic strength (unpublished results) which show ΔH values significantly different from those at $I = 0{\cdot}05$.

There are several outstanding features of the data in Table 1 and these will be considered separately. First, there is the variation in ΔG as compared with that in ΔH and in $T\Delta S$. The maximum variation in ΔG is only 400 cal. mole^{-1}, whereas the maximum variation in both ΔH and $T\Delta S$ is 6000 cal. mole^{-1}. This is a consequence of the almost exact compensation of changes in ΔH and $T\Delta S$, which is clearly demonstrated in Fig. 2, where $T\Delta S$ is plotted against ΔH. The slope of the line, as determined by a least-squares analysis, is $0{\cdot}995 \pm 0{\cdot}025$. A slope of unity corresponds to exact compensation of changes

[7] George and Hanania, *Biochem. J.*, 1953, **55**, 236.

in ΔH and $T\Delta S$. The linearity of this plot, and the fact that its slope is close to unity, are both of interest, and we shall deal with these two points in turn.

Following the argument given in Part III, we conclude from the linearity of the plot of $T\Delta S$ against ΔH, and the fact that the points for methæmoglobins A, S, and C fall on the line, that (i) the differences between the $T\Delta S$'s and ΔH's of ionization are due to differences in electrostatic interactions in each of the hæmoglobins arising as a result of different charge distributions in each case; (ii) the charge changes which differentiate the hæmoglobins do not occur at positions closer than 10 Å to the iron atoms. It seems reasonable from these observations to suggest that within the pH region 7·8—9·2, where the pK measurements are made, there is a charge configuration within 10 Å of each of the iron atoms that is characteristic of a hæmoglobin, and that variations in charged amino-acids

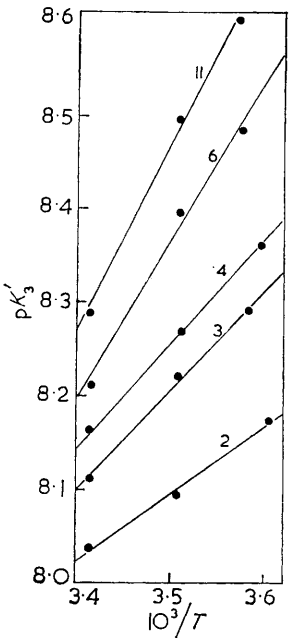

FIG. 1. Examples of the plots of pK_3' against $1/T$ used to determine ΔH. The numbers on the graph refer to the key given in Table 1.

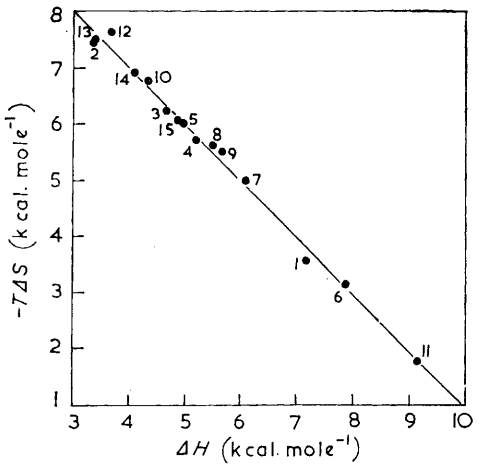

FIG. 2. $T\Delta S$ against ΔH, for the hæmoglobins in Table 1.

in this region would lead to drastic modification of the properties of hæmoglobin, for instance in the oxygen-binding capacity.

These conclusions depend only on the fact that the plot of $T\Delta S$ against ΔH is linear, and do not depend on the slope. This will depend on the ionic strength. Using the experimentally determined m_0 for human methæmoglobins A, S, and C, calculation shows [1] that $\bar{m}_0 = 1{\cdot}16$ and $\bar{m}_{0{\cdot}05} = 1{\cdot}08$. This is to be compared with the value of $\bar{m}_{0{\cdot}05} = 0{\cdot}995 \pm 0{\cdot}25$ obtained in this work. The discrepancy between the calculated and experimental values of $\bar{m}_{0{\cdot}05}$ may be attributed to either: (a) the assumption that the ΔH's of ionization at $I = 0{\cdot}05$ for human methæmoglobins A, S, and C are equal to the ΔH's at $I = 0$, or (b) the assumption that simplifications made to the Kirkwood equations are strictly valid at $I = 0{\cdot}05$.

A calculation of $\bar{m}_{0{\cdot}05}$ or $\bar{m}_0$ from a knowledge of the dielectric constant of water and its temperature coefficient is not possible because, as Westheimer and Kirkwood [8] pointed out,

[8] Westheimer and Kirkwood, *Trans. Faraday Soc.*, 1947, **43**, 77.

the Kirkwood theory does not take into account electro-restriction of the solvent near charges. This limitation, in relation to the data on the ionization of human methæmoglobins A, S, and C, has been discussed by us in Part II. The question arises, however, as to whether the slope of the line in Fig. 2 is characteristic of hæmoglobins, or is characteristic of water as a solvent for reactions involving charged species. Data on the ionization of the bound water molecule in factor B of vitamin B_{12} compared with those of the similar ionization in the mono-acid derivative, Factor V_{1A}, favour the latter alternative (unpublished results). A preliminary survey of the available data on the association of small ions also confirms the view that $m \simeq 1$ is characteristic of water as a solvent for charged species. This argument will be developed in a later Paper.

We have mentioned earlier that the variation in ΔH for the series of hæmoglobins is of the order of 6000 cal. mole^{-1}. This large variation of ΔH between different hæmoglobins has further significance. It is presumed in this study that the ionizing group is the same for all the hæmoglobins, the closely similar spectra of all the acid forms and all the alkaline forms making this a reasonable assumption. Thus we have the situation of a particular group (presumed to be the water bonded to the iron atom) ionizing with an enthalpy of ionization that varies between 3340 and 9160 cal. mole^{-1}, depending on the charge environment. This observation shows the danger of attempting to identify ionizable groups in a protein solely by its enthalpy of ionization. Other workers [9,10] have also demonstrated the effect of environment on the thermodynamics of an ionizable group, and our present study confirms their observations.

Effect of Ionic Strength.—In Table 2 are recorded values of pK_3' for the different

TABLE 2.

Values of pK_3' at various ionic strengths at 20·0°.

	Ionic strength, I							$\Delta G^\circ_{20^\circ}$
	0·0055	0·00928	0·0132	0·0174	0·00258	$-q$	pK_3	cal. mole^{-1}
Mouse	8·491	8·388			8·193	16·6 ± 0·8	9·22 ± 0·05	12,362
Rat	8·381	8·328	8·220		8·120	13·1 ± 0·5	8·98 ± 0·02	12,040
			(I = 0·0141)					
Baboon	8·417	8·357			8·222	11·1 ± 0·9	8·93 ± 0·04	11,973
Patas monkey ...	8·560		8·348	8·315	8·250	13·3 ± 0·9	9·06 ± 0·05	12,148
(I = 0·0031)								
Mona monkey ...	8·350		8·295	8·265	8·230	6·5 ± 0·4	8·66 ± 0·03	11,611
Pig	8·650		8·478	8·450	8·316	15·1 ± 2·4	9·37 ± 0·07	12,563
Dog	8·572	8·500		8·407	8·356	10·3 ± 0·4	9·02 ± 0·02	12,094
		(I = 0·00716)						
Hyaena	8·682	8·583			8·435	13·8 ± 0·5	9·16 ± 0·03	12,282
Tantalus monkey	8·564	8·484			8·392	9·1 ± 0·4	8·98 ± 0·02	12,040
Cat	8·630	8·565			8·405	11·7 ± 0·6	9·03 ± 0·03	12,107
Pigeon	8·364	8·330			8·320	1·8 ± 0·6	8·48 ± 0·03	11,370
Horse [7]						7·8 ± 0·4	9·01 ± 0·02	12,082
Human [3] A						9·0 ± 1·0	8·81 ± 0·05	11,812
Human [3] S						7·5 ± 1·2	8·73 ± 0·06	11,709
Human [3] C						6·8 ± 0·5	8·66 ± 0·05	11,611

The values of pK_3' were obtained at the ionic strength indicated at the top of each column except where a different ionic strength is indicated in parentheses beneath the pK_3' value. Values of pK_3 and q, the total charge on the protein, were obtained from plots of pK_3' against f(I). All values of pK_3' are subject to an error of between ±0·01 and ±0·02.

hæmoglobins at various ionic strengths between 0·026 and 0·0055. We obtained pK_3, the value of pK_3' at zero ionic strength, by extrapolating to zero ionic strength the plot of pK_3' against f(I) = $I^{\frac{1}{2}}/(1 + 10\ I^{\frac{1}{2}})$. The choice of this function for the hæmoglobin molecule has been discussed in Part I. The net charge, q, of a methæmoglobin in its acidic form is obtained from the graph by using the expression $q = (S + A)/2A$ (see Part I), where S is the slope of the f(I) plot, and A is the Debye–Hückel coefficient. Values of q, pK_3, and ΔG° for the different hæmoglobins are also recorded in Table 2.

[9] Hanania and Irvine, *J.*, 1962, 2745.
[10] Hanania and Irvine, *J.*, 1962, 2750.

Earlier in this Paper we concluded that the different thermodynamics of ionization of the various methæmoglobins originate from different charge effects. The different effects are now seen to be the result of differences in the number as well as in the configuration of the charges. For the human methæmoglobins A and S the difference between their free-energies of ionization (see Part II) is given by the equation

$$\Delta G_A^\circ - \Delta G_S^\circ = \frac{2N}{4{\cdot}2 \times 10^7} \frac{e_q e_f}{r_{f,q} D_E} \text{ cal. mole}^{-1} \qquad (2)$$

where e_q, e_f, $r_{f,q}$, and D_E have been defined in Part I. In general, for any hæmoglobin this equation is replaced by

$$\Delta G_A^\circ - \Delta G_x^\circ = \frac{N}{4{\cdot}2 \times 10^7} \sum_{l=1}^{l=n} \frac{e_l e_f}{r_{l,f} D_{E(e,f)}} \text{ cal. mole}^{-1} \qquad (3)$$

where $r_{l,f}$ and $D_{E(e,f)}$ are dependent on the relative positions of e_l and e_f and are defined in Part III. If a hæmoglobin differs from hæmoglobin A by n charges, and these occur at

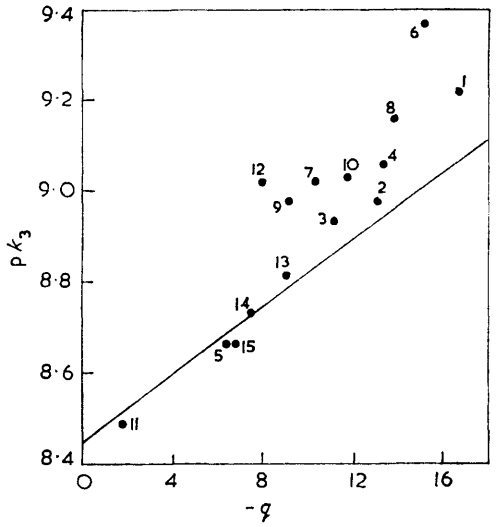

FIG. 3. Plot of pK_3 against q for the fifteen hæmoglobins in Fig. 2. The line is the "theoretical" line of slope 0·0375 drawn through the points for human hæmoglobins A, S, and C.

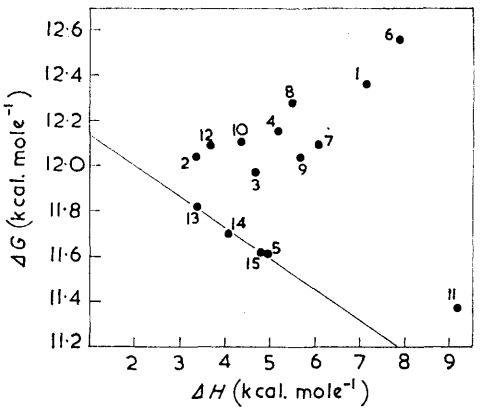

FIG. 4. Plot of ΔG° against ΔH for the fifteen hæmoglobins in Fig. 2. The line is drawn through the points for human hæmoglobins A, S, and C.

positions such that for all these charges $r_{l,f} = r_{f,q}$ and $D_{E(e,f)} = D_E$, then equation (3) becomes

$$\Delta G_A^\circ - \Delta G_x^\circ = \frac{nN}{4{\cdot}2 \times 10^7} \frac{e_q e_f}{r_{f,q} D_E} \text{ cal. mole.}^{-1} \qquad (4)$$

Hence for hæmoglobins of this type a plot of ΔG° (or pK_3) against q should be linear. Fig. 3 shows such a plot for the various vertebrate hæmoglobins. A "theoretical" line has been drawn through the points for hæmoglobins A, S, and C, the slope of which is based on the measured pK_3 values and the known charge differences between hæmoglobins A, S, and C. The plotted points for hæmoglobins A, S, and C are those obtained when the value of q is derived from the plot of pK_3 against f(I), and, within experimental error, they fall on the "theoretical" line. The hæmoglobins of rat, mona monkey, and pigeon, within experimental error, also fall on the line, indicating that the charge changes which differentiate

them from hæmoglobin A are in similar positions to those which differentiate hæmoglobins S and C from A. That is to say, they are remote from all of the iron atoms.

All the other hæmoglobins are seen to fall above the line. This is a consequence of two factors. Firstly, the charge changes in hæmoglobins A, S, and C occur at positions remote from the four iron atoms, and hence for any hæmoglobin for which equation (3) rather than equation (4) is applicable, all the $r_{l,f}$'s must be less than $r_{f,q}$. This also implies a smaller value of D_E. Secondly, all the other hæmoglobins are more negatively charged than A, S, or C. Thus the value of $\Delta G_A^\circ - \Delta G_S^\circ$ for these hæmoglobins must be greater than that for a hæmoglobin with the same number of charge changes but at distance $r_{f,q}$. Hence, in the plot of pK_3 versus q, no points are found significantly below the line. There is an interesting corollary to the above, namely, that for a point to fall below the line the charge changes must occur nearer to the iron atom than those in A, S, and C and must be such as to make the net charge on the hæmoglobin more positive than on A.

The linearity of the plot of $T\Delta S$ against ΔH seems to imply that a plot of ΔG against ΔH should also be linear. However, this is not strictly true, as is evident from an inspection of Table 1 of Part II. This shows that whereas m_0 (the slope of the plot of $T\Delta S^\circ$ against ΔH°) is constant to within $\pm 5\%$ for $45^\circ < \theta < 180^\circ$, m_0' (the slope of the graph of ΔG° against ΔH°, which is equal to $1 - m_0$) varies by a factor of 2. Hence we cannot define an average m_0' that will remain constant for a series of hæmoglobins which differ by charges at many positions in the molecule. The large variation in m_0', relative to that in m_0, is a consequence of the value of m_0 being close to unity, and this in turn is due to the relative values of D_E and $\partial(1/D_E)/\partial T$, which are themselves functions of θ. It is apparent that a plot of ΔG° against ΔH° will be linear only for hæmoglobins where D_E and $\partial(1/D_E)/\partial T$ are strictly constant, and this situation is most closely approximated to when the charge changes which differentiate the hæmoglobins occur at the same point in the molecule. Thus the graph of ΔG° against ΔH° is linear for the human methæmoglobins A, S, and C.

To summarize, we conclude that the differences in the thermodynamics of ionization of the fifteen methæmoglobins studied are due to differences in the charge configuration on the globins, which arise from the various amino-acid replacements.

The question now arises whether these conclusions also apply to: (1) the reaction of methæmoglobins with other ligands over the entire pH range in which hæmoglobin is stable; (2) the ionization of hæmoglobins of animals classified as fish, amphibia, or reptiles; (3) the reactions of reduced hæmoglobin, in particular the oxygenation reaction. Experiments designed to answer these three questions are in progress.

Experimental

Methods.—The procedure described in Part I was followed for determinations of pK_3', except that a Pye Dynacap pH meter was used for the pH measurements. The manual temperature control was used.

Material.—The hæmoglobins were prepared by the method described in Part I. The animals have been referred to in the text by their common names. More precise names are as follows: Rat—*Wistar* strain *albino*; baboon—*Papio anubis*; patas monkey—*Erythrocebus patas*; tantalus monkey—*Cercopithecus aethiops*; mona monkey—*Cercopithecus mona*; pigeon—*Columbia livia*. Dog, pig, and cat were specimens of unknown types of domestic species. Mouse was from an unknown strain of laboratory mouse. Mouse and rat hæmoglobin was obtained from pooled samples, and the remainder from single animals. No tests were performed to establish the homogeneity of the samples. However the presence of more than one component in any hæmoglobin does not invalidate in any way the conclusions drawn in this Paper, since the measured pK_3''s are an average of those for the pure components.

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