Reactivity Difference between Haemoglobins.   Part XIX[1]

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The formation constants of the azide complex of two methaemoglobins with widely different pH_{ch} values, guinea pig and pigeon methaemoglobin, have been measured as a function of ionic strength.   These results, combined with previous results for human methaemoglobin A and C, confirm that the ionic-strength dependence of the formation constant can be quantitatively accounted for in terms of a dielectric cavity model for the protein except over a narrow range of pH in the region of the characteristic pH for each methaemoglobin.   This breakdown of the model is interpreted in terms of a pH-dependent configurational change involving charged groups on the surface of the molecule.

The formation constants of the azide complex of the haem groups attached to the α and β polypeptide chains within the human methaemoglobin tetramer have been shown to differ by a factor of 5.   It is shown that this difference can quantitatively account for the value of the Hill constant of 0.91 $\pm$ 0.01 obtained when measurements are made at 405 nm, a wavelength which indicates reaction at either the α or β haems.   It is concluded that there is no positive or negative co-operativity between the haem groups when methaemoglobin reacts with azide ion.

The formation constants for the reaction of several methaemoglobins and sperm whale metmyoglobin with formate ion have been measured as a function of pH and temperature at $I = 0.25$M.   Formate ion as a ligand resembles fluoride ion in that all the haemoglobin species have closely similar affinities for the ligand, confirming that differences between species in the affinity for a particular ligand is attributable to the configuration change which accompanies the spin-state change.   The pH dependence of ΔH° resembles that for azide and cyanide ion showing that factors other than the spin-state change determine the type of ΔH° behaviour shown by a ligand.

The apparent paradox that the large pH variations in the enthalpy of formation of methaemoglobin complexes are not accompanied by large temperature variations of the number of protons released upon ligand binding, as would be required by the Wyman relationship, is re-examined.   The Wyman relationship is derived by an alternative procedure, and a tacit assumption is made explicit.   It is shown that the large pH variation in ΔH° can arise if the configuration of charged groups on the surface of the molecule is different in methaemoglobin and its complex.   When such a difference in configuration exists the addition of the same number of protons to methaemoglobin and its complex will give rise to different changes in the partial molar entropy of the two species.

The formation constant for the azide complex of glycera methaemoglobin has been determined as a function of pH and temperature.   The affinity of this haemoglobin for azide ion is much lower than that for a typical mammalian methaemoglobin.   This difference is attributed to the close proximity to the iron atom of an aspartic acid residue in postion E5(57) in glycera methaemoglobin.   The pH variation of the enthalpy of complex formation is similar to that observed for a typical mammalian methaemoglobin in spite of the absence of the distal imidazole group in glycera methaemoglobin.   This is accounted for in terms of the proximity to the iron atom of the E5(57) aspartic acid residue.

The formation constants of the azide complex of the isolated polypeptide chains of human and dog methaemoglobins have been determined as a function of pH and temperature.   The variation of the enthalpy of complex formation resembles that of a typical methaemoglobin, the characteristic pH for the α chains of human methaemoglobin and the β chains of human and dog methaemoglobin falling on the previously noted correlation line between pH_{ch} and a function of the charged amino-acid in the molecule.   The deviation of the α chain of dog methaemoglobin from the correlation line is discussed.

The formation constants of the azide complex of the valency hybrids of human haemoglobin and the hybrids of dog and human methaemoglobin have been determined as a function of pH and temperature.   The valency hybrids of human haemoglobin have the same characteristic pH as human methaemoglobin showing that pH_{ch} is a property of the haemoglobin tetramer even if only one pair of haems is reacting with ligand.   The dog–human hybrid methaemoglobins show the typical pH variation of the enthalpy of complex formation, there being a characteristic pH for the tetrameric species which can be calculated from the characteristic pH values of the isolated chain.   That a tetrameric methaemoglobin shows a single characteristic pH rather than two, corresponding to those of the isolated α and β polypeptide chains, is accounted for by structural constraints imposed by one haem on the other leading to a concerted transition from the acid to the alkaline configuration at the characteristic pH.

The formation constants for the reaction of azide, formate, and fluoride ions with various methaemoglobins chemically modified in different ways have been determined as a function of pH and temperature.   Chemical modification has two distinct effects on the pH variation of the enthalpy of complex formation.   (i) The characteristic pH of a modified methaemoglobin shifts towards that of the unmodified polypeptide chain.   This is explained in terms of a reduction in the constraint imposed by the modified chain on the unmodified chain, such that the pH at which the concerted configurational change occurs (pH_{ch}) is determined primarily by the unmodified chain.   (ii) The form of the variation may change, the extreme case being where the customary maximum in the $-\Delta H^\circ$ against pH plot becomes a minimum for methaemoglobin modified with iodoacetamide reacting with formate ion.   This variable behaviour is interpreted in terms of an equilibrium between the ' hydrogen in ' and ' hydrogen out ' configuration on the distal imidazole group.

The magnitude of the Bohr effect for a number of chemically modified and hybrid haemoglobin species has been determined as a function of pH.   The dog–human hybrid haemoglobins fit the previously noted correlation between the magnitude of the acid Bohr effect, $\Delta h^+_{5,3}$, and the characteristic pH of a haemoglobin, implying that no special explanation is required for the very small acid Bohr effect of $\alpha^A \beta_2{}^{dog}$.   The $\Delta h^+_{5,3}$ for the vacancy hybrid human haemoglobin, $\alpha^+ \beta_2(O_2)_2$, falls on the correlation with pH_{ch} but that for the hybrid $\alpha_2(O_2)_2\beta_2{}^+$ does not. $\Delta h^+_{5,3}$ Values for human methaemoglobins enzymatically digested with carboxypeptides A and B fall on the correlation line.   A mechanism is proposed to account for the correlation between $\Delta h^+_{5,3}$ and pH_{ch}.

THE development over the past 50 years of our understanding in structural terms of the functional properties of haemoglobin, and indeed of enzymes, can largely be described in terms of attempts to identify the amino-acid residues which, by virtue of their properties, environment, and movement, play a crucial role in determining the functional properties of the molecule.

The determination of the amino-acid sequence of

[1] J. G. Beetlestone, R. O. Igc, and D. H. Irvine, *J. Chem. Soc.* (A), 1970, 1368.

haemoglobins from many species and the discovery and characterization of the abnormal human haemoglobins has led to a reformulation of this problem; namely, which of the positions in the polypeptide chains must be occupied by specific amino-acids for the molecule to exhibit the functional characteristics of haemoglobin, *e.g.* co-operativity in oxygen binding, a Bohr effect, and sensitivity to diphosphoglycerate ion. We suggest the term *functionally crucial residues* to describe such amino-acids. The evidence available at present suggests that the gross features of the functional properties of haemoglobin are explicable in terms of a small number of such groups,[2-4] and indeed a molecular pathology can be based on this assumption.[5]

This approach exploits the fact that the magnitude of many of the functional properties of mammalian haemoglobins are very similar and it is, therefore, meaningful to speak of *the* mechanism of the Bohr effect *etc.* However the magnitudes, as opposed to the presence or absence, of these functional properties do differ from one species to another, and as Barcroft[6] pointed out these differences must be ascribed to differences in the composition of the protein part of the molecule. Are then these species differences to be explained in terms of variability at a few sites in the protein, that is to say, are most of the amino-acid residues in the molecule *functionally inoperable*; or, using this suggested nomenclature, are they *functionally operable*? The former is the assumption of those who maintain that a large proportion of the variability in the amino-acid composition in related proteins arises as a consequence of genetic drift rather than selection.[7-9]

A further question is whether, for each position in the molecule, and for each type of amino-acid substitution, there is a unique mechanism by which an amino-acid substitution affects the functional properties; or whether there exist a limited number of more general mechanisms by which changes in structure produce changes in reactivity. The latter alternative is analogous to the situation in physical organic chemistry where a large amount of the data on the effect of structural changes in small organic molecules on the thermodynamic and kinetic parameters of reactions of these molecules can be rationalized in terms of independent and additive contributions from inductive, mesomeric, and similar effects.

This argument has been framed in terms of haemoglobin but it is applicable to any discussion of the behaviour of isoenzymes or the species variation of enzymatic activity. However at the present time the problem is best tackled by a study of haemoglobin for not only is there a wealth of structural information but also there exist a large number of well characterized abnormal human haemoglobins in which the amino-acid composition differs from haemoglobin A by only one amino-acid in each α or β polypeptide chain.[10] Our general aim then in this series of papers is to determine which are the functionally operable amino-acid residues in haemoglobin and to establish the mechanisms by which amino-acid substitutions affect reactivity. In tackling this problem our strategy over the past 10 years has been to investigate the effects of the simplest possible structural changes on the thermodynamics of the simplest reactions of haemoglobin. The reactions of the deoxy-form are complicated by the presence of large homotropic and heterotropic interactions[11] and we have, therefore, concentrated our attention on the reactions of methaemoglobin where these interactions are small although more recently we have been able to extend the studies to the functionally significant reactions.

In this series[12-28] we have described the thermodynamics of the reaction of different methaemoglobins with various ligands and have postulated mechanisms by which the differences in behaviour shown by different methaemoglobins and ligands may be accounted for in structural terms. The discussion has been formulated in terms of K_L, the equilibrium constant for the reaction in which a ligand replaces the water molecule in the sixth co-ordination position of the iron atom in the ring.

Under some conditions for certain ligands, K_L can be directly measured by routine spectrophotometric procedures. Under most conditions an observed equilibrium constant K_{obs} is measured spectrophotometrically and K_L is calculated from this and known values of the pK values for the ionization of the conjugate acid of the

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free ligand and the ionization of the water molecule in the sixth co-ordination position of the iron atom. This can be formally represented:

$$Hb^+OH_2 + L^- \xrightleftharpoons{K_L} HbL + H_2O$$

$$Hb^+OH_2 \xrightleftharpoons{K_a} HbOH + H^+$$

$$HL \xrightleftharpoons{K_i} L^- + H^+$$
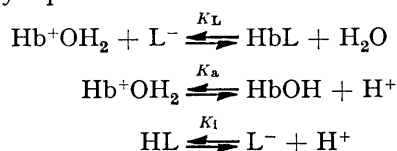
where Hb^+OH_2, $HbOH$, and HbL respectively represent methaemoglobin, alkaline methaemoglobin, and the complex of methaemoglobin with the ligand L^-. The ligand is represented as L^- since most of the work has been carried out with negatively charged ligands. However, the same set of equilibria may be used to describe the reaction with a neutral ligand such as methylamine. We define the following equilibrium constants

$$K_a = \frac{[HbOH][H^+]}{[Hb^+OH_2]} \qquad (1)$$

$$K_L = \frac{[HbL]}{[Hb^+OH_2][L^-]} \qquad (2)$$

$$K_i = \frac{[H^+][L^-]}{[HL]} \qquad (3)$$

$$K_{obs} = \frac{[HbL]}{([Hb^+OH_2] + [HbOH])([L^-] + [HL])} \qquad (4)$$

where $([Hb^+OH_2] + [HbOH])$ is the total methaemoglobin concentration referred to a standard state of 1 mol l^{-1} of haem iron and $([L^-] + [HL])$ is the total concentration of unbound ligand irrespective of its state of ionization. K_{obs} At constant ionic strength and known pH is measured spectrophotometrically. From known values of K_a and K_i, K_L may be readily calculated from the equation

$$K_L = K_{obs}([H^+] + K_a)([H^+] + K_i)/K_i[H^+] \qquad (5)$$

The standard free-energy change of complex formation, $\Delta G°$, is given by $\Delta G° = -RT\ln K_L$ and the corresponding enthalpy change $\Delta H°$ is calculated from the variation of log K_L with temperature.

K_{obs} Is determined spectrophotometrically, by measuring the optical density as a function of ligand concentration at a wavelength of maximum difference between the extinction coefficients of methaemoglobin and its complex. For all ligands investigated so far the Hill coefficient, n, is slightly less than or equal to unity indicating that homotropic interactions between the four haems are absent. However it must be noted that K_{obs} determined in this way is an average for the haems bound to the α and β polypeptide chains. That K_{obs} is not markedly different for the two haems is indicated by the value of n close to unity. However, Klapper [29] has carefully analysed the formation curve of the imidazole complex of methaemoglobin obtained by very precise spectrophotometric methods, and has shown that the formation constants for the haems bound to the α and β polypeptide chains differ by a factor of seven. We have

* $\Delta G°$ For the formation of the hydroxyl ion complex is calculated from K_a, as defined above, and the ionic product of water.

shown by a different method described below that a similar difference exists for the azide complex. We also show that the hypotheses that we have made in this series are not invalidated by the fact that the thermodynamic parameters on which they are based are average values for the two types of haems.

The most important experimental observations in Parts I—XVIII [1,12-28] may be summarized as follows:

(i) At low ionic strength ($I < 0.05$M) the variations of log K_L with ionic strength, pH, and type of haemoglobin for azide ion can be accounted for quantitatively in terms of electrostatic interactions using the Kirkwood dielectric cavity model for the protein except over a narrow range of pH in the region of the characteristic pH of the methaemoglobin.[18,27] This is investigated further in this paper. The remaining summary statements which follow are concerned with results carried out at $I = 0.05$M where most of the effects on the reaction at the iron atom with charged ligands of charged groups anywhere on the molecule are screened out.

(ii) $\Delta G°$ Values for the formation of the fluoride complex are very similar for all the methaemoglobins studied. At the isoelectric points, where any residual electrostatic effects are minimized, the $\Delta G°$ values for the methaemoglobins differ at most by only 80 cal mol^{-1} which is approximately twice the standard error of the observations. At the isoelectric point $\Delta G°$ for sperm whale metmyoglobin is ca. 250 cal mol^{-1} lower than that for the methaemoglobins. To a first approximation we may generalize and say that the $\Delta G°$ values for the formation of the fluoride complex of all methaemoglobins and sperm whale metmyoglobin are the same.[26] Results presented in this paper suggest that this generalization applies to all high-spin complexes.

(iii) In contrast to this behaviour the $\Delta G°$ values for the formation of the hydroxy,* azide, cyanide, hydrosulphide, and methylamine complexes of sperm whale metmyoglobin are approximately 1 100, 900, 700, 1 200, and 1 500 cal mol^{-1} below those for the corresponding methaemoglobin complexes. Similarly the $\Delta G°$ values for methaemoglobins reacting with these ligands differ from one another by up to several hundred cal mol^{-1}. For example $\Delta G°$ for the formation of the hydrosulphide complex of guinea pig methaemoglobin is 500—600 cal mol^{-1} less negative than that for human methaemoglobin A. Furthermore, there is a correlation between the $\Delta G°$ values for any two ligands as the haemoglobin type is varied.[1,15,16,26]

(iv) For all the ligands studied, variations in the $\Delta H°$ of complex formation, either as the species, or the pH, is varied, are much greater than the variations in $\Delta G°$.[12,15,16,19,26,27]

(v) This behaviour is shown most dramatically for the formation of the azide complex where the $\Delta H°$ values for different methaemoglobins at constant pH may differ by up to 12 kcal mol^{-1}, and where over the pH region 5.8—9 $\Delta H°$ for one particular methaemoglobin may vary

[29] H. Uchida, J. Heystek, and J. H. Klapper, *J. Biol. Chem.*, 1971, **246**, 2029.

by up to 8 kcal mol^{-1}. In all cases a plot of $-\Delta H^\circ$ against pH is a bell-shaped curve with a sharp maximum. We have defined this pH of maximum $-\Delta H^\circ$ as the characteristic pH, pH$_{ch}$, of a haemoglobin. For the haemoglobins so far studied pH$_{ch}$ has values in the range 5.8—8.6.[20,27] Sperm whale metmyoglobin[25] and glycera methaemoglobin (see below) show similar behaviour to the methaemoglobins, indicating that the existence of pH$_{ch}$ is not contingent on the presence of a tetramer.

In Part VIII[19] we suggested that this type of behaviour was associated with ligands which form low-spin complexes. However doubt was thrown on this by subsequent work in which we showed that the type of behaviour shown by fluoride ion depends on the particular haemoglobin [see (x) below] and that SH$^-$ as a ligand behaves differently from azide ion although it forms a low-spin complex.[25] That the spin state of the complex does not play a crucial role in determining the form of the pH dependence of ΔH° is demonstrated below where it is shown that formate ion shows similar behaviour to azide ion as a ligand.

(vi) Cyanide ion shows similar ΔH° behaviour to azide ion.[19]

(vii) ΔH° For the formation of the hydrosulphide complex shows large variations between methaemoglobins and with pH, but the form of the variation is entirely different from that for azide and cyanide ions. At pH$_{ch}$, $-\Delta H^\circ$ for hydrosulphide ion passes through a minimum in contrast to the maximum shown by azide ion.[26]

(viii) In the one case where it is accessible to experimental test, sperm whale metmyoglobin, hydroxyl ion shows similar ΔH° behaviour to hydrosulphide ion.[25]

(ix) Methylamine as a ligand shows ΔH° behaviour similar to hydrosulphide ion rather than azide ion. It is not possible to determine ΔH° for methylamine as a function of pH below 8.5 because of side reactions which occur under these conditions and hence this statement of similarity between hydrosulphide ion and methylamine as ligands is not based on the similarity of the form of the pH variation of ΔH°, but rather on the observed correlation at pH 8.5 between ΔH° for methylamine and hydrosulphide ion as ligands, and the lack of correlation between ΔH° for methylamine and azide ion complex formation.[1]

(x) Values of ΔH° for the formation of the fluoride complex are smaller than those observed for the other ligands (0—3 kcal mol^{-1} as opposed to 8—20 kcal mol^{-1}). In contrast to other ligands the form of the variation of ΔH° with pH is not similar for all methaemoglobins. For example $-\Delta H^\circ$ for dog methaemoglobin shows a variation of ca. 2 kcal mol^{-1} over the pH range 6—9 showing a distinct maximum at pH$_{ch}$. By contrast ΔH° for pigeon methaemoglobin hardly varies and that for sperm whale metmyoglobin shows a variation of 2 kcal mol^{-1} with a minimum at pH$_{ch}$.[19,26]

(xi) There is an approximately linear correlation between the characteristic pH of a methaemoglobin and its isoelectric point. However, pH$_{ch}$ varies over the range 5.8—8.6 while the isoelectric point varies only from 7.3—8.1 suggesting that the correlation is an indirect one, both pH$_{ch}$ and the isoelectric point being different functions of the content of charged amino-acids. There is indeed a linear correlation between pH$_{ch}$ and the difference between the number of lysine and arginine residues and the number of glutamic and aspartic acid residues (Lys + Arg — Glu — Asp).[27]

(xii) There is a correlation between the magnitude of the acid Bohr effect (the number of mole equivalents of hydrogen ion released when one mole equivalent of oxygen combines with haemoglobin) at pH 5.3 and pH$_{ch}$ suggesting that pH$_{ch}$ has relevance for the understanding of the variability of haemoglobin as well as methaemoglobin reactions.[28] This is further investigated in this paper.

We have attempted to account for these observations in the following way. The large variations in ΔH° values in contrast to the much smaller variations in ΔG° values, that is to say the compensation of ΔH° and ΔS°, we attribute in general terms to changes in protein configuration and hydration (the latter of necessity being dependent on the former) which accompany ligand binding. The former would be expected to produce enthalpy and entropy changes which would compensate to a certain extent and the latter would be expected to produce exactly compensating enthalpy and entropy changes.[19] We have postulated three mechanisms by which such changes could arise:

(1) The change of spin state on the iron atom which occurs when L$^-$ is hydroxyl, azide, cyanide, or hydrosulphide ion is known to be accompanied by small changes in the protein structure.[27] Hence there will be a contribution to the enthalpy and entropy of formation of the complex from this configurational change which may vary from one methaemoglobin to another as a consequence of differences in amino-acid composition.

(2) When the ligand L$^-$ replaces the water molecule bonded to the iron atom the hydrogen atom on the imidazole ring of the distal imidazole which 'sees' the solvent may move to the nitrogen atom on the same ring which is inside the molecule to form a hydrogen bond with the ligand.[1,19,26] This shift will produce changes in the structure of the hydration water in the vicinity of the distal imidazole, the magnitude of the thermodynamic consequences of which will depend on this structure which in turn will be dependent on pH and the type of haemoglobin.

(3) The replacement of the neutral water molecule by charged ligands may remove polarization on groups in the vicinity of the iron atom, particularly the distal imidazole,[19] thus producing changes in the hydration structure.
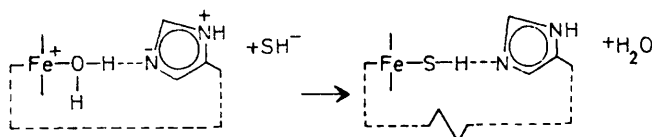
Thus we have formulated the reaction with
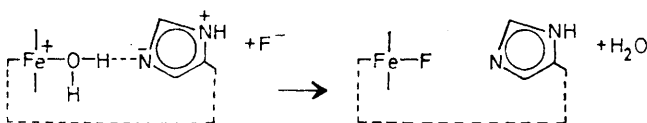
(a) azide and cyanide ion as
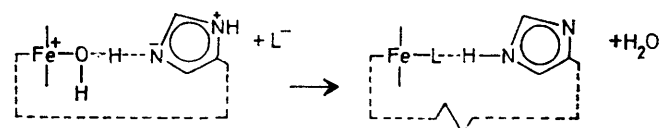
These ligands are charged, form low-spin complexes (the human methaemoglobin azide complex is *ca.* 95% low spin[30]), and do not carry a hydrogen atom on the ligand atom, and hence all three mechanisms are present. The difference between $\Delta G°$ values for different methaemoglobins and sperm whale metmyoglobin we ascribe to the variability of the contribution to $\Delta G°$ of the configuration change which accompanies the spin-state change. There will also be corresponding variable contributions to the enthalpy change. The large pH-dependent variations in $\Delta H°$ which are observed for these two ligands we ascribe to the effect on the hydration structure, which itself will be pH dependent, of the removal of the hydrogen atom from the external nitrogen of the distal imidazole.

(b) Hydrosulphide and hydroxyl ions as

$$\left[\begin{array}{c} \mathrm{Fe-O-H \cdots N} \\ | \\ \mathrm{H} \end{array} \!\!\! \bigcirc\!\!\!\!- NH^{+} \right] + SH^{-} \longrightarrow \left[\begin{array}{c} \mathrm{Fe-S-H \cdots N} \end{array} \!\!\! \bigcirc\!\!\!\!- NH^{+} \right] + H_2O$$

Hydrosulphide ion forms a low-spin complex and hydroxyl ion forms a complex which is *ca.* 50% low spin for methaemoglobin[30] and 30% low spin for sperm whale metmyoglobin,[31] and hence we observe $\Delta G°$ values which are variable from one haemoglobin to another as is observed with azide and cyanide ions. However, hydroxyl and hydrosulphide ions carry a hydrogen atom on the ligand atom which can form a hydrogen bond with the distal imidazole. Hence mechanism (2) is absent and we observe $\Delta H°$ behaviour which differs from that of azide and cyanide ions. We ascribe the observed variations of $\Delta H°$ with pH to mechanism (3). These variations are small at higher pH and hence we find the correlation between $\Delta H°$ for hydrosulphide ion and that for methylamine for which only mechanism (1) is present.

(c) Fluoride ion as

$$\left[\begin{array}{c} \mathrm{Fe-O-H \cdots N} \\ | \\ \mathrm{H} \end{array} \!\!\! \bigcirc\!\!\!\!- NH^{+} \right] + F^{-} \longrightarrow \left[\begin{array}{c} \mathrm{Fe-F} \end{array} \quad N \!\!\! \bigcirc\!\!\!\!- NH \right] + H_2O$$

We originally postulated that no hydrogen bond is formed between the ligand and the distal imidazole as illustrated and hence only mechanism (3) should be present. However this cannot account for the observed variability of the form of the pH variation of $\Delta H°$. We, therefore, speculate that for fluoride ion there is an equilibrium between the hydrogen 'in' and 'out' configurations. We re-examine these hypotheses in this paper.

While these three mechanisms can adequately account in a qualitative way for the behaviour of different ligands they in no way account for the particular form of the variation of $\Delta H°$ with pH for each methaemoglobin, that is, why the maximum or minimum value of $\Delta H°$ occurs at a particular pH, pH_{ch}, for each methaemoglobin. In order to account for this we have postulated that over a narrow pH range around pH_{ch} a structural perturbation of the protein occurs such that the charged groups on the surface of the molecule adopt different configurations above and below pH_{ch}. As a result the hydration structure will be different above and below pH_{ch} thereby giving rise to a different pH variation of the hydration structure by the three mechanisms summarized above. The reorientation of charged groups in the region of pH_{ch} can account for the observed breakdown of the dielectric cavity model at this pH.

It is the purpose of this paper to examine some of the assumptions underlying our approach and to test specific aspects of the various hypotheses summarized above. Each of the following sections will be concerned with a different aspect of this problem.

The Variation with Ionic Strength of the Formation Constant for the Azide Complex of the Methaemoglobins of Guinea Pig and Pigeon (with K. O. Okonjo)

In Part XVI[27] we showed that Kirkwood's dielectric cavity model for a protein could quantitatively account for the difference between the standard free energy of ionization to the alkaline form of three human abnormal methaemoglobins differing by a single amino-acid substitution from haemoglobin A. Previously in Part VII[18] we had shown that, except over a narrow pH range, the variation with ionic strength of the formation constant of the azide complex of human methaemoglobins A and C could also be described quantitatively by using this model for the methaemoglobin molecule. The breakdown of the dielectric cavity model close to what we subsequently defined as the characteristic pH was one of the observations which led to our postulate that a pH-dependent configurational change occurs at pH_{ch}. However our conclusion that the model is unsatisfactory at pH_{ch} was based on two experimental points for two haemoglobins which differ by only two amino-acids in the tetramer and for which pH_{ch} differs by only 0.4. We have, therefore, examined the ionic-strength variation of the formation constant of the azide complex of two methaemoglobins with widely different characteristic pH values: guinea pig ($pH_{ch} = 5.8$) and pigeon ($pH_{ch} = 8.0$).

Using the dielectric cavity model to obtain expressions for the activity coefficients of methaemoglobin and its complex with a negatively charged ligand we have shown (Part VII[18]) that the formation constant of the azide complex at zero ionic strength, $K°_L$, is given by

$$\log K°_L = \log K_L + \frac{A\sqrt{I}}{1 + \sqrt{I}} +$$
$$A \times (2q_I - x)\frac{\sqrt{I}}{1 + Ba\sqrt{I}} \quad (6)$$

where q_I is the net charge on methaemoglobin at a particular pH, x is the difference between q_I and the charge on the complex at the same pH, A and B are the customary

[30] P. George, J. G. Beetlestone, and J. S. Griffith, *Rev. Mod. Phys.*, 1964, **36**, 441.

[31] J. G. Beetlestone and P. George, *Biochemistry*, 1964, **3**, 707.

Debye–Hückel constants, I is the ionic strength, and a is the distance of closest approach of the protein molecule and a small ion. The second term is an expression for the activity coefficient of the azide ion.

$$\text{Defining } \log K'_L = \log K_L + \frac{A\sqrt{I}}{1 + \sqrt{I}}$$

equation (6) implies that a plot of $\log K'_L$ at constant pH against $f(I) = \dfrac{\sqrt{I}}{1 + Ba\sqrt{I}}$ should be linear with a slope equal to $-Ax(2q_I - x)$. The azide ion carries a single negative charge and hence x is given by $x = -1 + y$ where y is the number of hydrogen ions taken up when one mole of azide ion reacts with acid methaemoglobin. Values of y, corrected for the percentage of alkaline methaemoglobin in the solution were determined by a refinement of the procedure described in Part VI [17] details of which are given in the Supplementary Publication * together with values of y. Hence q_I as a function of pH can be calculated from values of the slope and compared with values of the net charge calculated from the acid–base titration curve q_T (see the Supplementary Publication for the calculation of q_T from acid–base titration curves of methaemoglobin.

Figures 1 and 2 of the Supplementary Publication show plots of $\log K'_L$ against $f(I)$. The observed behaviour is similar to that obtained in Part VII [18] and is qualitatively in agreement with equation (6). At those pH values where the protein carries a net positive charge $\log K'_L$ increases with decreasing ionic strength, and at those pH values where the protein carries a net negative charge $\log K'_L$ decreases with decreasing ionic strength.

In order to test the model quantitatively, that is to assess objectively whether the value of 'a' that gives agreement between q_I and q_T is independent of the species of haemoglobin and consistent with the known dimensions of the haemoglobin molecule, and to test whether the breakdown of this agreement at pH values close to pH$_{ch}$, suggested by the data for haemoglobins A and C, is a general phenomenon, the following procedure was adopted.

A computer was programmed to calculate values of q_I from the slopes of the plots of $\log K'_L$ against $f(I)$ using values of 'Ba' from 9 to 13 at intervals of 0.1. For each value of 'Ba' the slope of the plot of q_I vs. q_T as a function of pH was calculated by a least-squares procedure. The value of 'Ba' that gave a slope of unity was obtained by inspection of the computer print out of corresponding values of 'Ba' and the slope of q_I vs. q_T. In this analysis values of q at pH values close to pH$_{ch}$ were omitted. The best values of 'Ba' for the two methaemoglobins investigated in this paper together with those obtained from the data given in Part VII [18] were 11.4, 10.6, 10.0, and 9.8 for human methaemoglobins A and C, guinea pig and pigeon methaemoglobins respectively.

From the experimental error in $\log K_L$ we estimate the standard error in the best value of 'Ba' to be ± 0.5

* SUP No. 21710 (84 pp.). For details of the Supplementary Publications scheme, see Notice to Authors No. 7, *J.C.S. Dalton*, 1975, Index Issue.

which is to be compared with the standard error of the mean of the calculated values for the four haemoglobins of ± 0.5. Thus no significance can be attached to the difference between these values since the slope of the plot of q_I vs. q_T varies only slowly as 'Ba' is changed. The mean of the best values of 'Ba' $= 10.5 \pm 0.5$ gives 'a' $= 32 \pm 1.5$ Å which corresponds to the sum of the average radius of the molecule and a small ion plus the diameter of one water molecule.

An alternative method of assessing the best value of 'Ba' was also used. In this method the value of 'Ba' was varied as in the previous method and for each value $\Sigma(q_I - q_T)^2$ was calculated. The best value of 'Ba' was defined as that which gave the minimum value of this function. The average value of the best values of 'Ba'
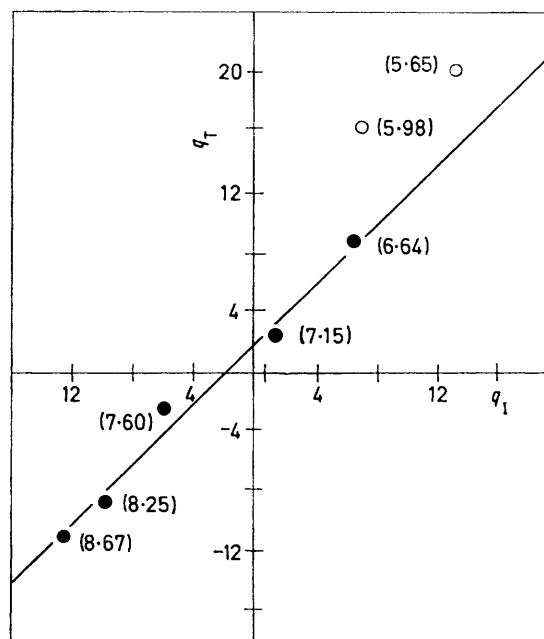

FIGURE 1 A plot of q_T against q_I for guinea pig methaemoglobin. Values of q_I were calculated using a value of $Ba = 10.0$. The figures in brackets indicate the pH corresponding to each point. Ringed points were omitted in the calculation of the best value of Ba

was found to be 11.0 which is in agreement with the values obtained by the first method within experimental error. The difference between the values of 'Ba' for human methaemoglobins A and C and that given in Part VII [18] arises from the refinements in the method used to calculate q_T. These methods are described in the Supplementary Publication. Figure 1 shows q_I plotted against q_T for guinea pig methaemoglobin. The plotted values of q_I were calculated using the best value of 'Ba' for guinea pig haemoglobin. The pH values for each point are shown on the graph and points corresponding to pH values close to pH$_{ch}$ are included but shown as ringed points. It is readily seen that these points show a much greater deviation from the line of unit slope than those corresponding to pH values away from pH$_{ch}$. Similar plots for the other three haemoglobins show similar be-

haviour. The significance of these deviations may be assessed as follows.

The random errors on q_T are much smaller than those on q_I (the titration curves on which q_T is based are given in Part VII [18] for human haemoglobins A and C and in Tables 2 and 3 of the Supplementary Publication for the haemoglobins of pigeon and guinea pig) and hence the deviations of the points from the straight lines in these figures are to be ascribed either to the inadequacy of the model or to random errors in q_I. We assess to what extent random errors are likely to account for the deviation in the following way. The error on q_I as assessed from the scatter of points on the plots of log K'_L against pH is between ± 1 and ± 2. If we exclude the points for pH values close to pH_{ch} the average of the squares of the deviations of q_I from the straight lines, $(\Delta q_I)^2$, is 2 with a range of 0—6 (Table 1) and we conclude, therefore, that at pH values not close to pH_{ch} the model is valid within the precision of the measurements. By contrast the deviations of those points corresponding to pH values close to pH_{ch} are at least an order of magnitude greater (Table 1). These large deviations clearly show the breakdown of the dielectric cavity model at pH values close to

TABLE 1

Values of the squares of the deviations of q_I from the straight lines shown in Figure 1 and Figures 3—5 of the Supplementary Publication, $(\Delta q_I)^2$

Guinea pig $pH_{ch} = 5.8$		Pigeon $pH_{ch} = 8.2$	
pH	$(\Delta q_I)^2$	pH	$(\Delta q_I)^2$
5.65	33.64	5.95	0.04
5.98	73.94	6.55	0.01
6.64	0.04	7.15	0.42
7.15	1.00	7.65	0.01
7.60	4.00	7.95	3.61
8.25	0.36	8.32	14.40
8.67	0.04	8.65	0.25

Haemoglobin A $pH_{ch} = 7.1$		Haemoglobin C $pH_{ch} = 7.5$	
pH	$(\Delta q_I)^2$	pH	$(\Delta q_I)^2$
6.0	1.21	6.0	4.84
6.5	4.00	6.5	9.61
7.0	12.96	7.0	0.16
7.5	1.69	7.5	23.0
8.0	1.69	8.0	0.36
8.5	0.00	8.5	4.41
9.0	2.89	9.0	0.49

pH_{ch}, thus supporting our hypothesis that a pH-dependent configurational change involving charged groups occurs in the region of pH_{ch}. The values for pigeon methaemoglobin would suggest that pH_{ch} for this haemoglobin is slightly higher than the previously estimated value. Inspection of Figure 2 of Part IX [20] shows that a higher value of ca. 8.2 is consistent with the data presented there for the variation with pH of $\Delta H°$ for the formation of the azide complex.

[32] R. Banerjee, Y. Henry, and R. Cassoly, *European J. Biochem.*, 1973, **32**, 173.
[33] A. C. Anusiem and J. G. Beetlestone, unpublished observations.
[34] R. Banerjee and R. Cassoly, *J. Mol. Biol.*, 1969, **42**, 337.

Haem Heterogeneity and the Reaction of Methaemoglobin with Azide Ion

The 'Hill constant', n, for the reaction of azide ion, or indeed many other ions, with methaemoglobin has been reported to be close to unity by many authors. It has, therefore, been concluded that there is no co-operativity in the binding of ligands to methaemoglobin. The one report by Banerjee and his co-workers of a value of n greater than 1 for the binding of azide ion [32] has been shown by Anusiem and Beetlestone [33] to be the consequence of structural perturbations of the haemoglobin molecule over the long equilibration times used by these workers. These structural perturbations give rise to non-isosbestic behaviour in the formation of the azide complex which in turn invalidates the method of calculating n. Later in this paper we report closely similar values of log K_L for azide binding to the isolated α and β met chains. In contrast to this observation the redox potentials of the isolated chains have been shown to be widely different from one another.[34] These results could be explained if the configurations of the isolated chains differ in the deoxy but not in the met form. In the absence of positive co-operativity upon ligand binding to methaemoglobin the closely similar values of log K_L for the isolated α and β met chains would lead us to expect a value of n close to unity unless the relative affinities for azide ion of the chains in the tetramer differ from that of the isolated chains. The experiments of Gibson et al.[35] showing that the rate of reaction with azide ion of the α and β met haems within the tetramer differs by a factor of ca. 6 suggest that the relative affinities may differ, presumably because the configurations of the met chains within the tetramer differ one from another. Further, the work of Uchuda et al.[29] shows that the affinities for imidazole of the α and β haems within the tetramers differ by 600 cal mol^{-1}. It could be argued that the smaller azide ion might not give a similar difference since it is known that the difference between the affinity for ligands of the isolated α and β deoxy-chains is dependent on the size and shape of the ligand.[36]

The precision of the experiments reported so far in this series of papers is not such as to allow a decision to be made as to the magnitude of the difference between the affinity for azide ion of the α and β chains within the tetramer by analysing the form of the formation curve. Furthermore, there is also the possibility that the binding of azide ion to methaemoglobin is co-operative but that this co-operativity is not reflected in the value of n because it is compensated for by a difference in the intrinsic affinity for azide ion of the α and β haems. This suggestion is particularly attractive in view of the suggestion of Perutz that co-operativity in ligand binding is found whenever there is an 'out of haem plane' to 'in haem plane' motion of the iron atoms upon binding of ligand.[37] The iron atom in the high-spin methaemoglobin is 'out of

[35] Q. H. Gibson, L. J. Parkhurst, and G. Geraci, *J. Biol. Chem.*, 1969, **244**, 4668.
[36] B. Talbot, M. Brunori, and E. Antonini, unpublished observations quoted in ref. 11.
[37] M. Perutz, *Nature*, 1972, **237**, 495.

plane ' while it is ' in plane ' in the low-spin methaemo-globin azide.

The work described in this part was carried out in order to determine (i) whether the intrinsic affinities for azide ion of the α and β chains within the tetramer are significantly different and (ii) whether there is co-operativity in the binding of azide ion to methaemoglobin. This was done by adopting an experimental procedure which minimizes experimental errors and by measuring optical densities at the wavelengths determined by Gibson [35] as reflecting reaction at the α and β haems. Details of this procedure are given in the Supplementary Publication.

Optical density measurements for an experiment at pH 6.0 are shown in Table 4 in the Supplementary Publication. Values of log K_L can be obtained in the usual manner from the values of the optical density at 405 nm using the expression

$$K_L = \frac{(D_0 - D)}{(D - D_\infty)[N_3]_u} \quad (7)$$

where D_0 is the optical density of methaemoglobin. D_∞ Is the optical density of methaemoglobin azide and D is the optical density when the unbound azide concentration is $[N_3]_u$. $[N_3]_u$ is calculated from the expression

$$[N_3]_u = \frac{(D_0 - D)}{(D_0 - D_\infty)}[Hb]_T$$

where $[Hb]_T$ is the total concentration of haemoglobin in mol l^{-1} of Fe. The value of K_L obtained in this way is an
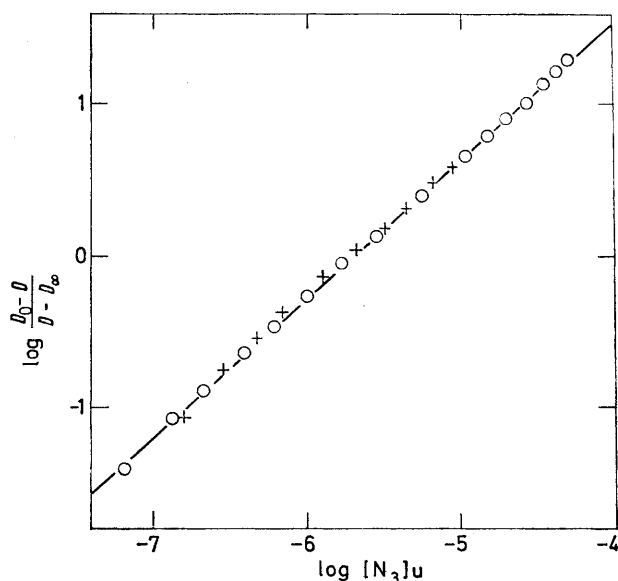

FIGURE 2 A ' Hill ' plot for the reaction of azide ion with human methaemoglobin A at 20 °C and pH 6, $I = 0.05$ as monitored at 405 nm. The crosses refer to the value of log $[(D_0 - D)/(D - D_\infty)]$, calculated from the observed values of D_0, D_∞, and D at 405 nm. The open circles refer to values of log $[(D_0 - D)/(D - D_\infty)]$ calculated from D_0 and D_∞ at 405 nm together with the values of K_L for the α and β haems determined from the measurements made at 412.3 and 414.3 nm

average of the equilibrium constants for the reaction of azide ion with the α and β haems in the tetramer. Values of K_L obtained in this way are given in the Sup-

plementary Publication. They show a steady decrease (by 35% between 15 and 80% formation) with increasing azide ion concentration suggesting that the affinities of the two haems are different. This can be illustrated in terms of a plot of log $[(D_0 - D)/(D - D_\infty)]$ against log-$[N_3]_u$, the slope of which would be unity, in the absence of co-operativity, if the affinities of the haems are the same, and less than unity if they are different. The points shown by crosses in Figure 2 show the values calculated from the optical densities at 405 nm. The slope is 0.91 ± 0.01 indicating either that the haems have different affinities for azide ion or that there is negative co-operativity. However, we cannot imply from this result that there is no positive co-operativity since the affinities of the two haems may be sufficiently different more than to compensate for some degree of positive co-operativity.

We turn to a consideration of the observations at the other wavelengths. According to Gibson,[35] in the region of 412.3 nm we are observing the reaction of the haem
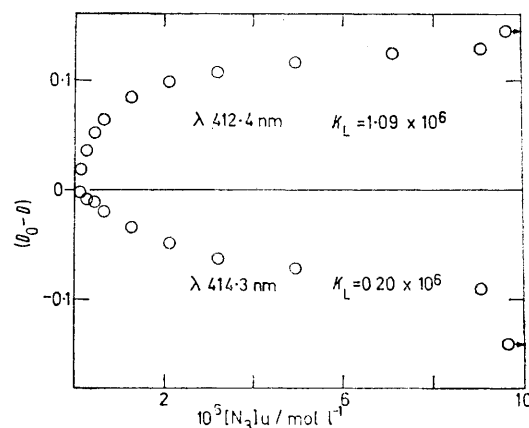

FIGURE 3 The variation of the optical density of a methaemo-globin solution with the concentration of free azide ion in solution at 412.3 and 414.3 nm. 412.3 nm is the isosbestic point for the met α chain and its azide complex and hence at this wavelength we are observing the progressive formation of the azide complex of the β chain. Similarly at 414.3 nm we are observing the formation of the azide complex of the α chain

attached to the β polypeptide chains since this wave-length is an isosbestic point of the spectra of the met and met azide forms of the α haems. Similarly at 414.3 nm we are observing the reaction of the α haems. Figure 3 shows the variation of the optical densities at 412.4 and 414.3 nm as a function of $[N_3]_u$. We note the much steeper rise at low values of $[N_3]_u$ of the optical density at 412.4 nm compared to that at 414.3 nm. The plots of the optical densities obtained at 412.2 and 412.3 nm are very similar to that at 412.4 and those at 414.2 and 414.4 nm correspond closely to that at 414.3 nm. These observations suggest, in conformity with the kinetic results of Gibson, that the affinity of the β haems is much greater than that for the α haems. This may be quanti-tatively described by calculating for each of the points at each wavelength an equilibrium constant from equation (7). The average values of log K_L at 412.4 and 414.3 nm

are respectively 6.02 and 5.36 respectively which, following Gibson, indicates that log $K_L^\beta = 6.02$ and log $K_L^a = 5.36$, corresponding to a difference in the ΔG° values of 880 cal mol^{-1}. This is to be compared to Klapper's value of 600 cal mol^{-1} for the reaction of imidazole.

Values of the Hill constant, n, for reaction at each of the haems may be calculated from the data in Figure 3 in the usual way. For the α haem the value is unity within experimental error indicating no co-operativity between the α haems. For the β haem $n = 0.92 \pm 0.01$ suggesting that the reaction at one β haem decreases the affinity of the other haem for azide ion. However, in view of the finite slit widths used and the small optical-density changes involved, it would be premature to lay too much stress on this point. Thus we conclude that (i) there is no positive co-operativity between the α chains or between the β chains within the met tetramer and (ii) that the affinity of the α and β haems differ significantly.

It remains to be shown whether the value of $n = 0.91$ obtained from the optical-density measurement at 405 nm is consistent with the observed difference between the affinities of the α and β haems, or whether some positive co-operativity between α and β chains must be postulated. We can decide between these two alternatives by calculating the optical density at 405 nm as a function of $[N_3]_u$ using the known values of K_α, K_β, and the observed values of D_0 and D_∞ at 405 nm.

It is readily shown that the optical density at 405 nm is given by

$$\frac{[N_3]_u}{2}\left\{\frac{K_\alpha}{(1 + K_\alpha[N_3]_u)} + \frac{K_\beta}{(1 + K_\beta[N_3]_u)}\right\}(D_\infty - D_0) + D_0$$

Similarly it can be shown that $[N_3]_u$ is given by

$$K_\alpha K_\beta [N_3]_u^3 +$$
$$\{(K_\alpha + K_\beta) - K_\alpha K_\beta[N_3]_T + K_\alpha K_\beta[Hb]_T\}[N_3]_u^2 +$$
$$\left\{1 - (K_\alpha + K_\beta)[N_3]_T + \frac{(K_\alpha + K_\beta)}{2}[Hb]_T\right\}[N_3]_u$$
$$- [N_3]_T = 0$$

$[N_3]_u$ was obtained for various values of total azide ion $[N_3]_T$ by using a computer to find the real positive root of this cubic equation for $[N_3]_u$.

Values of log $[(D_0 - D)/(D - D_\infty)]$ against log $[N_3]_u$ calculated in this way are shown as open circles in Figure 2. It is seen that there is very close agreement between the value of log $[(D_0 - D)/(D - D_\infty)]$ obtained from the observed values of D and those obtained using the value of D calculated from D_0, D_∞, K_α, and K_β. This close agreement indicates the absence of any co-operativity between α and β haems in the tetramer in the reaction of methaemoglobin with azide ion.

Having determined log K_L for azide ion reactions for the α and β chains in the tetramer it is of interest to ask whether we can account for the different values of log K_L for the α and β chains in different molecular environments. The values of log K_L for α^A, β^A, and values for valency hybrid haemoglobins (see below) $\alpha_2{}^+\beta_2(CO)_2$ and $\alpha_2(CO)_2\beta_2{}^+$ are given in Table 2 together with the values

corrected to 20 °C and phosphate buffer to allow direct comparison with the values reported above. The correction to 20 °C of the data for the valency hybrids was made using the known ΔH° and the correction from 1M (glycine + phosphate) buffer $I = 0.05$M to phosphate buffer $I = 0.05$M was made by using the known difference between log K_L for human methaemoglobin A in the two media.

We have previously attributed differences between values of log K_L for azide ion reacting with different haemoglobins to different contributions to the ΔG° of the configurational change which accompanies the spin-state change associated with the formation of the azide complex.[26] The spectrum of the isolated met α chain indicates that the iron atom is in a high-spin state as it is in the normal tetramer. Similarly the spectrum of the hybrid $\alpha_2{}^+\beta_2(CO)_2$ corresponds closely to the spectrum obtained by averaging the spectra of methaemoglobin and (carbon monoxide)haemoglobin. Thus we should expect that the affinity for azide ion should be the same for the α chain within the normal tetramer, in the isolated α chain and in the hybrid $\alpha_2{}^+\beta_2(CO)_2$. The observed values in phosphate buffer at pH 6.0 and 20 °C are 5.36, 5.37, and 5.43 (Table 2) which are the same

TABLE 2

Values of log K_L at pH 6 and 20 °C for azide binding to various methaemoglobin species

Species	log K_L	log K_L corrected to 20 °C and phosphate buffer
HbA	5.56 (In 1M-glycine) (pH 6, 20 °C)	5.71
α^A	5.22 (In 1M-glycine) (pH 6, 20 °C)	5.37
β^A	5.43 (In 1M-glycine) (pH 6, 20 °C)	5.58
$\alpha_2{}^+\beta_2(CO)_2$	5.55 (pH 6.2 and 16 °C)	5.43
$\alpha_2(CO)_2\beta_2{}^+$	5.64 (pH 6.1 and 16 °C)	5.56
α^A in $\alpha_2{}^A\beta_2{}^A$	5.36 (pH 6, 20 °C)	5.36
β^A in $\alpha_2{}^A\beta_2{}^A$	6.02 (pH 6, 20 °C)	6.02

within the error inherent in the observations and the correction procedure for the hybrid.

In contrast to the α chain the isolated met β chain has a spectrum which indicates the presence of a significant proportion of a low-spin form. Magnetic susceptibility measurements indicate that the proportion of low spin is 34%.[38] In the normal met tetramer the haems are high spin and therefore we should expect that the affinity for azide ion of the isolated β chains would be lower than those in the tetramer since for only 66% of the molecules will there be a contribution to ΔG° for the spin-state change. This is indeed observed, K_L for the β chain in the normal tetramer being 6.02 compared with 5.58 for the isolated β chain. Similarly the spectrum of the $\alpha_2(CO)_2\beta_2{}^+$ hybrid is consistent with a significant proportion of the β haems being in the low-spin state with the consequence that we find a value of 5.56 for log K_L for this hybrid.

[38] J. G. Beetlestone and J. B. Kushimo, unpublished observations.

Thermodynamics of the Reaction of Formate Ion
with Various Methaemoglobins and Sperm Whale
Metmyoglobin (with J. B. Kushimo)

We suggested in Part VIII [19] that there was a correlation between the magnetic susceptibility of a complex and the magnitude of the pH variation of the enthalpy of complex formation $[d(\Delta H°)/d(pH)]$ and we pointed out that such a correlation would arise if the ' hydrogen-in ' configuration was the stable one for the low-spin complexes and the ' hydrogen-out ' configuration for high-spin complexes. For complexes that are a mixture of spin states there would be an equilibrium between hydrogen-in and hydrogen-out configurations and hence intermediate $\Delta H°/pH$ behaviour. However subsequent work on the fluoride complexes of various haemoglobins showed that $d(\Delta H°)/d(pH)$ varied from species to species, even though all the fluoride complexes are high spin, suggesting that other structural features of the haemoglobin and the ligand may be involved in determining the nature of the pH variation of $\Delta H°$. In order to investigate this further we have examined the thermodynamics of the reaction of methaemoglobins with formate ion, an ion comparable in size to the azide ion but which forms a high-spin complex.

The complex between methaemoglobin and formate ion was first characterized spectrophotometrically by Scheler *et al.*[39] and in the present study we have confirmed the Soret and visible spectra obtained by these workers except for small differences in extinction coefficient which arise from our use of the value of 10.9×10^3 for the molar extinction coefficient of the methaemoglobin cyanide complex at 540 nm. The largest difference between the extinction coefficient of methaemoglobin and its formate complex is at 620 nm and accordingly the determinations of equilibrium constants were made at that wavelength.

Table 5 in the Supplementary Publication gives the values of $\log K_L$ as a function of temperature and pH at ionic strength $I = 0.25\text{M}$ for six methaemoglobins and sperm whale metmyoglobin. Control experiments have shown that the shape of the $\Delta H°$ against pH curve and the pH of maximum $-\Delta H°$ for the formation of the azide complex at the ionic strength of $I = 0.25\text{M}$ used for these measurements is the same as at $I = 0.05\text{M}$ although the absolute values differ slightly.[40] Values of $\Delta H°$ were obtained by plotting values of $\log K_L$ at constant pH (obtained by linear interpolation between adjacent experimental points) against $1/T$.

Figure 4 shows the variation of $\log K_L$ with pH for human methaemoglobin A reacting with formate ion at four different temperatures. Figure 5 shows the variation with pH of $\Delta H°$ for human methaemoglobins A and C, and the methaemoglobins of guinea pig and pigeon. The behaviour of sperm whale metmyoglobin and dog methaemoglobin is similar and is shown in Figure 6 of the Supplementary Publication. Comparison of these figures with those in Part XVI [27] shows that for the methaemo-

globins the form and the magnitude of the pH variation of $\Delta H°$ is closely similar to that of $\Delta H°$ for the formation of the azide complex. Within experimental error the maximum value of $-\Delta H°$ for each methaemoglobin occurs at the same pH as that for azide ion, *i.e.* at pH_{oh}, and the value of $d(\Delta H°)/d(pH)$ on either side of the maximum is the same as that obtained for azide ion. It is noteworthy that the maximum value of $-\Delta H°$ for human methaemoglobin C still occurs at pH 7.5 even
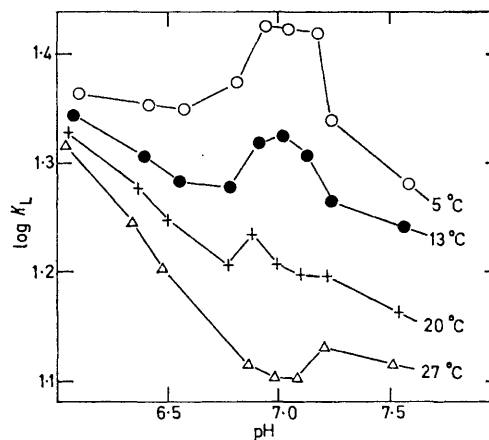

FIGURE 4 The variation with pH of $\log K_L$ for the reaction of formate ion with human methaemoglobin A at 5, 13, 20, and 27 °C in phosphate buffer $I = 0.25\text{M}$
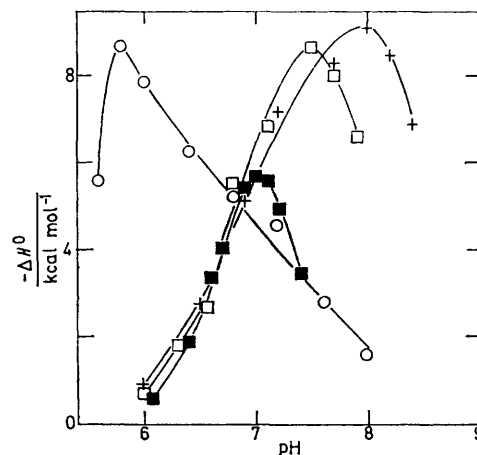

FIGURE 5 Plots of the variation with pH of the enthalpy of formation, $\Delta H°$, of the formate complex of human methaemoglobin A, ■; human methaemoglobin C, □; pigeon methaemoglobin, +; and guinea pig methaemoglobin, ○

though the amino-acid change which differentiates this methaemoglobin from human methaemoglobin A is more than 30 Å from all the iron atoms and the configurational change associated with the spin change is absent. For any particular haemoglobin the absolute values of $\Delta H°$ are less negative than those for azide ion, the magnitude of the difference being dependent on the particular methaemoglobin. These observations are consistent with the hypothesis that the mechanism which gives rise to the large bell-shaped variation of $-\Delta H°$ with pH for azide ion is also present in the reaction with formate ion.

[39] W. Scheler, A. Salweski, and F. Jung, *Biochem. Z.*, 1955, **326**, 288.
[40] J. B. Kushimo, M.Sc. Thesis, Ibadan University, 1972.

Since the formate complex is high spin this mechanism is not dependent on there being a spin-state change upon complex formation as we suggested earlier.

We have attributed variations in ΔG° for the formation of the azide complex between different methaemoglobins and sperm whale metmyoglobin to variations in the standard free-energy for the spin-state change. This was suggested by the fact that ΔG° for fluoride ion is the same for all methaemoglobins and sperm whale metmyoglobin, and that both methaemoglobin and its fluoride complex are largely in the high-spin form. Work in progress on the magnetic susceptibility of the azide complex of different haemoglobins suggests that this is indeed the case. The formate complex is also predominantly in the high-spin form and hence values of ΔG° for this ligand for the methaemoglobins and sperm whale metmyoglobin should be closely similar. Inspection of Figure 6 shows
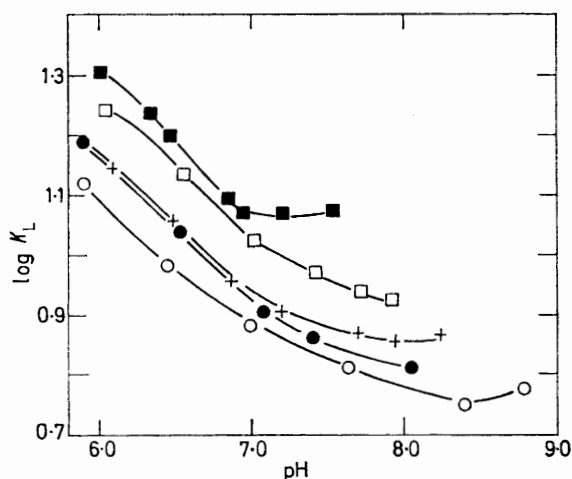
FIGURE 6   Plots of log K_L at 20 °C against pH for the reaction of formate ion with sperm whale metmyoglobin, ●; human methaemoglobin A, ■; human methaemoglobin C, □; guinea pig methaemoglobin, ○, and pigeon methaemoglobin, +

this to be the case. Particularly striking is the behaviour of sperm whale metmyoglobin which, as predicted, behaves as a typical methaemoglobin in contrast to its behaviour in those complexes where there is a contribution to ΔG° from the spin-state change.

Another interesting feature of the data shown in Figure 6 of the Supplementary Publication is the pH variation of ΔH° for sperm whale metmyoglobin. The magnitude of $d(\Delta H^\circ)/d(pH)$ on either side of the maximum is typical for a methaemoglobin reacting with either azide or cyanide ion. This is to be contrasted with its behaviour with azide ion where, although the form of the variation is the same, both the maximum and the minimum occurring at the same pH, the magnitude of $d(\Delta H^\circ)/d(pH)$ is much less.

The Wyman Linkage Relationships and the Thermodynamics of the Binding of Ligands to Methaemoglobin (with J. E. Goddard)

As outlined in the introduction, our original explanation of the large bell-shaped variation of ΔH° with pH

for the formation of the azide and cyanide complexes of methaemoglobin was in terms of a proton shift on the distal imidazole. However the demonstration that the variation with pH of ΔH° for the formation of the azide complex of glycera methaemoglobin (a haemoglobin in which the distal imidazole residue is replaced by leucine) closely resembles that of other methaemoglobins (see below) shows that the role played by the distal imidazole cannot be precisely that originally ascribed to it. Namely, that it cannot be essential for the existence of a large variation of ΔH° with pH although it may provide one mechanism by which a structural difference between methaemoglobin and its complex can arise. However, before we reconsider the question of what structural feature underlies the large variable compensating enthalpy and entropy change, in this part we shall put in more explicit terms just what has to be explained.

In Part VI [17] we showed that for the reaction of methaemoglobins A and C with azide ion the Wyman [41,42] relationship

$$\left(\frac{\partial \Delta H^\circ}{\partial pH}\right)_T = -2.303R \left\{ \partial\phi / \partial\left(\frac{1}{T}\right) \right\}_{pH} \qquad (8)$$

does not hold. ΔH° Is the enthalpy change for the reaction of methaemoglobin with a ligand and ϕ is the number of hydrogen ions released at a particular pH upon reaction with the ligand. The experimentally determined values of $(\partial \Delta H^\circ/\partial pH)_T$ imply values of $(\partial\phi/\partial T^{-1})_{pH}$ that are much larger than those observed. This can be illustrated in an alternative way in terms of the results presented in the previous section. Figure 4 shows the variation of log K_L for formate ion against pH at four temperatures. From the Wyman relationship

$$\left(\frac{\partial \log K_L}{\partial pH}\right)_T = \phi \qquad (9)$$

we should infer from Figure 4 that at 5 °C in the region of pH 6.7 ϕ is large and positive and at the same pH at 27 °C ϕ is large and negative. Similar arguments were given in Part VI [17] for the reaction with azide ion and as we showed there, such large variations in ϕ are not observed. What then is the origin of the discrepancy? In deriving equation (8) it is assumed that $d(\log \gamma_{HbN_3}/\gamma_{Hb})/dpH = 0$ where γ_{Hb} and γ_{HbN_3} are respectively the activity coefficients of methaemoglobin and its azide complex. In the application of this equation to the reaction of haemoglobin with oxygen no case has been found where this assumption has not been valid, although it must be noted that most tests have been made at 20 °C, a temperature at which the deviations are a minimum in the methaemoglobin reactions. However in an attempt to account for the pH dependence of log K_L in the pH region 5.5—6 for the reaction of azide ion with metmyoglobin in terms of ϕ it was necessary to include the activity coefficient terms.[25] The activity coefficients were calculated from the Kirkwood dielectric cavity

[41] J. Wyman, Adv. Protein Chem., 1964, **19**, 223.
[42] J. Wyman, Adv. Protein Chem., 1948, **4**, 407.

model for a protein.[43-45] Satisfactory agreement between the observed pH dependence of log K_L and that predicted from the observed value of ϕ was obtained at 20 °C when due account was taken of the effect on the activity coefficients of the variation with pH of the net charge on metmyoglobin and its azide complex. Similarly one could account for the variation of log K_L for formate with pH at 20 °C shown in Figure 4. However the minimum and maximum in the log K_L against pH curves at other temperatures cannot be accounted for in this way since the variation in the activity coefficient terms arising from the variation of the net charge with pH has only a small temperature dependence. Hence, in general, other factors must contribute to the variation of the activity coefficients with pH, but at 20 °C their contribution is fortuitously zero.

What then could be the origin of the large pH- and temperature-dependent variations in the activity coefficient ratio required to account for the observed variation in $\Delta H°$? Wyman in his review of linkage relationships notes that the derivation of the linkage relationship involves the assumption that the ratio of activity coefficients of the various protein species remains constant under different conditions, but goes further to state that this '. . . is equivalent to the assumption that there is no interaction between the sites in different macromolecules '.[41] It is our contention that the quoted statement is not necessarily correct.

The definition of an activity coefficient is dependent on the choice of standard state. Wyman chooses the standard state for reactant and product to be a solution in which the haemoglobin molecule is in the state where protons have been added to all possible sites on the molecule. Thus any variation with pH of the chemical potential of either reactant or product apart from those arising from loss of protons is subsumed in the activity coefficients defined in terms of the chosen standard state. However as the following analysis shows there can be a pH-dependent contribution to the standard free energy of the reaction which cannot be accounted for in terms of either linked hydrogen-ion effects or an intermolecular contribution to the activity coefficients.

In order to demonstrate the validity of this statement we shall derive the linkage relationship in a different, albeit more inelegant, way from that used by Wyman.

We write the reaction of methaemoglobin with a ligand X as
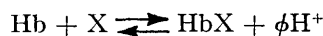
$$\text{Hb} + \text{X} \rightleftharpoons \text{HbX} + \phi\text{H}^+$$

ϕ Is the number of hydrogen ions released at a particular pH. The value of ϕ is determined by the differences between the pK values of ionizable groups on Hb and HbX and may of course be non-integral.

At equilibrium we have

$$\mu_{\text{Hb}} + \mu_{\text{X}} = \mu_{\text{HbX}} + \phi RT \ln a_{\text{H}^+} \qquad (10)$$

where μ_{Hb} and μ_{HbX} are the chemical potentials of Hb and

HbX under the particular conditions of pH and ionic strength.

We may express μ_{Hb} as

$$\mu_{\text{Hb}} = \mu°_{\text{Hb}} + RT \ln a_{\text{Hb}} \qquad (11)$$

where $\mu°_{\text{Hb}}$ is the standard chemical potential of Hb in its standard state. Following the procedure that we adopted in Part VII[18] we choose the standard state as a one molar solution with properties such that as the protein concentration and the ionic strength are reduced to zero the activity coefficient approaches unity assuming that the protein retains the same net charge and protein configuration (including configuration of charged groups on the surface of the molecules) that it has under the experimental conditions (i.e. in this case $I = 0.05\text{M}$ at the chosen pH).

$\mu°_{\text{HbX}}$ Is similarly defined and $\mu°_{\text{X}}$ is defined in terms of the customary hypothetical one molar solution.

Substituting from equation (11) and the equivalent equations for HbX and X into equation (10) we obtain

$$\mu°_{\text{HbX}} - \mu°_{\text{Hb}} - \mu°_{\text{X}} + \phi RT \ln a_{\text{H}^+}$$
$$= -RT \ln \frac{a_{\text{HbX}}}{a_{\text{Hb}}a_{\text{X}}} \qquad (12)$$

Defining activity coefficients by $a_{\text{HbX}} = [\text{HbX}]\gamma_{\text{HbX}}$ and $a_{\text{Hb}} = [\text{Hb}]\gamma_{\text{Hb}}$ and $a_{\text{X}} = [\text{X}]\gamma_{\text{X}}$ and substituting in equation (12) we have after rearranging terms

$$\log K_L = -(\mu°_{\text{HbX}} - \mu°_{\text{Hb}} - \mu°_{\text{X}})/2.303RT + \phi\text{pH}$$
$$+ \log \frac{\gamma_{\text{Hb}}\gamma_{\text{X}}}{\gamma_{\text{HbX}}}$$

which on differentiating with respect to pH gives

$$\frac{\text{d} \log K_L}{\text{dpH}} = -\frac{\text{d}(\mu°_{\text{HbX}} - \mu°_{\text{Hb}})}{2.303RT \, \text{dpH}}$$
$$+ \phi + \text{pH}\frac{\text{d}\phi}{\text{dpH}} + \frac{\text{d}}{\text{dpH}} \log \frac{\gamma_{\text{HbX}}}{\gamma_{\text{Hb}}\gamma_{\text{X}}} \qquad (13)$$

The last term on the right-hand side is not zero as we have shown in Part VII,[18] but it is not markedly temperature dependent and for the purpose of this analysis we shall neglect it. Equation (13) then reduces to the Wyman linkage relationship if

$$\frac{\text{d}(\mu°_{\text{HbX}} - \mu°_{\text{Hb}})}{2.303RT \, \text{dpH}} = \text{pH}\frac{\text{d}\phi}{\text{dpH}}$$

To simplify the formulation in the following argument we shall restrict ourselves to the situation where only one ionizable group is affected by the binding of the ligand X.

We, therefore, consider the four species Hb_u, HbX_u, Hb_i, and HbX_i corresponding to methaemoglobin and its complex with the linked group in the un-ionized and ionized states. If α_p and α_r are the fractions of the group ionized on HbX and Hb respectively at the chosen pH, then we have that

$$\mu°_{\text{HbX}} = \alpha_r\mu_{\text{Hb}i} + (1 - \alpha_r)\mu_{\text{Hb}u} \quad \text{and}$$
$$\mu°_{\text{HbX}} = \alpha_p\mu_{\text{HbX}i} + (1 - \alpha_p)\mu_{\text{HbX}u} \qquad (14)$$

[43] J. G. Kirkwood, *J. Chem. Phys.*, 1934, **2**, 351.
[44] C. Tanford and J. G. Kirkwood, *J. Amer. Chem. Soc.*, 1957, **79**, 5333.
[45] J. G. Kirkwood and F. H. Westheimer, *J. Phys. Chem.*, 1938, **6**, 507.

where μ_{Hb_u} is the chemical potential of the methaemoglobin without the ligand bound, with a proton bound to the linked ionizable group, and with the protein configuration on the rest of the molecule being that corresponding to the chosen pH. μ_{Hb_i}, μ_{HbX_u}, and μ_{HbX_i} are similarly defined for the respective species, the subscript i indicating that the linked group is ionized. If $\mu^{\circ}_{Hb_u}$, $\mu^{\circ}_{Hb_i}$, $\mu^{\circ}_{HbX_u}$, and $\mu^{\circ}_{HbX_i}$ are the corresponding standard chemical potentials we have

$$\mu_{Hb_i} = \mu^{\circ}_{Hb_i} + RT \ln \alpha_r$$
$$\mu_{Hb_u} = \mu^{\circ}_{Hb_u} + RT \ln (1 - \alpha_r) \quad (15)$$

and similar equations for μ_{HbX_i} and μ_{HbX_u} in terms of α_p. Combining equations (14) and (15) we obtain

$$\mu^{\circ}_{HbX} - \mu^{\circ}_{Hb} = \alpha_p(\mu^{\circ}_{HbX_i} - \mu^{\circ}_{HbX_u}) - \alpha_r(\mu^{\circ}_{Hb_i} - \mu^{\circ}_{Hb_u})$$
$$+ \mu^{\circ}_{HbX_u} - \mu^{\circ}_{Hb_u} + \alpha_p RT \ln \alpha_p$$
$$+ (1 - \alpha_p)RT \ln (1 - \alpha_p) - \alpha_r RT \ln \alpha_r$$
$$- (1 - \alpha_r)RT \ln (1 - \alpha_r)$$
$$= \alpha_p \Delta G^{\circ}_p - \alpha_r \Delta G^{\circ}_r$$
$$+ \mu^{\circ}_{HbX_u} - \mu^{\circ}_{Hb_u} + \alpha_p RT \ln \alpha_p + (1 - \alpha_p)RT$$
$$\ln (1 - \alpha_p) - \alpha_r RT \ln \alpha_r - (1 - \alpha_r) RT \ln (1 - \alpha_r)$$
$$(16)$$

where ΔG°_p and ΔG°_r are the standard free energies of ionization of the linked ionizable group on the reactant and product respectively when the charge and charge configuration correspond to the particular pH. The standard free-energy of ionization of the linked ionizable group on methaemoglobin is given by

$$\Delta G^{\circ}_r = \mu^{\circ}_{Hb_i} - \mu^{\circ}_{Hb_u}$$

with a similar equation for ΔG°_p.

Noting that $\Delta G^{\circ}_r = -2.303RT \log \alpha_r a_{H^+}/(1 - \alpha_r)$ and that $\alpha_p - \alpha_r = \phi$, equation (16) simplifies to

$$\frac{\mu^{\circ}_{HbX} - \mu^{\circ}_{Hb}}{2.303RT} = -\phi \log a_{H^+} + \log \alpha_p - \log \alpha_r$$
$$- \log K_p + \log K_r + \frac{\mu^{\circ}_{HbX_u} - \mu^{\circ}_{Hb_u}}{2.303RT}$$

Differentiating with respect to pH and assuming that K_p and K_r are not functions of pH we obtain

$$\frac{1}{2.303RT} \frac{d(\mu^{\circ}_{HbX} - \mu^{\circ}_{Hb_u})}{dpH} = \phi + pH \frac{d\phi}{dpH} + \frac{1}{\alpha_p} \frac{d\alpha_p}{dpH}$$
$$- \frac{1}{\alpha_r} \frac{d\alpha_r}{dpH} + \frac{1}{2.303RT} \frac{d}{dpH} (\mu^{\circ}_{HbX_u} - \mu^{\circ}_{Hb_u}) \quad (17)$$

Noting that

$$\alpha_p = \frac{K_p}{(1 + K_p)} \quad \text{and} \quad \alpha_r = \frac{K_r}{(1 + K_r)}$$

it is easily shown that

$$\frac{1}{\alpha_p} \frac{d\alpha_p}{dpH} - \frac{1}{\alpha_r} \frac{d\alpha_r}{dpH} = -\phi$$

and hence that

$$\frac{1}{2.303RT} \frac{d(\mu^{\circ}_{HbX} - \mu^{\circ}_{Hb})}{dpH}$$
$$= pH \frac{d\phi}{dpH} + \frac{d}{2.303RTdpH} (\mu^{\circ}_{HbX_u} - \mu^{\circ}_{Hb_u})$$

Substituting in equation (13) we obtain

$$\frac{d \log K_L}{dpH} = \phi - \frac{d}{2.303RTdpH} (\mu^{\circ}_{HbX_u} - \mu^{\circ}_{Hb_u}) \quad (18)$$

This reduces to the Wyman linkage relationship only if the second term on the right-hand side is zero. There is no necessity for this to be so. Differentiating this expression with respect to (T^{-1}) we get

$$\frac{d}{dpH} \left[\frac{\log K_L}{d(T^{-1})} \right] = \frac{d\phi}{d(T^{-1})} - \frac{1}{2.303R} \frac{d}{dpH} (\mu^{\circ}_{HbX_u} - \mu^{\circ}_{Hb_u})$$
$$+ \frac{T}{2.303R} \frac{d}{dpH} \left[\frac{d}{dT} (\mu^{\circ}_{HbX_u} - \mu^{\circ}_{Hb_u}) \right]$$

and hence

$$\frac{d}{dpH} (-\Delta H^{\circ}) = 2.303R \frac{d\phi}{d(T^{-1})} - \frac{d}{dpH} (\mu^{\circ}_{HbX_u} - \mu^{\circ}_{Hb_u})$$
$$- \frac{Td}{dpH} (S^{\circ}_{HbX_u} - S^{\circ}_{Hb_u}) \quad (19)$$

The first term in this equation is the same as the right-hand side of equation (8), i.e. the usual term in the Wyman relationship. The second term is the pH dependence of the difference between the standard chemical potentials of methaemoglobin and its complex, both species having the ionizable group that is thermodynamically linked to the ligand binding site in a specified state of ionization. Both $\mu^{\circ}_{HbX_u}$ and $\mu^{\circ}_{Hb_u}$ will be functions of pH since they were defined in terms of the net charge and protein configuration at a particular pH. As groups in the protein ionize so $\mu^{\circ}_{HbX_u}$ and $\mu^{\circ}_{Hb_u}$ will vary, and there is no necessity that their pH variation should be identical, since the charge configuration of reactant and product need not be the same. Hence in general this term will not be zero. Similar arguments apply to the entropy terms in equation (19). We have noted above that for the reaction of methaemoglobin with ligands $d\phi/d(T^{-1})$ is small and for all ligands the pH variation of ΔH° is much greater than this. Hence the large variation of ΔH° with pH arises primarily from the third term as the right-hand side of equation (19), i.e. the entropy term. How, in general, could a pH-dependent difference between $S^{\circ}_{HbX_u}$ and $S^{\circ}_{Hb_u}$ arise?

The Origin of Large Variable Entropy Changes.— Absolute values of partial molar entropies for protein molecules in solution are not known but since the typical mammalian haemoglobin tetramer has approximately 190 ionizable groups on its surface we may conclude that there will be a large contribution to the partial molar entropy from charged-group–solvent interactions.

Over the pH range 6—9 the ionization of various groups changes the net charge on the haemoglobin molecule by ca. 30. The detailed analysis of the titration curve of human methaemoglobin carried out by Orttung [46] suggests that the ionization of the imidazole side-chain of histidine residues accounts for slightly more than half of this change, the rest being accounted for by the carboxylic acid side-chains of aspartic and glutamic acid

[46] W. H. Orttung, *J. Amer. Chem. Soc.*, 1969, **91**, 162.

residues and the four N-terminal amino-groups. An analysis of the temperature dependence of the titration curve of haemoglobin shows that the enthalpy of ionization of carboxylic acid groups and the imidazole side-chain are similar to those observed for these groups in small molecules. On this basis we estimate that ionization of groups in the range pH 6—7 may lower the partial molar entropy of the haemoglobin molecule by up to 100—180 cal K^{-1} mol^{-1}. The entropy of formation of the azide complex varies by $ca.$ 10 cal K^{-1} mol^{-1} over the same pH range, $i.e.$ 7—10% of the total change in the partial molar entropy of the molecule; $i.e.$ we have to postulate that the loss of the same number of protons from methaemoglobin and its azide complex over the pH range 6—7 gives rise to a 7—10% greater decrease in the partial molar entropy of the azide complex than the methaemoglobin molecule. Thus quite small percentage changes in the partial molar entropy of the haemoglobin molecule upon complex formation could give rise to the observed behaviour. Such small changes could easily be accounted for in terms of (i) slight changes in charged-group-solvent interactions arising from changes in nearest-neighbour charged-group interactions; (ii) changes in the average distance of charged groups from the surface of the molecule, $i.e.$ distance from the region of low dielectric constant; (iii) changes in the distribution of protons among the various binding sites on the molecule, $e.g.$ a slight shift of protons from carboxylic acid sites to imidazole sites consequent upon slight changes in configuration would probably lead to a decrease in the partial molar entropy of the molecule. This latter mechanism has been mentioned briefly in a footnote by Wyman in connection with the variation with pH of the heat of proton oxygen interaction.[41]

In principle, therefore, we can account for the large variation of ΔH° for the formation of the azide complex of human methaemoglobin in the range pH 6—7, but nothing we have said so far would lead us to predict the sharp change in behaviour at pH 7.1 such that above this pH, pH_{ch}, ΔH° increases with pH.

However it will be recalled that in Part VII [18] and the first section of this paper we postulated the existence in the region of pH_{ch} of a pH-dependent configurational change involving charged groups. In this way the abrupt change in the ΔH° behaviour becomes explicable and we are led to conclude that for the protein configuration above pH_{ch} ionization of groups leads to a greater decrease in the partial molar entropy of methaemoglobin than its azide complex. At this stage we can say little about the nature of the configuration change occurring at pH_{ch} except to note that since the pH at which it occurs for a haemoglobin, pH_{ch}, seems to be dependent on the relative number of positive and negative charged amino-acids (excluding imidazoles) in the molecule,[27] and the partial molar entropy of the high and low pH configuration are affected differently by the ionization of groups, it would appear that it involves the concerted movement of charged groups on the surface from one average configuration to another.

We turn now to the question of how the replacement of the water molecule in methaemoglobin by azide ion could affect the configuration of charged groups on the surface of the molecule.

We know that the azide complex is low spin and that a slight configurational change in the protein accompanies the spin-state change upon complex formation. However this cannot be the effective mechanism since formate ion, which forms a predominantly high-spin complex shows the same ΔH° behaviour as azide ion.

We have postulated previously [19] that the variation of $-\Delta H^{\circ}$ with pH for the reaction of a methaemoglobin with ligands can be explained in terms of a proton-shift mechanism. According to this hypothesis, which we outlined in the introduction, methaemoglobin and its complexes exist as an equilibrium mixture between two structures which differ in the position of a hydrogen atom on the imidazole ring of the distal histidine. In one structure there is a hydrogen atom on the outer nitrogen of the imidazole ring which is on the surface of the molecule and ' sees ' the solvent, in the other this hydrogen shifts to the inner nitrogen of the ring to form a hydrogen-bond with the ligand which is bonded to the iron. The position of equilibrium between the two structures depends on the nature of the ligand. For ligands with a large variation of $-\Delta H^{\circ}$ with pH, such as azide, cyanide, and formate ion, we envisaged that the position of equilibrium in the complex is largely in the ' hydrogen in ' form, whereas aquomethaemoglobin is in the form in which the hydrogen is on the outer nitrogen of the imidazole ring. The shift of the proton from the outer to the inner nitrogen of the imidazole ring would be expected to result in a different arrangement of the charged groups on the surface of the molecule for the reactant and product, with a consequent different pH variation of S°. For ligands such as SH^{-}, OH^{-}, and methylamine we observed a smaller variation of ΔH° with pH. We suggested that since the ligand brings its own hydrogen atom into the complex, hydrogen bonding between the ligand and the hydrogen atom on the distal imidazole can occur while at the same time a hydrogen atom remains on the outer nitrogen in the complex. The smaller variation in ΔH° is thus seen as a consequence of the similarity of the surface configuration of methaemoglobin and the complex.

The small variation of ΔH° for SH^{-} and OH^{-} may arise from the fact that the ligand is charged whereas the ligand in methaemoglobin is the uncharged water molecule. This could affect the surface distribution of charged groups by a simple electrostatic interaction with or without the transfer of charge to the surface of the molecule as we suggest in Part XVI.[27] If this is the case we should predict that the variation of ΔH° with pH should be zero for methylamine as a ligand. The available data suggest that this is indeed the case but unfortunately experimental difficulties prevent the measurement of ΔH° over the complete pH range.[1]

It should be pointed out that the different behaviour shown by the two classes of ligands, $i.e.$ azide type and

sulphydryl type could be explained in an alternative way. It could be argued that, since the ligand does not bring a hydrogen atom into the complex, no hydrogen bond will be formed between the ligand and the distal imidazole and that as a consequence of the absence of the hydrogen bond the E helix, and hence groups on the surface, has a slightly different configuration. However there is no X-ray evidence for such a movement of the E helix and, as will be seen below, the pH variation of $\Delta H°$ for formate and azide ions reacting with chemically modified methaemoglobin is more readily understood in terms of the first hypothesis.

For fluoride ion the pH variation of $\Delta H°$ is small but the most significant feature is that the type of behaviour varies from one methaemoglobin to another. At one extreme, $-\Delta H°$ for the formation of the fluoride complex of dog methaemoglobin shows a maximum, and at the other, $\Delta H°$ for metmyoglobin shows a minimum. That for pigeon methaemoglobin shows hardly any variation at all.[26] On our hypothesis we should expect such behaviour if for fluoride ion the ' hydrogen in ' and ' hydrogen out ' configuration had comparable stability, the precise position of equilibrium depending on the haemoglobin species. If this is correct then replacement of hydrogen by deuterium in the solvent and hence in the exchangeable hydrogens should dramatically affect the form of the $\Delta H°$ against pH curve for fluoride ion as a ligand. Such behaviour is indeed observed [47] and will be reported in a subsequent paper.

The original basis for our postulated mechanism involving the shift of a proton from the outer to the inner nitrogen of the distal imidazole was the X-ray crystallographic work of Stryer et al.[48] on metmyoglobin. They found that the binding of azide ion to metmyoglobin results in the release of a sulphate ion from a position adjacent to the tertiary nitrogen of the distal imidazole ring which is exposed to the solvent. We have studied the effect of sulphate ion on the thermodynamics of azide binding to methaemoglobin.[49] We find that the values of log K_L differ little from those measured in phosphate buffer, but the behaviour of $\Delta H°$ is very different in the presence of sulphate, being invariant with pH. This behaviour is readily explicable if the sulphate ion is bound at a site adjacent to the outer nitrogen atom of distal imidazole in both methaemoglobin and its azide complex, for this would prevent operation of the mechanism by which we suggest azide binding affects the configuration of charged groups on the surface of the molecule. These results will be described in detail in a subsequent paper.

Thermodynamics of the Reaction of Glycera Methaemoglobin with Azide and Hydroxyl Ions
(with G. B. Ogunmola and Bette Seamonds)

As summarized in the introduction and elaborated above we have postulated that the transfer of a proton

from the ' outside ' to the ' inside ' position on the distal imidazole plays a crucial role in determining the variability of $\Delta H°$ for the formation of the azide, cyanide, and formate complexes of methaemoglobins.

If this particular mechanism for the variable $\Delta H°$ change is correct then we should expect to find markedly different $\Delta H°$ behaviour for the binding of azide-type ligands to a methaemoglobin where the distal histidine has been replaced by another residue which is incapable of the same type of proton shift. Haemoglobin Zürich, a haemoglobin variant which satisfies this requirement,[50] is unstable in the methaemoglobin form and hence it was not possible to obtain the thermodynamic parameters of the binding reaction.[24] Monomeric glycera methaemoglobin fulfils the same requirements and is quite stable in the range pH 5—10 and hence we are able to determine the thermodynamics of complex formation with simple anions as a function of pH.

Riggs and his co-workers [51] have determined the amino-acid sequence of monomeric glycera haemoglobin and showed the distal group to be leucine rather than histidine which is typical of mammalian haemoglobins. The haemoglobins obtained from the annelid polychete, *Glycera dibrachiata*, exist in both monomeric and polymeric forms.[52] The monomeric glycera haemoglobin contains a single haem and a single polypeptide chain having a molecular weight of *ca.* 18 000, analogous to myoglobin and the subunits of tetrameric haemoglobins. The oxygen affinity of monomeric glycera haemoglobin is lower than that of human α chain, but not as low as that of tetrameric haemoglobins.[53]

In the present work, we have studied the reactions of monomeric glycera methaemoglobin with azide and hydroxyl ions at different values of pH and temperature and we have shown that the replacement of the distal histidine by leucine does not affect the general form of the pH variation of $\Delta H°$ for azide complex formation. We have shown in the previous section that $\Delta H°$ variability arises when the configuration of charged groups on the surface of the molecule is different in methaemoglobin and its complex. Since the proton transfer cannot operate in glycera methaemoglobin an alternative mechanism must be operative.

The pK_a for the ionization of glycera acid methaemoglobin to the alkaline form at 20 °C is 9.53. Table 6 of the Supplementary Publication gives values of pK_a at various temperatures and a plot of pK_a *versus* $1/T$ is linear within the temperature range of 12.6—27.40 °C.

The ionization is accompanied by a free-energy change, $\Delta G°$, of $+12.80 \pm 0.03$ kcal mol^{-1} at 20 °C and an enthalpy of ionization $\Delta H°$ of 5.05 ± 0.02 kcal mol^{-1}. The pK_a for glycera methaemoglobin is higher than those for typical mammalian methaemoglobins and sperm

[47] R. O. Ige and J. G. Beetlestone, unpublished observations.
[48] L. Stryer, J. C. Kendrew, and H. C. Watson, *J. Mol. Biol.*, 1964, **8**, 96.
[49] S. L. Iwuagwu, Ph.D. Thesis, University of Ibadan, 1973.

[50] C. J. Muller and S. Kingma, *Biochem. Biophys. Acta*, 1961, **50**, 595.
[51] T. Imamura, T. O. Baldwin, and A. Riggs, *J. Biol. Chem.*, 1972, **247**, 2785.
[52] B. Seamonds, R. E. Forster, and A. J. Gottlieb, *J. Biol. Chem.*, 1971, 246, 1700.
[53] B. Seamonds, Ph.D. Thesis, University of Pennsylvania, 1969.

whale metmyoglobin, the ΔG° for the formation of the hydroxyl complex of glycera haemoglobin being *ca.* 1.9 kcal mol⁻¹ less favourable than that for human methaemoglobin and 0.71 kcal mol⁻¹ less favourable than that for sperm whale metmyoglobin.

Azide ions react stoicheiometrically with glycera methaemoglobin in the ratio 1 : 1; the Hill coefficient *n*, for the reaction being essentially unity (0.97 ± 0.04) in agreement with earlier work on tetrameric methaemoglobins [17,19,27] and sperm whale metmyoglobin.[25]

Table 6 of the Supplementary Publication shows log-K_{obs} and log K_{L} for the reaction of glycera methaemoglobin with azide ion at different values of pH and temperature. At 20 °C and pH 6.2 log $K_{\mathrm{L}} = 4.29$. The affinity for azide ion of glycera methaemoglobin is thus lower than that of either sperm whale metmyoglobin or a typical tetrameric methaemoglobin. This behaviour parallels that of the ionization to the alkaline form and this is illustrated in Figure 7 which shows a plot of pK_{a}
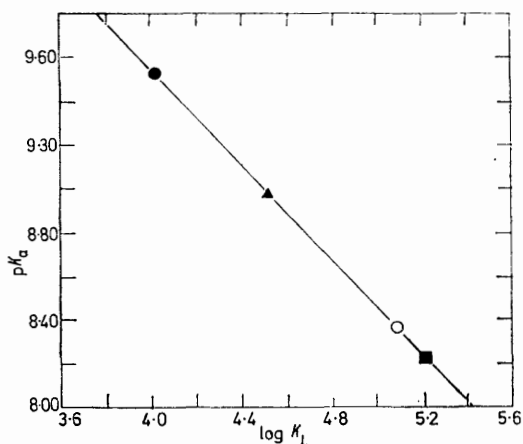
FIGURE 7 A plot of pK_{a} against log K_{L} at 20 °C for mammalian methaemoglobins, the α and β chains of mammalian methaemoglobin, sperm whale metmyoglobin, and glycera methaemoglobin: mean values of log K_{L} and pK_{a} for different mammalian methaemoglobin, ■; mean value of log K_{L} and pK_{a} of the α and β chain of mammalian methaemoglobin, ○; sperm whale metmyoglobin, △; and glycera methaemoglobin, ●

versus log K_{L} at pH 8.0 for glycera methaemoglobin, sperm whale metmyoglobin, and the average of several mammalian methaemoglobins and their subunits. Apart from small differences within the methaemoglobins there is a linear correlation between pK_{a} and log K_{L} the slope of the plot being very close to −1. What then are the structural factors which give rise to these differences in ligand affinity? We consider various possibilities:

(1) In an earlier paper we suggested that the differences between ΔG° for the formation of low-spin complexes of vertebrate methaemoglobins and sperm whale metmyoglobin could be ascribed to differences between the contribution to ΔG° of the change in configuration known to accompany the spin-state change. However this is unlikely to be the explanation of the different behaviour of glycera methaemoglobin since the percentage of high- and low-spin forms in acid and alkaline glycera

methaemoglobin, as determined approximately from the visible spectra, would lead one to expect the affinity of glycera methaemoglobin for azide and hydroxyl ions to be intermediate between that of sperm whale metmyoglobin and a typical vertebrate methaemoglobin.

(2) Whether a methaemoglobin is a monomer or a tetramer clearly cannot be a major factor determining ligand-binding behaviour since the constituent chains behave similarly to the parent vertebrate tetramers and differ from metmyoglobin by as much as metmyoglobin differs from glycera methaemoglobin.

(3) At the ionic strength at which the ligand-binding experiments were carried out most of the interaction between charged groups on the surface of the protein and the formal positive charge on the iron atom will be screened out and hence is unlikely to be responsible for the observed difference in ΔG° for the binding of charged ligands. However, in glycera methaemoglobin an aspartic acid residue occupies position E5(57) which is adjacent to leucine E6(58), the group which replaces the distal histidine found in most haemoglobins. The presence of this negatively charged group in a position close to the haem iron would be expected to produce a marked decrease in the affinity for charged ligands. An estimate of the magnitude of this effect can be obtained from the ligand binding studies carried out on methaemoglobin Norfolk in which the group adjacent to the distal histidine in the α chain is an aspartic acid residue rather than a glycine residue which occupies this position in human haemoglobin A. Beetlestone and Irvine [24] estimated that the affinity for fluoride ion of the abnormal α chain was approximately 30 times lower than that for the normal β chain. In the absence of other factors such as spin-state differences this would lead one to expect the equilibrium constant for the formation of the azide complex of glycera methaemoglobin to be *ca.* 30 times less than that for methaemoglobin A, that is to say log-$K_{\mathrm{L}} = 4.16$ rather than the value for methaemoglobin A of 5.64 at pH 6.0. The observed value for glycera methaemoglobin is 4.30 in good agreement with the predicted value.

Similarly we would predict a pK_{a} value of 9.61 which is in good agreement with the observed value of 9.53. We conclude, therefore, that coulombic interaction between the negatively charged group on the E5(57) aspartic acid residue and the formal charge of +1 on the haem group in glycera acid methaemoglobin is sufficient to account for the observed difference between the affinity for negatively charged ligands of human and glycera methaemoglobins. This mechanism is independent of the chemical nature of the charged ligand and, therefore, produces the same effect on the affinity for azide and hydroxyl ion in agreement with the observed behaviour.

Figure 8 illustrates the variation of ΔH° with pH for the reaction of glycera haemoglobin with azide ion. In contrast to the ΔG° behaviour the variation of ΔH° with pH is large and has the typical bell-shaped form with a pH of maximum $-\Delta H^\circ$, *i.e.* pH_{ch}, occurring at pH 7.2

compared with 7.1 for human methaemoglobin A [17] and 8.6 for sperm whale metmyoglobin.[25]

Following the argument presented above we conclude that the charge configuration on the surface of glycera methaemoglobin differs from that of its azide complex. However, since the absence of the distal imidazole group precludes proton transfer on this group as a mechanism by which this difference is brought about, we must infer either that a different mechanism is operative in glycera methaemoglobin or that the proton transfer hypothesis is incorrect and that there is another mechanism common to both glycera methaemoglobin and mammalian methaemoglobins. The latter alternative seems unlikely in view of the evidence for the proton transfer mechanism, namely (i) the different $\Delta H°$ behaviour shown by those ligands with a proton bonded to the ligand atom and those without, and (ii) the specific effect of sulphate ion in eliminating the pH variation of $-\Delta H°$ for the binding of azide ion. In addition it should be noted that $d\Delta H°/dpH$ for glycera methaemoglobin in the pH range 6—7.2
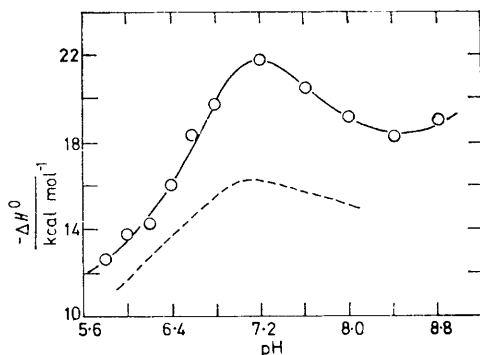
FIGURE 8   Plots of $\Delta H°$ of formation of the azide complex against pH for: monomeric glycera methaemoglobin, ○; human methaemoglobin A, — — —

is much greater than that for a typical mammalian haemoglobin (see Figure 8) implying that the variation of $\Delta H°$ may arise by a different mechanism in glycera methaemoglobin. What could this mechanism be?

We have concluded above that there is a large electrostatic interaction between the positive charge on the haem iron atom and the aspartic acid residue at position E5(57) in glycera methaemoglobin. Hence it is not unlikely that upon neutralization of this charge by the azide ion this group will move to a different orientation with a consequent readjustment of other charged groups on the surface of the molecule. If this contention is correct then the reaction of hydrosulphide ion with glycera methaemoglobin should show the same pH variation of $-\Delta H°$ as that for azide ion since the presence or absence of the proton on the ligand is not crucial.

The Thermodynamics of the Reaction with Azide Ion of the Separated Polypeptide Chains of Human and Dog Methaemoglobins (with O. S. Adeosun, J. E. Goddard, and M. M. Ogunlesi)

We have shown in Part XVI [27] that there is a linear correlation between the characteristic pH of a methaemo-

globin and the difference between the number of lysine and arginine residues and the number of glutamic and aspartic acid residues (Lys + Arg — Glu — Asp) in the tetramer. Since the amino-acid composition of the tetramer is the sum of that of the component chains, this would imply that one should be able to calculate the pH_{ch} for a tetramer by taking the average of the pH_{ch} values of the contributing chains. However we have also implied that the form of the pH variation of $\Delta H°$ is a function of the tetramer and does not arise simply by the addition of the ΔH contributions from the component chains since for all the methaemoglobins we have investigated so far the variation of $-\Delta H°$ with pH for the reaction with azide ion shows a smooth curve with a single discrete maximum. In this section and the next we look more closely at these two assumptions. We have determined the pH_{ch} for the reaction with azide ion of the isolated met chains of human and dog haemoglobins. We have already reported [23] the determination of the pH_{ch} for the isolated α chain of human methaemoglobin A, but subsequent work has shown that the native form of the met α chain is unstable and that our experiments were carried out on modified α chains. However it has been shown that the isolated met α and β chains are sufficiently stable in 1M-glycine solution to permit the determination of pH_{ch}.[34]

Haemoglobin Reconstituted from the Chains.—When either the oxy or the met forms of the two types of chain were recombined the product had the same spectral and electrophoretic properties as the corresponding haemoglobin A derivative.

The values of log K_L for the formation of the azide complex of the α and β met chains of dog and human haemoglobins in 1M-glycine are shown in Table 7 in the Supplementary Publication. The values of $\Delta H°$ calculated in the usual way from these data are shown in Figure 9. Each chain shows a typical variation of $\Delta H°$ with pH, there being in each case a smooth curve with a distinct maximum of $-\Delta H°$. It is worth noting that the form of the curve does not appear to be influenced by the state of polymerization of the species as both the monomeric human α chains and the tetrameric human β chains show the typical bell-shaped curves which we have found in the past for a number of haemoglobin tetramers and for the two monomeric species, sperm whale metmyoglobin and glycera haemoglobin.

The variation of $\Delta H°$ with pH for human A and dog haemoglobins reacting with azide ion in 1M-glycine was also determined and values of log K_L and $\Delta H°$ for these reactions are shown in Table 8 in the Supplementary Publication. Calculation of $\Delta H°$ as a function of pH from these values shows that in 1M-glycine the pH_{ch} is unchanged for both human A and dog haemoglobins. The values of log K_L are generally lower in the presence of 1M-glycine than in its absence, but the average difference is not more than *ca.* 0.1. The values of $-\Delta H°$ are also slightly lower in 1M-glycine the difference ranging from *ca.* 0.4 to 1.0 kcal mol⁻¹ for the two species across the pH range.

Assuming that pH_{ch} is dependent on the overall amino-acid composition of the tetramer, we have calculated the
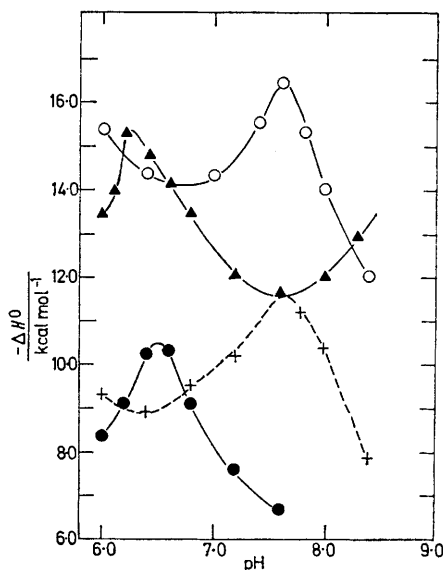

FIGURE 9   Plots of ΔH° against pH for the reaction of azide ion with the isolated chains of dog methaemoglobin and human methaemoglobin A in 1M-glycine: $\bigcirc$, β^+_{dog}; $\blacktriangle$, α^+_{dog}; $\bullet$, β^+_A; $+$, α^+_A

pH_{ch} for the human A and dog haemoglobins by taking the average of the pH_{ch} values of the α and β polypeptide chains

$$i.e. \quad pH_{ch}(\alpha_2\beta_2) = \tfrac{1}{2}[pH_{ch}(\alpha) + pH_{ch}(\beta)] \quad (20)$$

Our results are shown in Table 3 together with the experimentally observed pH_{ch} values for the two parent haemoglobins and the canine–human hybrids which we will discuss in the next section.

TABLE 3

Observed and calculated values of pH_{ch}. The calculated values were obtained from the equation $pH_{ch} = \tfrac{1}{2}[pH_{ch}(\alpha) + pH_{ch}(\beta)]$

	pH_{ch}(obs)	pH_{ch} (calculated)
α_{can}	6.3	
β_{can}	7.6	
α_A	7.7	
β_A	6.5	
Human	7.1	7.1
Canine	6.9	6.95
$\alpha_2^A\beta_2^{can}$	7.8	7.65
$\alpha_2^{can}\beta_2^A$	6.3	6.4

The observed and calculated values agree within experimental error showing that the characteristic pH of the tetramer can indeed be calculated from an average of those of the contributing chains, as would be expected from the correlation between pH_{ch} and the amino-acid composition.

We look now at the typical bell-shaped curve for the variation of ΔH° with pH. Figure 10 shows the curves obtained by averaging the ΔH° profiles for α and β chains for either human A or dog methaemoglobins together with the experimental curves for the parent methaemoglobins. The form of the curves calculated in this way

differs markedly from those found experimentally, showing that when they are in the tetramer the chains cannot be operating independently but must interact with one another in some way and operate in a concerted fashion. We discuss in the next section how this concerted interaction might arise and we also show there that pH_{ch} is still a function of the tetramer even if the contributing chains come from different species (human–dog hybrids) or if one of the chains is functionally inoperable and exists in the tetramer in its carbon monoxide form (human valency hybrids). It could be argued that ΔH° for the parent tetramer is calculated from values of log K_L which are themselves the average of different log K_L values for the constituent chains. In order to examine this possibility we have calculated values of ΔH° as a function of pH from values of log K_L obtained by averaging the values for the isolated α and β chains of human methaemoglobin. This curve is compared with the experimental curve in Figure 10, and confirms that the observed ΔH° profile is a function of the tetramer.

We look now at the apparent correlation between pH_{ch} and the function (Lys + Arg − Glu − Asp − 8) (Part XVI[27]). Since we first noted this correlation the primary structure of dog haemoglobin has been determined and Figure 7 in the Supplementary Publication shows a plot of pH_{ch} against the function (Lys + Arg − Glu − Asp − 8) on which the values for dog haemoglobin and the separated
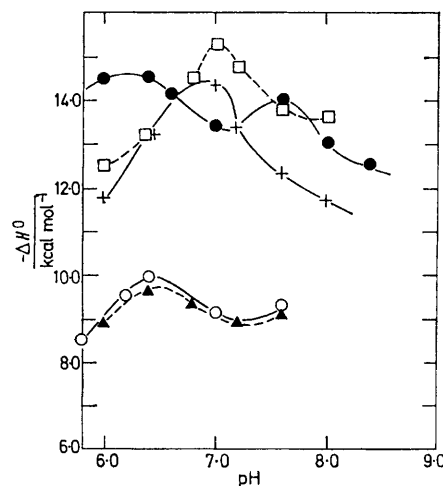

FIGURE 10   Plots showing the pH variation of values of $-\Delta H^\circ$ for dog and human methaemoglobin calculated from the equation

$$\Delta H^\circ = \tfrac{1}{2}\{\Delta H^\circ(\alpha^+) + \Delta H^\circ(\beta^+)\}$$

compared with the observed values of ΔH° for these methaemoglobins. Dog methaemoglobin: calculated value, $\bullet$; observed value, $+$. Human methaemoglobin A: calculated value, $\blacktriangle$; observed value, $\square$. Value of ΔH° calculated from the average values of log K_L for α^+_A and β^+_A, $\bigcirc$

chains of human and dog haemoglobins have been included. From this it is clear that whereas the correlation between pH_{ch} and the composition of charged amino-acids still holds for the human α and β chains it does not do so for the dog tetramer or for its separated α chain although the dog β chain does lie very close to the correlation line. The dog α and β chains have the same net (Lys + Arg −

Asp — Glu) as one another and yet have widely different characteristic pH values. The electrophoretic mobilities at pH 8.3 of the dog chains differ one from another as is shown in Table 4 which includes the electrophoretic

TABLE 4

Species	Electrophoretic mobility (pH 8.3; 300 V; 16 mA; 12 h)	pH$_{ch}$	(Lys + Arg — Asp — Glu — 8)
β^A	6.7 cm	6.5	−12
$\alpha_2^{dog}\beta_2^A$	6.5 cm	6.3	−8
α^{dog}	4.4 cm	6.2$_5$	−4
A	4.0 cm	7.0	−6
Dog	3.7 cm	6.9	−4
β^{dog}	2.5 cm	7.6	−4
$\alpha_2^A\beta_2^{dog}$	1.2 cm	7.8	−2
α^A	1.0 cm	7.6$_5$	0

mobilities and the characteristic pH values of the separated chains of dog and human haemoglobins together with those of the parent tetramers and of the dog–human hybrids which will be discussed in the next section. The observed pH$_{ch}$ values for the dog chains agree qualitatively with the values that would be expected from their electrophoretic mobilities at pH 8.6, the chain with the higher electrophoretic mobility having the lower pH$_{ch}$ and vice versa.

On the face of it, it seems as if these results, particularly those with the dog α chain, invalidate the correlation between pH$_{ch}$ and the function of charged amino-acid residues in the molecule. A comparison of the primary structure of each of the dog chains with that of the equivalent chain of haemoglobin A shows that apart from 1 aspartic acid replacing 1 glutamic acid and 1 lysine replacing 1 arginine in the β chain and 2 aspartic acids replacing 2 glutamic acids in the α chain and several substitutions of neutral amino-acids, the dog α chain differs from the human α chain in having one extra aspartic acid residue, Asp αA13 (α15) while the canine β chain has one extra lysine at Lys βF3 (β87) and another at Lys βE20 (β76). One extra acidic residue on the α chain therefore shifts the pH$_{ch}$ from pH 7.65 for α HbA to pH 6.3 for α Hb dog. This is qualitatively in the direction which would be expected from an extra negative charge, but quantitatively the difference is greater than would be expected. Similarly for the β chain the extra two lysine residues in the dog β chain give rise to a shift in pH$_{ch}$ from pH 6.5 for the human β chain to pH 7.6 for the dog β chain. This is in reasonable agreement with what one would expect from our observed correlation. In each case the charged substitutions are in what are regarded as functionally inoperable positions in the molecule, being on the surface and far from the haem or the interchain contacts. If, however, the configurational change underlying pH$_{ch}$ does involve the concerted re-arrangement of charged groups on the surface of the molecule then the pH at which this change takes place will depend not only on the overall number of ionizable groups on the molecule but also on their relative positions. Thus it could well be argued that the substitution of only one extra positive or negative charge on the protein

surface at a crucial position could alter the whole pattern of interaction of charged groups and thus drastically alter pH$_{ch}$.

Thus the pH$_{ch}$ can still be regarded as being governed by the composition of charged amino-acid residues in the haemoglobin even if the apparent direct correlation obtained for the first few haemoglobins for which the complete sequence was known was fortuitous. That it is not governed only by charged residues on the surface of the protein has been shown in this section by the fact that the form of the $\Delta H°$ vs. pH profile cannot be obtained by averaging the curves for the contributing chains. This shows that the chains must interact in some way and we will present further evidence for this in the following two sections when we look at the human A valency hybrids, the dog–human hybrids, and various chemically modified haemoglobins.

In conclusion it should be noted that, although the spectrum of the met β chains of human haemoglobin is different from that of human methaemoglobin in a manner that suggests a significant proportion of a low-spin form, the spectrum of the met β chain of dog haemoglobin is identical with that of dog haemoglobin. Details of the spectra are discussed in the Supplementary Publication.

The Thermodynamics of the Reaction of Azide Ion with the Valency Hybrids of Human Haemoglobin A and the Met-hybrids of Dog and Human Haemoglobins
(with O. S. Adeosun, J. E. Goddard, and M. M. Ogunlesi)

In this section we look at the valency hybrids of human haemoglobin A and the dog–human methaemoglobin hybrids and show that the contributions of the α and β chains to determining pH$_{ch}$ are not independent one of another. In the valency hybrids the pH$_{ch}$ remains unchanged whichever chain is reacting with ligand and thus it must be determined by the overall structure of the tetramer even if one pair of chains is functionally inoperable. The results obtained with the dog–human hybrids demonstrate that in the same way as in the natural haemoglobin species the form of the variation of −$\Delta H°$ with pH is a smooth curve with a single maximum and we show that the same curve cannot be obtained by adding the contributions due to the individual chains. We also look at the postulated configurational change underlying pH$_{ch}$ for which we consider two possibilities: (1) that the configurational change involves only charged groups on the surface of the molecule without affecting the tertiary structure and (2) that the configurational change involves changes in the tertiary structure. Since pH$_{ch}$ is a function of the tetramer the latter possibility implies that the configurational change involves a concerted perturbation of the tertiary structure of the α and β polypeptide chains. This would depend on the presence of crucial interchain interactions.

The Binding of Ligands by the Met Haems of the Valency Hybrids.—We examine first the thermodynamics of the binding of azide ion to the valency hybrids of human

haemoglobin A [$\alpha_2(CO)_2\beta_2$ and $\alpha_2{}^+\beta_2(CO)_2$]. The values of log K_L are shown in Table 9 of the Supplementary Publication and $-\Delta H°$ as a function of pH in Figure 11.*
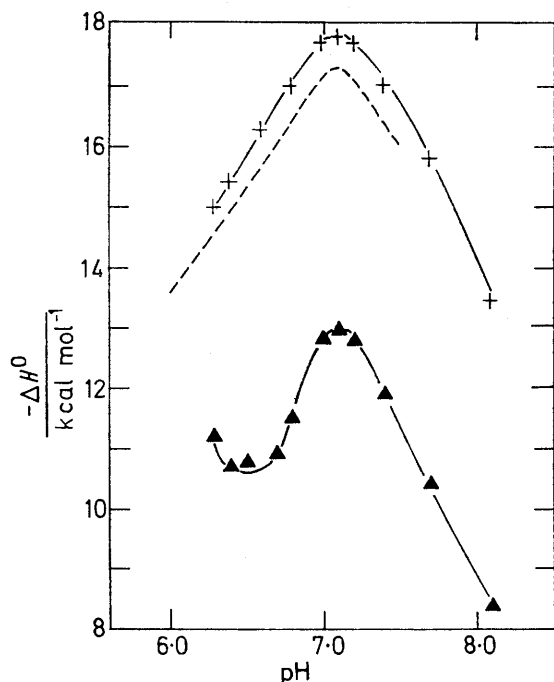

FIGURE 11 Plots of $\Delta H°$ for the formation of the azide complex of: $\alpha_2{}^+\beta_2(CO)_2$, ▲; $\alpha_2(CO)_2\beta_2{}^+$, +; $\alpha_2{}^+\beta_2{}^+$ (*i.e.* human methaemoglobin A), — — —

The pH_{ch} of both hybrids is the same as that for haemoglobin A, whereas if the chains had been operating independently in the tetramer we should expect to find a pH_{ch} of *ca.* 7.6 for $\alpha_2{}^+\beta_2(CO)_2$, since the pH_{ch} of the isolated α chain has been shown to be 7.6 and a pH_{ch} of 6.5 for $\alpha_2(CO)_2\beta^+$ — the pH_{ch} of the isolated β chain. Thus when either chain reacts with ligand in the environment of the tetramer it does not undergo the configurational change which gives rise to pH_{ch} at a pH determined by its own amino-acid composition, but at a pH determined by the composition of the tetramer. Thus both chains must interact one with another in some way to give rise to a concerted configurational change which is manifested in the fact that the pH_{ch} is the same whether $\Delta H°$ refers to the action of azide ion with the haem in the α or the β polypeptide chain. These results also mean that the structural change underlying pH_{ch} is largely unchanged by the presence of half of the chains in the tertiary oxy-form. This conclusion is supported by the fact that haemoglobin in which only some of the haems have been oxidized (by adding less than stoicheiometric amounts of potassium ferricyanide) has the same pH_{ch} as methaemoglobin.[54] The interaction between the chains which gives rise to a pH_{ch} which is a function of the amino-acid composition of the tetramer breaks down however, if the tertiary structure of either of the chains is

* The values at 20 °C are substantially in agreement with those of R. Banerjee, F. Stetzkowski, and Y. Henry, *J. Mol. Biol.*, 1973, **73**, 455.

seriously disrupted as is shown in the following part. We also note that the absolute values of $\Delta H°$ for $\alpha_2(CO)\beta_2{}^+$ are the same as those for normal human methaemoglobin A whereas those for $\alpha_2{}^+\beta_2(CO)_2$ are much lower. This would suggest that the configuration of $\alpha_2(CO)_2\beta_2{}^+$ more closely resembles methaemoglobin than does $\alpha_2{}^+\beta_2(CO)_2$.

The Binding of Ligands by the Dog–Human Hybrids.— We next look at the thermodynamics of binding of azide ion to the dog–human hybrid haemoglobins in order to investigate the effect of changing the structure of one of the polypeptide chains while at the same time retaining the interchain interactions as far as possible. Table 10 in the Supplementary Publication gives the values of log K_L for azide binding to the hybrids and Figure 12 shows the variation of $\Delta H°$ with pH for the two hybrids and the parent methaemoglobins. The following features of this figure are noteworthy.

(i) The hybrids show the typical behaviour of a native methaemoglobin with a well defined pH_{ch}. (ii) In spite of the similarity of pH_{ch} of the parent methaemoglobins (6.9 and 7.1) the hybrids have widely different pH_{ch} values at 6.2 and 7.8. This is in qualitative agreement with the observed electrophoretic mobilities of the hybrids and the parent haemoglobins at pH 8.3 (included, together with their pH_{ch} in Table 4) and with the amino-acid compositions of the tetramers, although, as was discussed above, the correlation appears to be less direct than we at first thought.

The characteristic pH values for the hybrids calculated from $pH_{ch}(\alpha_2\beta_2) = \frac{1}{2}[pH_{ch}(\alpha) + pH_{ch}(\beta)]$ are included in
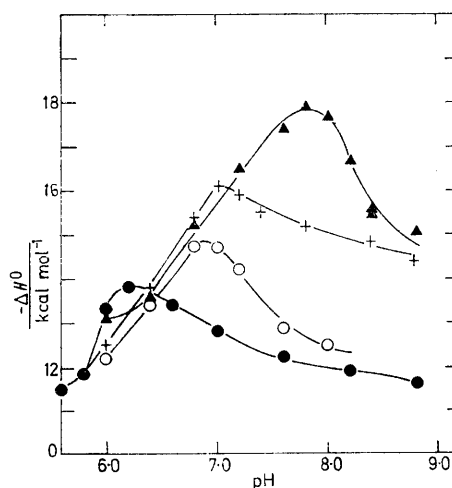

FIGURE 12 Plots of the enthalpy of formation, $\Delta H°$, of the azide complex of the hybrids of dog and human methaemoglobin together with those of the parent methaemoglobins in phosphate buffer, $I = 0.05$M; methaemoglobin A ($\alpha_2{}^A\beta_2{}^A$), +; dog methaemoglobin ($\alpha_2{}^{dog}\beta_2{}^{dog}$), ○; $\alpha_2{}^{dog}\beta_2{}^A$, ●; $\alpha_2{}^A\beta_2{}^{dog}$, ▲

Table 4. Once again the observed and calculated values agree within experimental error.

Both the possibilities suggested at the beginning of this section for the nature of the postulated configurational

[54] G. B. Ogunmola, Ph.D. Thesis, University of Ibadan, 1968.

change underlying pH_{ch} could lead to results such as the above. If, however, the second possibility is correct, that is, tertiary structural changes are involved as well as charged groups on the surface of the molecule, then under certain conditions the interactions could break down, as the following argument shows. Consider human methaemoglobin A at pH 6.0. If we increase the pH to 6.5 we reach the pH_{ch} of the β chains. However, in the tetramer a configurational change in the tertiary structure of the β chain could be constrained by the presence of the α chain. Further increase of pH to 7.1 could give rise to a situation where the configurational change of both the α and β polypeptide chains could occur together if the decrease in the free energy arising from the release of the β chains from their constrained configuration was equal to the free energy required to force the α chains into their high pH form 0.6 pH units below their pH_{ch}. That is to say the chains in the tetramer change configuration in a concerted manner. A mechanism of this type would account for the observed dependence of pH_{ch} on the properties of the tetramer rather than the individual chains. Whether a mechanism of this type which involves tertiary structural changes rather than just a change in the configuration of charged groups on the surface of the protein is indeed the mechanism underlying pH_{ch} can be demonstrated by a consideration of the pH_{ch} of a haemoglobin in which the native structure of one of the chains has been disrupted in some way. If this is done we should no longer expect the energy required to force one pair of chains into their high pH form to be equal to the decrease in free energy arising from the release of the other pair of chains from their constrained configuration at a pH approximately midway between the pH_{ch} values of the contributing chains. Rather we should expect the characteristic pH of the tetramer to be closer to that of the unmodified chain, which would have the greatest constraining influence. We show in the following section that this is in fact what is observed.

The Reactions of Azide, Formate, and Fluoride Ions with Chemically Modified Methaemoglobins (with J. E. Goddard and K. O. Okonjo)

In the previous section we noted that the postulated configurational change, of which pH_{ch} is the manifestation, could be of two types. One possibility is that the change involves the tertiary, or perhaps even the quaternary structure of the protein, the other that it involves only charged flexible side-chains on the surface of the molecule without the remainder of the structure being affected. If the former alternative is the correct one then if the haemoglobin is chemically modified to produce changes in the tertiary or quaternary structure which give rise to changes in functional properties, then the pH_{ch} might also be expected to be affected. If, however, pH_{ch} results from a configurational change involving only charged groups on flexible side-chains on the surface of the molecule, the interior of the molecule being effectively a structureless region of low dielectric con-

stant, small perturbations of the tertiary or quaternary structure would not be expected to produce large effects on pH_{ch}.

In order to investigate these alternatives we examined the ligand-binding behaviour of various chemically modified methaemoglobins: human haemoglobin A modified by reaction with cystine, cystamine, and iodoacetamide at the $\beta93$ sulphydryl group, human haemoglobin C reacted with iodoacetamide, human haemoglobin A in which the carboxyl terminal amino-acid residues of the β chains have been removed by digestion with carboxypeptidase, A and the product of the digestion of dog haemoglobin with carboxypeptidase A. Previous work on human haemoglobin A in which the reactive $\beta93$-SH groups have been blocked by treatment with the above three reagents has shown that the modified haemoglobins have very similar properties (stability, spectra, and molecular weight) to unmodified haemoglobin A,[55] and we show in the next part that their alkaline Bohr effects are similar to those of unmodified haemoglobin, although their acid Bohr effects are markedly altered. Removal of the C-terminal tyrosine and histidine of the β chain has
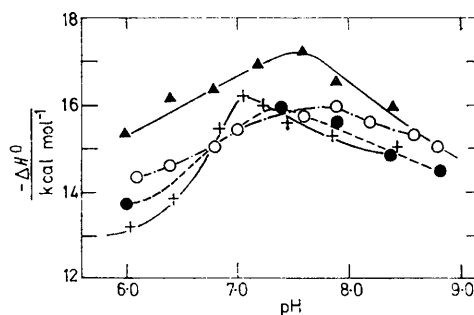

FIGURE 13   Plots of $-\Delta H°$ for the formation of the azide complex of various methaemoglobins at $I = 0.05\text{M}$: unmodified human methaemoglobin A, $+$ —— $+$; human methaemoglobin chemically modified at $\beta93$ with cystamine ● —— ●; human methaemoglobin chemically modified at $\beta93$ with cystine, ▲—▲; human methaemoglobin A chemically modified at $\beta93$ with iodoacetamide, ○ — ○

been shown by Perutz [56] to produce a dislocation extending throughout most of the β subunits. No work on the met reactions of these modified haemoglobins has yet been reported.

We look first at the results we obtained with haemoglobin A modified chemically by modification of its $\beta93$-SH groups. Values of $\log K_L$ for the reaction of these chemically modified methaemoglobins with azide, formate, or fluoride ion were determined in the usual manner and are shown in Tables 11, 12, 13 in the Supplementary Publication. The variation of $-\Delta H°$ with pH for the reaction of the three modified haemoglobins with each of the ligands is shown in Figures 13—15. The corresponding curves for methaemoglobin A are shown for comparison. For each of these ligands the

[55] J. F. Taylor, E. Antonini, M. Brunori, and J. Wyman, *J. Biol. Chem.*, 1966, **241**, 241.
[56] M. F. Perutz, *Nature*, 1969, **222**, 1243.

figures show two obvious differences between the be-
haviour of the modified methaemoglobins on the one
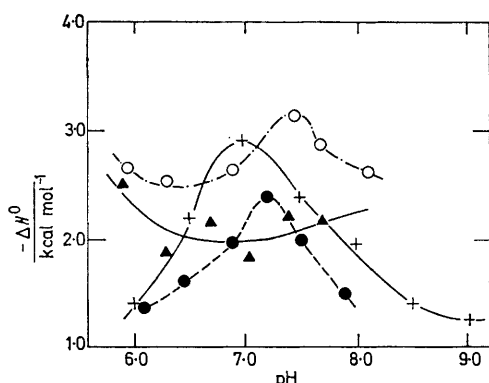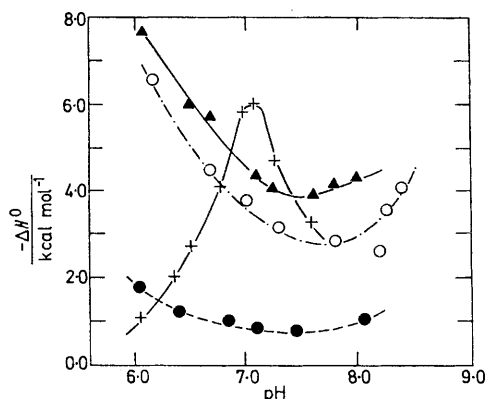hand and methaemoglobin A on the other. The first

FIGURE 14   Plots of $-\Delta H^\circ$ of formation of the fluoride complex
of various methaemoglobins at $I = 0.05\text{M}$: unmodified human
methaemoglobin A, $+ \text{——} +$; human methaemoglobin A
chemically modified at $\beta 93$ with cystamine, $\bullet \text{———} \bullet$;
human methaemoglobin A chemically modified at $\beta 93$ with
cystine, $\blacktriangle \text{—} \blacktriangle$; human methaemoglobin A chemically
modified at $\beta 93$ with iodoacetamide, $\bigcirc \text{— • — • —} \bigcirc$

difference lies in the shape of the curves. For their
reaction with azide ion the three modified haemoglobins
still show a curve of $-\Delta H^\circ$ against pH which goes
through a maximum (although, as we will discuss later,
the position of the pH_{ch} is altered from that of methaemo-
globin A), but the curves are much less steep than those
for methaemoglobin A reacting with azide ion. The
steepness of the curves can be conveniently expressed in
terms of $d(-\Delta H^\circ)/d\text{pH}$ at pH 6.5. The values of
$d(-\Delta H^\circ)/d\text{pH}$ for cystamine, cystine, and iodoacetamide
modified methaemoglobins are $+1.95$, $+1.35$, and 1.15
kcal mol^{-1} pH unit^{-1} respectively compared with a value

FIGURE 15   Plots of $-\Delta H^\circ$ of formation of the formate complex
of various methaemoglobins at $I = 0.05\text{M}$: unmodified human
methaemoglobin A, $+ \text{——} +$; human methaemoglobin A
chemically modified at $\beta 93$ with cystamine, $\bullet \text{———} \bullet$;
human methaemoglobin A chemically modified at $\beta 93$ with
cystine, $\blacktriangle \text{—} \blacktriangle$; human methaemoglobin A chemically
modified at $\beta 93$ with iodoacetamide, $\bigcirc \text{— • — • —} \bigcirc$

of 3.35 kcal mol^{-1} pH unit^{-1} for methaemoglobin A. A
similar effect is found for reaction with fluoride ion,
except that here the cystine- and iodoacetamide-modified

methaemoglobins have variations of $-\Delta H^\circ$ with pH
which are approximately zero. The modified haemo-
globins reacting with formate ion show behaviour which
is even more markedly changed from that of methaemo-
globin A as here cystamine haemoglobin has a negligible
variation of $-\Delta H^\circ$ with pH while the other two haemo-
globins have minima at pH values similar to those at
which they have maxima for their reaction with the other
ligands. Figure 16 shows that there is a linear correl-
ation between $d\Delta H^\circ/d\text{pH}$ for any two ligands.

Thus irrespective of the ligand the magnitude of the
effect of the various reagents is in the sequence iodo-
acetamide $>$ cystine $>$ cystamine. However, the be-
haviour of formate ion shows the greatest sensitivity to
chemical modification of the protein, and for iodoacet-
amide- and cystine-modified haemoglobin the variation of
ΔH° with pH resembles the behaviour of SH$^-$ with un-
modified methaemoglobin A. As the following argument
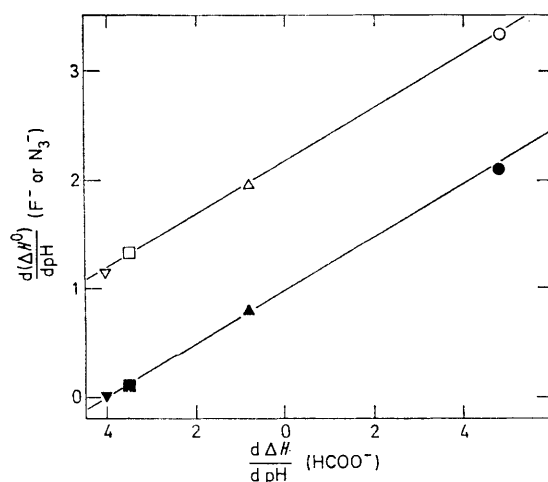shows this behaviour is readily explicable in terms of the

FIGURE 16   Plots of $-d\Delta H^\circ/d\text{pH}$ at pH 6.5 for azide ion (open
symbols) and fluoride ion (solid symbols) as ligands against
$-d\Delta H^\circ/d\text{pH}$ at 6.5 for formate as a ligand: unmodified
human methaemoglobin A, $\triangle$ and $\blacktriangle$; cystine-modified
human methaemoglobin A, $\square$ and $\blacksquare$; iodoacetamide-modified
human methaemoglobin A, $\triangledown$ and $\blacktriangledown$

hydrogen-transfer mechanism involving the distal imid-
azole.

No detailed structural information based on X-ray
crystallography is available for the modified species used
in this study but the X-ray results of Moffat [57] on haemo-
globin A modified with a spin label on the $\beta 93$-SH group
indicate that this modification results in extensive struc-
tural changes in the molecule including displacements of
the proximal and distal histidines of the β chain and a
displacement of the α-chain haem group away from its
proximal histidine, similar to but smaller than its dis-
placement in the β chain.

Any modification of the distance between the distal
histidine and the iron atom will inevitably affect the
stability of a hydrogen bond between the ligand and the

[57] J. K. Moffat, *J. Mol. Biol.*, 1971, **55**, 135.

imidazole ring. If the distance becomes too small no hydrogen bond will be formed and the hydrogen will remain on the ' outside ' nitrogen even for ligands such as azide and formate ion, and we should expect a pH variation of $-\Delta H°$ resembling that for SH^-. For less drastic modification of the position of the distal imidazole we would expect an equilibrium between the ' hydrogen in ' and ' hydrogen out ' configurations, which would give rise to a pH variation of $\Delta H°$ intermediate between that observed for azide and sulphydryl ion with unmodified methaemoglobin A. This is the type of behaviour exhibited by the chemically modified methaemoglobins shown in Figures 13—15. It is of interest at this point to recall that in contrast to azide and formate ion, fluoride ion shows wide variability in its $\Delta H°$ behaviour for different unmodified methaemoglobins. Thus for dog methaemoglobin a maximum is observed in the plot of $-\Delta H°$ against pH, for metmyoglobin a minimum, and for pigeon methaemoglobin no variation of $\Delta H°$ with pH. This behaviour resembles the behaviour of formate ion for different modified methaemoglobin described above and suggests that for fluoride ion as a ligand the position of equilibrium between the ' hydrogen in ' and ' hydrogen out ' configurations varies from haemoglobin to haemoglobin. We have previously noted that the form of the pH variation of $\Delta H°$ for the formation of the fluoride and sulphydryl complexes of metmyoglobin are identical.[58]

It should be noted that in the extreme cases, the ' hydrogen out ' or ' hydrogen in ' configuration will be overwhelmingly favoured for both the haem attached to the α polypeptide chain and for that attached to the β chain. However, where the two forms have comparable stability the position of equilibrium between the two forms may be different for the α and β haems. In such a case the observed, *i.e.* average, $d\Delta H°/dpH$ would have different contributions from the two haems.

In Part XV [26] we suggested an alternative mechanism to account for the different behaviour of azide and hydrosulphide ion as ligands; namely, that in the azide complex a hydrogen bond is not formed between the ligand and the distal imidazole thus allowing the E helix to adopt a different configuration from that in methaemoglobin. Unfortunately, we cannot eliminate this alternative hypothesis on the basis of the data presented here.

Inspection of Figures 13—15 shows that the second major difference between the modified methaemoglobins on the one hand and methaemoglobin A on the other is the shift to higher pH of the pH_{ch} for the modified haemoglobins. The values of pH_{ch} for cystamine-, cystine-, and iodoacetamide-modified methaemoglobins are respectively 7.3, 7.5, and 7.7 (taking average values for the three ligands in each case) compared with a value of 7.1 for methaemoglobin A and 7.7 for the isolated met α chain. All three modified methaemoglobins have a greater anodic mobility on electrophoresis in starch gel at pH 8.3 than haemoglobin A. This would be expected to give rise to a lower pH_{ch} than haemoglobin A if the configurational change underlying pH_{ch} was a function only

of the overall net charge on the protein as manifested by the isoelectric point. The fact that the pH_{ch} for these modified methaemoglobins is shifted to higher pH values, towards the pH_{ch} of the unmodified chain, shows that modification of the tertiary structure of one, or both, of the chains does affect the pH_{ch} and hence we must conclude that the configurational change underlying pH_{ch} involves not only the charged groups on the surface of the molecule, but also the tertiary, and possibly the quaternary, structure.

In attempting to interpret these results we wished to determine whether in the case of iodoacetamide-modified methaemoglobin the value of 7.7 for pH_{ch} could be identified with the pH_{ch} of 7.7 found for the isolated α chain or whether the correspondence was purely coincidental, *i.e.* that the modifying reagent would have shifted pH_{ch} by 0.6 pH units regardless of the value of pH_{ch} for the α chain. To decide between these two possibilities we determined pH_{ch} for human methaemoglobin C modified
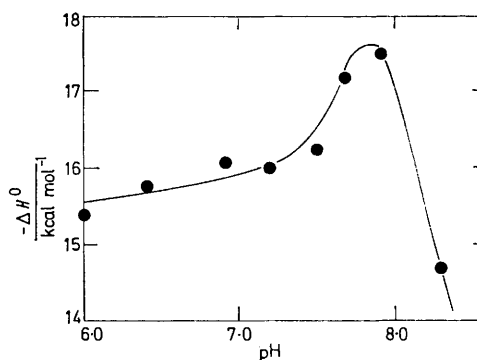
FIGURE 17 A plot of $-\Delta H°$ against pH for the reaction of iodoacetamide modified human methaemoglobin C with azide ion in phosphate buffer $I = 0.05M$

by reaction with iodoacetamide. The values of log K_L for reaction of this species with azide ion are shown in Table 14 in the Supplementary Publication and the variation of $\Delta H°$ with pH is shown in Figure 17. The characteristic pH is 7.8, the same, within experimental error, as that obtained for the iodoacetamide-modified haemoglobin A. The pH_{ch} for unmodified haemoglobin C is 7.5 and this haemoglobin has the same α chains as haemoglobin A, but different β chains. If iodoacetamide always shifted pH_{ch} by 0.6 of a pH unit, the value of pH_{ch} for iodoacetamide HbC would be 8.1. The fact that it is the same as that of the corresponding HbA derivative suggests that in these modified haemoglobins the pH_{ch} of the α chain determines the pH_{ch} of the tetramer.

In the light of these results we look again at the explanation for the position of pH_{ch} discussed above. For both human A and dog haemoglobins, the human valency hybrids and the human–dog hybrids, the pH_{ch} of the tetramer has been shown to be an average of the

[58] J. G. Beetlestone and D. H. Irvine in ' Probes of Structure and Function of Macromolecules and Membranes: Vol. II Probes of Enzymes and Hemoproteins,' eds. B. Chance, T. Yonetani, and A. S. Mildvan, Academic Press, New York, 1971.

pH$_{ch}$ values of the contributing chains and also the form of the plot of $\Delta H°$ against pH has been shown to be a function of the tetramer and not merely an average of the contributions from the two types of chains. This suggests that the configurational change in each chain is subject to the influence of the partner chain. For example, as discussed above, in the human haemoglobin A tetramer the β chain cannot change from its low pH to its high pH configuration at pH 6.3 because of the constraint imposed on it by the α chain which at this pH is more stable in its low pH configuration. At pH 7.1 the constraints imposed on the β chain forcing it to remain in its acid configuration become sufficiently great that it becomes energetically more favourable to have the α chain in its alkaline configuration at a pH lower than its own pH$_{ch}$. Hence the configurations of both chains change simultaneously to give the alkaline configuration of the tetramer. If this hypothesis is correct then the pH$_{ch}$ of the tetramer could only be expected to be an average of the pH$_{ch}$ of the contributing chains if both chains have energetically similar constraining effects on one another in the environment of the tetramer. The fact that for both human A and dog haemoglobins pH$_{ch}$ can indeed be calculated from pH$_{ch}$($\alpha_2\beta_2$) = $\frac{1}{2}$\{pH$_{ch}$(α) + pH$_{ch}$(β)\}, indicates that this is true of the unmodified haemoglobins.

If, however, the energy required to constrain one chain is considerably reduced, say by chemical modification, the pH$_{ch}$ of the tetramer will be determined by the other chain. In an intermediate situation where the energy required to constrain one chain is diminished slightly then the pH$_{ch}$ for the tetramer will lie between the pH$_{ch}$ of the unmodified chain and the average for the two isolated chains.

It is just this behaviour that we have described above for the chemically modified human methaemoglobin A. Moffat's results show that chemical modification at β93 considerably disrupts the structure of the β chain and it is not unreasonable to assume that this will facilitate its constraint by the less disrupted α chain. Furthermore the structural consequences of modification of methaemoglobin A at β93, as assessed by the effect on d$\Delta H°$/dpH, or by the effect on the magnitude of the Bohr effect, are greatest for iodoacetamide, less for cystine, and least for cystamine. This is the same sequence as the pH$_{ch}$ values: 7.3, 7.5, and 7.7.

It would appear that in these modified haemoglobins the α chain has, to an increasing degree, been released from the constraints imposed on it by the β chain, to the point where in iodoacetamide-modified haemoglobin the constraining influence of the β chain on the α chain is negligible. The same would, of course, apply to iodo-acetamide-modified haemoglobin C where the pH$_{ch}$ is again that of the α chain.

Further evidence in favour of this explanation for the shift in pH$_{ch}$ comes from the results obtained with the products of the enzymic digestion of haemoglobin A with carboxypeptidase A. The product of the digestion with

carboxypeptidase A (HbCPA) has had the two carboxyl terminal residues of the β chain (Tyr–His) removed. For this haemoglobin species the Hill constant, $n = 1$ for the oxygenation reaction (our value agrees with that of Antonini et al.[59]) and X-ray analysis has shown that the tertiary structure of the β chain has been drastically altered.[60,61] The values of log K_L for azide and fluoride ion binding for met HbCPA are shown in Tables 15 and 16 in the Supplementary Publication and plots of $-\Delta H°$ vs. pH are shown in Figures 18 and 19. For both
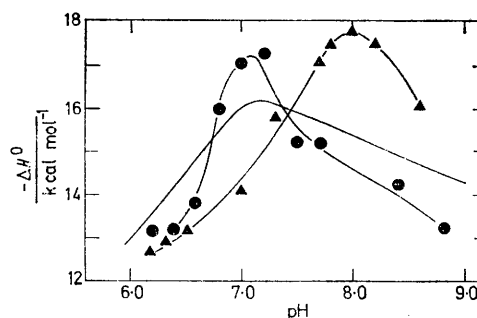

FIGURE 18 Plots of $-\Delta H°$ against pH for the reaction of enzymatically digested human methaemoglobin A with azide ion in phosphate buffer $I = 0.05$M: HbCPA, A ▲——▲; HbCPB, ●——●; undigested human methaemoglobin, A, ——
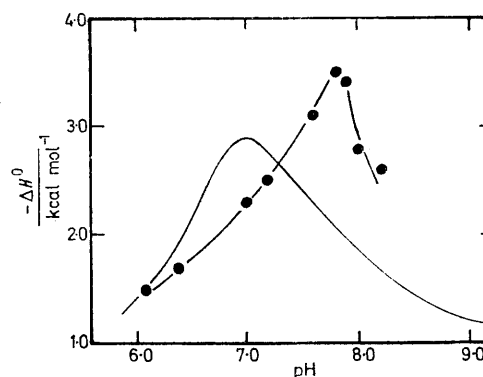

FIGURE 19 Plots of $-\Delta H°$ against pH for the reaction of fluoride ion with enzymatically digested human methaemoglobin A in phosphate buffer $I = 0.05$M: HbCPA, ●——●; unmodified human methaemoglobin A, ——

ligands the value of d($-\Delta H°$)/dpH is somewhat larger than that for methaemoglobin A reflecting a greater difference in structure between the aquomethaemoglobin and its azide or fluoride complex than in unmodified methaemoglobin A. The more marked difference between HbA and HbCPA, however, lies in their pH$_{ch}$ values, the pH$_{ch}$ for HbCPA being at pH 7.9 (taking the average of the values obtained with azide and fluoride ion) compared with pH 7.1 for HbA and pH 7.7 for the isolated met α chain. The close similarity between the pH$_{ch}$ of HbCPA and that of the isolated α chain, together with the X-ray results, which show a drastic alteration of

[59] R. Zito, E. Antonini, and J. Wyman, J. Biol. Chem., 1964, 239, 1804.

[60] M. F. Perutz, Nature, 1969, 222, 1240.
[61] J. K. Moffat, J. Mol. Biol., 1971, 58, 69.

the structure of the β chain, provide strong support for the hypothesis that the pH$_{ch}$ of this species is indeed being determined by the α chain.

It may be asked whether it is not simply that any modification of a haemoglobin shifts the pH$_{ch}$ to higher pH values and it is only fortuitous that pH$_{ch}$ approaches that of the α chain. The result for chemically modified methaemoglobin C suggests that this is not so but to further test our hypothesis we require a haemoglobin modified in the chain with the higher pH$_{ch}$ as this should shift the pH$_{ch}$ of the tetramer to lower pH values.

We, therefore, chose to examine the properties of dog haemoglobin digested with carboxypeptidase A (dog HbCPA).

Digestion of dog haemoglobin with carboxypeptidase A removes the two carboxylic terminal residues as in human methaemoglobin A. Assuming that this brings about similar widespread disruption of the β polypeptide
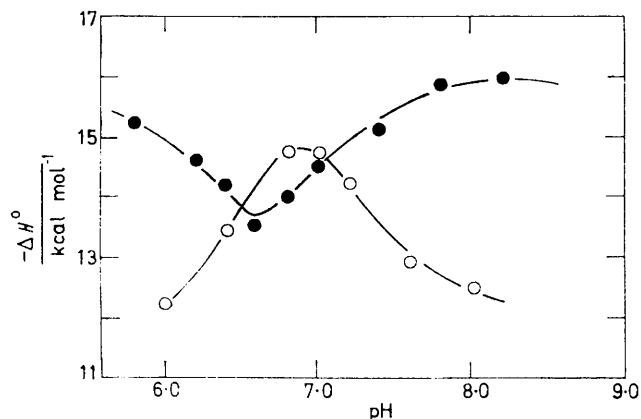
FIGURE 20 Plots of $\Delta H°$ against pH for the reaction with azide ion of dog methaemoglobin digested with carboxypeptidase A, ●; and for comparison, of unmodified dog methaemoglobin ○——○

chain as it does in human haemoglobin we should predict that the pH$_{ch}$ for this species will be lower than the pH$_{ch}$ for unmodified dog methaemoglobin (6.9) since, as we have shown above, the pH$_{ch}$ for the α chain of dog methaemoglobin is 6.3. Table 17 in the Supplementary Publication shows the values of log K_L and Figure 20 shows the plot of $-\Delta H°$ versus pH for dog HbCPA reacting with azide ion. In this case the $-\Delta H°$ against pH plot shows a minimum rather than a maximum, and the pH$_{ch}$, as defined by the minimum, is at pH 6.6. Thus although the pH$_{ch}$ has not been shifted completely to that of the α chain it has gone in the predicted direction, towards lower pH values, thus lending support to our hypothesis that the position of the pH$_{ch}$ is governed by the relative magnitudes of the constraining influences of one chain upon the other. The occurrence of a minimum rather than a maximum in the $-\Delta H°$ vs. pH curve for this species is noteworthy. The behaviour of this species resembles that of iodoacetamide-modified methaemoglobin A reacting with formate ion and following the

same argument used above we infer that the ' hydrogen in ' configuration has been destabilized.

The Acid Bohr Effect (with O. S. Adeosun, J. E. Goddard, M. M. Ogunlesi, and K. O. Okonjo)

In this section we are concerned with the Bohr effect, which is a measure of the pH dependence of the affinity of haemoglobin for oxygen. It has been shown that the relationship between the oxygen pressure when a haemoglobin is half saturated with oxygen ($p_{\frac{1}{2}}O_2$) and the pH of the solution can be expressed as follows

$$\frac{d \log p_{\frac{1}{2}}O_2}{dpH} = -\Delta h^+$$

where Δh^+ is the number of mole equivalents of hydrogen ion produced when one mole equivalent of oxygen reacts with haemoglobin, the production of hydrogen ion arising from the effect of the oxygenation of haemoglobin on the pK of ionizing groups on the protein.[41,62] For the haemoglobins which have been investigated so far Δh^+ is positive above a pH of ca. 6, reaching a maximum around pH 7, and becomes negative below pH ca. 6. The former phenomenon is commonly referred to as the alkaline Bohr effect and the latter as the acid Bohr effect. Perutz and his co-workers [2] have identified the groups which are responsible for a large fraction of the alkaline Bohr effect and the structural change which gives rise to their change in pK. We have shown (Part XVII [28]) that for the reaction of five vertebrate haemoglobins with oxygen the magnitude of the maximum alkaline Bohr effect varies very little provided that the haemoglobins are stripped, i.e. free from 2,3-diphosphoglyceric acid.

The mechanism of the acid Bohr effect is much less well understood. The groups which give rise to it have been tentatively identified as carboxyl groups [2,62] and we have shown (Part XVII [28]) that, in contrast to the alkaline Bohr effect, the magnitude of the acid Bohr effect varies markedly from one haemoglobin to another. We have also shown that for five vertebrate haemoglobins there is a correlation between the magnitude of the acid Bohr effect at pH 5.3 and the characteristic pH of the haemoglobin. In this section we look more closely at this correlation by investigating the Bohr effect for a number of artificial haemoglobin species. We have measured the magnitude of the Bohr effect by determining Δh^+ as a function of pH for each of the species and we look further at the relationship between their characteristic pH values and acid Bohr effects at pH 5.3 and suggest possible explanations for the variation of the magnitude of the acid Bohr effect between species.

We look first at the two haemoglobins formed by hybridising dog and human A haemoglobins ($\alpha_2^{dog}\beta_2^A$ and $\alpha_2^A\beta_2^{dog}$). As we showed above, these two species have widely separated characteristic pH values and in each case their pH$_{ch}$ can be calculated by taking an average of those of the contributing chains. In both

[62] E. Antonini, J. Wyman, M. Brunori, C. Fronticelli, E. Bucci, and R. Rossi Fanelli, *J. Biol. Chem.*, 1965, **240**, 1096.

cases, particularly the $\alpha_2^{dog}\beta_2^A$ hybrid, the characteristic pH shows only a qualitative correlation with the composition of charged amino-acids in the tetramer. We
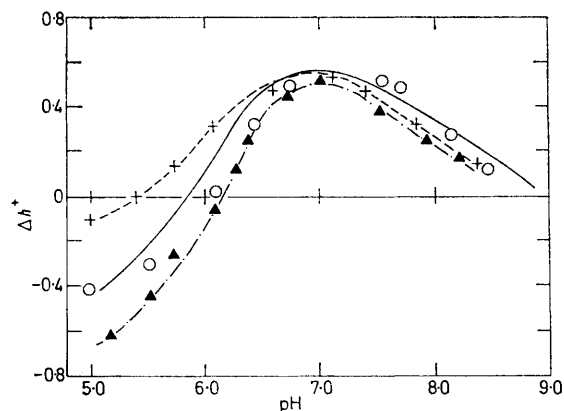

FIGURE 21 Plots of Δh^+ against pH for the reaction of the dog–human hybrid haemoglobins with oxygen at $I = 0.05$M at 25 °C: $\alpha_2^A\beta_2$ dog, + — — — +; $\alpha_2^{dog}\beta_2^A$, ▲ — · — ▲; human A, $\alpha_2^A\beta_2^A$ —— (data from Part XVII); dog haemoglobin $\alpha_2^{dog}\beta_2^{dog}$, ○ (data from Part XVII)

have measured the Bohr effect by determining Δh^+ as a function of pH for these two hybrid haemoglobins and the results, together with those for the two parent haemoglobins, are shown in Table 18 in the Supplementary Publication and in Figure 21. In both cases the maximum value of the Bohr effect at pH 7 is much the same as that for other mammalian haemoglobins, but the acid Bohr effects are very different from those of the parent haemoglobins, $\alpha_2^A\beta_2^{dog}$ having a much smaller acid Bohr effect, and $\alpha_2^{dog}\beta_2^A$ a much larger one than dog and human haemoglobins. These are in qualitative agreement with the results of Antonini and his co-workers [63] and Enoki and Tomita [64] who found that the acid Bohr effect was completely absent in $\alpha_2^A\beta_2^{can}$ whereas we find it to be present but much reduced. These authors, however, determined the Bohr effect from the variation of the oxygen affinity of the species with pH. This is less precise than the method which we have used which involves the direct measurement of the number of mole equivalents of hydrogen ion released when one mole equivalent of oxygen binds to haemoglobin, and the difference between our results and theirs is probably within experimental error. In Figure 22 we show a plot of the magnitude of the acid Bohr effect at pH 5.3 against the characteristic pH for a number of haemoglobins including the two dog–human hybrids. It can be seen that the dog–human hybrids fall very close to the correlation line and that the very small acid Bohr effect observed for the $\alpha_2^A\beta_2^{dog}$ hybrid is just what would be expected as a result of its high pH_{ch}. Hence there is no need for a special explanation of the type provided by Antonini et al.[63] as to why the acid Bohr effect for the

[63] E. Antonini, J. Wyman, E. Bucci, C. Fronticelli, M. Brunori, M. Reichlin, and A. Rossi Fanelli, Biochim. Biophys. Acta, 1965, 104, 160.
[64] Y. Enoki and S. Tomita, J. Mol. Biol., 1968, 32, 121.

$\alpha_2^A\beta_2^{dog}$ hybrid is so small; it arises as a consequence of its high pH_{ch}.

The values of the Hill constant n for the dog–human hybrid haemoglobins are shown in Table 18 in the Supplementary Publication. The hybrid $\alpha_2^{dog}\beta_2^A$ shows values of n which are significantly lower than normal at all four pH values at which they were measured with an irregular change dependent on pH. This agrees with the observations of Enoki and Tomiti.[64] The hybrid $\alpha_2^A\beta_2^{can}$ has almost constant and only slightly lowered values of n at all four pH values at which they were measured. This is also in agreement with the results of Enoki and Tomita, but differs from the results obtained by Antonini et al.,[63] who found the value of n for $\alpha_2^A\beta_2^{can}$ haemoglobin decreases significantly with decreasing pH.

In Part XVII [28] we discussed two possible general mechanisms for the observed variation in the magnitude of the acid Bohr effect between haemoglobin species.

We favoured the hypothesis in which the specific ionizable groups which give rise to the acid Bohr effect are common to a group of haemoglobins, but the environmental change which gives rise to the shift in the pK values of these groups on oxygenation is different. We shall now make the hypothesis more specific. We suggest that (i) below a certain pH deoxy- and oxy-haemoglobin have similar structures (at least similarity in that part of the structure relating to the acid Bohr effect) so that upon oxygenation in this pH range no protons are released or taken up. (ii) With increasing pH the oxy- or the deoxy-haemoglobin undergoes a configurational change so that at high pH the configurations
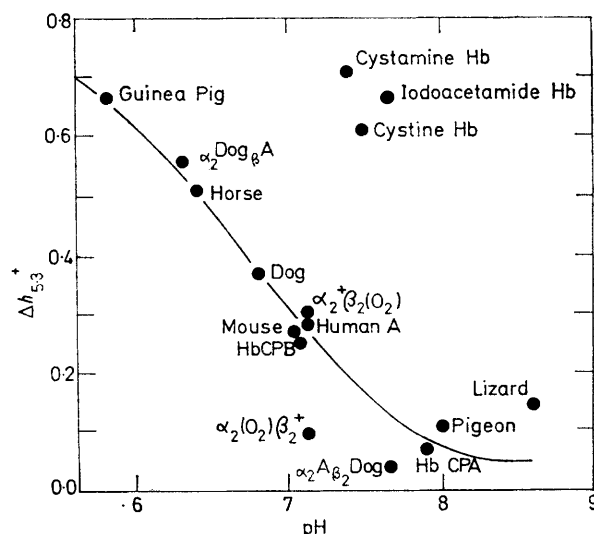

FIGURE 22 A plot of the magnitude of the acid Bohr effect at pH 5.3 ($\Delta h^+_{5.3}$) against pH_{ch} for various haemoglobins

of deoxy- and oxy-haemoglobin in that part of the molecule relevant to the acid Bohr effect are different. (iii) The pH at which this configurational change occurs depends on the species of haemoglobin in the same

manner as pH_{ch}. This does not imply that the configurational change occurs at pH_{ch} but requires that it varies from species to species in the same manner as pH_{ch}.

The observed variability in the magnitude of the acid Bohr effects would then arise in the following way. For a haemoglobin with a very high pH_{ch} the configurational change underlying the acid Bohr effect would occur at a high pH, i.e. in a pH range where the acid Bohr effect group(s) would be completely ionized. In such a case the acid Bohr effect would be zero. For a haemoglobin with a low pH_{ch} the configurational change underlying the acid Bohr effect would occur at low pH and hence in the pH range where the Bohr effect group(s) ionize the configurational difference between deoxy- and oxy-haemoglobin, and hence Δh^+, will be maximal. For a haemoglobin where the configurational change occurs over the same pH range as that in which the acid Bohr effect groups ionize Δh^+ will have intermediate values. These consequences of the proposed model are in qualitative agreement with the data given in Figure 22 and lend support to the contention that the variability of the acid Bohr effect does not arise from a different number and type of ionizable groups for each haemoglobin. In the case of the dog–human hybrid haemoglobins the hybrid $\alpha_2^A\beta_2^{dog}$ has a high pH_{ch} (pH 7.65), hence the pH-dependent configurational change in the oxy- or deoxy-form will also occur at a high pH. Thus at pH 5.3, where we measure the magnitude of the acid Bohr effect, only a small proportion of the molecules will have undergone the configurational change which results in the shift in the pK values of the acid Bohr effect groups and hence the uptake of protons on oxygenation. Hence the acid Bohr effect is small. In the case of the $\alpha_2^{dog}\beta_2^A$ hybrid, which has a low pH_{ch} (pH 6.3), the configurational change underlying the acid Bohr effect will also occur at a low pH such that it has largely occurred at pH 5.3 so that the proportion of molecules with different configurations in the oxy- and deoxy-forms will be large and hence the acid Bohr effect is large.

We next consider the valency hybrids of human haemoglobin A, [$\alpha_2^+\beta_2(O_2)_2$ and $\alpha_2(O_2)_2\beta_2^+$]. Both these hybrids have a pH_{ch} which is the same as that for the fully oxidised methaemoglobin A, which shows that the configurational change underlying the pH_{ch} is a function of the composition of the tetramer even if only one type of chain is undergoing reaction. Table 19 in the Supplementary Publication and Figure 23 show the Bohr effect as a function of pH for these two valency hybrids. For both hybrids the alkaline Bohr effect expressed in terms of the number of reactive haems is very similar to that for haemoglobin A in agreement with the results of Antonini et al.[65] but not Banerjee and Cassoly[66] who reported that $\alpha_2^+\beta_2(O_2)_2$ has a Bohr effect which is two thirds normal and that $\alpha_2(O_2)_2\beta_2^+$ has a Bohr effect which is four thirds normal. Our finding that the two hybrids have the same alkaline Bohr effect confirms that the α and β chains must make equal contributions to providing the protons released on oxygen binding. It also shows

that the structural change underlying the alkaline Bohr effect can still occur in the chains which are reacting with oxygen even if the partner chains are in the met form and appear to make no contribution to furnishing the protons released on oxygen binding. In other words the configurational change which takes place on the binding of oxygen to a particular chain appears to be independent of the state of the partner chain.

We note that the value of the acid Bohr effect for $\alpha_2^+\beta_2(O_2)_2$ falls on the correlation line in Figure 22, whereas that for $\alpha_2(O_2)_2\beta_2^+$ does not. Before attempting to interpret this result and the results for the chemically modified haemoglobins described below we must make more explicit some tacit assumptions that we made above in framing the explanation for the acid Bohr effect–pH_{ch} correlation. We assumed that (i) the number and pK of the acid Bohr effect group(s) is the same
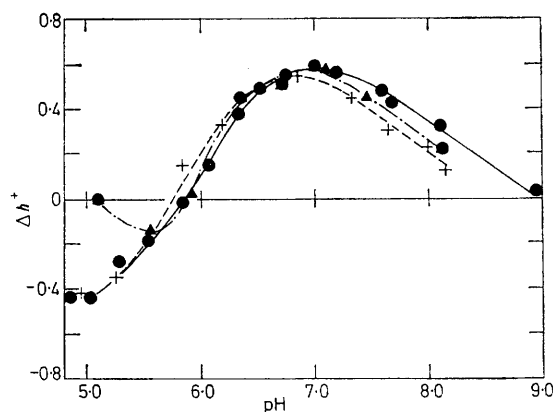

FIGURE 23 Plots of Δh^+ against pH for the reaction of haemoglobin A and its valency hybrids with oxygen at 16 °C, $I = 0.05M$: $\alpha_2(O_2)_2\beta_2(O_2)$, ———; $\alpha_2^+\beta_2(O_2)_2$, + — — — — +; $\alpha_2(O_2)\beta_2^+$, ▲ – • – ▲. Values of Δh^+ are expressed per mole of reactive haems

for all haemoglobins. (ii) The relative contribution to the acid Bohr effect of the groups on the α and β chains remains the same from one haemoglobin to another. We have made no assumption about the magnitude of the relative contribution since the observed Δh^+ is the average of the contributions from the α and β chains. (iii) The configurational change relevant to the acid Bohr effect that takes place between deoxy- and oxy-haemoglobin has the same effect on the pK of the acid Bohr effect groups, i.e. the variation of Δh^+ with pH is to be ascribed entirely to the variation between species of the pH at which the pH-dependent configurational change on the oxy- and deoxy-haemoglobin occurs.

Even if these assumptions are indeed valid for normal haemoglobins we cannot assume that they will be so for the valency hybrid haemoglobins. That Δh^+ for $\alpha_2^+\beta_2(O_2)_2$ falls on the line suggests that the assumptions are valid for this haemoglobin. For $\alpha_2(O_2)_2\beta_2^+$ we must

[65] M. Brunori, G. Amiconi, E. Antonini, J. Wyman, and K. H. Winterhalter, J. Mol. Biol., 1970, 49, 461.
[66] R. Banerjee and R. Cassoly, J. Mol. Biol., 1969, 42, 351.

conclude that one at least of these assumptions is invalid. That the point for the $\alpha_2^+\beta_2(O_2)_2$ hybrid falls on the correlation line suggest that the contributions of the α and β chains to the acid Bohr effect are equal. Hence we conclude that assumption (ii) is not valid for the $\alpha_2(O_2)_2\beta_2^+$ hybrid.

We look now at the Bohr effect for human haemoglobin modified by reaction with carboxypeptidase A (HbCPA). This enzyme removes the two carboxyl terminal residues of the β chain (Tyr-His). The Bohr effect as a function of pH is shown for this species in Table 20 of the Supplementary Publication and in Figure 24 and the acid Bohr effect is included in the correlation in Figure 22. In HbCPA the alkaline Bohr effect is *ca.* one-third normal, in agreement with Antonini *et al.*[59] In this species the structure of the β chain has been disrupted and the pH_{ch} is close to that of the α chain. The acid Bohr effect is very small, as if it too was showing only the contribution arising from a configurational change occurring at a pH characteristic of that of the α chain. The value of the Hill constant n for this species was unity at both pH 6.3 and 7.0 and 20 °C in agreement with the findings of Antonini *et al.*[59] and Churnish *et al.*[67]

Our results with the haemoglobins which have been modified at the $\beta93$ sulphydryl group by reaction with cystine, cystamine, or iodoacetamide are shown in Table 21 of the Supplementary Publication and in Figure 25 and agree quite closely with those previously reported by Taylor *et al.*[55] except that our alkaline Bohr effect for
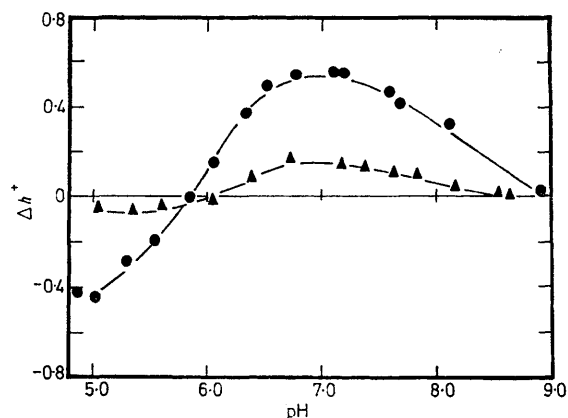

FIGURE 24   Plots of Δh^+ for the reaction with oxygen of enzymatically digested human haemoglobin A at 25 °C, $I = 0.05$M: HbCPA, ▲——▲; unmodified human haemoglobin A, ●—●

cystamine-treated haemoglobin is higher than theirs. The values of the Hill constant n which we found are identical with those found by the above authors for all three modified haemoglobins. The acid Bohr effects which we obtained for these species are included in the correlation with the pH_{ch} in Figure 22. These are the

first haemoglobins we have come across so far which deviate markedly from the correlation. The alkaline Bohr effect for these compounds differs little from that of haemoglobin A, the value for cystine-modified haemoglobin being slightly lower. The acid Bohr effect, on the
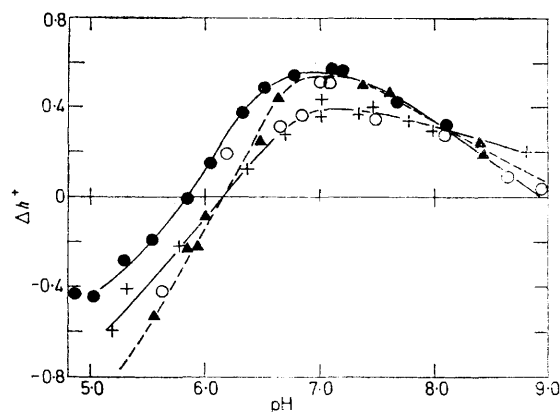

FIGURE 25   Plots of Δh^+ for the reaction with oxygen of chemically modified human haemoglobin at 25 °C, $I = 0.05$M: cystamine Hb, ▲————▲; cystine Hb, +———+; iodoacetamide Hb, ○; unmodified Hb, ———

other hand, is markedly increased in all cases despite the fact that pH_{ch} is shifted to higher pH values, *i.e.* towards that of the α chain, the unmodified chain. Enough is not known about the mechanism of the acid Bohr effect to enable us to explain this and we can only conclude that the acid Bohr effect is particularly sensitive to alterations to the structure in the region of the $\beta93$ cystine residue. Perutz *et al.*[2] have shown that reaction of the $\beta93$ cystine residue with N-ethylmaleimide prevents the formation of salt bridges between His 146β and Asp 94β which are normally present in deoxyhaemoglobin, and one can only speculate as to whether our modifying reagents have a similar effect and whether one of these two groups is crucially involved in the acid Bohr effect.

EXPERIMENTAL

The reagents, preparative techniques and physicochemical procedures used in this paper were the same as, or closely similar to, those used in earlier papers in this series or to previously published work by other workers. Details are given in the Supplementary Publication.

[67] R. H. Churnish and A. Chanutin, *Arch. Biochem. Biophys.*, 968, **123**, 163.