595. *Reactivity Differences between Hæmoglobins. Part V.*[1] *Further Studies on the Ionisation of Methæmoglobins*

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The ionisation of eleven vertebrate methæmoglobins to their alkaline form have been measured at various ionic strengths and temperatures. Large variations are observed in the enthalpy and entropy of ionisation, but the free energy of ionisation 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 interaction originating from different charge configurations on each of the hæmoglobins. Values of the average net charge on each of the methæmoglobins in the pH range 8—9 are obtained from the variation of the ionisation constants with ionic strength. A good correlation is obtained between the charges obtained by this method and those obtained from titration curves.

IN Part IV [1] of this Series we reported data on the ionisation to their alkaline forms of eleven vertebrate methæmoglobins. For these methæmoglobins there are large variations in ΔH and $T\Delta S$ of ionisation which nearly compensate each other, such that the free energies of ionisation vary only slightly. These variations in ΔH and $T\Delta S$ were attributed to different electrostatic interactions arising from the different charge configurations of the hæmoglobins.

In order to test the generality of our conclusions we have extended the ionisation studies to include the methæmoglobins of some species of birds, and of cow, guinea pig, rabbit, bat, shrew, and lizard. An investigation of the thermodynamics of ionisation of a number of methæmoglobins at high ionic strength has also been carried out, and the implications of the results are discussed.

Throughout this Series of Papers we have extrapolated to zero ionic strength values of the ionisation constant of methæmoglobin at finite ionic strengths (pK_3') using an extrapolation function derived from Kirkwood's expression for the activity coefficient of a spherical protein molecule.[2] The validity of this procedure can be tested, for, as shown Part I,[3] values of the net charge on the protein at a given pH can be obtained from the

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[1] Part IV, J. G. Beetlestone and D. H. Irvine, *J.*, 1964, 5090.

[2] J. G. Kirkwood, *J. Chem. Phys.*, 1934, **2**, 351.

[3] J. G. Beetlestone and D. H. Irvine, *Proc. Roy. Soc.*, 1964, *A*, **277**, 401.

slope of the plot of pK_3' against the ionic strength function. These values may then be compared with those obtained directly from acid–base titration curves. We now report a comparison of the values for the net charge at pH 8·5 on seven methæmoglobins as determined by the two methods.

Ionisation Studies at Low Ionic Strength.—Tables 1 and 2 summarise the results of investigation on eleven methæmoglobins, and Figure 1 shows how closely the new data follow the plot of $T\Delta S$ against ΔH obtained in earlier studies. Thus, following the arguments given in Parts III and IV, we conclude that the origin of the variations in ΔH and $T\Delta S$ of ionisation for these methæmoglobins is also the result of different electrostatic interaction arising from different charge configurations.

TABLE 1

Values of pK_3' at various temperatures and I = 0·0506

Species	pK_3' (I = 0·0506)			ΔG (cal. mole^{-1})	ΔH (cal. mole^{-1})	$-T\Delta S$ (cal. mole^{-1})
Guinea pig (16)	8·460(4·4)	8·330(12·5)	8·213(20·0)	11,012	5860	5150
Lizard (17)	8·404(6·8)	8·338(12·3)	8·260(20·0)	11,075	4120	6960
Bat (18)	8·610(6·05)	8·455(12·3)	8·288(20·0)	11,113	8340	2770
Shrew (19)	8·470(12·0)	8·280(20·0)	8·098(26·9)	11,102	10,220	880
Duck (20)	8·500(6·2)	8·395(11·5)	8·260(20·0)	11,075	6550	4530
Turkey (21)	8·508(6·0)	8·380(11·5)	8·204(20·0)	11,000	8110	2890
Chicken (22)	8·490(6·4)	8·390(11·5)	8·247(20·0)	11,058	6650	4410
Guinea fowl (23)	8·385(12·7)	8·244(20·0)	8·124(27·7)	11,054	6830	4220
Cow (24)	8·259(12·6)	8·230(20·0)	8·203(27·3)	11,035	1570	9460
Mangabey monkey (25)	8·180(12·8)	8·090(20·0)	8·025(27·3)	10,847	4120	6730
Rabbit (26)...............	8·064(12·45)	8·020(20·0)	8·000(27·3)	10,753	1760	8990

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

An interesting feature of Figure 1 is the positions of the different methæmoglobins on the line which, except in a few cases, bear no correlation with the net charges of the methæmoglobins. For the human methæmoglobins A, S, and C, a correlation is observed, and this is expected since the charge changes which differentiate these hæmoglobins occur at the same site in the molecule. The site at which these charge changes occur are as remote as possible from the iron atoms,[4] and this fact, together with the known charge changes and the positions of these hæmoglobins on the line in Figure 1, may be used to

TABLE 2

Values of pK'_3 at various ionic strengths at 20°

	Ionic strength, I					
	0·0055M	0·0092M	0·0258M	$-q$	pK_3'	ΔG_{20° (cal. mole^{-1})
Guinea pig	8·630	8·500	8·354	14·0	9·22	12,364
Lizard	8·500	8·420	8·317	7·6	8·81	11,818
Bat	8·373	8·333	8·296	2·4	8·48	11,369
Shrew	8·395	8·375	8·310	4·6	8·64	11,586
Duck	8·432	8·413	8·300	6·2	8·72	11,686
Turkey	8·300	8·286	8·235	3·1	8·45	11,330
Chicken	8·452	8·397	8·305	6·6	8·74	11,712
Guinea fowl	8·438	8·397	8·332	6·3	8·72	11,689
Cow	8·662	8·564	8·358	14·3	9·11	12,213
Mangabey monkey ...	8·365	8·313	8·152	8·9	8·73	11,708
Rabbit.....................	8·205	8·169	8·058	6·4	8·49	11,382

Values of pK_3' and q were obtained from pK_3' versus f(I) plots

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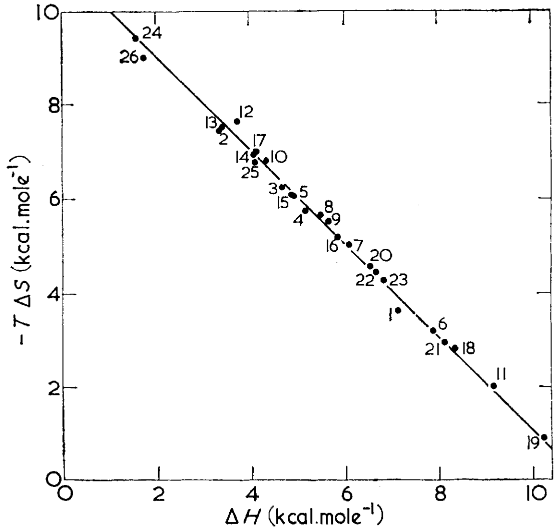
[4] A. F. Cullis, H. Muirhead, M. F. Perutz, M. G. Rossman, and A. C. T. North, *Proc. Roy. Soc.*, 1962, *A*, **264**, 161.

derive information about the charge distribution of the other methæmoglobins. To take but one example. The position of pig hæmoglobin (6) is anomalous in relation to hæmoglobins A and C ($q = -9{\cdot}0$ and $-6{\cdot}8$, respectively), since the net charge on pig methæmoglobin ($q = -15{\cdot}1$) is considerably more negative than that on human methæmoglobin A, and, other things being equal, this hæmoglobin might have been expected to fall on the line on the opposite side of A to C. This suggests that in pig hæmoglobin both negative and positive charge substitutions occur, the fomer predominating, but the latter occurring on the average much closer to the iron atoms so as to produce a net electrostatic effect corresponding to positive charge substitutions relative to hæmoglobin A. More detailed analysis of this type must be deferred until the net charges in the methæmoglobins are firmly established, although the values quoted in Table 2 are unlikely to be seriously in error, as is shown later in this Paper where the net charges on a few hæmoglobins, as obtained from ionisation studies, are compared with those from acid–base titration studies.

FIGURE 1. $T\Delta S$ against ΔH at $I = 0{\cdot}05$ for the hæmoglobins in Table 1, together with those in Table 2 of Part IV [1]



Ionisation Studies at High Ionic Strength.—In Part I [3] we showed that, at high ionic strength, the differences between the free-energies and enthalpies of ionisation of methæmoglobins A, S, and C become less than experimental error, and we attributed this to the screening of the long-range electrostatic interactions which gave rise to the observed differences at low ionic strength. It is of interest to consider to what extent the differences between the ionisation behaviour of methæmoglobins of different species are screened out at high ionic strength.

Table 3 summarises the results of ionisation studies on ten hæmoglobins at ionic strength 1·0 and various temperatures, and Figure 2 shows a plot of $T\Delta S$ against ΔH for those hæmoglobins. The human hæmoglobins A, S, and C, and that of horse,[5] are included for comparison. The results show that the spread of ΔH values is still large (5 kcal. mole^{-1}) compared with that (6 kcal. mole^{-1}) for ionic strength 0·05. This implies that the major contribution to the different electrostatic interactions of the hæmoglobins originates from charged groups which are not screened out even at ionic strength 1·0. We may conclude that these groups are much closer to the iron atoms than the ASC position. It is of interest that the slope of the plot of $T\Delta S$ against ΔH is close to unity, paralleling the behaviour at low ionic strength.

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[5] P. George and G. I. H. Hanania, *Biochem. J.*, 1953, **55**, 236.

Comparison of the Net Charge, q, *obtained by Two Different Methods.*—In this and in previous discussions we have used values of the net charge, q, obtained from the slope of the plot of pK_3' against an ionic-strength function derived from Kirkwood's expression for the activity coefficient of a protein molecule. In view of the significance attached to q, it is necessary to test the validity of this procedure. We have accordingly compared values of q obtained by this method with those obtained from acid–base titration studies. The results are shown in Figure 3, in which the net charge on each of the methæmoglobins obtained from titration curves is plotted against q obtained from the slope of the plot of pK_3'

TABLE 3

Values of pK_3' at ionic strength 1·0M

Species	pK_3' (I = 1·0M)			$\Delta G_{20°}$ (cal. mole^{-1})	ΔH (cal. mole^{-1})	$-T\Delta S$ (cal. mole^{-1})
Mouse (1)	8·491(5·3)	8·330(12·1)	8·145(20·0)	10,921	8700	2220
Rat (2)	8·438(7·0)	8·333(12·6)	8·157(19·7)	10,937	8400	2540
Baboon (3)	8·403(6·5)	8·333(12·1)	8·207(20·0)	11,004	5630	5370
Patas monkey (4) ...	8·446(5·7)	8·322(12·2)	8·205(20·0)	11,001	6240	4760
Mona monkey (5) ...	8·435(5·4)	8·345(11·7)	8·215(20·0)	11,015	5640	5380
Pig (6)	8·615(6·65)	8·547(12·0)	8·388(20·0)	11,247	6500	4750
Dog (7)	8·644(4·1)	8·493(12·5)	8·356(20·0)	11,204	6750	4450
Hyaena (8)	8·502(7·17)	8·436(12·2)	8·320(20·0)	11,155	5500	5660
Tantalus monkey (9)	8·530(5·15)	8·412(11·5)	8·230(20·0)	11,035	7500	3540
Cat (10)..................	8·605(5·5)	8·515(11·5)	8·404(20·0)	11,268	5150	6120
Pigeon (11)	8·542(8·0)	8·482(12·0)	8·315(20·0)	11·149	6960	4190

The figures in parentheses beside the pK_3' values are the temperatures of measurement. The number in parentheses beside the name of each animal gives the key to Figure 2

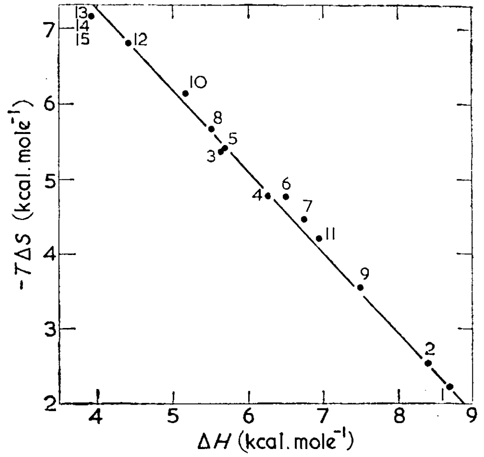

FIGURE 2. $T\Delta S$ against ΔH at I = 1·0 for the hæmoglobins in Table 3

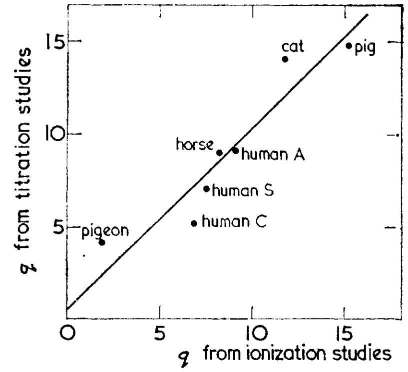

FIGURE 3. The charge q on a methæmoglobin, as obtained from titration-curve studies, plotted against q obtained from the variation of pK_3' with ionic strength

against f(I). It should be noted that the values of q obtained from the latter method represents the average value of q between pH 8·0 and 9·0, since pK_3' was determined from measurements in this range of pH. Also, q from titration curves was calculated from measurements on oxyhæmoglobins using the known values of pK_3' for the methæmoglobins. Points for human hæmoglobins S and C are also included in Figure 3. The q from titration curves for these hæmoglobins was computed from the measured value for hæmoglobin A and the known amino-acid differences between the three hæmoglobins, hæmoglobin S being known to have two less negative charges than hæmoglobin A, and hæmoglobin C two more positive charges than hæmoglobin A. The value of q from titration curves for horse methæmoglobin was calculated from the work of Wyman and Ingalls,[6] and the corresponding q from pK_3 studies from the work of George and Hanania.[5]

The line in Figure 3 represents the best line as obtained from a least-squares analysis of the data, with a slope of $1{\cdot}05 \pm 0{\cdot}14$ and intercept equal to $0{\cdot}5 \pm 1{\cdot}4$. Exact agreement between the two methods of obtaining q would require the line to have a slope of unity and to pass through the origin. The agreement between the two methods is good, bearing in mind the experimental errors involved in the determination of q (estimated to be ±1 charge unit for each method) and also the possible error introduced by the procedure of calculating the methæmoglobin charge from the titration curve of oxyhæmoglobin rather than directly. The good agreement between values of q from titration curves and those from ionisation studies is strong support for the validity of the extrapolation function used in the latter method, as well as for the Kirkwood electrostatic model on which it is based. It also serves to expose the limitations of the simple or extended Debye–Hückel functions that have been used in protein studies in the past by other workers.[7]

EXPERIMENTAL

Ionisation constants were determined by the procedure described in Part I.[3] A Pye Dynacap pH meter was used for the pH measurements. Acid–base titration of hæmoglobin was carried out at 20° and $I = 0{\cdot}05$ under an atmosphere of oxygen using CO_2-free solutions of oxyhæmoglobin, hydrochloric acid, and sodium hydroxide. Hæmoglobin concentration was about 10^{-4}M.

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: guinea pig—*Cavia porcellus*; lizard—*Agama agama*; bat—*Epomophorus gambiansis*; shrew—*Crocadura manni*; duck—*Cairina moschata*; turkey—*Meleagris gallopavo*; chicken—*Gallus domestica*; guinea fowl—*Numida meleagris*; cow—*Bos* (domestic cow of unspecified breed); mangabey monkey—*Cercocebus torquatos*; rabbit—*Oryctolagus cuniculus*.

We thank Mr. E. O. Banigo for carrying out the titration curves on pig, rabbit, human, and pigeon hæmoglobins.

This work was supported in part by a grant from the United States Public Health Service.

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[*Received, November 13th*, 1964.]

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[6] J. Wyman and E. N. Ingalls, *J. Biol. Chem.*, 1941, **139**, 877.

[7] R. K. Cannan, *Cold Spr. Harb. Symp. Quant. Biol.*, 1938, **6**, 1; A. Gronwall, *C.R. Lab. Carlsberg* (*Ser. Chem.*), 1942, **24**, 185; P. George and G. I. H. Hanania, *Biochem. J.*, 1952, **52**, 517.