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Substituted benzaldehydes designed to increase the oxygen affinity of human haemoglobin and inhibit the sickling of sickle erythrocytes

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1 Substituted benzaldehydes have been designed to bind preferentially to the oxy conformation of human haemoglobin at a site between the amino terminal residues of the α-subunits. Such compounds should stabilize the oxygenated form of haemoglobin and thereby increase its oxygen affinity.

2 The compounds produce the expected effect, left-shifting the oxygen saturation curve of dilute haemoglobin solutions and of whole blood, although the binding pattern to haemoglobin is more complex than envisaged by the design hypothesis.

3 The predicted best compound is also a potent inhibitor, at low oxygen pressure, of the sickling of erythrocytes from patients homozygous for sickle cell disease, and may prove to be a clinically useful anti-sickling agent.

Introduction

Previous studies in this laboratory (Beddell *et al.*, 1976) have demonstrated the feasibility of designing novel compounds to interact with a receptor site of known structure on a protein molecule and produce a defined physiological effect. The binding site for the natural effector substance, 2,3-diphosphoglycerate (DPG) (Benesch & Benesch, 1967; Chanutin & Curnish, 1967) in the deoxy conformation of human haemoglobin (Arnone, 1972) was chosen as the model receptor site, and a series of bibenzyl dialdehyde derivatives was designed, synthesized and shown to produce the desired effect, i.e. a stabilization of the deoxy conformation of haemoglobin and a consequent right-shift of its oxygen saturation curve. It was also demonstrated that the relative binding energies of DPG and the designed compounds to certain mutant and species different haemoglobins could be predicted from a knowledge of the changes in amino acid residues in these haemoglobins in the vicinity of the DPG binding site (Beddell *et al.*, 1979).

The studies with these variant haemoglobins had no obvious direct therapeutic relevance but we have now carried out a similar exercise aimed at designing compounds to alleviate the clinical symptoms caused by one specific mutant haemoglobin, namely sickle haemoglobin, HbS (for a recent review see Dean & Schechter, 1978). HbS differs from normal haemoglobin, HbA, at a single amino acid position in each β-chain of the $\alpha_2\beta_2$ haemoglobin tetramer (Ingram, 1956). The change from an acidic residue (glutamic acid) in HbA to a neutral, hydrophobic residue (valine) in HbS at position β^6 results in a decreased solubility of the haemoglobin tetramer, but only in the deoxy conformation which predominates at low oxygen pressures. Under such conditions the HbS within sickle erythrocytes precipitates as extended fibrous structures which eventually physically distort the cell membrane leading to the characteristic sickle cell shape (Herrick, 1910) and the symptoms of the disease which include a chronic haemolytic anaemia. The impaired oxygen transport due to this chronic anaemia is partially compensated for by a right-shifted oxygen saturation curve. However, this has the unfortunate effect of increasing the proportion of the insoluble deoxy form further, leading to increased polymerization.

One approach to the treatment of sickle cell disease might therefore be to antagonize the action of DPG by shifting the saturation curve back to the left and decreasing the relative amount of the insoluble

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deoxy conformation at a given oxygen pressure. This could be achieved either by a direct competitive antagonism at the DPG binding site or by binding at some alternative site on the haemoglobin tetramer in the oxy conformation and producing an allosteric, non-competitive antagonism. Knowledge of the structure and mechanism of haemoglobin function suggested that the latter effect might be more readily achieved in practice and this paper describes the design, synthesis and testing of a series of compounds to this end.

Methods

Oxygen saturation curve measurements

Human haemoglobin solutions of low phosphate content were prepared as described previously (Beddell *et al.*, 1976; Paterson *et al.*, 1976) and stored as 2 cm^3 aliquots at −20°C. Saturation curve measurements on dilute haemoglobin solutions (23 μM on a tetramer basis, 5.4 ml of solution) were carried out at 37°C in the dissociation mode using a commercially available automatic apparatus (HEMOX-ANALYZER, T.C.S. Medical Products Division, Southampton, Pa, U.S.A.). This instrument records oxygen pressure using a Clark oxygen electrode and oxygen content of the haemoglobin using dual-wavelength spectrophotometry. Haemoglobin solutions containing the required concentration of compound in 100 mM HEPES buffer at pH 7.40 and 35 mM KCl were prepared in the optical cell and equilibrated for 10 min before bubbling nitrogen (British Oxygen Company 'white spot') through the stirred solution to deoxygenate it, which typically required ca. 30 min; 20 μl of a 1.5% silicone emulsion (Hopkin & Williams), was also added to prevent frothing. The dissociation curves were recorded as continuous curves on an X-Y plotter.

Oxygen saturation curves of whole blood were measured in the association mode at 37°C using a different commercially available apparatus (HEM-O-SCAN, Aminco, Silver Springs, Maryland, U.S.A.). The operation of this apparatus is similar to the HEMOX except that the sample to be measured is prepared as a thin film on a glass cover slip covered by a thin, gas-permeable membrane. Heparinised normal human blood (Wellcome donor panel) was centrifuged, the appropriate amount of compound was dissolved in the plasma fraction and the erythrocytes were then resuspended in the plasma. The samples were incubated for 30 min at 37°C under an atmosphere of humidified air/5% CO_2 and were then prepared for oxygen saturation curve measurement. After equilibration in the apparatus for ca. 20 min the sample was deoxygenated by passing nitrogen/5% CO_2 over it and the oxygen association curve was monitored continuously as the proportion of oxygen in the humidified atmosphere was slowly increased.

Measurement of anti-sickling activity

Samples of sickle blood were obtained from informed donors by antecubital venepuncture and were taken into lithium-heparin anticoagulant using the 'Vacutainer' system. For short-term storage up to five days, the blood was mixed 4:1 with standard acid-citrate-dextrose (ACD) anticoagulant and was well oxygenated prior to refrigeration at 4°C. Homozygous sickle genotype (HbSS) was verified by cellulose acetate electrophoresis of a crude lysate using tris-EDTA-borate buffer (10.2 g, 0.6 g, 3.2 g l^{-1}) pH 8.4 and incorporating known standards. Sickle blood was adjusted to a haematocrit of 5% with an incubation mixture containing NaCl 128 mM, $NaHCO_3$ 25 mM, glucose 5 mM and 1% w/v bovine serum albumin (Fraction V, Sigma) and various concentrations of the compound under investigation. Each mixture (2 ml) was incubated in a 25 ml Erlenmeyer flask at 37°C on a water bath with continuous shaking under an atmosphere of humidified air/5% CO_2. The pH was checked and found, typically, to lie within the range 7.26–7.31; no further adjustment was made. After 60 min, measurements of the partial pressure of oxygen (P_{O_2}: mmHg) were made in the control mixtures using a Clark oxygen electrode thermostatted to 37°C and coupled to a PHM 71 acid-base analyzer (Radiometer). The P_{O_2} was found typically to be in the range 135–140 mmHg, indicating adequate aeration. Samples (ca. 200 μl) from controls (no compound) were taken and the cells fixed by the admixture of glutaraldehyde solution to a final concentration of 1%. Incubation of the mixtures was then continued under a humidified atmosphere of 4% O_2/91% N_2/5% CO_2 and after 60 min the equilibration P_{O_2} of ca. 28 mmHg was ascertained in each flask. The cells were then fixed *in situ* by the addition to each flask of glutaraldehyde solution to a final concentration of 1%. Cell fixation under these conditions was found to be both reliable and reproducible. Unstained wet-mount slides were prepared and photo-micrographs were taken of two fields for each sample (each ca. 500 cells) using Nomarski interference optics. Photomicrographs were counted by two independent observers and erythrocytes (400/field) were scored as either 'normal' (discoid), 'sickle' (classical crescent or holly-leaf forms) or 'bizarre' (irreversibly sickled cells or echinocytes, if present). Observer concordance was checked by correlation analysis and percentage cell type was calculated from the mean of two fields each counted by two observers.

Compound design

DPG binds at a site between the N-termini of the β-chains which is specific for the deoxy conformation of haemoglobin (Arnone, 1972). This results in a stabilization of the deoxy form and a right-shift of the oxygen saturation curve. A competitive antagonist in the classical pharmacological sense would be a compound which bound at this same site, thereby competing with DPG, but with comparable affinities for both deoxy and oxy conformations. In fact, the knowledge of the structure of this site in both conformations (Perutz *et al.*, 1968; Bolton & Perutz, 1970) suggests that the design of such a compound with an affinity high enough to compete effectively with DPG would be very difficult. An alternative strategy of designing compounds to bind at a different site on the haemoglobin tetramer was therefore adopted. Such compounds should then be non-competitive antagonists and exert their antagonism through an allosteric mechanism by binding preferentially to the oxy conformation and stabilizing this conformation relative to the deoxy conformation. This conformationally selective binding would then be expected to produce a left-shifted oxygen saturation curve.

Because of the success in designing dialdehyde compounds to react at the β-chain terminal amino groups (Beddell *et al.*, 1976) and a report in the literature of certain pyridoxal derivatives reacting at either α- or β-chain terminal amino groups (Benesch *et al.*, 1973) we turned our attention to the α-chain terminal amino region as a design site. Examination of the crystal structure data available at the time (Perutz *et al.*, 1968; Bolton & Perutz, 1970) for horse deoxy and horse met (oxy type) haemoglobins, indicated considerable differences in structure at the α-terminal amino regions of the two forms, although the differences are not as pronounced as at the corresponding β-terminal amino regions. In particular, there appeared to be a large difference in distance between the terminal amino groups themselves which was reported as 20.7 Å in the deoxy form and 12.4 Å in the met (oxy) form. By analogy with the design of dialdehydes to bind to the β-terminal amino groups we again elected to design bifunctional agents to link the two α-terminal amino groups. The particular spatial orientation of these amino functions was not easy to match in a dialdehyde and we therefore decided on a structure containing one aldehyde to form a Schiff base with one amino group and an acidic grouping to form a salt bridge with the other. This also carried the advantage of promoting aqueous solubility. The correct distance and geometry for this bridging interaction in the oxy form but not in the deoxy form (at either α- or β-terminal amino regions) was then achieved by attaching these functions to a biphenyl template to give the structure shown as

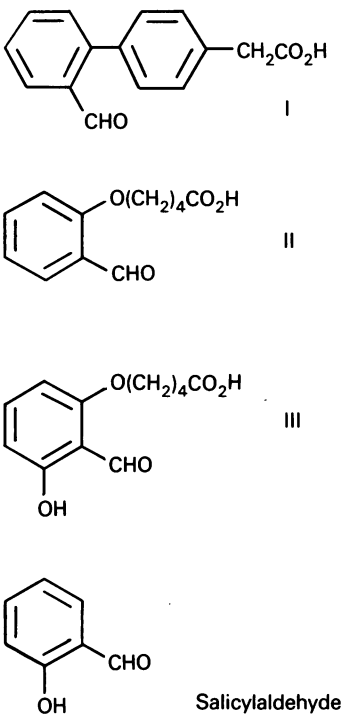

Figure 1 Structure of compounds used.

compound I (Figure 1). In addition, the α-terminal region, unlike the β-terminus, contains hydrophobic residues, particularly α^{77} proline, and it was therefore hoped that the hydrophobic central portion of the biphenyl structure might form a favourable hydrophobic interaction with these residues. The postulated binding mode for compound I is shown schematically in Figure 2a. It is likely, of course, that additional binding may occur in both oxy and deoxy conformations via Schiff base formation with other amino groups, particularly with the β-terminal amino groups, but also with the amino groups of lysine residues. However, such binding should be weaker than the intended mode shown in Figure 2a if the design strategy is effective.

Aromatic aldehydes form stable Schiff bases with amines when they contain a hydroxyl group ortho to the aldehyde (Zaugg *et al.*, 1977), presumably due to the formation of a hydrogen bond with the nitrogen of the Schiff base imine linkage. Attempts were made to increase the binding of compound I by introducing such an *ortho* hydroxyl group but this proved difficult synthetically. A search was made, therefore, for alternative structures which would maintain the correct spatial orientation of the aldehyde and acid functions as in compound I but would allow easier introduction of the *ortho* hydroxyl group. The correct spatial

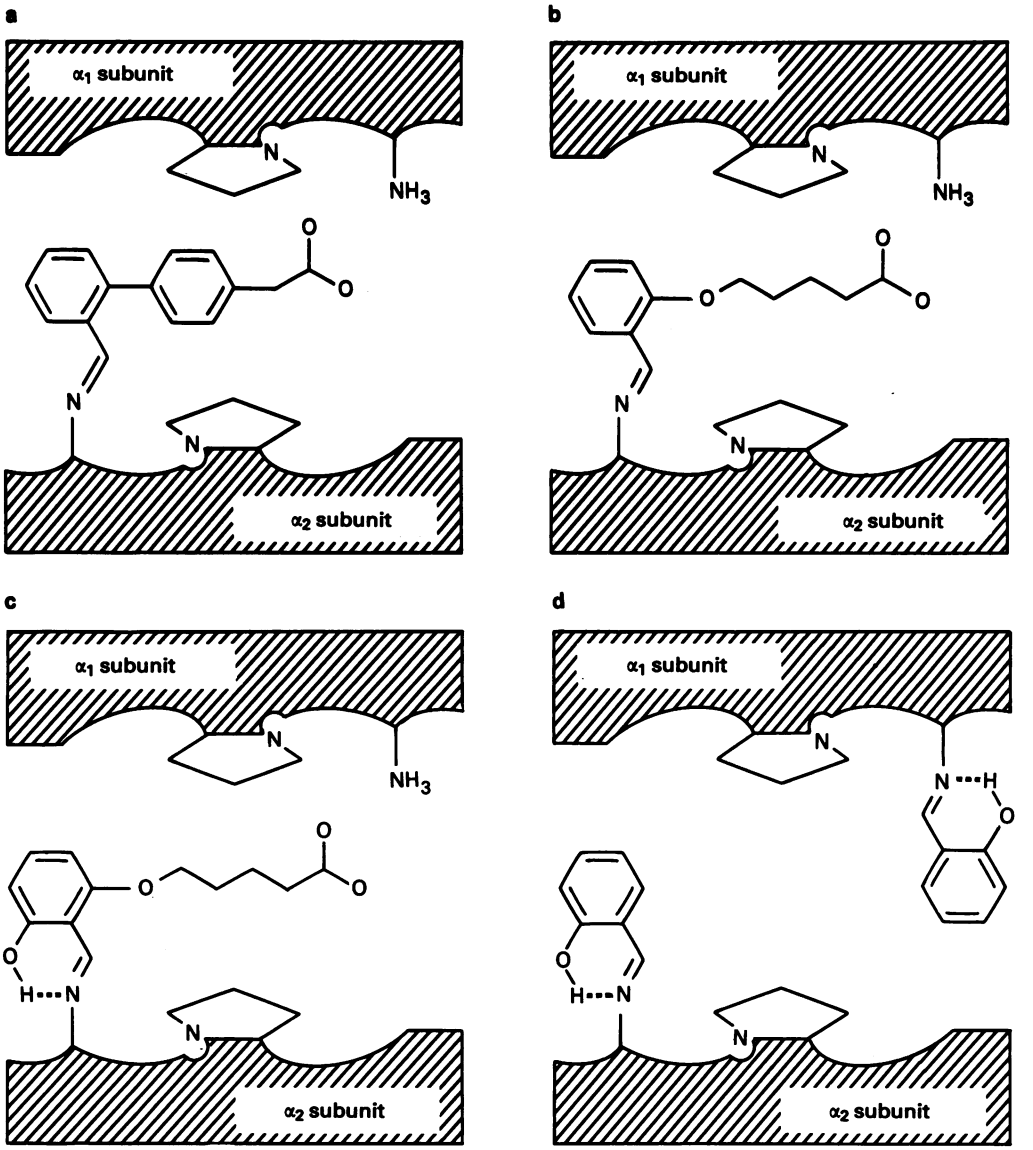

Figure 2 Schematic representation of the postulated binding modes for the four compounds at the α-chain terminal amino region of human oxyhaemoglobin; (a) compound I; (b) compound II; (c) compound III; (d) salicylaldehyde.

arrangement (in its extended form) was achieved in compound II (Figure 1) and the postulated binding mode for this compound is shown in Figure 2b. Compound III (Figure 1) containing the required *ortho* hydroxyl group was then synthesized and its predicted binding mode is shown in Figure 2c. Finally, salicylaldehyde, a known (Zaugg *et al.*, 1977; Beddell *et al.*, 1978) left-shifting, anti-sickling compound, was included within the series for comparative purposes. Examination of the α-terminal amino region shows that there is room for 2 molecules of salicylaldehyde at this site, and its postulated 2:1 binding is shown schematically in Figure 2d.

Preparation of compounds

2'-Formyl-4-biphenylacetic acid (compound I) was prepared by Friedel-Craft acetylation of 2-nitrobiphenyl followed by reduction of the nitro-group to give 4-acetyl-2'-aminobiphenyl. A Will-

gerodt reaction then gave the corresponding acetic acid which was converted to compound I by diazotisation and reaction with formaldoxime. 5-(2-Formylphenoxy)pentanoic acid (compound II) was prepared by alkylation of salicylaldehyde with ethyl 5-bromopentanoate and subsequent saponification of the ester. 5-(2-Formyl-3-hydroxyphenoxy) pentanoic acid (compound III) was prepared as described in published UK patent specification No. 2053218, Kneen, G. (The Wellcome Foundation Limited). Salicylaldehyde was purchased from BDH and fractionally distilled at reduced pressure under nitrogen.

Results

The effects of a range of concentrations of the compounds on the oxygen saturation curves of dilute haemoglobin solutions are shown in Figure 3 (a to d). For clarity only certain of the measured curves are plotted in each case. Salicylaldehyde has previously been shown to left-shift the oxygen saturation curve of erythrocyte suspensions (Zaugg *et al.*, 1977) and the present result confirms that this is due to a direct left-shifting effect on haemoglobin. Compounds I and II also left-shift the saturation curve as predicted, and exert their effect over a similar concentration

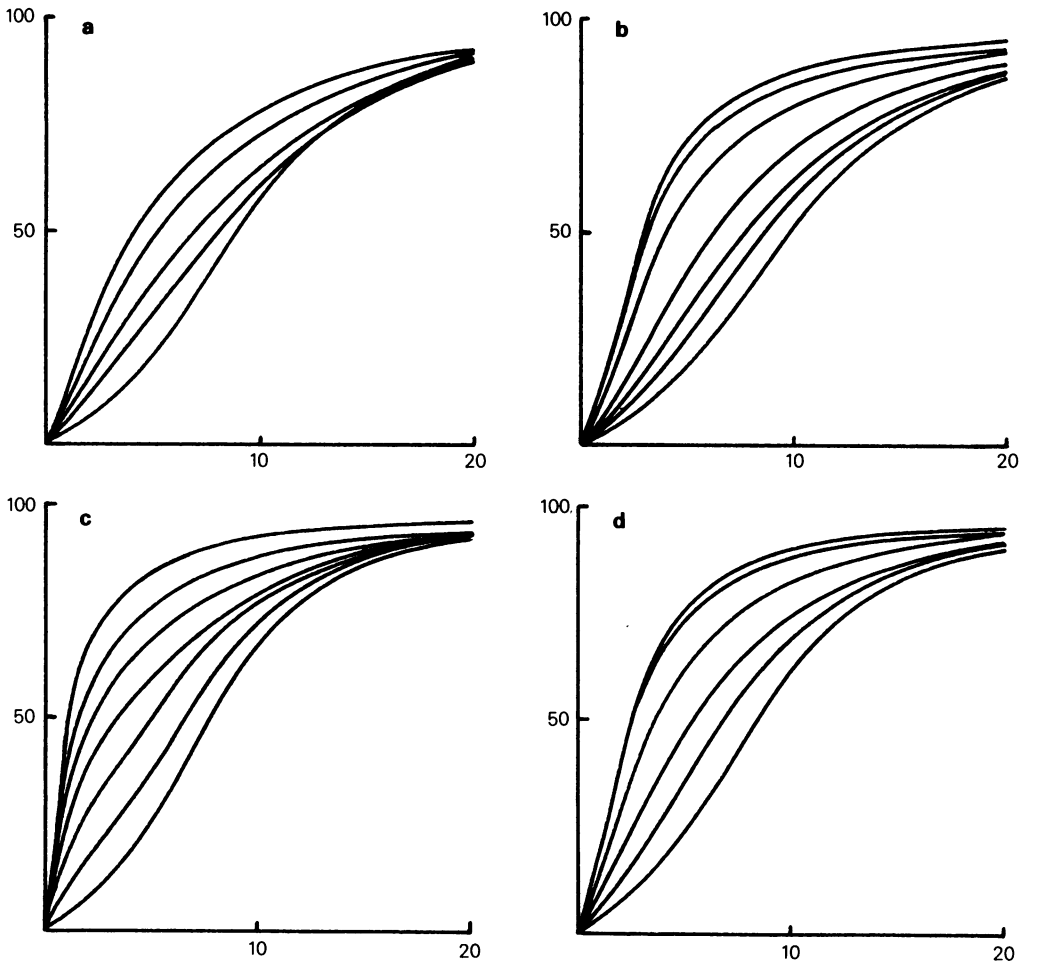

Figure 3 Oxygen dissociation curves of normal haemoglobin solutions for various concentrations of the compounds. Abscissae, the partial pressure of oxygen in mmHg. Ordinates, the percentage saturation of the haemoglobin with oxygen. In each case the right-most curve is the control curve and the progressively left-shifted curves are for the concentrations indicated; (a) compound I at 0.1, 0.2, 0.5 and 2 mM; (b) compound II at 0.05, 0.1, 0.2, 0.5, 1 and 2 mM; (c) compound III at 0.01, 0.02, 0.03, 0.04, 0.05 and 0.1 mM; (d) salicylaldehyde at 0.05, 0.1, 0.2, 0.5 and 2 mM. The reproducibility of measurement of individual curves is within ± 5% of the respective P50 value.

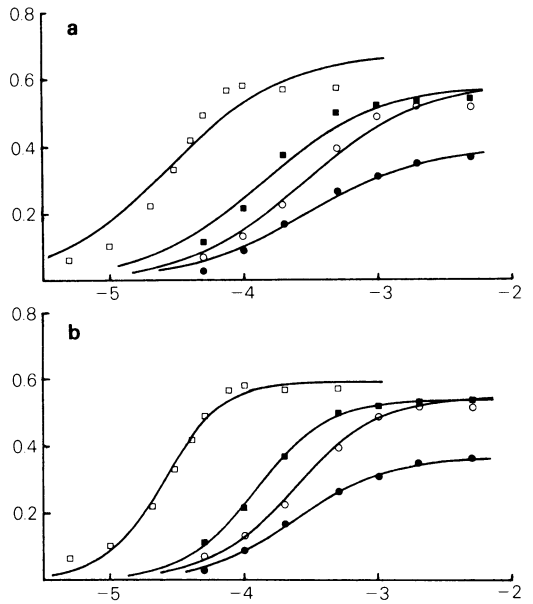

Figure 4 Log concentration-response curves for the compounds. Abscissae, log of the molar compound concentration (including the amount bound to haemoglobin). Ordinates, the differences in fractional saturation at 5 mmHg between the control curve and the curves in the presence of the various compound concentrations. (●) Compound I; (○) compound II; (■) salicylaldehyde; (□) compound III. The solid lines are the best-fit curves from fitting equation (1) with n = 1 (a) and with n allowed to vary (b).

range to salicylaldehyde indicating comparable potencies for these three compounds. Compound III produces its effects at much lower concentrations showing that the presence of the *ortho* hydroxyl group results in a large increase in potency, as predicted. A preliminary account of the left-shifting effect of compound III (also referred to as BW12C) on whole blood has been published (Kneen & White, 1981).

The relative potencies of the compounds are illustrated more clearly in Figure 4 in which the differences in saturation (ΔS) at 5 mmHg between the control curves and the curves at each compound concentration (x) are plotted against log x. Such curves may be described empirically using equation (1), a form of the Hill equation (Rang, 1971):

$$\Delta S = \frac{ax^n}{b^n + x^n} \quad (1)$$

where a is the asymptotic response at high x, b is the concentration giving 50% effect and n is a measure of the deviation of the slope of the Hill plot from unity. For simple 1:1 binding of compound to haemoglobin n will be 1 and equation (1) is then exact for any given partial pressure of oxygen (Goodford *et al.*, 1980); 5 mmHg was chosen in the present case to give maximum discrimination between curves. The lines in Figure 4 are the calculated curves obtained by the non-linear fitting of equation 1 to the data with n set at 1 (Figure 4a) or allowed to vary (Figure 4b), and Table 1 gives the corresponding best-fit parameter values for the latter. In all cases a significantly better fit, as judged by partial F-tests, is obtained with $n > 1$. For compound III this may be due, wholly or in part, to the fact that the free concentrations of compound are generally much lower than the total concentration plotted in Figure 4, but for the other compounds this is not the case. This then suggests that the binding is more complex than described in the design hypothesis and that at least two binding sites per haemoglobin tetramer are involved. In addi-

Table 1 Results of fitting equation (1)* to the concentration-response curves for the compounds

Compounds	a	b (mM)	*n*	*Sum of*† *squares*	*F*+
I	0.369 ±0.009	0.245 ±0.017	1.31 ±0.10	.00031	12.8 (5%)
II	0.548 ±0.017	0.239 ±0.022	1.35 ±0.14	.00127	9.3 (5%)
III	0.596 ±0.019	0.0248 ±0.0017	1.97 ±0.24	.00521	31.2 (0.1%)
Salicylaldehyde	0.539 ±0.008	0.121 ±0.005	1.63 ±0.12	.00061	39.4 (1%)

*The best-fit values for the parameters obtained by the non-linear fitting (Beddell *et al.*, 1976) of equation (1) to the concentration-response data where response is taken as the increase in oxygen saturation at a partial pressure of 5 mmHg. Estimated standard errors, calculated from the matrix of partial derivatives used in the fitting routine, are given in parentheses. No correction is made for the amount of compound bound to the haemoglobin.

†The sum of squares of residuals between calculated and measured response values.

+F values for partial F-tests of the reduction in sum of squares obtained by allowing n to vary compared to setting n = 1. Associated signigicance levels are given in brackets.

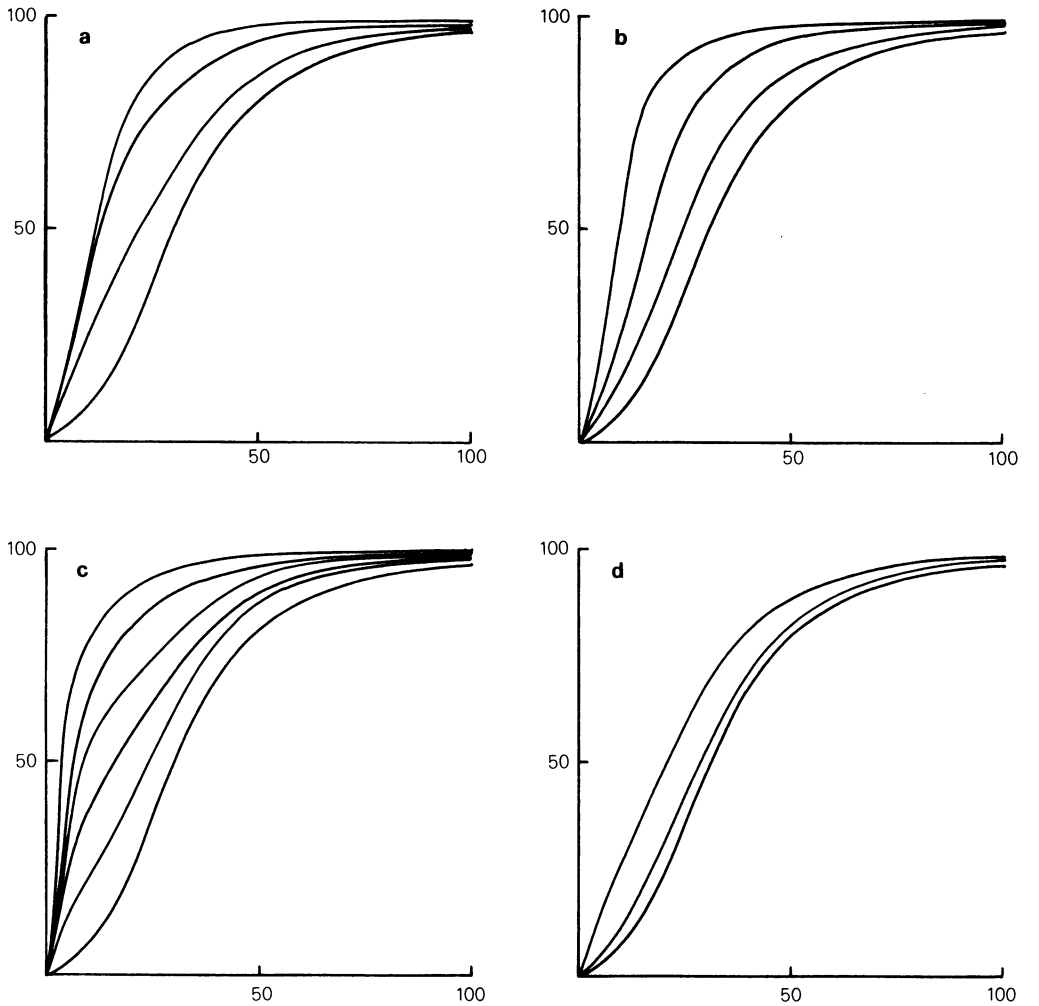

Figure 5 Oxygen association curves of normal whole blood for various concentrations of the compounds. Abscissae, the partial pressure of oxygen in mmHg. Ordinates, the relative percentage saturation of the blood with oxygen. In each case the right-most curve is the control curve (no compound present) and the progressively left-shifted curves are for the concentrations indicated: (a) compound I at 3, 10 and 30 mM; (b) compound II at 3, 10 and 30 mM; (c) compound III at 2, 4, 6, 8 and 10 mM; (d) salicylaldehyde at 10 and 30 mM. The reproducibility of measurement of individual curves is within ± 5% of the respective P50 value.

tion, although an exact analysis of the oxygen saturation curves is difficult due to the complexity of the binding, it is clear that binding to the deoxy conformation also contributes to the left-shift since the oxygen affinity of the deoxy conformation is increased on compound binding (Goodford *et al.*, 1978).

The compounds also produce left-shifts in whole blood and Figure 5 gives normal (HbA) whole blood oxygen saturation curves measured in the presence of various concentrations of the compounds. In agreement with the findings for haemoglobin solution, compound III again gives the largest left-shifts of the oxygen saturation curves, although the difference in potency between compound III and the other compounds is less marked in whole blood due to the high concentrations of haemoglobin present. It is evident from the whole blood data for compound III that more than 3 moles of compound per mole of haemoglobin tetramer are required to effect a full left-shift of the oxygen saturation curve and a similar situation pertains for the haemoglobin solution data. Furthermore, the curves measured at intermediate concentrations of compound show a pronounced biphasic

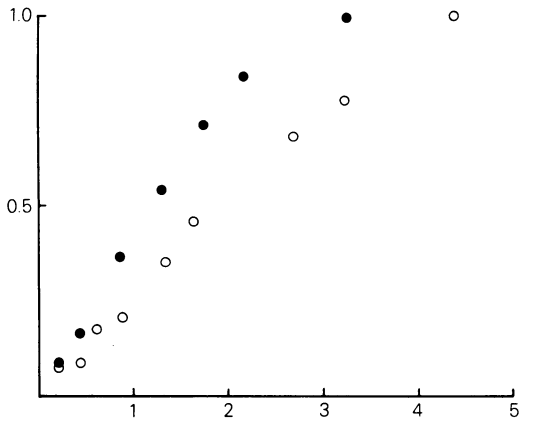

Figure 6 Plots of fraction, f, of haemoglobin modified by compound III (ordinate) against the molar ratio of compound to haemoglobin (abscissa) for haemoglobin solution (●) and whole blood (○). The points were derived from experimental data, partly plotted in Figures 3c and 5c, as discussed in the text.

character which suggests that they are a composite of curves for two functionally different haemoglobin forms with different affinities for oxygen. The curves were therefore analyzed according to this model to determine the fraction of reacted haemoglobin as a function of the molar ratio of compound to haemoglobin. Twelve oxygen partial pressure values, P, were selected, and the corresponding saturation values, S, were measured from the saturation curves of haemoglobin solutions or whole blood in the presence of various concentrations of compound III. To estimate the fraction, f, of haemoglobin reacted at each compound concentration it was assumed that f is independent of pressure. The value of f is 0 in the absence of compound and the control curves provided saturation values $S_0(P)$ for each of the selected values of P. It was assumed that f is 1 when the curves are fully left-shifted by 15 mM compound III in whole blood and 500 μM compound III in haemoglobin solution and the corresponding curves under these circumstances provided saturation values $S_1(P)$. For each intermediate concentration of compound, f was determined by fitting to equation (2) where $S_f(P)$ are the saturation values measured from the curve for that concentration of compound.

$$S_f(P) = f.S_1(P) + (1 - f).S_0(P) \qquad (2)$$

Figure 6 shows the results of this analysis for both haemoglobin solution and whole blood oxygen saturation curve data. The previous analysis of the solution data in terms of differences in saturation at 5 mmHg oxygen pressure was equivocal because of the lack of knowledge of the amounts of compound III bound to the haemoglobin. In fact equilibrium dialysis measurements (R.D. White, unpublished results) show that the association constant for compound III binding to oxyhaemoglobin is in excess of $10^6\,mol^{-1}$ and the reaction is therefore essentially stoicheiometric under whole blood conditions. The present analysis, thus, clearly demonstrates the presence of more than one binding site for compound III on the haemoglobin tetramer. There also appears to be more compound bound in the case of whole blood which may be due to a greater amount of weak 'non-specific' binding at the higher haemoglobin concentrations in whole blood, but could also be due to weak binding to other components of whole blood such as plasma proteins.

The compounds were designed to bind preferentially to the oxy conformation of haemoglobin and thereby increase the amount of this form at a given partial pressure of oxygen. They were therefore tested for their anti-sickling action using suspensions of sickle cells incubated at a fixed low partial pressure of oxygen. In aerated samples in the absence of compound, almost all the cells appear as normal, discoid erythrocytes. On partial deoxygenation to an oxygen pressure of ca. 28 mmHg, the proportion drops to about 50% and there is a concomitant rise in the proportion of reversibly sickled forms. The actual proportion varies with donor and is dependent upon, *inter alia*, intraerythrocytic haemoglobin concentration and membrane damage (both related to cell age) and the compounds were therefore tested on the same sample of blood (similar results were obtained for other blood samples). All four compounds cause a concentration-related inhibition in the generation of reversibly sickled cells (Figure 7). Compound III is the most potent and is effective over a three fold lower concentration range than the other compounds: at a concentration of 0.5 mM compound III, the field of cells is very similar in appearance to the aerated control. The approximate linearity of the response-curve for compound III is in accordance with the postulated stoicheiometric binding of the compound to haemoglobin, and comparison with other experiments (Kneen & White, 1981) shows that the required concentration of compound for equilibrium anti-sickling activity is in proportion to the haematocrit of the cell suspension.

Discussion

Previous rational drug design exercises have often relied on the modification of the structure of an existing enzyme substrate or receptor hormone with the aim of producing, for example, an enzyme inhibitor or a receptor antagonist. However, if the structure of the receptor site itself is known then it becomes feasible to design compounds of different

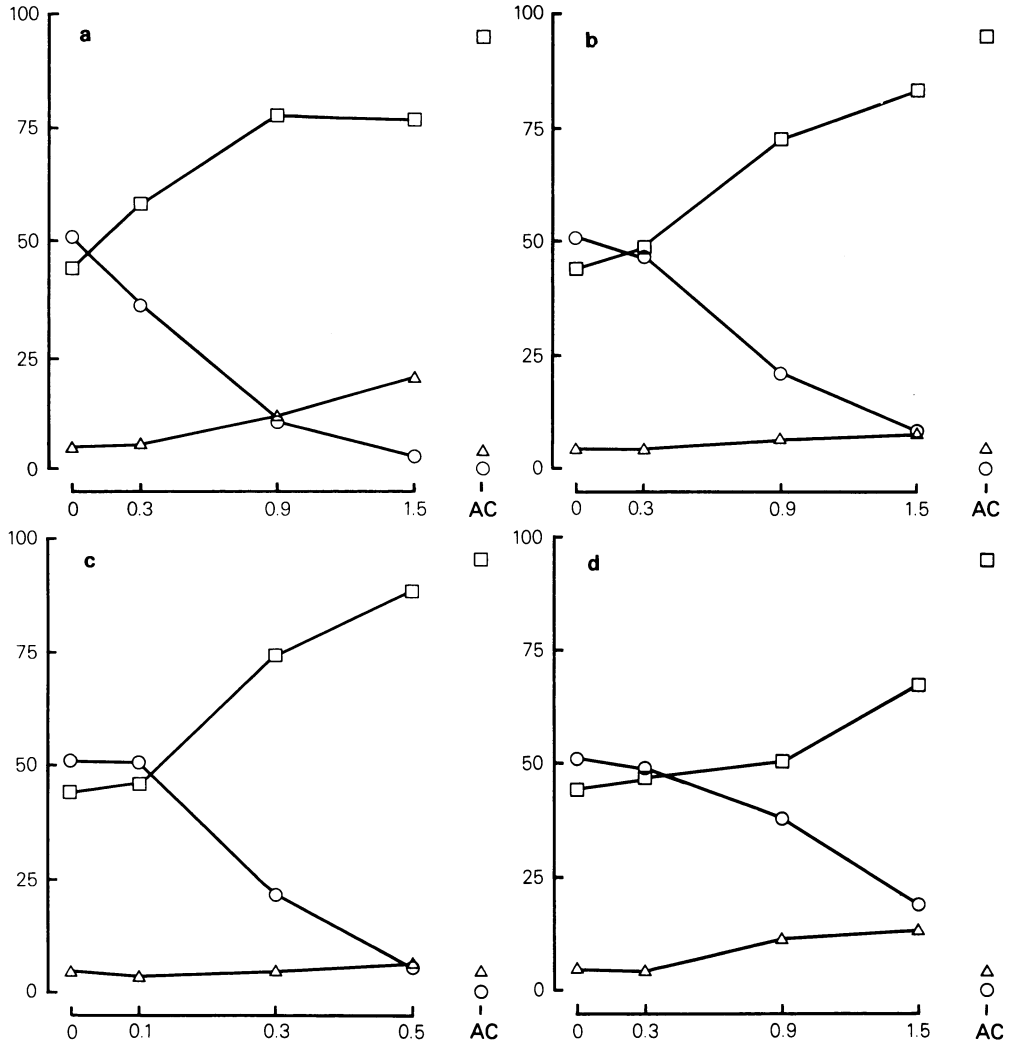

Figure 7 Equilibrium anti-sickling action of the compounds at various concentrations in suspensions of sickle cells at a partial pressure of oxygen of ca. 28 mmHg. Abscissae, the concentration of compound in mM (AC: aerated control). Ordinates, percentage of cell type as determined by photomicrography. (□) Normal cells; (○) sickled cells; (△) bizarre cells. (a) Compound I; (b) compound II; (c) compound III; (d) salicylaldehyde. The reproducibility of measurement of cell type is typically in the range of ± 5% of the total cell count.

chemical type and thus produce completely novel lead structures. These may then be modified to optimise receptor fit and other properties, such as transport and metabolism, in order to gain full expression of *in vivo* biological activity.

In the design of compounds to mimic the effect of DPG and right-shift the oxygen saturation curve (Beddell *et al.*, 1976) the choice of design site was obvious. In the present case there are no known, naturally-occurring, left-shifting compounds and the choice of design site is not obvious. However, the detailed knowledge of the structure of haemoglobin and the changes that occur in going from deoxy to oxy haemoglobin (Baldwin & Chothia, 1979) made the choice less difficult than might perhaps have been expected. The α-chain terminal amino region was selected based on the crystal structure information for horse deoxy and methaemoglobin which was available at the time (Perutz *et al.*, 1968; Bolton & Perutz, 1970). In fact, more recent interpretations of data for human deoxy (Fermi, 1975) and carbonmonoxy haemoglobin (Baldwin, 1980) suggest much

smaller differences in structure between the deoxy and oxy conformations than found in the earlier work. The discrepancy arises largely because the terminal amino groups are not firmly anchored in the oxy conformation and the first two residues may adopt more than one position, although there also appear to be significant differences in the relative positions of the entire first helical section of the α-chains. This highlights one of the major problems in such a practical design exercise in that the structure of the receptor site is not rigid and it is often difficult to take account of the flexibility of both the designed ligands and the receptor site itself in predicting the properties of the ligand-receptor complex. The more recent techniques of sophisticated molecular graphics combined with limited potential energy calculations may aid this crucial step considerably (see for example Blaney *et al.*, 1982).

It is perhaps partly for these reasons that the original intention, the design of a compound highly selective for a single site in the oxy conformation, has only been met in part; additional binding to both oxy and deoxy conformations occurs and probably contributes to the observed effects. Nevertheless, the compounds do left-shift the oxygen saturation curve and exert an anti-sickling effect as predicted and compound III shows high potency in its actions. Indeed, there is as yet no evidence to indicate that the primary action of the compounds is not as predicted in the original design hypothesis. The exercise has therefore been successful and compound III (BW12C) is now showing promise as a therapeutic anti-sickling agent in that it appears to show high specificity for binding to haemoglobin *in vivo*.

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