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SPECIES DIFFERENCES IN THE BINDING OF COMPOUNDS DESIGNED TO FIT A SITE OF KNOWN STRUCTURE IN ADULT HUMAN HAEMOGLOBIN

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1 Oxygen dissociation curves are reported for human haemoglobins A_1, F_{II}, F_I, A_{1C} and Raleigh (β1 valine → acetylalanine) and for horse haemoglobin in the absence and presence of 2,3-diphosphoglycerate (DPG), or 4,4′-diformyl-2-bibenzyl oxyacetic acid, or the bisulphite addition compound of the latter.

2 These haemoglobins were selected because their amino acid sequences are different at the DPG receptor site of human adult deoxyhaemoglobin.

3 The size of the shifts of the dissociation curves are in the sequence expected from the postulated numbers of interactions made by each compound with each haemoglobin type, based on the assumption of a common receptor site for the three compounds.

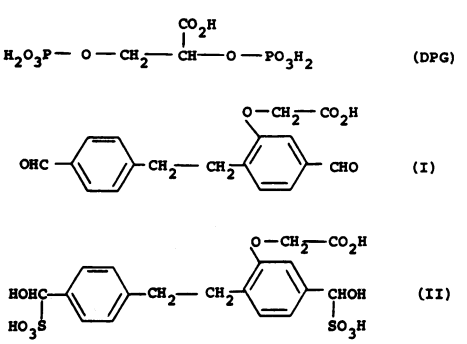
4 Multiple linear regression analysis shows that the free energies of interaction of the compounds with the haemoglobins may be predicted, to a first approximation, by summing the number of ionic and covalent bonds predicted for each effector-receptor combination, a reversible covalent bond contributing about twice as much energy (-6.78 kJmol^{-1}) as an ionic interaction (-3.14 kJmol^{-1}).

Introduction

The haemoglobin tetramer exists in an equilibrium between two quaternary protein conformations, the deoxy (T) state with a low oxygen affinity and the oxy (R) state with a high oxygen affinity (Perutz, Muirhead, Cox & Goaman, 1968; Bolton & Perutz, 1970; Perutz, 1970a). The natural effector substance of human red blood cells, 2,3-diphosphoglycerate (DPG), binds preferentially to the T state thereby lowering oxygen affinity and causing the physiological right shift of the oxygen dissociation curve (Benesch & Benesch, 1967; Perutz, 1970b). The binding of DPG at its receptor site on deoxyhaemoglobin has been observed crystallographically (Arnone, 1972), but DPG binding to oxyhaemoglobin is too weak (Szabo & Karplus, 1976; Goodford, St-Louis & Wootton, 1978) to be seen by this method.

The binding site for DPG on deoxyhaemoglobin has been used as a model drug receptor site to design novel compounds of a different chemical type to mimic the DPG effect (Beddell, Goodford, Norrington, Wilkinson & Wootton, 1976). The designed compounds gave the expected right shift of the oxygen dissociation curve of human adult haemoglobin, and recent n.m.r. evidence supports the predicted modes of binding (Brown & Goodford, 1977).

The present work concerns the interaction of DPG and two of the novel compounds, 4,4′-diformyl-2-bibenzyl oxyacetic acid (I) and its bisulphite addition compound (II), with a number of haemoglobins which have different amino acid sequences at the DPG receptor site. The results provide further support for a common receptor site for the compounds, and also suggest that it may be possible to predict the binding energies of novel compounds at receptors of known structure. A preliminary communication of some of this work has been published (Goodford, 1978).



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Methods

Preparation of haemoglobins and compounds

Horse haemoglobin was prepared by the method of Paterson, Eagles, Young & Beddell (1976) except that the horse red blood cells were washed with a solution which was 5 mmol dm^{-3} in HEPES buffer, 117 mmol dm^{-3} in NaCl and 13 mmol dm^{-3} in KCl, and the fractionation stage was omitted.

Human foetal haemoglobins were purified from cord blood haemolysates by a modification of the method of Efremov & Huisman (1974) involving elution from a DEAE-cellulose column (5 × 100 cm) by a pH gradient of 0.05 M Tris-HCl, pH 8.3 to pH 7.8. Haemoglobin A_1 was purified from human adult blood haemolysates by the same method.

Haemoglobin Raleigh was separated from haemoglobins Russ, Russ-Raleigh and A_1 by the method of Moo-Pen, Bechtel, Schmidt, Johnson, Jue, Schmidt, Dunlap, Opella, Bonaventura & Bonaventura (1977) using carboxy-methyl cellulose. This method was also used to prepare haemoglobin A_{1C}.

The homogeneity of eluted haemoglobin peaks was determined by isoelectric focusing in thin layer polyacrylamide gels using an LKB Multiphor. Purified fractions were concentrated by ultrafiltration to give 5% (w/v) haemoglobin solutions. These were dialysed against 0.1 mol dm^{-3} NaCl, 1 mmol dm^{-3} HEPES at pH 7.2 to remove any remaining organophosphates. The levels of phosphate and methaemoglobin were determined as described by Paterson *et al.* (1976). Phosphate levels were generally below 0.05 mol total phosphate per mol of haemoglobin and methaemoglobin levels below 5%, except for horse haemoglobin which had a methaemoglobin level of 7%.

Compound I was prepared as described previously (Beddell *et al.*, 1976). Compound II was formed *in situ* before oxygen dissociation curve measurements by using solutions which were 5 mmol dm^{-3} in sodium metabisulphite.

Oxygen dissociation curve measurements and analysis of data

Oxygen dissociation curves for the various haemoglobins were measured at 37°C as described previously (Beddell *et al.*, 1976) in the absence and presence of 2.5 mmol dm^{-3} of DPG, compound I or compound II. Solutions were 0.03 mmol dm^{-3} in haemoglobin, 100 mmol dm^{-3} in HEPES buffer (pH 7.35), with an ionic strength contribution from NaCl plus test compound of 35 mmol dm^{-3}.

Results were analysed using the relationship between oxygen saturation, Y, of haemoglobin, and oxygen pressure, p, at a concentration, d, of effector:—

$$Y = \frac{\alpha(1+\alpha)^3(1+dK_O) + Lc\alpha(1+c\alpha)^3 + Lb\alpha(1+b\alpha)^3\,dK_D}{(1+\alpha)^4(1+dK_O) + L(1+c\alpha)^4 + L(1+b\alpha)^4\,dK_D} \quad (1)$$

α is the normalised oxygen pressure ($\alpha = p.K_R$ where K_R is the oxygen affinity of the R state of haemoglobin), K_O and K_D are the affinities of the effector for the oxy and deoxy conformations of the haemoglobin tetramer and L, c and b are constants (Goodford, Norrington, Paterson & Wootton, 1977). Simpler expressions have been proposed (Benesch, Benesch, Renthal and Gratzer, 1971; Baldwin, 1975) but these are less general and there is some confusion over the most appropriate simplification to use in practice (Szabo & Karplus, 1976; Benesch, Edalji & Benesch, 1977).

Previous oxygen dissociation curve measurements in this laboratory (Norrington, unpublished observations) using adult human haemoglobin A_1 and a range of concentrations of the compounds gave K_O values of 470, 600 and 1170 mol^{-1} for DPG, compound I and compound II respectively. These values were used in fitting equation (1) to the present data on the assumption that the K_O values do not vary greatly between the different haemoglobins. A constant value for K_R of 17.5 kPa^{-1} was also used (Goodford *et al.*, 1977; Groth, Garby & de Verdier, 1978). Thus for each combination of haemoglobin and compound the control dissociation curve and the curve in the presence of compound (twelve points in all) were 'least squares' fitted as a pair (Powell, 1970) using equation (1) to estimate the parameters, L, c, b and K_D. The function minimised was the sum of squares of the differences between measured and calculated oxygen saturation values. The free energies of binding of the compounds to the deoxy conformation, ΔG, were then calculated directly from the K_D values using the relationship:—

$$\Delta G = -RT \ln K_D \quad (2)$$

As a test of the dependence of the analysis on the assumed values of K_O, equation (1) was refitted to the data using a constant value for K_O of 600 mol^{-1} for DPG, compound I and compound II. This had the effect of raising the ΔG values for DPG by about 0.4 $kJmol^{-1}$ and lowering the ΔG values for compound II by about 1.2 $kJmol^{-1}$, but these changes were not sufficient to affect the conclusions reached

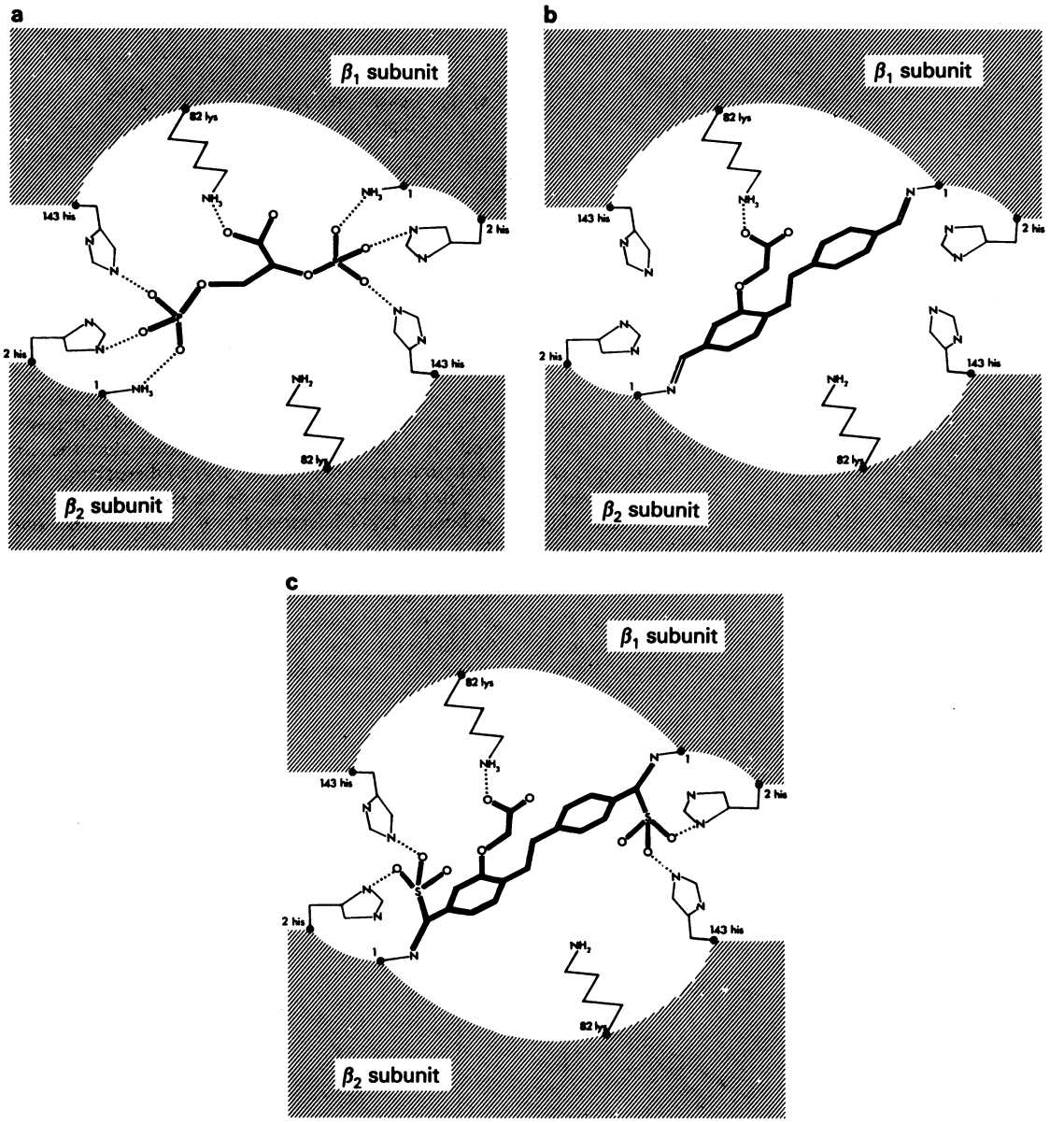

Figure 1 Schematic representations of the 2,3-diphosphoglycerate (DPG) receptor site in human deoxyhaemoglobin A_1. (a) With DPG bound as observed by Arnone (1972); (b) the postulated mode of binding of Compound I; (c) the postulated mode of binding of Compound II.

materially, since the total range of ΔG was over 30 $kJmol^{-1}$.

Results

The observed mode of binding of DPG to human adult deoxyhaemoglobin (Arnone, 1972) is shown schematically in Figure 1a. A total of seven ionic interactions is formed. The amino acid residues involved in binding are valine 1β (via the terminal amino group) and histidines 2β and 143β of both β subunits and the lysine 82β residue of one or the other β subunit. Compounds I and II were designed to interact with these same residues (Beddell *et al.*, 1976) and their postulated modes of binding are

Table 1 Modifications at the 2,3-diphosphoglycerate (DPG) receptor site in the haemoglobins studied

Haemoglobin	*Modifications at the DPG receptor site*
Horse	β2 histidine → glutamine
Foetal Major (HbF_{II})	β143 histidine → serine
Foetal Minor (HbF_{I})	β143 histidine → serine
	β1 valine acetylated
HbA_{1C}	β1 valine forms Schiff base with glucose
Raleigh	β1 valine → acetylalanine

shown schematically in Figure 1b and c. Both compounds were thought to form covalent bonds with the N-terminal amino groups (valine 1β) of both β chains and to interact ionically with one of the lysine 82β residues. In addition for compound II, the sulphonate groups were thought to form ionic interactions with histidines 2β and 143β. Thus, compound I should form two covalent and one ionic interaction and compound II should form two covalent and five ionic interactions on the basis of these original predictions.

The alterations at the DPG binding site in the various haemoglobins studied are given in Table 1 (see Dayhoff, 1972 and references therein; Bunn, Haney, Gabbay & Gallop 1975; Moo-Penn *et al.*, 1977). If it is assumed that DPG and the compounds bind to the deoxy form of each haemoglobin in the same way as they bind to human adult deoxyhaemoglobin, then it is possible to predict the number and types of interaction for each combination of compound and haemoglobin. For example, horse haemoglobin has histidine 2 of the β chains replaced by glutamine, and this will reduce the number of possible ionic interactions with DPG and compound II by two, leaving five and three ionic interactions respectively. It would therefore be expected that the effect of these compounds on the oxygen dissociation curve of horse haemoglobin would be less than their effect on human adult haemoglobin A_1. On the other hand, these same histidine residues are not involved in the predicted mode of binding of compound I, so the effect of this compound on the oxygen dissociation curves of horse haemoglobin and haemoglobin A_1 should be the same. Similar reasoning may be applied to the other haemoglobins studied to determine the expected numbers of ionic and covalent interactions in each case.

Figures 2a to f shows the oxygen dissociation curves for the various haemoglobins in the absence and presence of 2.5 mmol dm^{-3} DPG, compound I or compound II. Inset in each figure are the postulated number of ionic, n_I, and covalent, n_C, interac-

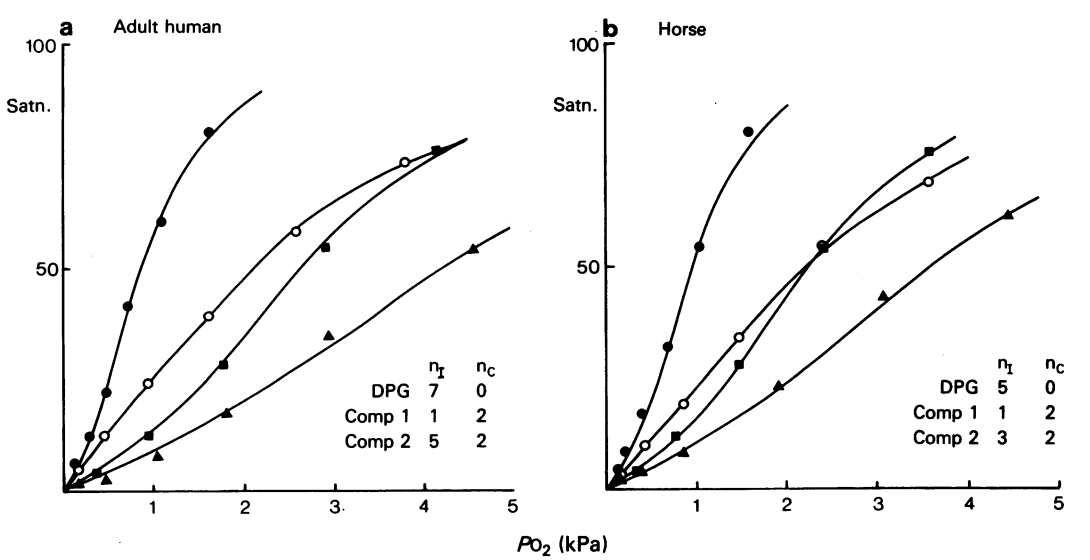

Figure 2 a and b.

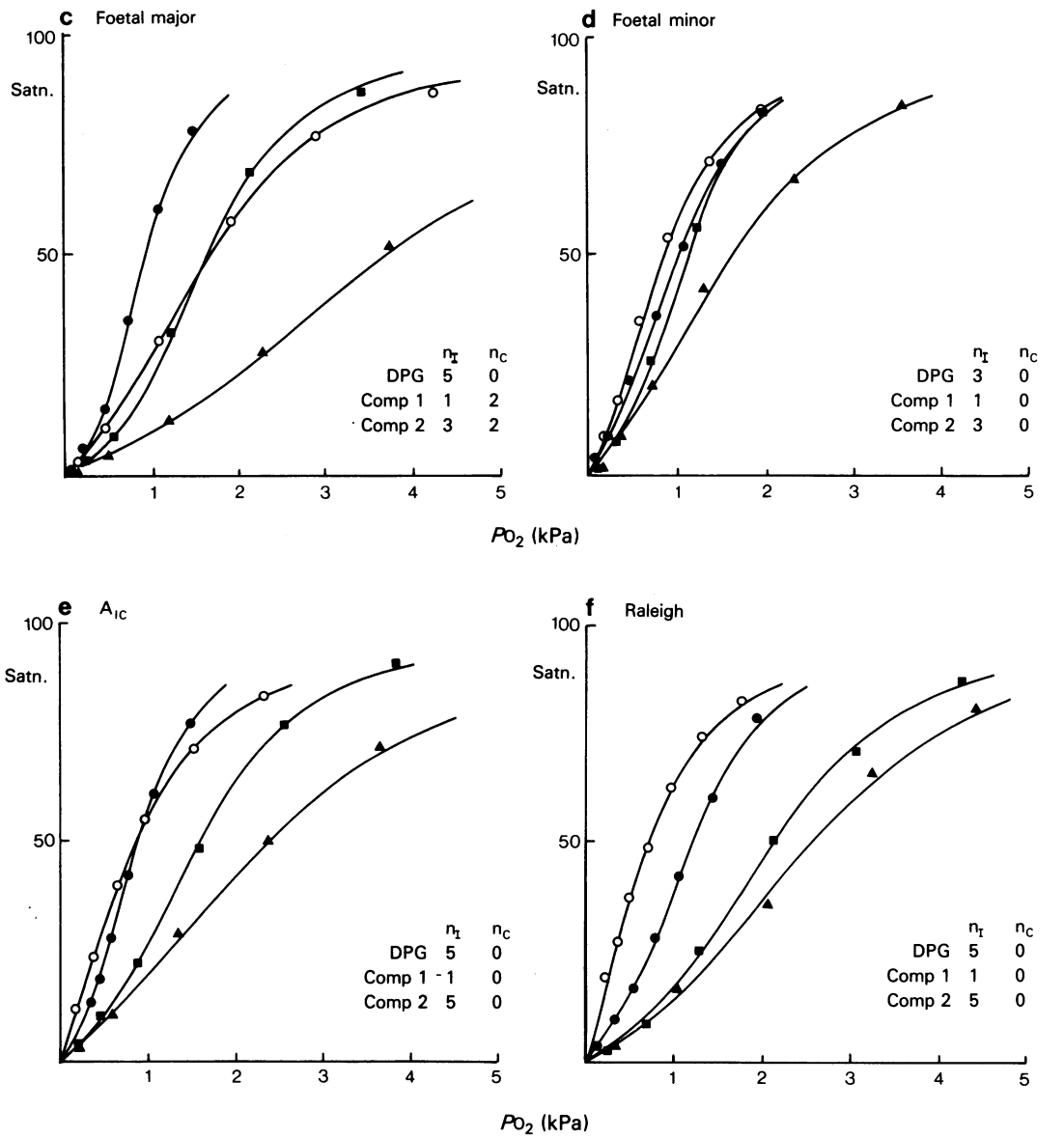

Figure 2 Oxygen dissociation curves for the different haemoglobins (a–f) in the absence (●) and presence of 2.5 mmol dm^{-3} DPG (■), 2.5 mmol dm^{-3} compound I (○), and 2.5 mmol dm^{-3} Compound II (▲). The n_I and n_C values are the postulated numbers of ionic and covalent interactions for each combination of haemoglobin and compound.

tions for each combination, based on the assumption of a common receptor site for the compounds and their expected modes of binding at this site. Taking horse haemoglobin as an example again (Figure 2b) and comparing the results with human adult haemoglobin A_1 (Figure 2a), it can be seen that, as expected, the effects of DPG and compound II on horse haemoglobin are both reduced compared to human adult, but the effect of compound I is about the same for both haemoglobins. In general there is good agreement between the observed shifts of the oxygen dissociation curves and the postulated numbers of ionic and covalent interactions in each case. This not only supports the assumption of a common binding site,

Table 2 The postulated number of ionic, n_I, and covalent, n_C, interactions for each combination of haemoglobin and compound, the observed affinities, K_D, of the compounds for the deoxyhaemoglobins, the associated free energies of binding, ΔG, and the calculated binding energies, ΔG_{calc}, predicted from equation 3

Haemoglobin	*Compound*	n_I	n_C	K_D mol^{-1}	ΔG $kJmol^{-1}$	ΔG_{calc} $kJmol^{-1}$
Human adult	DPG	7	0	71,400	−28.8	−30.3
		7	0	55,700	−28.2	−30.3
		7	0	93,600	−29.5	−30.3
	Comp. I	1	2	33,000	−26.8	−25.0
		1	2	16,900	−25.1	−25.0
		1	2	45,200	−27.7	−25.0
	Comp. II	5	2	774,500	−35.0	−37.6
		5	2	886,000	−35.3	−37.6
		5	2	1,164,000	−36.0	−37.6
Horse	DPG	5	0	26,100	−26.2	−24.0
	Comp. I	1	2	28,200	−26.4	−25.0
	Comp. II	3	2	282,600	−32.4	−31.3
Foetal Major	DPG	5	0	6790	−22.8	−24.0
	Comp. I	1	2	7320	−23.0	−25.0
	Comp. II	3	2	292,600	−32.5	−31.3
Foetal Minor	DPG	3	0	878	−17.5	−17.7
	Comp. I	1	0	115	−12.2	−11.4
		1	0	75.6	−11.2	−11.4
		1	0	13.5	−6.7	−11.4
	Comp. II	3	0	9020	−23.5	−17.7
HbA_{1C}	DPG	5	0	7990	−23.2	−24.0
	Comp. I	1	0	623	−16.6	−11.4
	Comp. II	5	0	67,200	−28.7	−24.0
Raleigh	DPG	5	0	8030	−23.2	−24.0
		5	0	8430	−23.3	−24.0
	Comp. I	1	0	1.22	−0.5	−11.4
		1	0	76.1	−11.2	−11.4
	Comp. II	5	0	33,800	−26.9	−24.0
		5	0	31,250	−26.7	−24.0

but suggests that it might be possible to predict the magnitude of the effect of a particular compound on the oxygen dissociation curve of a particular haemoglobin simply by considering the numbers and types of interactions involved in binding.

This idea was tested further by regressing the free energies of binding of the compounds to the haemoglobins against the postulated numbers of ionic and covalent interactions. The ΔG values are tabulated in Table 2; 18 combinations of the three compounds with the six haemoglobins were possible and replication increased the number of observations, n, to 29. The resulting regression equation and statistics are:—

$$\Delta G = -3.14n_I - 6.78n_C - 8.29 \quad (3)$$
$$(\pm 0.30)\ \ (\pm 0.68)\ \ (\pm 1.37)$$
$$n = 29;\quad r = 0.928;\quad F_{2,26} = 81.15$$

Standard errors for the regression coefficients are given in brackets, r is the multiple correlation coefficient and $F_{2,26}$ is the variance ratio. The associated statistics for equation (3) are all significant at the 0.1% level and the equation explains 86% of the variation in the measurements. Thus, to a first approximation, the energies of binding of the compounds to the various haemoglobins may be predicted by summing the numbers of ionic and covalent interactions present, a reversible covalent bond contributing about twice as much energy (-6.78 kJmol^{-1}) as an ionic interaction (-3.14 kJmol^{-1}).

The measured ΔG values are plotted against the values calculated from equation (3) in Figure 3. Agreement is worst for low energy values, and this may be due to experimental error, since these low energies correspond to only slight shifts of the oxygen dissociation curves. However, estimation of the error from replicate determinations suggests an overall experimental error of measurement of only 7% which may be compared with the residual error from the regression of 14%. This shows that the present model, which only takes account of the most obvious interactions, still accounts for a surprisingly high proportion of the overall variance, although some lack of fit remains as shown by the variation between different

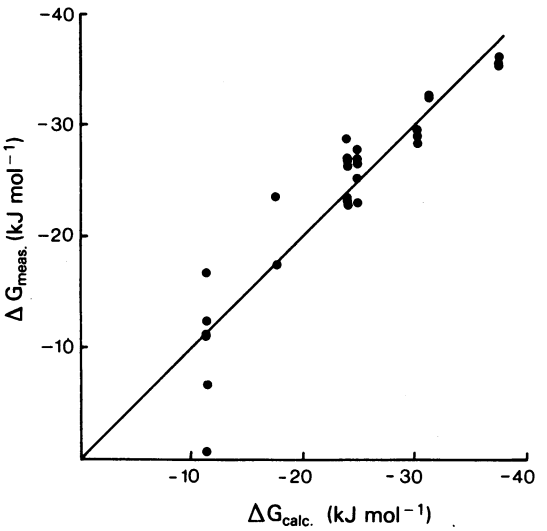

Figure 3 Plot of measured ΔG values against ΔG values calculated using equation (3).

haemoglobins when the postulated interactions are the same. However, this represents only a second order effect.

Discussion

The present results are compatible with previous studies of the oxygenation of the various haemoglobins and the effect of DPG (Tyuma & Shimizu, 1969; de Verdier & Garby, 1969; Bunn & Briehl, 1970; Bunn, 1971; McLean & Lewis, 1975; Moo-Penn *et al.*, 1977). In each case the oxygen dissociation curves for the stripped haemoglobins resemble that of normal human adult haemoglobin A_1, and the effect of DPG on the curves is significantly reduced as expected from the differences in the DPG receptor site of the different haemoglobins. We have shown further that the magnitude of the effects of the bibenzyl compounds, I and II, on the oxygen dissociation curves of the haemoglobins are also well correlated with the changes at the DPG binding site. This provides further support for the assumption of a common binding site for DPG and the compounds to the deoxy form of the haemoglobins, since it is unlikely that such a significant correlation would be obtained if the compounds bound elsewhere. In fact, the results show that although horse and the foetal haemoglobins contain many more differences in amino acid sequence from normal human adult haemoglobin, it is apparently only the differences at the DPG site which significantly affect the binding of the compounds and the magnitude of their effect on the oxygen dissociation curves. This is an important finding and suggests that in favourable cases it may be sufficient to consider only the structure of the receptor site itself in the general design of species-selective therapeutic agents.

It is generally believed that the energies of interaction of small molecules with proteins are a function of the number of interactions made on binding (Weber, 1975), but there is very little evidence available concerning the nature of this postulated relationship. Studies of ligand binding to lysozyme (Johnson, Phillips & Rupley, 1968) showed that the binding energies increased with the number of van der Waals contacts between ligand and protein, provided that the ligand was not distorted to a high energy conformation on binding. The approximate linear free energy relationship observed in the present study (equation (3)) for disparate ligands and haemoglobins suggests that such distortions are not quantitatively important in the present case, although the binding of DPG has been shown to cause slight distortions of the haemoglobin tertiary structure (Arnone, 1972).

Jencks (1975) has suggested that an approximate linear free energy relationship may be expected, provided that the ligand which interacts most weakly is already 'anchored' to the protein and does not have to overcome the decrease in translational and overall rotational entropy involved in the anchoring process. However, in the present study, compound I is postulated to bind to certain haemoglobins via only one interaction, and the decrease in translational and rotational entropy on binding might therefore be expected to lead to a relatively smaller energy of interaction in this case compared to others where a greater number of interactions can occur. Also, there may be a smaller number of bound counter-ions or solvent molecules displaced by the ligand when one only interaction is present, and this would again lead to a smaller intrinsic energy for the single attachment case compared to others. The relationship between binding energy and the number of interactions may therefore be non-linear when the number of interactions is small, and this may explain the slight lack of fit of the linear model used. Unfortunately, as mentioned in the results section, the error in the measurements is largest for numerically small values of ΔG, and a break in linearity is not readily seen in the data. However, the substantial value of the intercept of equation (2) of -8.29 (± 1.37) kJmol^{-1} might result from such non-linearity, and if all the data corresponding to the single interaction case are omitted from the regression, the intercept is in fact numerically larger (-13.42 ± 1.52 kJmol^{-1}).

On the other hand, the model used here is based on a number of crude assumptions which may well be unjustified, and could also contribute towards the lack of fit. In particular, it is assumed that all ionic

interactions formed are of equal energy regardless of the chemical nature or charge of the interacting groups. In fact the contribution of a single ionic interaction of -3.14 kJmol^{-1} is somewhat lower than might be expected (Perutz, 1970a; Szabo & Karplus, 1972) and this may reflect the diverse nature of the interacting groups which may be ionised to different degrees at pH 7.4 (Bucci, Salahuddin, Bonaventura & Bonaventura, 1978). Further, the C=N of the Schiff base linkage is assumed to be of equal energy to the C—N linkage postulated for compound II. Information on the expected energies of these interactions is sparse, but Metzler (1957) has determined energies of Schiff base formation between amino acids and pyridoxal in the range -5.5 to -10 kJmol^{-1} with a value of -9.2 kJmol^{-1} for valine. The value of -6.78 kJmol^{-1} for a covalent contribution determined here therefore seems reasonable. Finally, the assignment of the numbers of interactions may not be correct in every case, since it is assumed that each change at the binding site will result in the complete elimination of the corresponding interaction.

There are therefore many reasons why the simple linear free energy model used here may be theoretically unsound. However, despite all these, it has been shown that in practice the energies of binding of the compounds and the magnitude of their effect on the oxygen dissociation curves can actually be predicted from a simple consideration of the numbers of ionic and covalent interactions involved. This is an encouraging finding for drug design by the method of receptor fit (Beddell *et al.*, 1976) since it suggests that it may be possible to predict the relative strengths of binding of compounds to receptors, and in certain cases potency, not only within congeneric series but also for compounds of diverse chemical type.

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