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COMPOUNDS DESIGNED TO FIT A SITE OF KNOWN STRUCTURE IN HUMAN HAEMOGLOBIN

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1 The three-dimensional coordinates of the atoms in human haemoglobin are known, and there is a specific site in the deoxygenated form of the protein at which 2,3-diphosphoglycerate (DPG) interacts.

2 Molecular models of this site have been constructed and used to design compounds which should bind to the deoxy conformation and stabilize it. These compounds should thereby promote oxygen liberation, as does DPG.

3 The compounds so designed were found to promote oxygen liberation. Their relative potencies, as assessed by sigmoidal dose-response curves, are in the predicted sequence.

Introduction

It has long been accepted (Langley, 1878) as a working hypothesis that some drugs may interact with specific receptors of complementary structure. Attempts have been made to deduce the properties and structures of such receptors from the properties of the interacting drugs (e.g. Clark, 1937; Belleau, 1967; Smythies, 1975). There are also direct biochemical observations on receptors suggesting, for example, that the membrane-bound acetylcholine receptor from *Torpedo electroplax* is a protein (Eldefrawi & Eldefrawi, 1973). Similarly, the cardiac glycoside receptor site in mammalian cell membranes may be situated on a tetrameric protein (Stein, Lieb, Karlish & Eilam, 1973). Haemoglobin is also a tetrameric protein, and has been singled out as a model of unique value for the study of drug actions (Clark, 1937).

Detailed knowledge of the structure of any receptor had to await the advent of high-resolution X-ray crystallography. Sperm whale myoglobin was the first biological macromolecule for which the three-dimensional positions of individual atoms were established (Kendrew, Watson, Strandberg, Dickerson, Phillips & Shore, 1961). The molecular structure of horse haemoglobin was determined by Perutz, Muirhead, Cox, Goaman, Mathews, McGandy & Webb (1968). The haemoglobin tetramer contains two α and two β subunits, each consisting of a single polypeptide chain which resembles one myoglobin molecule.

There is a site of known structure on the human deoxyhaemoglobin tetramer at which the small molecule 2,3-diphosphoglycerate (DPG) binds and stabilizes the deoxy conformation. The resulting liberation of oxygen can be measured to provide a quantitative biological response. Thus the DPG-haemoglobin interaction has some characteristics of a 'drug-receptor system', and human haemoglobin was therefore selected as a model pharmacological receptor for the present study. The appropriateness of this choice cannot yet be fully assessed. It is open to the criticism that the haemoglobin molecule is not subject to the same constraints and interactions with neighbouring molecules as a membrane-bound pharmacological receptor. Haemoglobin is also present in greater amounts by several orders of magnitude. On the other hand the choice offers a number of advantages. Haemoglobin can be prepared in a relatively pure form, and its structure and function are well established as is the mode of action of DPG.

Greenwald (1925) found DPG to be present in erythrocytes in millimolar concentrations, but the function of these large amounts remained unknown for 40 years although Sutherland, Posternak & Cori (1949) discovered that micromolar concentrations were needed in order to catalyze the phosphoglyceromutase reaction. In 1963 Sugita & Chanutin observed electrophoretically that DPG formed reversible complexes with haemoglobin, and Chanutin & Curnish (1967) showed that it altered the dissociation curve of haemoglobin, decreasing the oxygen affinity. At about this time Monod, Wyman & Changeux (1965) published their theory of macromolecular properties citing haemoglobin as an example, and Benesch & Benesch (1967) proposed that DPG complexed with haemoglobin as an allosteric regulator. Finally Arnone (1972) obtained crystals of deoxyhaemoglobin which incorporated DPG, and showed by X-ray crystallography how this compound fitted its receptor site in the protein.

A scale model (Beddell, Goodford, Norrington &

Wilkinson, 1973) of the complete haemoglobin tetramer has been constructed as the basis for designing compounds to fit the DPG site. It was predicted that such compounds, like DPG, would diminish the affinity of haemoglobin for oxygen, and their specific effects on the dissociation curve are now described.

Methods

Preparation of human haemoglobin

Human haemoglobin of low phosphate content was prepared by counterflow dialysis (Paterson, Eagles, Young & Beddell, 1976). All preparative work was conducted at 4°C. Red blood cells from 2–5 weeks old blood were washed with 0.9% w/v NaCl solution (saline) and haemolysed in distilled water. The red cell ghosts were removed by centrifugation, and the proteins were fractionated by ammonium sulphate precipitation. Haemoglobin precipitated between 33%–65% saturation, and this precipitate was redissolved and dialysed using a sterilized hollow cellulose fibre dialyser (Dow Chemical Company, Miniplant C/HFD-15). The dialysing buffer was passed through the fibres, and the surrounding solution was analysed for total haemoglobin, methaemoglobin and phosphate after 48 hours. Separate aliquots were stored sterile at −20°C.

Determination of the dissociation curves

The method of Allen, Guthe & Wyman (1950) was modified as follows: 5 cm^3 of the test solution was placed in a cylindrical all-glass tonometer of approximately 100 cm^3 capacity to one end of which was fused a spectrophotometer cell. The air pressure was reduced to 8 kPa while a small flow of nitrogen (British Oxygen Company oxygen-free 'white spot' nitrogen) was admitted. Lower pressures induced frothing and were therefore avoided. Atmospheric pressure was then restored by readmission of nitrogen, and the tonometer was rotated horizontally for 3 min in a water bath at 37°C while the thin film of test solution on the tonometer walls equilibrated with the gas phase. The tonometer was next placed upright in the sample position of a thermostated Pye-Unicam SP 800 spectrophotometer, and the spectrum from 500 to 650 nm was observed. After three or four successive evacuations followed by readmission of nitrogen a constant spectrum of deoxyhaemoglobin was recorded. A small measured volume of air was admitted to the tonometer through a 'Rotaflow' tap, equilibrated with the test solution as before, and a second spectrum observed. After successive readmissions of air a series of spectra were generated which were completed by a final observation of the test solution in pure oxygen. Measurement of each spectrum allowed the proportions of oxy and deoxy haemoglobin to be calculated, and the corresponding oxygen pressure was determined from the total volume of air admitted. Appropriate corrections were applied, and the results were expressed as a dissociation curve showing the proportion of oxyhaemoglobin as a function of oxygen pressure.

Curve fitting

The method of Powell (1970) was used to provide iterative least squares fits to the observations. The equation of Monod *et al.* (1965):

$$Y = \frac{Lc\alpha(1+c\alpha)^3 + \alpha(1+\alpha)^3}{L(1+c\alpha)^4 + (1+\alpha)^4}$$

was used to fit the dissociation curves (e.g. in Figure 2), where L and c are the fitted constants, α is the normalized oxygen pressure and Y is the fractional oxygen saturation of the haemoglobin. Following the suggestion of Edelstein (1971), the normalized oxygen pressures were calculated by multiplying the observed oxygen pressures in kPa by the constant value of 17.5 kPa^{-1}, corresponding to the average oxygen binding constant for the isolated α and β chains as determined by Brunori, Noble, Antonini & Wyman (1966).

The dose-response curves shown in Figure 4 were fitted (Powell, 1970) using the equation $R = ax/(b+x)$ where a and b are fitted constants, R is the oxygen liberated at 2 kPa divided by the maximum possible oxygen release at the same pressure, and x is the concentration of drug.

Molecular modelling

The working model (Beddell *et al.*, 1973) was at first used as a basis for designing compounds. However, it became clear that a more accurate representation was needed of the DPG receptor site of human deoxyhaemoglobin. This was constructed (Norrington, 1974) at a scale of 2×10^8: 1 from atomic coordinate positions kindly supplied by Dr M.F. Perutz, and was used to make accurate measurements of the distances between atoms in the protein and interacting atoms in models of trial molecules.

Choice of compounds

The DPG receptor site of human deoxyhaemoglobin is an exceedingly basic region in the protein. Each β chain contributes its terminal amino group and three basic amino acid residues ($His^{\beta 2}$, $Lys^{\beta 82}$ and $His^{\beta 143}$), giving a total of eight basic nitrogen functions in the immediate site. Two more basic residues ($Lys^{\beta 144}$ and $His^{\beta 146}$) from each β chain are nearby. Arnone (1972)

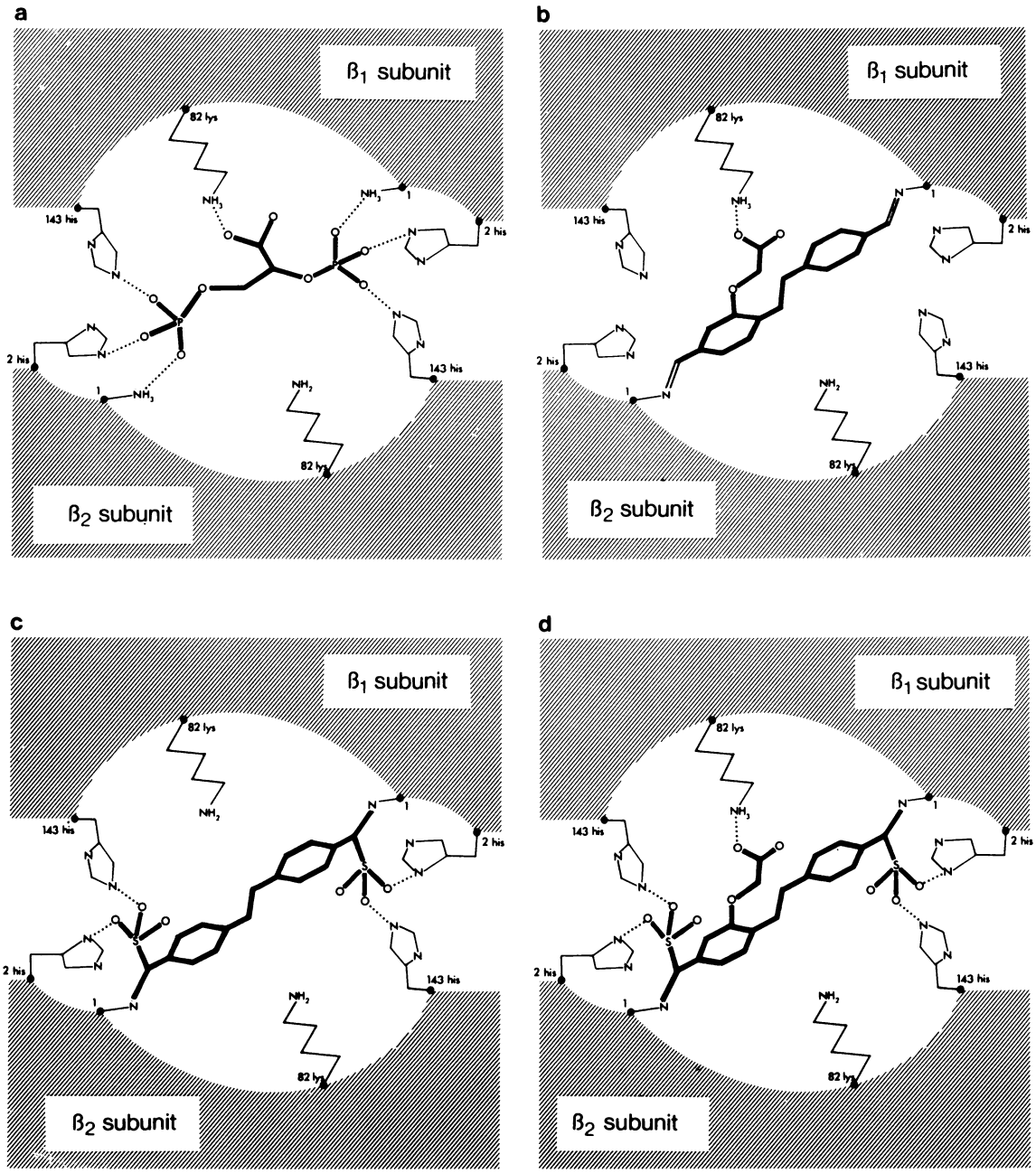
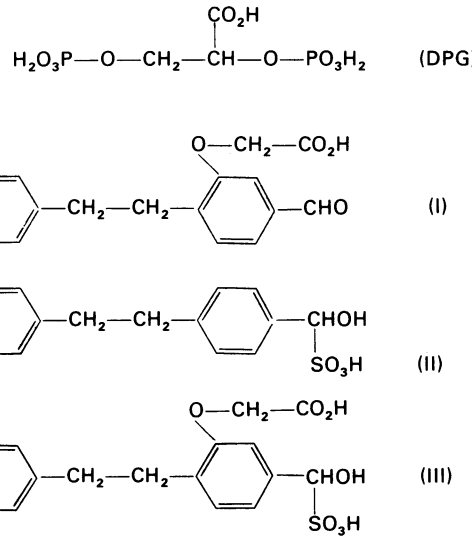


Figure 1 Schematic representations of the 2,3-diphosphoglycerate (DPG) receptor site: (a) with DPG bound as observed by Arnone (1972); (b) the postulated mode of binding of Compound I; (c) the postulated mode of binding of Compound II; (d) the postulated mode of binding of Compound III.

showed that each of six oxygen atoms in the phosphate groups of DPG is in a position to interact with one of the basic nitrogen functions of the receptor, while a seventh carboxyl oxygen might be interacting (Figure 1a). In attempting to design alternative types of compound to react with this region of the protein it was desirable to avoid highly ionized molecules like DPG itself, because their ionic character would inhibit passage across the red cell membrane. This property enables DPG to accumulate in the red cell after its metabolic formation, but would prevent exogenous compounds from reaching their intended intracellular site of action. We were also anxious, if possible, to design compounds of a completely different chemical type from DPG, and it was desirable to impose certain limitations on the conformational flexibility of the compounds. For these and other reasons attention was directed to the terminal amino groups of the β chains, and it was noted that the bibenzyl structure, in the conformation observed by Robertson (1934), would nearly span the distance between these amino groups in human deoxyhaemoglobin. The introduction of two aldehyde substituents to give bibenzyl-4,4′-dialdehyde provided reactive groups which were sterically matched to react with the terminal amino groups of the deoxy-protein by Schiff base formation. Moreover the aromatic rings of the bibenzyl structure imparted the intended degree of rigidity to bibenzyl-4,4′-dialdehyde, and model-building studies confirmed that it could not remain similarly bound when the protein was in the oxy conformation. It was therefore reasonable to hope that this compound would bias the haemoglobin equilibrium in favour of the deoxy form, and thereby promote the liberation of oxygen in a manner analogous to DPG itself.

High aqueous solubility had not been expected for bibenzyl-4,4′-dialdehyde, but it was actually found to be so insoluble in water that it could not be tested on haemoglobin solutions. It was therefore necessary to modify the structure in an attempt to increase solubility, and if possible provide more reactive groups to react with the protein. Compound I was therefore prepared, and this was sufficiently soluble for testing at concentrations up to 2.5 mmol dm^{-3}. It also seemed possible that the carboxylate group could interact (Figure 1b) with $Lys^{\beta 82}$, since it was in a favourable position to do so and Arnone (1972) had suggested that the carboxylate group of DPG might interact in this manner.



An alternative method of solubilizing bibenzyl-4,4′-dialdehyde was also investigated, by converting it to the bisulphite derivative (Compound II). This was soluble and could be tested at concentrations up to 5 mmol dm^{-3}, but it was necessary to add 5 mmol dm^{-3} sodium metabisulphite to the solution because some dialdehyde tended to precipitate unless free bisulphite ions were also present. Two possible modes of interaction between Compound II and the DPG receptor site may be considered. The bisulphite derivative might be in equilibrium with a sufficiently high concentration of the free dialdehyde for that to react by Schiff base formation with the terminal amino groups of the β chains as originally envisaged. However, it is well established (Lauer & Langkammerer, 1935; Shriner & Land, 1941; Caughlan & Tartar, 1941; Sheppard & Bourns, 1954) that the bisulphite derivatives of aldehydes are α-hydroxysulphonic acids, and Compound II might therefore interact with the terminal amino groups and

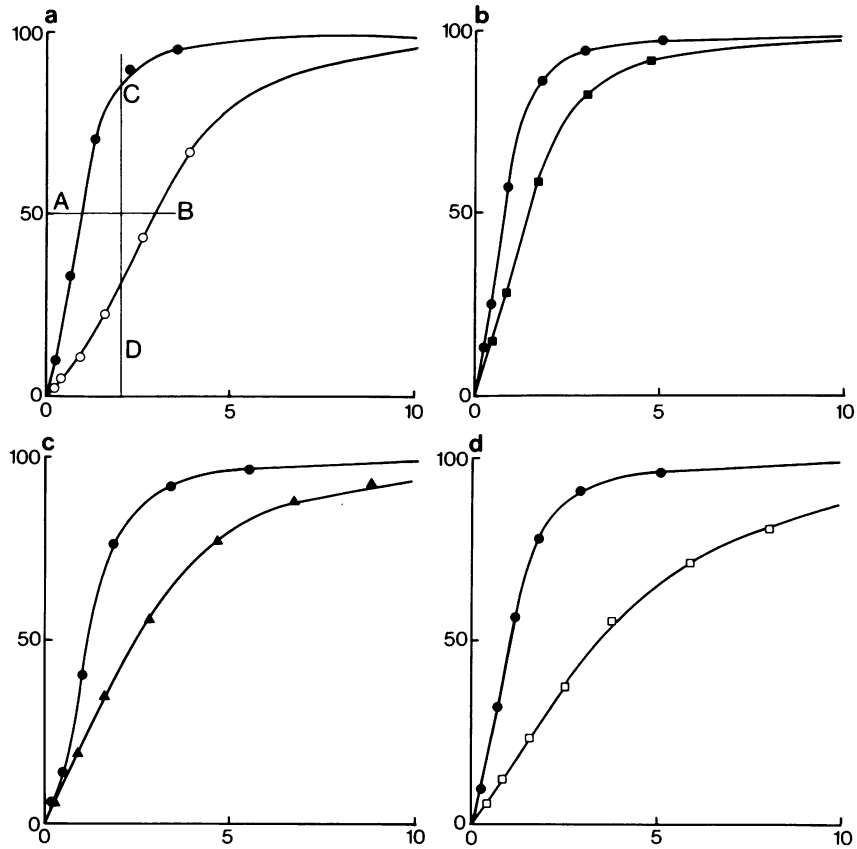


Figure 2 The oxygen dissociation curves of human haemoglobin in the presence and absence (●) of the compounds at a concentration of 2.5 mmol dm^{-1}. Abscissae, the gas pressure of oxygen in kPa. Ordinates, percent of oxyhaemoglobin. (a) Observations with 2,3-diphosphoglycerate (○); (b) observations with Compound I (■), note the diminished right shift and reduced oxygen liberation at constant pressure; (c) observations with Compound II (▲); (d) observations with Compound III (□).

some of the other basic residues of the DPG site, perhaps as illustrated schematically in Figure 1c. The bisulphite derivative (Compound III) of Compound I was also prepared, and since this has the extra carboxyl group available for interaction, we expected it to be the most potent of the compounds studied. Figure 1d illustrates one possible way in which it might interact.

Preparation of compounds

Bibenzyl-4,4'-dialdehyde was prepared according to the method of Reichstein & Oppenauer (1933). 4,4'-Diformyl-2-bibenzyl-oxyacetic acid (Compound I) was obtained by carboxymethylation of the corresponding phenol prepared by reduction of the stilbene obtained from the Wittig reaction (cf. Reimann, 1969) between triphenyl-(4-carbethoxybenzyl)-phosphonium bromide and 3-hydroxy-4-formyl benzoic acid. The bisulphite derivatives (Compounds II and III) were prepared by addition of an aqueous solution of sodium metabisulphite to an alcoholic solution of the dialdehyde.

Free 2,3-diphosphoglyceric acid was obtained by ion exchange on Amberlite IR-120(H) from the pentacyclohexylammonium salt (Calbiochem A Grade), and a 25 mmol dm^{-3} solution of the potassium salt was prepared by titration to pH 7.35 with potassium hydroxide solution. Aliquots were stored at −20°C.

Test solutions were prepared containing either 2,3-diphosphoglycerate or the appropriate test compound. Sodium metabisulphite 5 mmol dm^{-3} was also added to solutions of bisulphite derivatives in order to stabilize these derivatives. HEPES buffer (Good, Winget, Winter, Connolly, Izawa & Singh, 1966) at pH 7.35, haemoglobin solution, sodium chloride and water were added to give a final haemoglobin con-

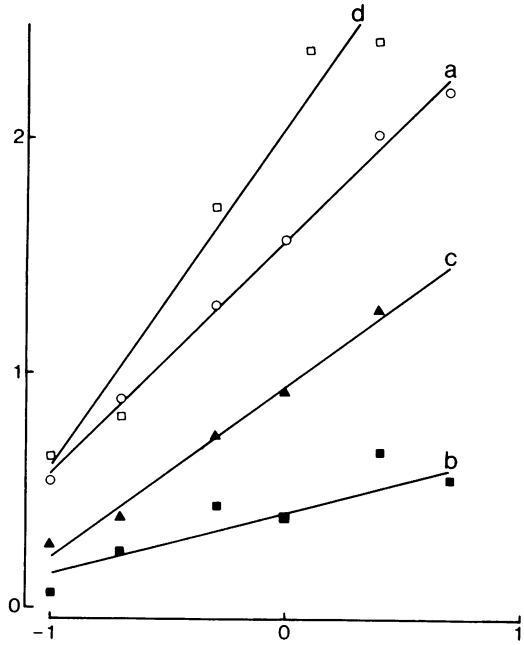


Figure 3 Logarithmic abscissae, concentration of compound in mmol dm^{-3}. Ordinates, right shift in the P_{50} value of the haemoglobin dissociation curves (kPa). (a) Observations with 2,3-diphosphoglycerate (○); (b) observations with Compound I (■); (c) observations with Compound II (▲); (d) observations with Compound III (□).

centration of 30 μmol dm^{-3}, and an ionic strength contribution from test compound, sodium metabisulphite and sodium chloride of 35 mmol dm^{-3}.

Results

Control observations with DPG

Figure 2a shows oxygen dissociation curves for human haemoglobin in the absence and presence of 2.5 mmol dm^{-3} DPG. The shift to the right demonstrates the decreased oxygen affinity which is caused by this compound. It may be measured by the oxygen pressure at equilibrium with a mixture of 50% oxyhaemoglobin and 50% deoxyhaemoglobin (the P_{50} value). This is given by the intersection of line AB with the curves. Six different DPG concentrations have been studied, and Figure 3a shows the shift of P_{50} as a function of the logarithm of the concentration of DPG.

The observations may also be expressed as the amount of oxygen liberated from haemoglobin by different concentrations of DPG at a constant partial pressure of oxygen. Such an interpretation is more

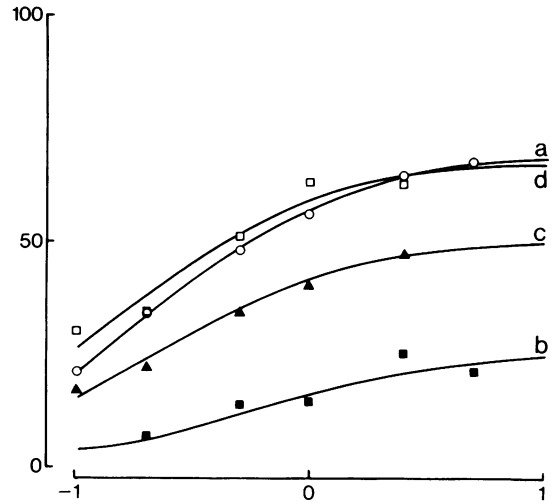


Figure 4 Logarithmic abscissae, concentration of compound in mmol dm^{-3}. Ordinates, oxygen liberated from the haemoglobin, at a constant pressure of 2 kPa, expressed as a percentage of the maximum amount of oxygen which could be liberated from the haemoglobin at that pressure. (a) Observations with 2,3-diphosphoglycerate (○); (b) observations with Compound I (■); (c) observations with Compound II (▲); (d) observations with Compound III (□).

relevant to the physiological function of DPG, which is to control the amount of oxygen liberated from the blood at the partial pressure of the capillaries. The intersections of the curves with the line CD, which corresponds to an arbitrary pressure of 2 kPa, are plotted against the logarithm of the concentration of DPG in Figure 4 (line a). The distance CD represents the total amount of oxygen carried by the haemoglobin in the absence of DPG at a gas pressure of 2 kPa, and is thus the maximum response which could be measured by this test system under these physiological conditions. It represents 100% response in Figure 4, and the effect of DPG has been fitted to a sigmoid which provides an adequate fit to the observations.

Observations with compound I

Figure 2b shows the dissociation curve in the presence and absence of 2.5 mmol dm^{-3} of Compound I. This compound is less potent than DPG, as shown by the smaller shifts of the P_{50} plotted against log concentration in Figure 3 (line b). The amount of oxygen liberated at a constant pressure of 2 kPa is also much smaller (Figure 4, line b), and the sigmoid fitted to the observations shows an asymptotic maximum at only 26% response, which may be compared with the fitted asymptote of 70% for DPG. One may conclude that this compound does promote the liberation of oxygen

as intended, but that it does so weakly. A lower potency was expected, however, since the compound was designed to interact with fewer groups in the receptor site than DPG itself (Figure 1b).

Observations with compound II

Sodium metabisulphite 5 mmol dm^{-3} was added to solutions containing Compound II, in order to prevent the free dialdehyde from precipitating. Control observations were therefore carried out to test whether this inorganic salt had any effect on the dissociation curve by itself. Although the addition of 5 mmol dm^{-3} sodium metabisulphite slightly modified the dissociation curve, the effect was always smaller than that caused by only 0.1 mmol dm^{-3} DPG.

Compound II was less potent than DPG but more potent than Compound I as judged by the right shift of the P_{50} value (Figure 3, line c), or the amount of oxygen liberated at 2 kPa oxygen pressure (Figure 4, line c). The greater potency would be expected if some of the oxygen atoms of the sulphonate groups were able to interact with the basic nitrogen atoms in the DPG site, and this type of interaction is shown schematically in Figure 1c.

Observations with Compound III

Sodium metabisulphite 5 mmol dm^{-3} was also added to solutions of Compound III. This compound had a substantial effect on the dissociation curves (Figure 2d), showing a comparable potency to DPG itself as measured both by the right shift (Figure 3, line d) and the oxygen liberated at 2 kPa pressure (Figure 4, line d). Once again this was to be expected if the compound interacted with several of the basic nitrogen atoms at the DPG receptor site, as shown schematically in Figure 1d.

Discussion

The present experiments were carried out in order to test the feasibility of designing drugs to interact with a receptor site of known molecular structure, and produce predefined biological effects. The work is open to many criticisms, and it is still incomplete. In particular, there is as yet no direct evidence on the mechanism by which the novel compounds influence the haemoglobin-oxygen dissociation curve, nor the site at which they interact with the protein. However, we believe that a study such as the present investigation should be carried out prospectively because it is very easy to justify the choice of compounds *post hoc propter hoc* once the mode of molecular interaction has been established. It is improbable that the predicted interactions as illustrated schematically in Figure 1 are correct in every detail. However, when the true mechanisms have been established it should be instructive to analyse the errors in the present predictions.

It has been common practice to design new drugs by modifying the chemical structure of a known substance which has the desired biological properties, and this procedure has imposed severe restraints on the choice. However, it is not necessary for the novel compounds to be related to the original substance when the structure of the receptor site is already known. In the present case the starting substance was a sugar phosphate, and we were rapidly and logically led to the consideration of aromatic dialdehydes from a study of the receptor site. In our opinion it is most unlikely that we would have made this transition from one chemical class to another, if we had applied the more conventional procedures. Designing new compounds by the method of receptor fit therefore seems to offer a substantial advantage in this respect.

Interaction with a biological receptor is not the only important factor in designing drugs. It might also be necessary to optimize uptake or distribution *in vivo*, and such aspects are often related to the physicochemical properties of compounds (Hansch & Fujita, 1964). In the present work, for example, the solubility of bibenzyl-4,4′-dialdehyde was not adequate and various modifications of this structure were therefore considered. The present approach again shows an advantage over traditional procedures in this respect, because it was possible to select compounds for synthesis which should not only have greater solubility, but should also interact more favourably with the receptor site.

In the present study the selected compounds produced the desired biological effect, and to this extent at least the approach has been successful. Moreover it was possible to design novel compounds which were not closely related to the natural starting substance DPG, and the first structure chosen could be modified logically in order to improve both its fit to the receptor and its physicochemical properties at the same time. This suggests that the method may prove to be both efficient and flexible. In fact it was not even necessary to postulate a unique mechanism of receptor interaction for the designed compounds, and we have not yet established whether they form Schiff bases, or interact without covalent bond formation, or even just rely on hydrogen bonding to the protein. It is now necessary to determine the mode of binding in order to optimize receptor fit, but the structure of native haemoglobin is already known and Fourier difference maps should identify the location of each compound unless it grossly distorts the crystal structure. The method of designing drugs to fit their receptors may therefore prove to be more economical in this respect than would be expected at first sight, and should be widely applicable because new protein structures are being published with increasing

frequency. It would now seem appropriate for more of these structural studies to be specifically directed at enzymes which were selected in the first place as potential targets for therapy.

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