mg, 0.214 mmol) in dichloromethane (1 mL) was cooled to 0 °C. To this mixture was added a solution of **28c** (16.8 mg, 0.058 mmol) in dichloromethane (1.5 mL). After stirring at 0 °C for 3.5 h, the solution was washed with saturated sodium bicarbonate and sodium thiosulfate. The separated organic layer was dried over anhydrous magnesium sulfate and the solvent was removed under reduced pressure. Flash chromatography (hexane–ethyl acetate, 1:1) gave **29** as a colorless oil: 6.5 mg, 49%; $^1$H NMR ($CDCl_3$) $\delta$ 3.70 (m, 1 H), 3.13 (m, 2 H), 3.00–1.20 (m, 11 H), 0.91 (d, 3 H, $J$ = 6 Hz), 0.76 (s, 3 H); IR ($CHCl_3$) 3605, 1790, 1012 cm$^{-1}$.

**3a$\alpha$,4,4a,5,6,7,7a$\alpha$,8,9,9a$\alpha$-Decahydro-5$\beta$-hydroxy-4a$\beta$,8$\alpha$-dimethylazuleno[6,5-*b*]furan-2(3*H*)-one (30).** To a solution of **29** (6.5 mg, 0.027 mmol) in ethyl acetate (3 mL) was added 5% rhodium on alumina (7.9 mg). The mixture was hydrogenated at 58 psi by using a Parr hydrogenation apparatus for 4.5 h. The catalyst was removed by filtration, and the solvent was removed under reduced pressure. Flash chromatography (ethyl acetate) gave **30** as a colorless oil (3.8 mg, 58%): $^1$H NMR ($CDCl_3$, 200 mHz) $\delta$ 4.78 (7 line m, 1 H), 3.67 (t, 1 H, $J$ = 8.5 Hz), 2.89 (dd, 1 H, $J_1$ = 18.2, $J_2$ = 10.5 Hz), 2.74–2.50 (m, 1 H), 2.17 (dd, 1 H, $J_1$ = 18.2, $J_2$ = 2.7Hz), 2.10–1.10 (m, 10 H) 0.98 (d, 3 H, $J$ = 6.9 Hz), 0.84 (s, 3 H); IR ($CHCl_3$) 3605, 3010–2830, 1762 cm$^{-1}$. Spectral data for this material compared favorably with those data reported in the literature.[26]

**Acknowledgment.** We thank the National Institutes of Health for financial support through Grants CA25787 and more recently GM33061.

**Registry No.** (±)-**1**, 83097-02-3; (±)-**2**, 60426-81-5; **6a**, 99783-47-8; **6b**, 83096-98-4; **7b**, 56522-18-0; **7c**, 92243-46-4; **7d**, 83096-99-5; **8**, 83096-97-3; **9**, 99783-48-9; (±)-**10**, 99783-49-0; **11**, 99783-50-3; (±)-**12**, 83096-96-2; (±)-**13**, 99783-51-4; **15**, 99783-52-5; **16a**, 83097-01-2; (±)-**16b**, 99783-54-7; **16b** (3-bromo deriv active), 99783-55-8; **17**, 99783-53-6; (±)-**18**, 83097-03-4; (±)-**19a**, 83097-04-5; (±)-**19b**, 99783-56-9; (±)-**19c**, 99783-57-0; (±)-**19d**, 83097-05-6; (±)-**19e**, 83097-06-7; (±)-**19f**, 83097-07-8; (±)-**20**, 99783-59-2; (±)-**21**, 99783-60-5; (±)-**22**, 99783-61-6; (±)-**23**, 99783-58-1; (±)-**24a**, 83097-08-9; (±)-**24b**, 72341-84-5; (±)-**25a**, 72341-86-7; (±)-**25b**, 72341-85-6; (±)-**26a**, 99783-62-7; (±)-**26b**, 99783-63-8; (±)-**27a**, 99783-64-9; (±)-**27b**, 99783-65-0; (±)-**28a**, 99783-66-1; (±)-**28b**, 99783-67-2; (±)-**28c**, 99783-68-3; (±)-**29**, 99783-69-4; (±)-**30**, 76156-91-7; 2-methyl-1,3-cyclopentanedione, 765-69-5.

# Hemoglobin as a Receptor of Drugs and Peptides: X-ray Studies of the Stereochemistry of Binding

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**Abstract:** In an attempt to establish a stereochemical rationale for the development of drugs against sickle cell anemia, we have crystallized human deoxyhemoglobin with the antihyperlipoproteinemia drug bezafibrate (I), with the diuretic drug ethacrynic acid (III), with succinyl-L-tryptophan-L-tryptophan (IV), and with *p*-bromobenzyloxyacetic acid (V) and have determined the structures of the complexes by X-ray analysis. Our results show that these compounds seek out niches in the protein where their stereochemistry of binding is determined by the available van der Waals space and, within that space, by a tendency to maximize electrostatic interactions. These range from strong hydrogen bonds to weakly polar interactions between halogens and aromatic quadrupoles. Another large part of the binding energy is due to hydrophobic effects. The binding site of *p*-bromobenzyloxyacetic acid lies in the interior of the $\alpha$-chain, in a position hitherto believed to be filled by close-packed side chains of the globin. The binding of these varied compounds induces small distortions in the hemoglobin molecule which affect the solubility of deoxyhemoglobin S. Ethacrynic acid, *p*-bromobenzyloxyacetic acid, and succinyl-L-tryptophan-L-tryptophan increase and bezafibrate reduces the solubility of deoxyhemoglobin S. Ethacrynic acid increases while bezafibrate and succinyl-L-tryptophan-L-tryptophan lower the oxygen affinity of hemoglobin A. *p*-Bromobenzyloxyacetic acid does not affect it.

The stereochemistry of drug binding to proteins is an almost virgin field, because most receptors are probably membrane proteins of unknown structure. The only crystallographically analyzed complexes are those of myoglobin and hemoglobin with the anaesthetic gas $CH_2Cl_2$,[2,3] of carbonic anhydrase with sulfanilamide,[4] and of dihydrofolate reductase with methotrexate.[5] $CH_2Cl_2$ occupies crevices in the globin, while the two drugs are analogues to the enzymes' natural substrates and act as inhibitors at their active sites. In a search for agents that would alleviate sickle cell anemia, we have made X-ray studies of complexes of hemoglobin with several compounds, three of them commonly used drugs. Their binding sites are diverse and coincide neither with the active site at the heme nor with the site of the natural allosteric effector 2,3-diphosphoglycerate (DPG). The compounds either inhibit or promote the polymerization of deoxyhemoglobin S in ways not yet fully understood.

Hemoglobin is a tetramer made up of two $\alpha$-chains, each containing 141, and two $\beta$-chains, each containing 146 amino acid residues. Each chain carries one heme. The $\alpha$-chains contain 7 and the $\beta$-chains 8 helical segments, interrupted by nonhelical ones. Each chain also carries short nonhelical segments at the N- and C-termini. Starting from the N-terminus the helical segments are denoted A to H and the nonhelical ones NA, AB, BC, etc., to HC, which denotes a segment of three nonhelical segments at the C-terminus. Residues within each segment are numbered from the amino end A1 to A16 etc. We label each residue by its structural position followed by its position in the sequence, e.g.,

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Glu A3(6)$\beta$. The tetramer has pseudotetrahedral symmetry. A true dyad relating the chains $\alpha_1$ to $\alpha_2$ and $\beta_1$ to $\beta_2$ runs along an $\sim$10 Å wide water-filled central cavity. Pseudo-dyads at right angles to the true dyad and to each other relate $\alpha_1$ to $\beta_1$ and $\alpha_1$ to $\beta_2$. The hemes are linked to the globin by covalent bonds between their irons and $N_\epsilon$ of histidines F8, known as the proximal ones. Histidine F8$\beta$ is followed by a reactive cysteine F9$\beta$ which faces the surface of the molecule. The oxygen-combining side of the heme faces another histidine, E7, known as the distal one[6] (Figure 1).

Hemoglobin is an allosteric protein in equilibrium between two alternative structures: the deoxy or T structure with low, and the oxy or R structure with high oxygen affinity. The oxygen affinity of the R structure is similar to that of free $\alpha$ and $\beta$ subunits or of dimers composed of one $\alpha$ and one $\beta$ subunit, while the oxygen affinity of the T structure is lower by the equivalent of $\sim$3.5 kcal/heme, due mainly to the constraints of additional bonds between the four subunits. These constraints take the form of salt bridges, i.e., hydrogen bonds between carboxylates and cationic amino, guanidinium, or imidazolium groups. Rupture of any of these salt bridges raises the oxygen affinity of the T structure and biases the allosteric equilibrium toward the R structure.[6] In this paper we shall be concerned only with the salt bridges linking the carboxylate of the C-terminal His HC3(146)$\beta$ to Lys C5(40)$\alpha$ and of its imidazole to Asp FG1(94)$\beta$.

Sickle cell anemia is caused by a mutation that leads to the replacement of the pair of glutamates A3(6)$\beta$ by valines. The glutamates are external, so that the replacement leaves the internal structure of the molecule intact, but the creation of the extra hydrophobic patch causes sickle cell deoxyhemoglobin (deoxyhb S) to polymerize in the red cell, which leads to sickling. Mammalian hemoglobins form linear aggregates with the dyad axis perpendicular to their length and with contacts between the GH segments and B helices of neighboring molecules. These linear aggregates form the basic structural units of crystals of normal mammalian deoxyhb's and of fibres of deoxyhb S. The crucial difference between them lies in the aggregation of deoxyhb S into double filaments, stabilized by a contact between valines A3(6)$\beta$ in one filament and phenylalanine F1(85)$\beta$ and leucine F4(88)$\beta$ in the heme pockets of the neighboring filament. Seven or eight double filaments aggregate further to form fibres $\sim$200 Å thick. Drugs might inhibit polymerization either by biasing the allosteric equilibrium toward the more soluble R structure or by altering the T structure in a way that destabilizes interactions between neighboring molecules in the deoxyhb S fibre.[6]

There have been several attempts to influence the allosteric equilibrium of hemoglobin by synthetic compounds designed to fit the atomic model. Bedell et al. made three substituted bifunctional benzaldehydes to fit into the DPG binding site. They all lowered the oxygen affinity, but no X-ray work was done to determine their stereochemistry of binding; one of them, the bisulfite derivative of diformyl-2-dibenzyloxyacetic acid was shown by NMR to affect the same two C2 histidine resonances as DPG.[7,8] Walder et al. have used bifunctional acylating agents of variable length to cross-link Lys EF6(82)$\beta_1$ and $\beta_2$; they studied the cross-linked deoxyhemoglobins by X-ray analysis and related their structures to the antigelling activity of the different linkers.[9,10] Bedell et al. designed substituted benzaldehyde derivatives to bind preferentially at a site between the amino termini of the $\alpha$-subunits. One of these 5-(2-formyl-3-hydroxyphenoxy)pentanoic acid was antigelling and raised the oxygen affinity of hemoglobin, but its binding site is not known (see below).[55] Kuyper et al. designed analogues of the dihydrofolate reductase inhibitor trimethoprim,

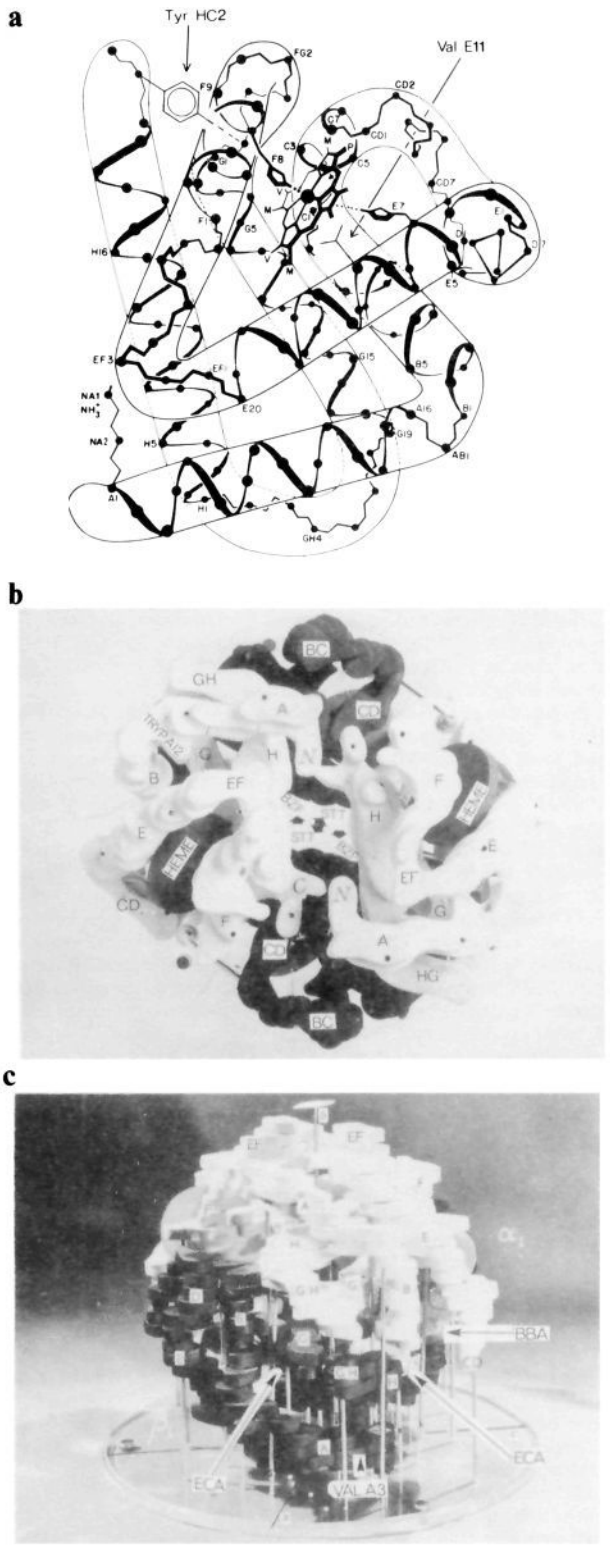


**Figure 1.** Structure of hemoglobin. (a, top) Tertiary structure of the $\alpha$-chain, showing the notation of helical and nonhelical segments, the heme pocket with the proximal histidine F8 forming a covalent link to the iron, and the distal histidine E7 and valine E11; also the hydrogen bond between the penultimate tyrosine HC2 and the carbonyl of valine FG5 that stabilizes the tertiary structures of both oxy- and deoxyhemoglobin. Parts b (middle) and c (bottom) show a model of the quaternary structure. Part b is taken parallel to the dyad axis that runs through the central cavity of the molecule, halfway between the STT signs. Part c is taken perpendicular to the dyad, marked *b* at the top of the model. The principal binding sites of the different compounds are marked by arrows. [BZF, bezafibrate; ECA, ethacrynic acid; STT, succinyl-L-Trp-L-Trp; BBA, *p*-bromobenzyloxyacetic acid.]

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Table I. Crystallographic Data

| Hb derivative | compound added | [Cpd]/[Hb] | unit cell dimensions a, Å | b, Å | c, Å | β, deg | space group | $d_{lim}$,[a] Å | $R_{int}$,[b] % | $R_{d-n}$,[c] % |
|---|---|---|---|---|---|---|---|---|---|---|
| HBCO | | | 53.8 | | 194.3 | | $P4_32_12$ | 3.0 | 6.0 | |
| | bezafibrate | 3.3 | 54.0 | | | | $P4_32_12$ | 4.85 | 5.1 | 5.2 |
| | ethacrynic acid | 3.3 | 54.1 | | 193.6 | | $P4_32_12$ | 3.5 | 7.3 | 23.0 |
| Hb | | | 63.15 | 83.59 | 53.80 | 99.34 | $P2_1$ | 1.74 | 3.6 | |
| | bezafibrate | 13 | 63.3 | 83.5 | 53.9 | 99.3 | $P2_1$ | 2.33 | 9.0 | 6.1 |
| | ethacrynic acid | 3.3 | 63.3 | 83.2 | 53.7 | 99.5 | $P2_1$ | 2.80 | 8.6 | 14.0 |
| | STT | 33 | 63.2 | 83.2 | 53.8 | 100.1 | $P2_1$ | | | |
| | | | 63.4 | 83.4 | 54.0 | 100.4 | $P2_1$ | 2.50 | 6.9 | 21.0 |

[a] $d_{lim}$ = (radius)$^{-1}$ of limiting sphere in which data were collected. [b] $R_{int}$ = agreement factor of intensities between Friedel pairs within each set of data: $R_{int} = \sum_{hkl}(|I_{hkl} - I_{\bar{h}\bar{k}\bar{l}}|)/\sum_{hkl}(I_{hkl})$. [c] $R_{d-n}$ = agreement factor of structure amplitudes between native and derivative data: $R_{d-n} = \sum_{hkl}(|F_d| - |F_n|)|/\sum_{hkl}|F_d|$.

co-crystallized the enzyme-drug complexes, determined their structure by X-ray analysis, and published a summary of their results, without giving details.[11]

## Experimental Section

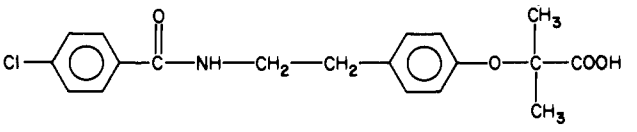
**Compounds Investigated**: Bezafibrate (I) (BZF), an antihyperlipoproteinemia drug made by Boehringer, Mannheim; Clofibric acid (II) (CFA), the active hydrolysis product of the antihyperlipoprotenemia drug clofibrate which is its ethyl ester; ethacrynic acid (III) (ECA), a diuretic marketed by Merck, Sharpe & Dohme, Inc.; succinyl-L-tryptophan-L-tryptophan (IV)[12] (STT), a compound synthesized by Dr. J. R. Votano at the Dept of Biology, Massachussetts Institute of Technology; and *p*-bromobenzyloxyacetic acid (V)[13] (BBA).

**Preparation of Crystals and Collection of X-ray Data.** Hemoglobin (Hb) was prepared from human blood, unsuitable for transfusion, supplied to us by the British National Blood Transfusion Service, and crystals of deoxyhemoglobin (deoxyhb) and carbomonoxyhemoglobin (HbCO) were grown as described in ref 14. Aliquots of the drugs ranging from 1 to 10 mol/mol of Hb were added to the Hb solutions before crystallization. If the ratio was too low, too little of the drug was bound; if it was too high, the crystals were disordered or crystallization was inhibited. Good crystals were sometimes found only after several trials. Most of the drugs reduced the solubility of Hb; ref 14 recommends a series of ammonium sulfate solutions buffered with diammonium orthophosphate for the growth of large crystals, dilution increasing in steps of 0.1 M. When crystallizing the drug complexes, dilutions had to be extended sometimes by several steps beyond those recommended in ref 14, before crystals suitable for X-ray analysis were obtained. X-ray data were collected on either a Nonius CAD 4 or a Hilger 4-circle diffractometer with $\omega$ scans. The crystallographic data are summarized in Table I.

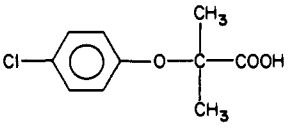
Crystals ranged from 0.5 to 1.5 mm in linear dimensions. For each derivative, corrections for radiation damage, absorption, Lorentz, and polarization factors were applied and the intensities from several crystals and from symmetry-related reflexions were merged to form a unique set. Table I shows the agreement factors between symmetry-related reflexions within each set and the agreement factors between reflections from native and derivative crystals. With one exception, to be referred to below, derivative crystals proved isomorphous with native ones. In the analysis of deoxyhb with ECA and STT, resolution was limited by the availability of good crystals. In the analysis of complexes with HbCO it was limited by the geometry of the diffractometer which failed to resolve reflexions *hkl* and *hk*($1 + l$) below 3.5 Å spacing, due to the long *c* axis (194 Å). In the analysis of deoxyhb + BZF we found that extension of the resolution beyond 2.5 Å did not produce any further improvement of the difference map, probably because of the high temperature factors of the bezafibrate molecule.

Difference electron density maps were usually plotted to include positive and negative contours only with $|\Delta\rho| > 3\sigma$; sometimes the $\pm 2\sigma$ levels were also examined. Fourier maps, $2F_o$(derivative) $- F_c$(native), were also studied. Models of the drugs with standard bond length and bond angles were fitted to these maps by means of an Evans & Sutherland Multipicture System with use of a modified version of the FRODO program, which allows manual rotations and translations of the drug molecules as a whole and rotations about single bonds within the drugs

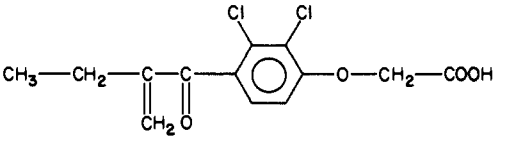
Chart I. Formulae of Compounds Used



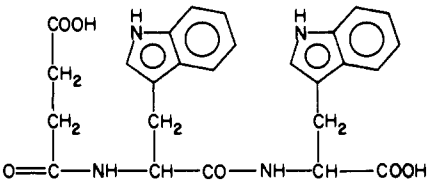
I (BZF)



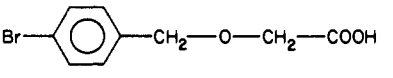
II (CFA)



III (ECA)



IV (STT)



V (BBA)

or the protein. Except in the case of STT, the protein was altered only in the vicinity of drug binding sites, in accord with indications in the difference map (paired positive and negative peaks) or with stereochemical requirements. For BZF and CFA, the protein atoms were not moved; for BBA only a single peptide plane was rotated; for ECA several side chains were moved. In the case of STT, the large *R* factor against the native data suggested large-scale movements of the protein. Although these might well have been only rigid-body movements due to small differences in molecular packing, they could have hindered the interpretation of difference-maps. Hence the native Hb model with 3 STT molecules attached was refined by least squares with energy-like constraints, with minimization of the residual $\Delta\Delta F = (|F_{do}| - |F_{no}|) + (|F_{nc}| - |F_{dc}|)$, where o = observed, c = calculated, n = native, and d = derivative.[15] Only two cycles of refinement were applied, during which the difference *R* factor ($\sum\Delta\Delta F/\sum F_o$) dropped from 21% to 16%, and the root-mean-square shift of protein atoms was 0.4 Å.

Oxygen equilibria of purified HbAo solutions (0.2 mM heme) were measured with a Hemox Analyzer (TCS, Southampton, PA, USA) interfaced with an HP 85 microcomputer which allows the storage of up to 500 points on the deoxygenation curves as described in ref 16. Titrations of HbAo with bezafibrate were done in 0.02 or 0.05 M bis-Tris

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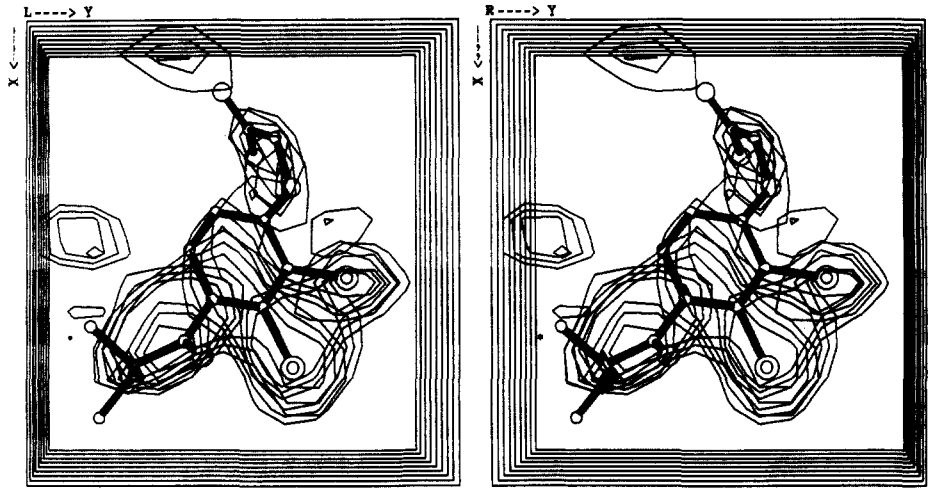


**Figure 2.** Stereoview of ECA, bound to the sulfhydryl of Cys F9(93)$\beta$ of human deoxyhb. Model fitted to the difference electron density.

buffer containing varying concentrations of the drug in the presence of increasing amounts of sodium chloride and/or diphosphoglycerate (Na Salt) at 25 °C.

In Paris binding of BZF to oxy and deoxy HbAo was measured according to Benesch et al.[17] with slight modifications, using $^{14}$C-labeled $10^{-6}$ M bezafibrate (SA 28 Ci/M) in 0.03 M bis-Tris buffer, 0.01 M NaCl, pH 7, and $10^{-5}$ to $10^{-3}$ M (tetramer) HbAo at 25 °C. For $HbO_2$ experiments the solutions were equilibrated with air. Solutions were deoxygenated by equilibration without dithionite under humidified argon until a pure deoxy spectrum was obtained. The Hb solutions were concentrated by centrifugation of 2-mL aliquots through Centricon 30 (Amicon) filters for 5 to 20 min. The filtrate was weighed and the free $^{14}$C BZF concentration was assayed on 0.1-mL aliquots diluted in 3 mL of an ACS liquid scintillation mixture (Amersham). Radioactivity was measured with an LKB 1212 scintillation counter. Samples were analyzed in triplicate and each counting was repeated four times. Hb concentrations measured in the retentate agreed within 2% with those calculated from the initial concentration corrected for the filtered volume. The $^{14}$C BZF concentration in the filtrate did indeed represent the free BZF concentration in the retentate, as preliminary experiments had shown the label was not retained by the Centricon 30 membrane. To measure BZF binding to deoxy Hb, the deoxygenated solutions were inserted into the Centricon device under paraffin oil in strictly anaerobic conditions. Spectra of these solutions recorded at the end of the experiments were identical with those determined before the centrifugation. All chemicals used were of analytical grade. 2,3-DPG was from Sigma. $^{14}$C BZF was kindly supplied by Boehringer Mannheim (FRG).

In Pittsburgh, equilibrium dialysis was used to determine the binding constants and the number of binding sites in deoxyhb and HbCO. The free drug concentrations were measured by UV for BZF with HbCO; for deoxyhb HPLC was used to separate the drug from dithionite and from $\alpha\beta$ dimers and to measure the free drug concentrations. Hb A was isolated according to ref 14 and equilibrated with 50 mM phosphate buffer pH 7.4 and 100 mM NaCl. Equilibrium dialyses were done by using an equilibrium micro volume dialyzer (Hoffer Scientific Instruments Model EMD101 0.5 mL) fitted with dialysis membranes (Hoffer Scientific Instruments EMD103) with a 3 in. diameter and 12–14000 daltons MW cut-off. The membranes were boiled with a 5% $Na_2CO_3$–50 mM EDTA solution for 5 min and then washed twice with distilled water and stored in 50% ethanol at 4 °C. They were rehydrated before use by soaking in dialysis buffer overnight. For binding to HbCO one side of the cell was filled with 0.3 mL of HbCO of known concentration and the other side with 0.3 mL of a given concentration of the drug. Control sets of cells were made by injecting the same volume of a given drug concentration (0.3 mL) into one side and 0.3 mL of plain buffer into the other side. After filling the chambers, the whole system was rotated at 4 °C for 20–24 h. For binding to deoxyhb a solution of 300 mM sodium dithionite was prepared in degassed pH 7.4 phosphate buffer, then 0.1 mL of this solution was added to each half-cell which contains either 0.2 mL of drug or 0.2 mL of HbA (or buffer was used to measure the amount of drug that equilibrates in each compartment) to raise the total volume in each half-cell to 0.3 mL with a final concentration of dithionite of 100 mM. The cells were immediately sealed with vaseline and parafilm and the system was again equilibrated for 20–24 h at 4 °C. The free drug concentrations were measured by UV for HbCO and HPLC (Waters) for deoxyhb studies.

The binding constants were estimated from Scatchard plots according to the equation $r/C_f = nK - Kr$, where $r$ = (moles of drug bound)/(moles of hemoglobin), $C_f$ is the molar concentration of free drug, $n$ is the number of drug molecules per tetramer, and $K$ is the equilibrium constant.

ECA was dissolved in 10 mM phosphate buffer pH 8.0 to a final concentration of 10 mM for crystallization studies and 3 mM for all other experiments. Purified carbonmonoxylated HbA was added to this solution at a concentration of 3.1 mM heme to give a molar ratio of ECA per $\alpha\beta$ dimers of approximately 6. The reaction was allowed to proceed overnight at 4 °C under mild stirring. Then the Hb solution was passed through ion exchange columns to remove unreacted ECA. The electrophoresis showed at least 95% reacted Hb. The reacted Hb was decarbonmonoxylated under pure $O_2$ and intense light at 0 °C until the ratio of the absorbances at the $\alpha$ and $\beta$ bands peaks equalled 1.065. The methemoglobin content was 4%. Oxygen binding experiments were performed as for BZF.

## Results

**Ethacrynic Acid (ECA).** Discovery that halogens attached to a benzyloxy or phenoxyacetic acid[19–24] impart strong antigelling activity to hemoglobin led to the study of a diuretic that incorporates these features: ethacrynic acid (ECA).[18] ECA was found to have the most potent antigelling properties of any antisickling compound yet investigated, to bind to Hb either covalently or by very strong secondary valency forces, to cross the erythrocyte membrane, and to shift the oxygen equilibrium to the left, toward higher affinity. The ability of acrylophenones to undergo Michael-type addition reactions with SH groups made it seem likely that ECA would react with the accessible cysteines F9(93)$\beta$, but titration of HbS-ECA with *p*-mercuribenzoate gave a normal value of 2.2 free SH groups per tetramer, suggesting either that reaction with ECA had left the SH groups free or that it had been displaced by the mercurial.[18] Binding studies of ECA with HbCO showed no free drug in solution, even at drug concentrations as high as 10 mM.

The difference electron density maps showed ECA to be bound to two different pairs of sites: one next to Cys F9(93)$\beta$ and the other next to HisG19(117)$\beta$ (Figures 2–7). When a model of

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(19) Abraham, D. J.; Mehanna, A. S.; Williams, F. L. *J. Med. Chem.* **1982**, *25*, 1015–1017.

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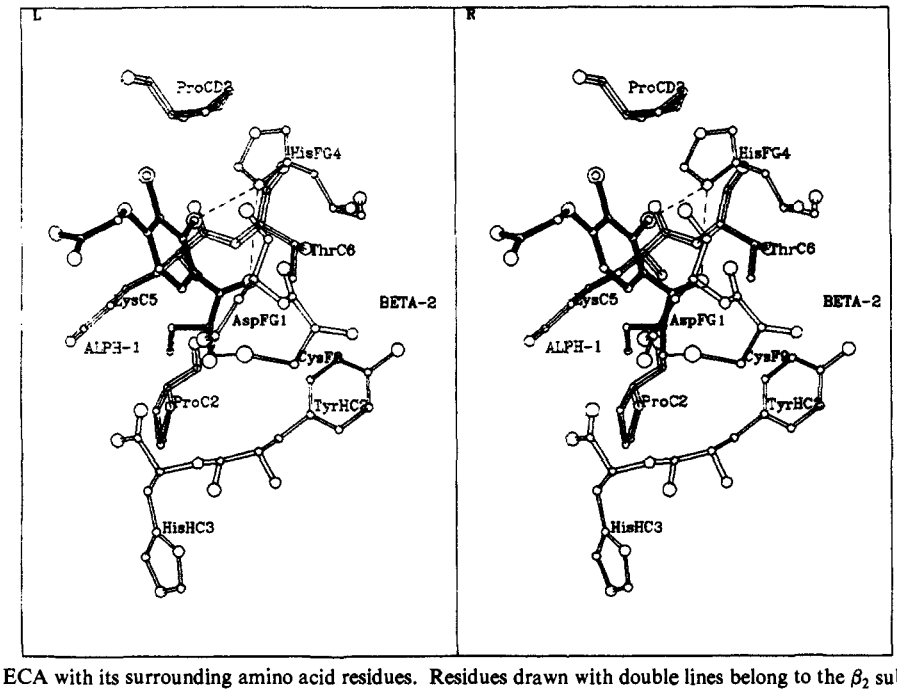
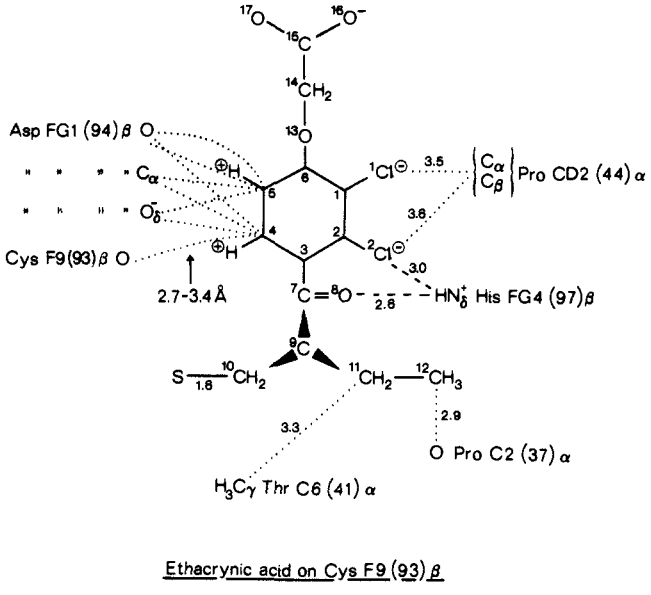



**Figure 3.** Stereoview of ECA with its surrounding amino acid residues. Residues drawn with double lines belong to the $\beta_2$ subunit and those with triple lines to the $\alpha_1$ subunit.




Ethacrynic acid on Cys F9 (93) β

**Figure 4.** Diagrammatic view of contacts between ECA and globin. ⊕ and ⊖ indicate fractional charges due to the dipole moment of the *o*-dichlorobenzene moiety. In this and subsequent diagrams broken lines indicate hydrogen bonds and dotted lines van der Waals contacts.

ECA had been fitted to the density next to the SH groups of Cys F9(93)$\beta$, it became clear that C(10) is linked to the sulfur by a covalent bond. The chlorines make contact with the imidazole sidechain of His FG4(97)$\beta$ and with several hydrogens of Pro CD2(44)$\alpha$. The hydrogens on C(4) and C(5) of the dichlorobenzene are within 2.0–2.5 Å of the carboxylate of Asp FG1(94)$\beta$. In the crystal structure of the free drug the dihedral angle between C(3)–C(7)–O(8) and C(1)–C(2)–C(3) is 61.1°,[25] but in the bound ECA molecules this is reduced to 31.5° and −34.5°, respectively. The carbonyl C(7)–O(8) accepts a hydrogen bond from NH of His FG4(97)$\beta$. C(11)$H_2$ is internal in contact with $CH_3$ of Thr C6(41)$\alpha$. The carboxylate is external and solvated. A large negative peak covering His HC3(146)$\beta$ shows that it has been displaced from its native position and that its salt bridges with Lys C5(49)$\alpha$ and Asp FG1(94)$\beta$ have been broken. The difference density exhibits a system of positive and negative peaks indicative of a shift of helix F toward helix E and a consequent narrowing of the pocket between them, which may distort the binding site that is complementary to ValA3(6)$\beta$ in the deoxyhb S gel.

*o*-Dichlorobenzene has a dipole moment of 2.25 D with the negative pole between the two chlorines and the positive pole between C(4)H and C(5)H.[26] His FG4(97)$\beta$ has a p$K_a$ of 7.85 in carbonmonoxy or oxy and of 8.1 in deoxyhemoglobin;[27] the suspension medium of the crystals has a pH of 6.5, so that the histidine is positively charged. ECA arranges itself with the chlorines and the carbonyl near the histidine and C(4)H and C(5)H near a carboxylate, so that its dipole is compensated by oppositely charged groups of the protein. Another weakly polar interaction is produced by the contact between the chlorines and the aliphatic hydrogens of Pro CD2(44)$\alpha$. Combination of ECA with the SH group creates a chiral center at C(9). Looking from the hydrogen on C(9) we proceed clockwise from CO to $C_2H_5$ to $CH_2$–S.

The second pair of ECA residues form covalent bonds with $N_\epsilon$ of His G19(117)$\beta$, but with very different occupancies at the two sites related by molecular symmetry. At the site with high occupancy, the carboxylate of ECA forms a salt bridge with $NH_3^+$ of Lys A12(14)$\alpha$ of a neighboring molecule; its carbonyl forms a hydrogen bond with the main chain NH of Asn B1(19)$\beta$ at a distance of 2.8 Å. There are also van der Waals interactions with other amino acid residues. The positive pole of the *o*-dichlorobenzene ring is compensated by a contact between C(5)H and the carboxylate of Glu B4(22)$\beta$; both chlorines are in contact with $CH_3$ groups of aliphatic side chains. The site with low occupancy that is related to the first by molecular symmetry is unrelated crystallographically and lacks a lysine from a neighboring molecule to bind to ECA's carboxylate.

We have also determined the structure of the complex obtained by reaction of HbCO with ECA, followed by chromatographic purification of the product and crystallization. The difference map shows a single peak between Cys F9(93)$\beta$ and His FG4(97)$\beta$. ECA can be fitted to this peak with C(10) linked covalently either to the sulfhydryl or to $N_\epsilon$ of the histidine. When C(10) is linked

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(26) Atkins, P. W. "Physical Chemistry", 2nd ed.; Oxford University Press: London, 1982; p 781.

(27) Perutz, M. F.; Gronenborn, A. M.; Clore, G. M.; Fogg, J. H.; Shi, D. T.-b. *J. Mol. Biol.* **1985**, *183*, 491–498.

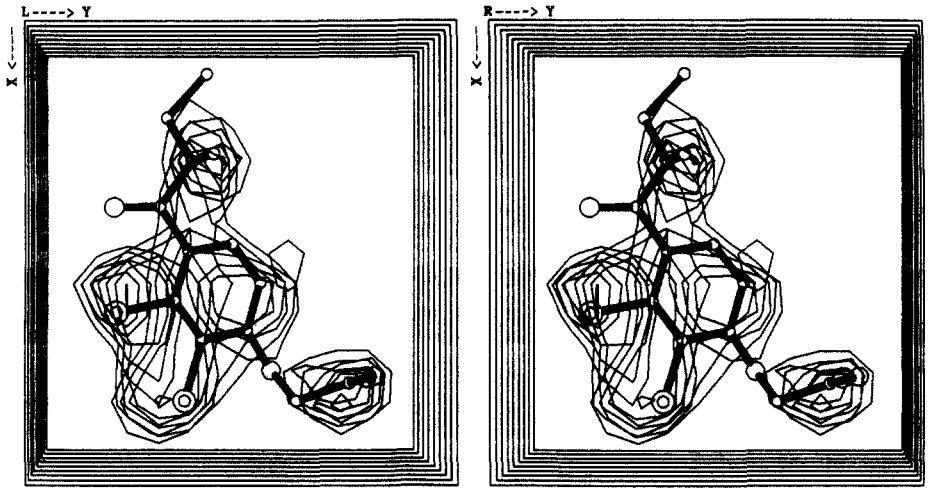


**Figure 5.** Stereoview of ECA bound to His G19(117)$\beta$. Model fitted to the difference electron density map.

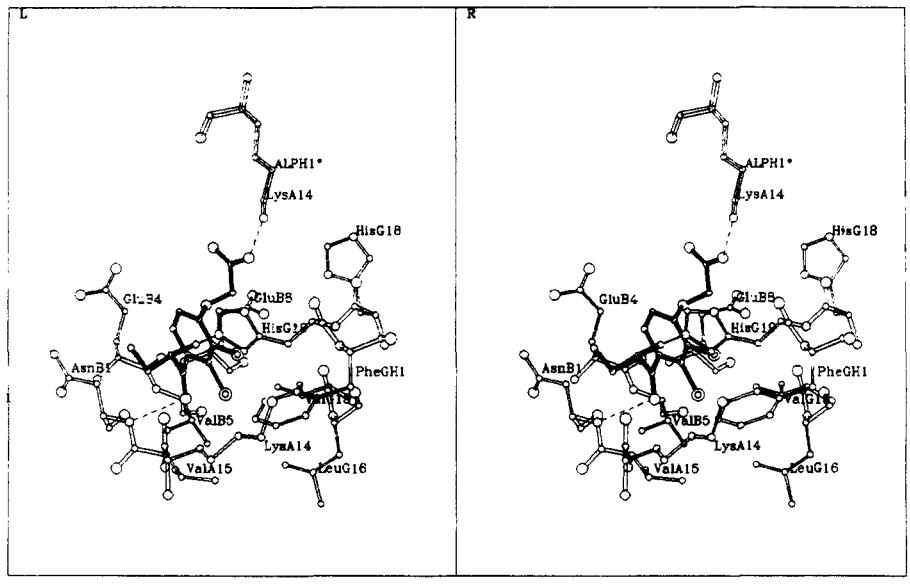


**Figure 6.** Stereoview of ECA bound to His G19(117)$\beta$ with its surrounding amino acid residues. Residues drawn in double lines belong to the $\beta$-subunit of one molecule. The lysine drawn in triple lines belongs to the $\alpha$-subunit of a neighboring molecule.

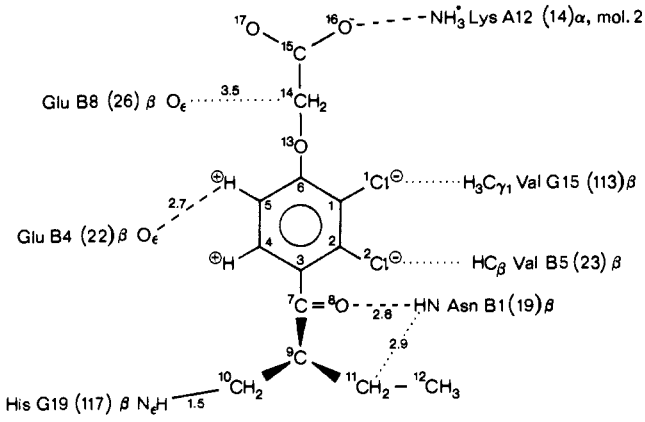


**Figure 7.** Diagrammatic view of contacts of ECA bound to His G19-(117)$\beta$ with the surrounding globin.

to the sulfhydryl, the carboxylate forms a salt bridge with the histidine; when C(10) is bound to the histidine, the carboxylate forms a salt bridge with Lys C5(40)$\alpha$. Thus both structures are chemically plausible, and an alternative method will be needed to decide between them.

**Bezafibrate (BZF).** Abraham et al.'s finding that the antihyperlipoproteinemia drug clofibric acid has antisickling properties[20] led us to investigate related drugs, among them bezafibrate. It resembles two molecules of clofibric acid in tandem and has roughly twice the pharmacological activity, but contrary to our expectation it lowered, rather than raised, the minimum gelling concentration of deoxyhb S. It proved to be a powerful allosteric effector of human hemoglobin, producing a right shift of the oxygen equilibrium and acting synergistically, rather than competing, with the natural effector DPG.[28] Our difference electron density map showed that it is bound in the central cavity by contacts with one $\beta$ and two $\alpha$ subunits. Figures 8–11 show the structure of the drug and its interactions with the globin. No covalent bond binds the drug to the globin but a multitude of secondary interactions, many of them polar. Chlorobenzene has a dipole moment of 1.57 D,[26] the amide side chain of asparagine one of 3.4 D,[29] and each CH of the phenyl side chain of phenylalanine one of about 0.5 D.[30] The electronegative chlorine is in contact with the electropositive $C_\zeta H$ of Phe C1(36)$\alpha_1$, with NH of His G10(103)$\alpha_1$, with $NH_2$ of Asn G10(108)$\beta_1$, and with $C_2H_3$ of Leu G7(100)$\alpha_1$. The amide hydrogens of the asparagine are also in close contact with the $\pi$ electrons of the benzene ring C(1)–C(6), suggestive of a hydrogen bond. The aliphatic side

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(29) Wada, A. *Adv. Biophys.* **1976**, *9*, 1–63.
(30) Levitt, M., private communication.

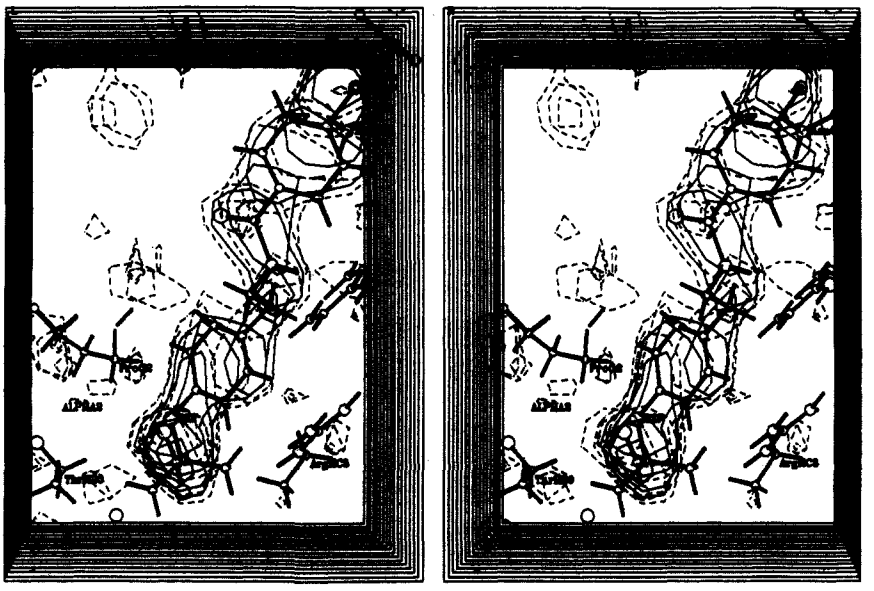

**Figure 8.** Stereoview of BZF fitted to the difference electron density found between the two $\alpha$-chains. Only positive difference electron density is shown; solid contours are drawn at intervals of $3\sigma$ or 0.06 e/Å$^3$ and broken contours at intervals of $2\sigma$ or 0.04 e/Å$^3$.

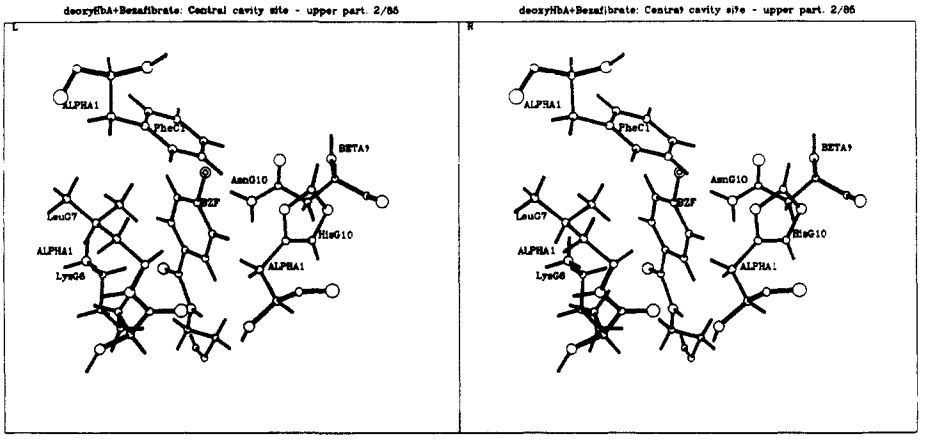


**Figure 9.** Stereoview of surroundings of chlorobenzene and amide moieties of BZF.

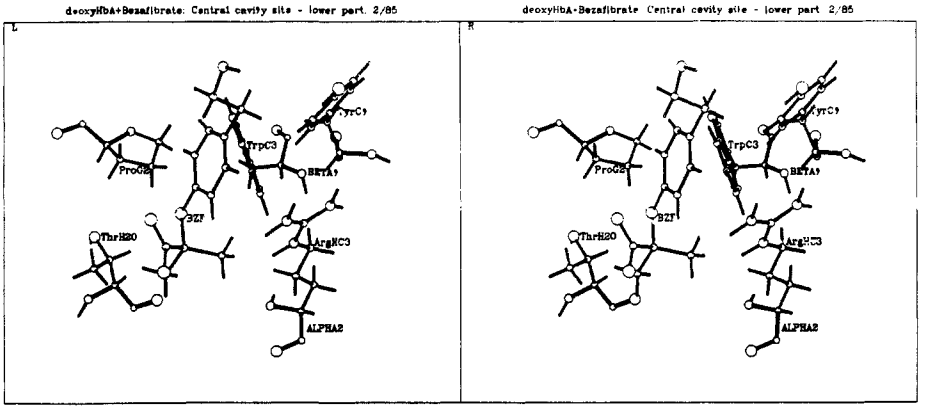


**Figure 10.** Stereoview of surroundings of the phenoxy and isobutyrate moieties of BZF.

chain of Lys G6(99)$\alpha$ is in contact with the flat face of BZF's amide group.

Two of the indol CH's of Trp C3(37)$\beta$ make contact with the $\pi$ electrons of the benzene ring C(10)–C(15). The isobutyl group appears in the electron density map as a single unresolved sphere, suggesting free rotation about the O(2)–C(16) bond; this surprised us, as the carboxylate is near enough to interact with the guanidinium group of Arg HC3(141)$\alpha_2$. Possibly that interaction is weak, because the positive charge of the guanidinium is compensated by its salt bridge with Asp H9(126)$\alpha_1$. Nonpolar interactions are seen between C(14)H of the drug and $CH_2$ of Pro G2(95)$\alpha_2$ and between C(2)H and C(3)H of the drug on the one hand and $C_\beta H_2$ of His G10(108)$\beta_1$ on the other hand.

The difference map also exhibits positive density inside the $\alpha$ subunit between the side chains of leucines G12(105), G16(109), H8(125), and H12(129), but there is not enough space to accommodate a bezafibrate molecule in that position. A system of negative peaks distributed spherically around the $\alpha$ subunit suggests that the positive peaks are due to compression of a loosely packed region of the globin.

**Succinyl-L-tryptophan-L-tryptophan (STT).**[12] The difference map shows one symmetry-related pair of difference peaks at the entrance to the central cavity between the $\alpha$ subunits (sites 1 and 2); a third difference peak between the $\alpha$-heme of one molecule

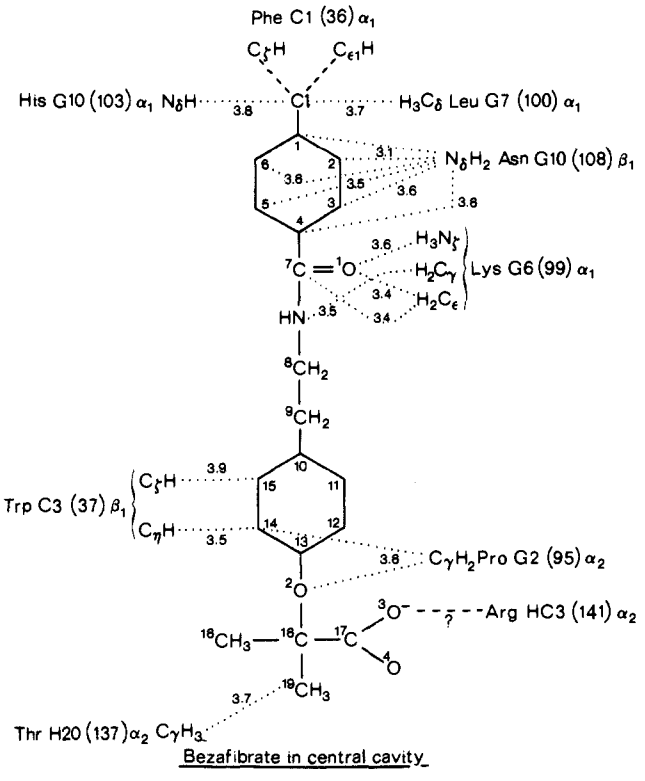


**Figure 11.** Diagrammatic view of contacts of BZF with the surrounding globin.

and the $\beta$-heme of its neighbor (site 3); a fourth within one of the $\alpha$ subunits (site 4); and two weaker, symmetry-related pairs of peaks flanking the center of the Hb molecule (sites 5–8). Only the difference peaks at sites 1, 2, and 3 are well enough resolved to fit molecular models to them (Figures 12–17). At sites 1 and 2 $C_{\zeta_3}H$ and $C_{\epsilon_3}H$ of Trp 2 are within less than 4 Å of the indole ring of Trp 1 within the same molecule, suggesting interaction between the CH's of one ring and the $\pi$ electrons of its neighbor. Each STT is bound to one $\alpha$ subunit by three hydrogen bonds and several van der Waals interactions. Interaction between the symmetry-related pair of STT's consists of van der Waals contacts between $C_\beta H_2$ and $C_\gamma$ of the two molecules and of contacts between $C_\beta H_2$ of one molecule and the $\pi$ electrons of the indole ring of the next. The STT molecule at site 3 is hydrogen bonded to one of the heme propionates and to the distal histidine of the $\alpha$ subunit of one molecule. It is also in van der Waals contacts with four other residues of that same $\alpha$ subunit and with two residues of the $\alpha$ subunit plus four residues of the $\beta$ subunit of the neighboring molecule. Some of these contacts are weakly polar: for example, that between $C_{\delta_2}H$ and $C_{\epsilon_2}H$ of Phe C7(41)$\beta_1$ and the $\pi$ electrons of the indol ring of Trp 1.

Site 4 lies within the $\alpha$ chain between Trp A12(14), Ala B2(21), Gly B3(22), Val G14(107), and Leu G16(109). It is represented by one or possibly two large undifferentiated peaks of positive density. One of these peaks is surrounded by pairs of positive and negative peaks that flank the side chains in contact with it, indicating that these side chains have been displaced. It is not clear whether the positive peaks represent the indoles of an STT molecule wedged within the $\alpha$ subunit or whether they arise from distortions of the $\alpha$ subunit induced by the STT molecules bound at the other sites.

***p*-Bromobenzyloxyacetic Acid (BBA).**[13] This compound binds to a site between the nonhelical CD segment and helix B of the $\alpha$ chain (Figures 19–21). It is held in place there by a salt bridge of its carboxylate with Lys C5(40)$\alpha$; by contacts of the bromine with one of the aromatic hydrogens of a phenylalanine and with several aliphatic hydrogens; and by contacts of its benzene ring with one leucyl side chain and one main chain oxygen. The difference map shows that this oxygen has moved away from the benzene ring; the distances shown on Figure 19 are for its new position.

**Compounds That Failed To Bind to Crystals.** We co-crystallized deoxyhb with several other compounds that had shown antigelling activity, including the antimalarial drugs chloroquine and qui-

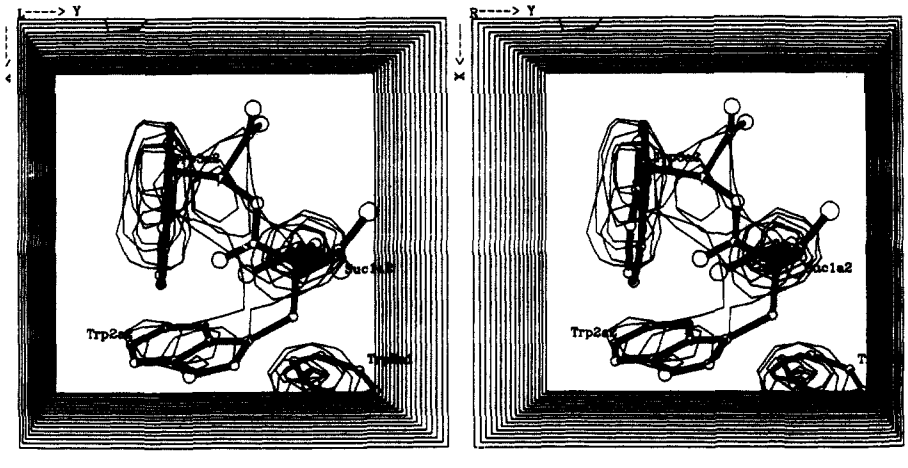

**Figure 12.** Stereoview of STT in the central cavity between the $\alpha$-chains fitted to the difference electron density.

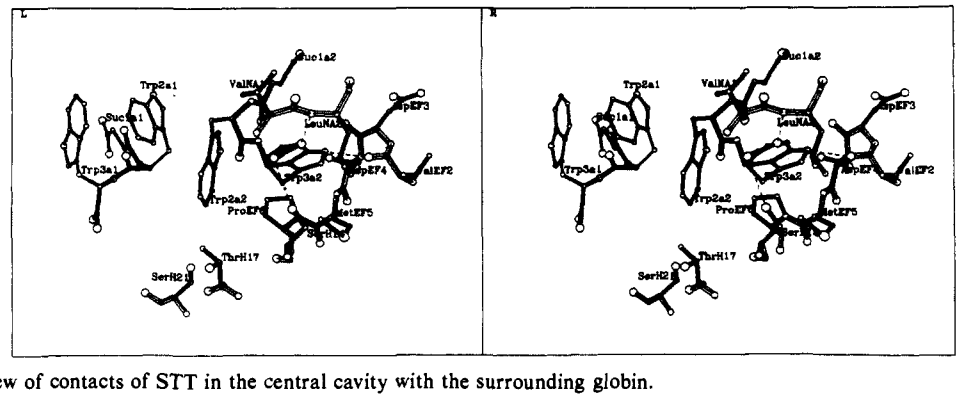

**Figure 13.** Stereoview of contacts of STT in the central cavity with the surrounding globin.

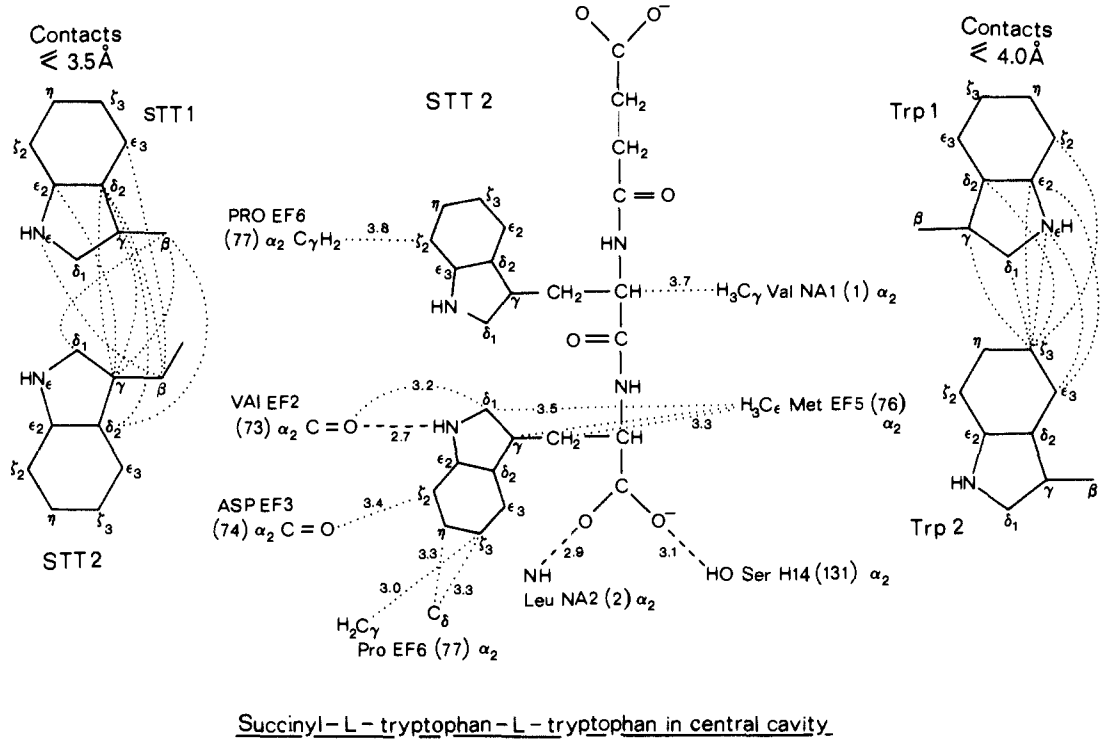


**Figure 14.** Diagrammatic view of STT in the central cavity. (a, left) Contacts between the two symmetry-related STT molecules. (b, middle) Contacts with the globin. (c, right) Contacts between two indol residues within the same STT molecule.

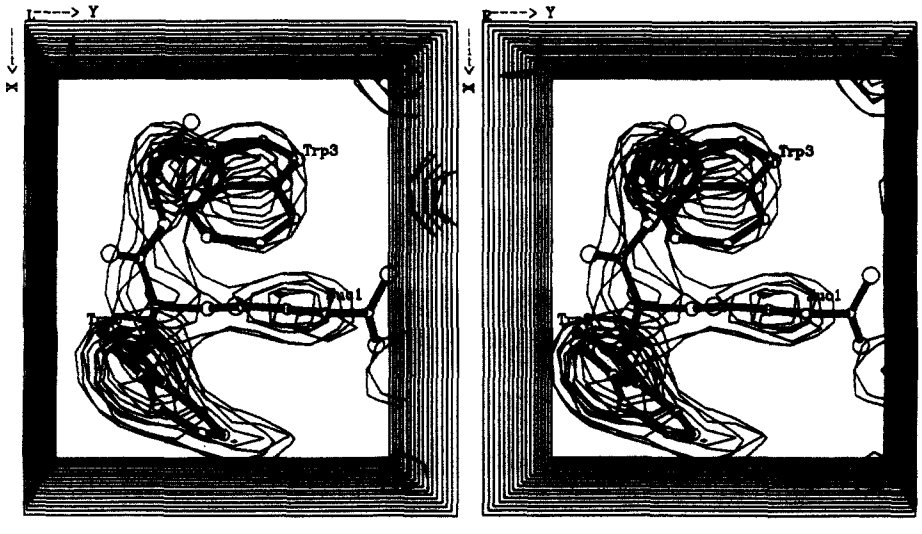


**Figure 15.** Stereoview of contacts of STT sandwiched between two Hb molecules fitted to the difference electron density.

nocrine, the dipeptides L-Arg-L-Trp[31] and *N*-acetyl-L-Phe-L-Phe,[32] and 5-(2-formyl-3-hydroxyphenoxy)pentanoic acid.[33,55] A difference electron density map of deoxyhb with *N*-acetyl-L-Phe-L-Phe at 5.5 Å resolution showed only one significant peak at a site between two molecules. The compound seems to make contact with His FG4(97)$\beta$, Lys FG2(95)$\beta$, Pro CD2(44)$\alpha$, and His CD3(45)$\alpha$ of one molecule and with Asp CD5(47)$\alpha$ and the carbonyl of His CD3(45)$\alpha$ of the neighboring molecule. We cannot exclude the possibility of its binding to deoxyhb in solution to the same four residues as those in the first molecule, but the more likely interpretation of our result is that this binding site is operative only in the crystal lattice, especially since no significant density was seen at the site related by the molecular twofold axis where the intermolecular contacts are different. In none of the other compounds did we observe any significant difference density. HbCO with 5-(2-formyl-3-hydroxyphenoxy)pentanoic acid crystallized in a new form, but the crystals were disordered and unsuitable for X-ray analysis. The compound had been given to us supposedly combined with oxyhemoglobin as a Schiff base that had been reduced with borohydride, thus forming a covalent link which should have remained stable in both carbonmonoxy and deoxy hemoglobin, but our X-ray results suggest that in fact the compound combines loosely with the R structure and dissociates from the T structure.

**Binding Constants of Drugs to Hemoglobin in Solution.** The method of Benesch et al. allows dissociation constants to be determined at low drug concentrations by measuring the amount of hemoglobin necessary to bind half the drug present in the solution.[17] In Paris, we used that method with $^{14}$C-labeled BZF and found the dissociation constant from deoxyhemoglobin $K_D$ = 0.115 ± 0.004 mM (30 mM bis-Tris, 10 mM NaCl, no $Na_2$-

(31) Luskey, K. L.; Pavone, V.; Noguchi, C. T.; Schechter, A. N. In "The Molecular Basis of Mutant Hemoglobin Dysfunction"; Sigler, P. B., Ed.; Elsevier/North Holland: New York, Amsterdam, Oxford, 1981; pp 213–224.
(32) Votano, J. R.; Altman, J.; Wilchek, M.; Gorecki, M.; Rich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3190–3194.
(33) Kneen, G.; White, R. D. *Br. J. Pharmacol.* **1981**, *74*, 965P.

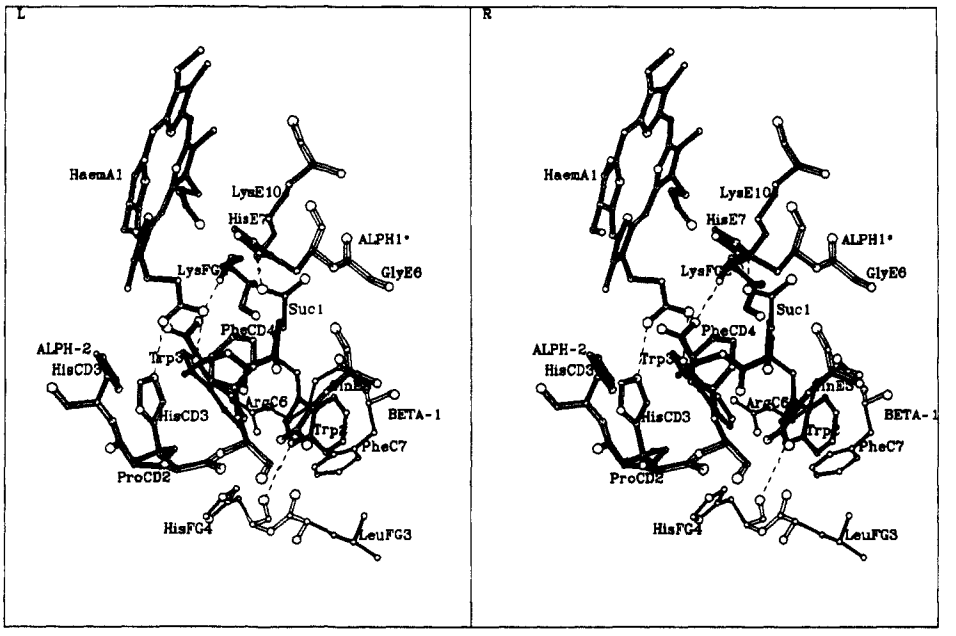


**Figure 16.** Stereoview of contacts of STT sandwiched between two Hb molecules. Residues marked with double lines belong to the β-chain of one molecule, residues with triple lines marked ALPH-2 to the α-chain of the same molecule, and those marked ALPH 1* and the heme to the α-chain of the neighboring molecule.

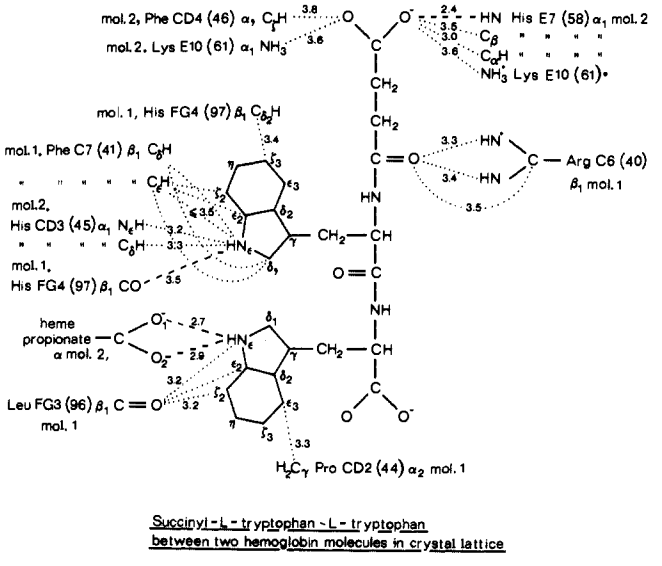


**Figure 17.** Diagrammatic view of contacts of STT with residues in two neighboring molecules.

$S_2O_3$, pH 7.0, 25 °C). In Pittsburgh, using the procedure described under Methods and unlabeled BZF, we found $K_D$ = 0.89 mM (50 mM phosphate buffer, 100 mM NaCl, 100 mM $Na_2S_2O_3$, pH 7.4). The difference is due to the competition between bezafibrate and chloride, and probably also phosphate, to be described below. A Scatchard plot indicated binding of 2 molecules per tetramer at major sites. Using the Pittsburgh method and unlabeled BZF we found weak nonspecific binding to HbCO, and using Benesch et al.'s method and labeled BZF we also found apparently weak binding; the amount bound increased with [Hb]. This behavior resembles that of pteroylglutamate found by Benesch et al. They ascribed it to the binding of the drug to $\alpha\beta$ dimers whose absolute concentration increases with the hemoglobin concentration. Figure 22 shows a Hill plot relating the fractional binding of BZF to concentrations of tetramer in deoxyhb and of $\alpha\beta$ dimer in $HbO_2$. The two sets of data lie on a straight line with a slope of 0.93 and a correlation coefficient of 0.97, showing that BZF binds to one and the same site on $\alpha\beta$ dimers, no matter whether they are free or assembled into $\alpha_2\beta_2$ tetramers, and showing also that there is no binding to $HbO_2$ tetramers. This is consistent with the X-ray results which showed no drug bound to crystals of HbCO (these are isomorphous with $HbO_2$). Moreover, the X-ray results show most of the residues binding BZF to the central cavity in deoxyhb

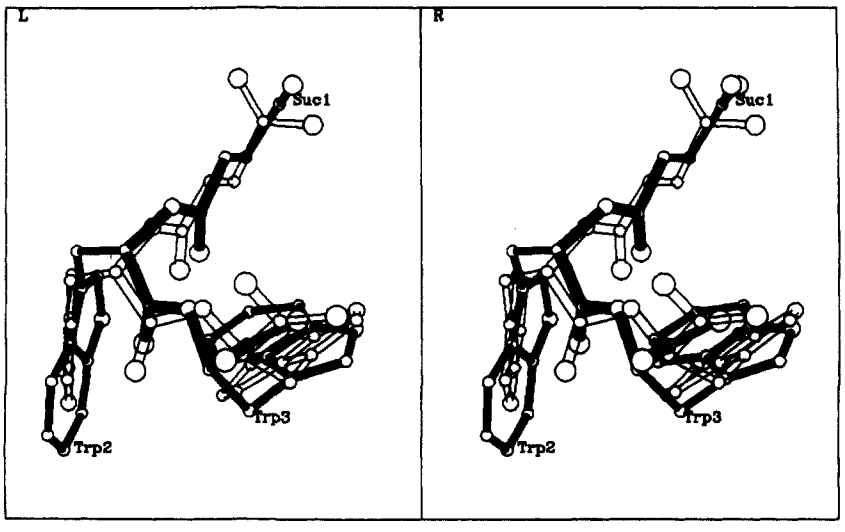


**Figure 18.** Comparison of STT conformation in the central cavity and between two hemoglobin molecules.

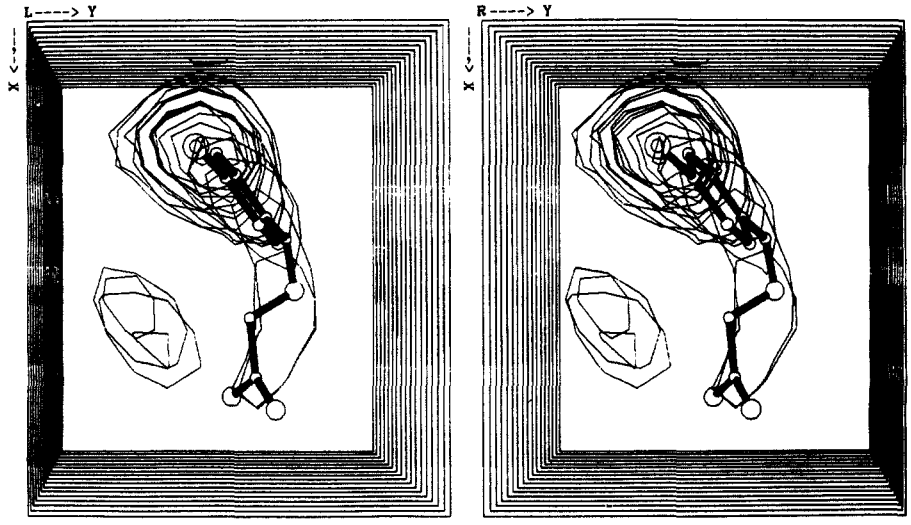


Figure 19. Stereoview of *p*-bromobenzyloxyacetic acid fitted to the difference electron density.

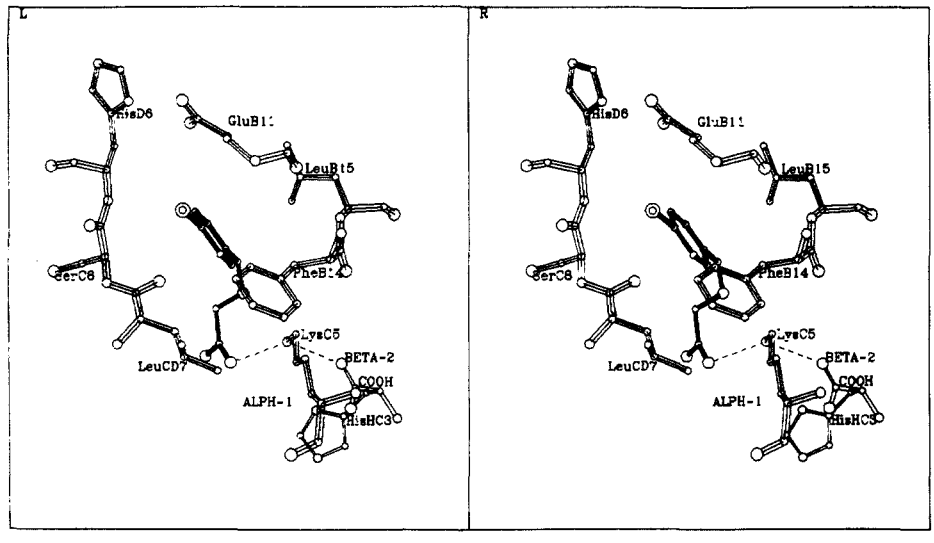


Figure 20. Stereoview of *p*-bromobenzyloxyacetic acid sandwiched between helix B and the nonhelical CD segment. Residues marked with double lines belong to the $\beta$-chain and those marked with triple lines to the $\alpha$-chain. For Ser C8 read Ser CD8.

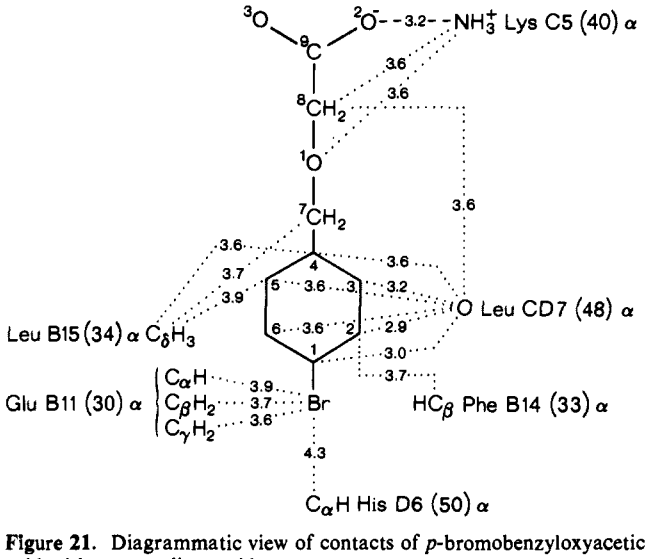


Figure 21. Diagrammatic view of contacts of *p*-bromobenzyloxyacetic acid with surrounding residues.

to belong to one $\alpha\beta$ dimer. The result is also consistent with the oxygen equilibria to be described below.

We have not been able to determine the dissociation constant of ECA, because we failed to detect any free drug in solution even a molar ratio as high as 10 mol of drug per mol of Hb. Votano and Rich have found the dissociation constant of STT from deoxyhemoglobin $D_D$ = 3.0 mM ([Hb] = 0.8 mM, 50 mM phosphate, 150 mM NaCl, 20 mM $Na_2S_2O_4$, pH 7.2, 22 °C). A Scatchard plot suggested binding of 2 mol of STT per tetramer at major sites.[12] *p*-Bromobenzyloxyacetic acid showed only weak nonspecific binding to deoxyhemoglobin.

**Effect of Minimum Gelling Concentration of Sickle Cell Hemoglobin (HbS).** Table II compares the effects of the compounds investigated here on the solubility of deoxyhb S in terms of the concentration ratios of saturated solutions of HbS with and without each compound. The ratios are compared with those given by phenylalanine as a standard. The largest ratio was obtained by incubating $HbO_2S$ with ECA prior to reduction with dithionite; in fact it is the largest ratio observed in any antisickling compound we have studied so far. BZF, on the other hand, promotes gelling at concentrations <30 mM.

**Effects on the Oxygen Equilibrium.** We have investigated the effects on the oxygen equilibrium of ECA, BZF, and STT. Figures 23 and Table III compare the oxygen affinities of native Hb and Hb previously reacted in the CO form with 3 mol of ethacrynic acid per mol of Hb tetramer. The reacted hemoglobin binds oxygen with reduced cooperativity with a two or three times lower $P_{50}$. The two dissociation curves diverge at the bottom and converge at the top, indicating a decrease in the stability and rise in oxygen affinity of the T structure. The Bohr effect is reduced by 40%, consistent with the disruption of the salt bridges formed by His HC3(146)$\beta$, while the effect of DPG is only slightly below normal.

**Table II.** Solubility Ratios of Deoxyhemoglobin S with Various Drugs

| compound | 5 mM | 10 mM | 20 mM | 40 mM | ref |
|---|---|---|---|---|---|
| ethacrynic acid | 1.106 | 1.166 | 1.317 | 1.375 | 18 |
| ethacrynic acid | 1.143[a] | | | | this work |
| STT[b] | 1.09 | 1.19 | 1.27 | 1.37 | 12 |
| *p*-BoBz[c] | 1.059 | 1.118 | 1.233 | 1.398 | 13 |
| bezafibrate | 0.954 | 0.932 | 0.981 | 1.198 | 13 |
| L-phenylalanine | 1.036 | 1.048 | 1.093 | 1.178 | 13 |
| quinacrine | 1.094 | 1.160 | 1.325 | 1.373 | this work |
| chloroquine | 1.072 | 1.150 | 1.294 | 1.394 | this work |

[a] This higher ratio was obtained when oxyhemoglobin was incubated with ethacrynic acid overnight and $Na_2S_2O_4$ was added afterwards. In our earlier published results reaction of ethacrynic acid with dithionite had reduced the solubility ratio. [b] Succinyl-L-tryptophan-L-tryptophan. [c] *p*-Bromobenzyloxyacetic acid.

**Table III.**[a]

| | HB-ECA | | HbAo | |
|---|---|---|---|---|
| pH | $P_{50}$ (mmHg) | $n_{50}$ | $P_{50}$ (mmHg) | $n_{50}$ |
| 6.81 | 3.2 | 1.44 | 8.9 | 2.95 |
| 7.13 | 2.4 | 1.54 | 5.9 | 2.90 |
| 7.49 | 1.9 | 1.60 | 3.8 | 2.90 |
| alkaline Bohr effect | −0.33 | | −0.54 | |
| 7.10 DPG 5 mM | 6.0 | 1.49 | 18.5 | 2.85 |

[a] 0.05 mM bis-Tris, 100 mM NaCl, 25 °C. DPG = 2,3 diphosphoglycerate. (COHbAo was reacted with a 3 mM excess ECA per mmol of tetramer concentration for 36 h at 4 °C and then stripped of the unreacted reagent on AG 11 $A_8$ and AG 501 × 8 columns. Electrophoresis showed the presence of at least 95% of Hb ECA.)

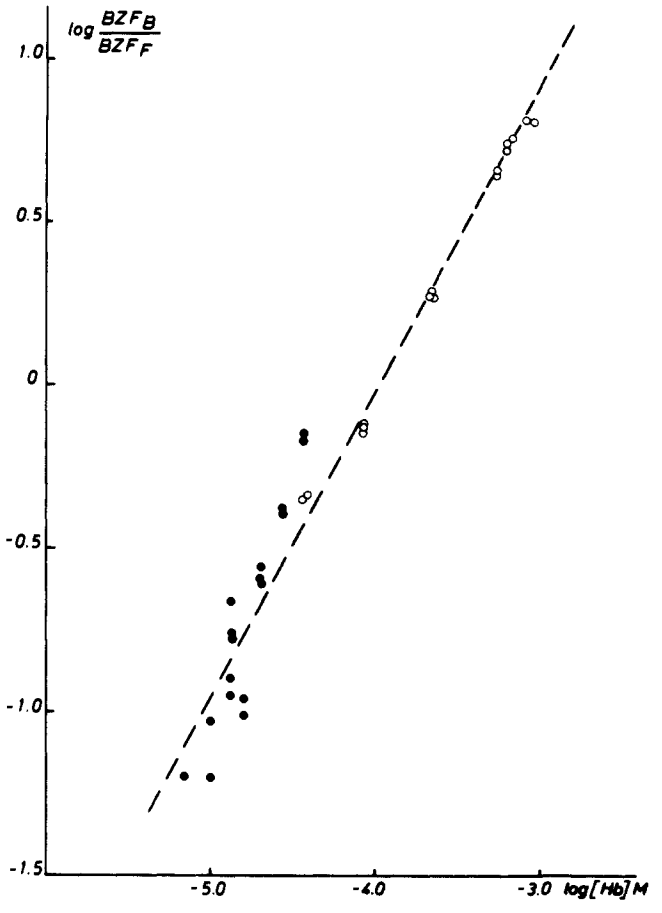


**Figure 22.** Hill plot of the ratio of bound to free bezafibrate vs. concentration of deoxyhb tetramers and $HbO_2$ dimers. The dimer concentration was calculated from $K_{4,2} = [D]^2/[T] = 1.5 \times 10^{-6}$ $M^3$, where $K_{4,2}$ is the dissociation constant; D and T stand for dimers and tetramers. For details see Methods.

BZF lowers the oxygen affinity more strongly than, and acts synergistically with, the natural allosteric effector 2,3-diphosphogylcerate.[28] A plot of log [BZF] against log $P_{50}$ shows a straight line with a slope of 0.61, indicating that rather more than two molecules per Hb tetramer are bound (Figure 24). The

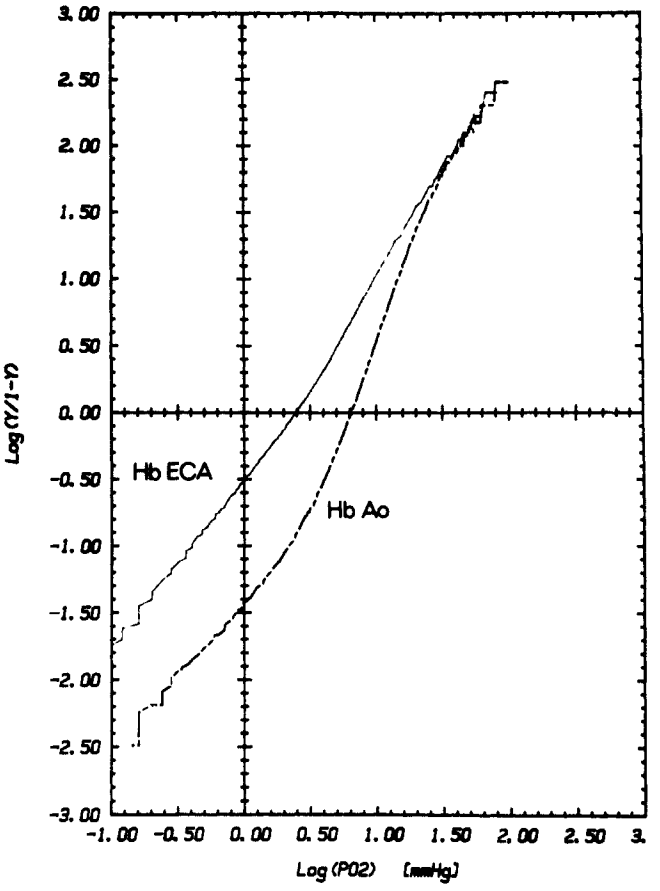


**Figure 23.** Hill plot of oxygen equilibrium curves of native human Hb compared to Hb reacted with 3 mol of ethacrynic acid per M tetramer (300 μM heme, 0.1 M $Cl^-$, 0.03 M bis-Tris buffer, pH 7.13, 25 °C).

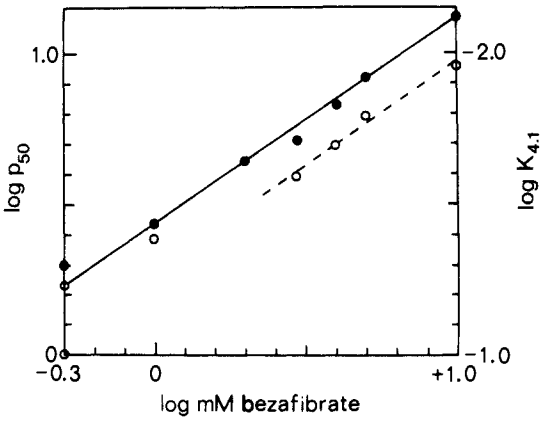


**Figure 24.** Dependence on bezafibrate of log p50 ($pO_2$ at half-saturation with oxygen) and of $K_{4,1}$ (association constant of the first heme to combine with oxygen) (200 μM heme, 0.01 M NaCl, 0.03 M bis-Tris buffer, pH 7.20, 25 °C).

same slope is shown by a plot of log $K_{4,1}$, the association constant of the first oxygen to combine with Hb in the T structure, against

log [BZF], when BZF is present in excess, while $K_{4.4}$, the association constant of the fourth oxygen, is independent of [BZF]. This is consistent with the crystallographic and direct binding studies showing that BZF binds only to the T structure. Unlike the natural allosteric effectors BZF was found to have no influence on the magnitude of the alkaline Bohr effect, but it competes with chloride because it occupies the oxygen-linked chloride binding site between Val NA1(1)$\alpha_1$ and Arg HC3(141)$\alpha_2$. In consequence, ($\Delta \log P_{50}/\Delta \log$ [BZF] is halved when the concentration of chloride is raised from 10 to 100 mM. A further rise in chloride concentration raised $P_{50}$, perhaps because chloride then binds to other sites not blocked by BZF (data not shown).

STT lowers the oxygen affinity of haemoglobin by an amount comparable to BZF, and like BZF it acts synergistically with DPG and competes with chloride. At 60–70 $\mu$M haem, 50 mM bis-Tris, 100 mM NaCl, and 25 °C, addition of 3 mM STT raises $P_{50}$ from 7.4 to 16.6 mmHg. The further addition of 3 mM DPG raises $P_{50}$ to 29 mmHg. Without added NaCl, addition of 3 mM STT raises $P_{50}$ from 2.3 to 20.5 mmHg, equivalent to a binding energy of at least 5.6 kcal per mol of haemoglobin tetramer.

## Discussion

**Nature of Interactions between Protein and Drug or Peptide.** In this discussion we might regard STT as a model of a peptide hormone, BZF and BBA as "unreactive" drugs that bind to proteins by secondary valency forces only, and ECA as a reactive drug that binds covalently. What generalizations can we draw from their binding to hemoglobin? Some could have been inferred from the rules of crystal packing and protein structure, while others are unexpected.

The activated CH=$CH_2$ bond of ECA is attacked by sulfhydryl groups of cysteine and the imidazole ring of histidine. We have found ECA bound to both reactive cysteines F9(93)$\beta$, forming covalent S–C(10) bonds. These bonds are stabilized by several polar and nonpolar interactions between the drug and the protein: the *o*-dichlorobenzene moiety of ECA has its dipole moment of 2.25 D so arranged that the positive pole interacts with a carboxylate, while one negative pole and the nearby carbonyl oxygen share a hydrogen bond to a histidine. There are also weakly polar interactions between the chlorines and aliphatic CH's (for interatomic distances see Figure 4).

In deoxyhb one of the two symmetry-related histidines G19(117)$\beta$ ($\beta_2$) exhibits a high and the other ($\beta_1$) a low occupancy of ECA. Why has the drug not reacted with any of the other 20 accessible histidines and why that asymmetry? The answer seems to be that this one particular pair of sites offers a unique constellation of polar groups which immobilize the drug in an orientation favorable for its reaction with the histidine. The carbonyl oxygen of the drug is hydrogen bonded to a main chain NH of the globin, the positive pole of the dichlorobenzene is compensated by a carboxylate ion, and the electronegative chlorines are in contact with the methyl side chains of two valines. The asymmetry is probably due to the salt bridge formed by the carboxylate of the drug with $NH_3^+$ of Lys A12(14)$\alpha$ of the neighboring Hb molecule at the site $\beta_2$ and the absence of that salt bridge at the site $\beta_1$.

All the compounds that bound to hemoglobin contained at least one negative charge. None of the positively charged drugs we tried bound to our crystals, probably because the cationic residues in the central cavity of hemoglobin outnumber the anionic ones by two to one (10 pairs of cations, 5 pairs of anions). There is no strong hydrogen bond attaching BZF to the globin, but several of the van der Waals contacts are markedly polar, such as those between the CH's of Phe C1(36)$\alpha_1$ and the chlorine, those between $NH_2$ of Asn G10(108)$\beta_1$ and the $\pi$ electrons of the first benzene ring suggestive of a hydrogen bond, and those between the CH's of Trp C3(37)$\beta_1$ and C(14) and C(15) of the second benzene ring. In each case hydrogens of the globin seem to point to high electron density of the drug.

Each STT molecule in the central cavity is attached to the globin by three strong hydrogen bonds but relatively few (~9) van der Waals interactions. On the other hand, we found such interactions, some of them again distinctly polar, between the two indole rings within each STT and between two indole rings of neighboring STT's. Note in Figure 14a the many contacts of $C_\beta H_2$ of one tryptophan residue with the $\pi$ electrons of the indole of the neighboring molecule and in Figure 14c the contacts between $C_{\zeta 3}H$ and $C_{\epsilon 3}H$ of one STT molecule and the $\pi$ electrons of the indole of its neighbors along the chain. The STT molecule that lies between two hemoglobin molecules is the one best defined in the electron density map, probably because all polar groups except the carbonyl between the two tryptophans form hydrogen bonds with polar groups of one or the other Hb molecule. Of the few van der Waals contacts, those between the CH's of Phe C7(41)$\beta_1$ and the $\pi$ electrons of indole 1 are again distinctly polar. A major portion of the binding energy of BZF and STT is likely to come from the hydrophobic effect, contributed by the respective displacement of about 13 and 21 partially immobilized water molecules in the central cavity and by the liberation of the partially immobilized water molecules surrounding BZF and STT in solution. Even though the surroundings of the STT molecules bound in the central cavity and at the contact between neighboring Hb molecules are quite different, Figure 18 shows that their conformations are similar. Proton NMR has shown that the stacking of the two indole rings of STT found in Hb crystals is in fact the same as in free STT and also in free L-Trp L-Trp in solution (A. M. Gronenborn, unpublished).

We were surprised by the penetration of the tertiary structure of the globin by molecules as large as BBA or *p*-bromobenzyl alcohol which binds in a pocket near Trp A12(14)$\alpha$.[20] Since the density of globular proteins is equal to that of the mean crystal density of its component amino acids,[34,35] we did not expect that a globin chain could find niches to accommodate these compounds with only minor adjustments.

**Comparison with Interatomic Contacts in Organic Crystal Structures and Proteins.** We expected aromatic moieites of drugs or peptides to form $\pi$–$\pi$ interactions with aromatic rings of the globin and were surprised to find none of these. A search of the crystallographic literature then revealed $\pi$–$\pi$ interactions to be dominant only in crystals of large polycyclic structures like coronene,[36] while smaller aromatic rings tend to interact quite differently. For example, in crystalline benzene a hydrogen of one molecule intermeshes with the hydrogens of one of its neighbors like the teeth of two bevel gearwheels whose axes are at 90° to each other: the H–H distances are 2.64 and 2.78 Å. Other hydrogens of the first molecule are in contact with the $\pi$ electrons of another neighbor.[37] Contacts of the latter kind are prominent also in the herringbone structures of anthracene and naphthalene, or in octaphenylcyclooctatetraene, with CH–C distances ranging from 2.6 to 3.2 Å.[38,39] They are the dominant ones found here between aromatic rings of the drugs and the globin and between the indole rings of STT, and they also seem to occur frequently between aromatic side chains in proteins.[53,54] Halogens seem to interact preferentially with aromatic hydrogens and more rarely with aliphatic ones. At intermolecular contacts in crystals of *p*-dichlorobenzene Cl···H = 2.96 Å,[40] implying, when C–H···Cl is linear, that the C–H···Cl distance equals 3.9 Å. The CH–Br distance found in a 1,1-diaryl-2-bromoethylene is 3.86 Å.[41]

Would the dipole moment of 1.57 D of chlorobenzene place sufficient negative charge on the chlorine to shorten this distance significantly? Neutron diffraction studies of amino acid hydrochlorides have shown CH···$Cl^-$ distances up to 0.35 Å shorter than the sum of the van der Waals radii,[42] but at a pole to pole sep-

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aration of 4.6 Å, the dipole moment of BZF corresponds to no more than 0.07 of an electronic charge on the chlorine. This is unlikely to produce a shortening of the CH···Cl distance measurable by our methods.

The intermolecular distances found in this study are listed in Figures 4, 7, 11, 14, 17, and 21. In trying to compare them with those found in the organic crystal structures just mentioned, we run up against two difficulties. The standard error in atomic coordinates in native human deoxyhb is 0.2 Å, but that of the drug atoms is probably larger because the resolution of the X-ray data was 2.3–2.8 Å, compared to 1.74 Å for the native data. Our second difficulty arose from the low occupancy of some of the drug binding sites (40–60% for BZF; 10–60% for ECA). When there is less than full occupancy, the X-ray data arise from the superposition of two protein structures, the native and the drug-bound conformation. In principle, it might be possible to refine a linear combination of two atomic models against the X-ray data, but this was not practicable at the available resolutions. Refinement against a single model proved unsatisfactory.

**Allosteric Effectors: Implications for Drug Action.** BZF and STT lower the oxygen affinity of Hb by combining specifically with the T structure. They must dissociate before the T → R transition occurs because the cavity between the $\alpha$ chain in the R structure becomes too narrow to accommodate them (see Figures D24 and 25, ref 6). Two of the four pairs of binding sites of CFA overlap with the sites of the two aromatic moieties of BZF.[13] CFA therefore has a similar, though weaker effect on the oxygen affinity than BZF.

All these compounds bind to the central cavity mainly at sites between the $\alpha$ chains, far removed from the receptor site for the natural allosteric effector for 2,3-diphosphoglycerate between the N termini of the $\beta$ chain. This implies that a drug may mimic the action of a natural allosteric effector, such as a hormone, on the allosteric equilibrium of a receptor without being chemically related to that hormone and by binding to a quite different site.

**Effects of Drugs on Polymerization of Sickle Cell Hemoglobin.** This study was begun with the aim of developing a rational stereochemical basis for the design of antisickling drugs. What has it taught us? It will be useful to take into account the X-ray studies of Walder et al. on the effects of cross-linking reagents,[43,44] our own previous X-ray study of clofibric acid and other compounds,[20] as well as our present results. Walder et al. introduced bifunctional reagents which link together the pair of lysines EF6(82) and then studied the gelling of deoxyhemoglobin S and the crystal structure of cross-linked deoxyhemoglobin A as a function of chain length between the reacting groups. They found the antigelling activity of the reagents to increase with the degree of distortion in quaternary structure they caused.

We are faced with the paradox that of the three compounds binding between the $\alpha$ chains, CFA and STT raise the minimum gelling concentration, while BZF lowers it, even though their binding sites overlap. Each of these compounds induces small changes in tertiary and quaternary structure, the smallest being probably those induced by BZF, but even the largest changes are too close to the standard error of our coordinates to determine them with confidence. Even if we could, we would find it difficult to pinpoint those small distortions that are the operative ones.

We have found a variety of other sites where the binding of drugs exerts antigelling effects: one between helices A and E near Trp A12(14)$\alpha$[20] that was also the binding site of $CH_2Cl_2$ found by Schoenborn,[3] another near Leu CD7(47)$\alpha$, a third near Cys F9(93)$\beta$, and possibly a fourth near His G19(117)$\beta$. Their modes of action are more easily interpreted. Trp A12(14)$\alpha$ and His G19(117)$\beta$ lie close to the contact between neighboring molecules along single chains of deoxyhb A or S (Figure 12, p 85 of ref 6). Since any distortions in this sensitive region of the molecule may shift the equilibrium between polymers and monomers, it would be desirable to develop drugs that bind more firmly to one of these sites. The binding site of BBA near Leu CD7(48)$\alpha$ is of great interest in view of Benesch et al.'s finding that Asp CD6 stabilizes the Hb S gel.[45]

We now come to ECA which inhibits formation of the C-terminal salt bridges of His HC3(146)$\beta$. Due to the rupture of these salt bridges it biases the allosteric equilibrium strongly toward the more soluble R structure. This may be sufficient to explain its antigelling activity, but we cannot rule out the possibility that the small displacements of helix F caused by the reaction of ECA with Cys F9$\beta$ may lower the affinity of Phe F1(85)$\beta$ and Leu F4(88)$\beta$ for Val A3(6)$\beta$ of a neighboring Hb S molecule. These residues form the vital binding site that determines the association of two single filaments of deoxyhb S into the double filament which constitutes the structural unit of the HbS gel.[46] We cannot be sure whether binding of ECA at the site near His G19(117)$\beta$ is pro- or antigelling, because we have no evidence that ECA reacts with that histidine in solution; reaction may have been induced in the crystal by the binding of the carboxylate of the drug to Lys A12(14)$\alpha$ of the neighboring molecule.

**Hydrophobic Interactions.** We have estimated the hydrophobic contribution of the free energy of binding, $\Delta G$, of BZF and ECA to deoxy hemoglobin. Water-accessible surface areas (ASA) were estimated by the method of Shrake and Rupley,[50] and the reduction in accessible surface area on binding was calculated from

$$\Delta ASA = ASA(\text{free drug}) + ASA(\text{free deoxyhb}) - ASA(\text{deoxyhb + drug})$$

For BZF, $\Delta ASA = 680$ Å.$^2$ For peptides $\Delta G$ has been estimated to be −(13 to 25) cal mol$^{-1}$ Å$^{-2}$ of ASA.[51] Assuming these values, $\Delta ASA$ for BZF represents −(9 to 17) kcal mol$^{-1}$ of hydrophobic binding energy. The major positive contribution to $\Delta G$ comes from the loss of translational and rotational entropy of the drug. For a molecule of the size of BZF, $T\Delta S$ (in vaccuo) would be about 25 kcal mol$^{-1}$, but in solution it cannot be estimated, so that it is not possible to calculate the binding constant of BZF. The hydrophobic effect probably represents the largest single negative contribution to the free energy of binding, but the specificity of binding depends on the detailed interactions described elsewhere in this paper. Their individual contributions may be small but even 1 kcal mol$^{-1}$ of binding energy represents a sevenfold change in equilibrium constant.

## Conclusions

1. Halogenated, aromatic derivatives of oxyacetic acid and certain aromatic peptides bind to the central cavity of Hb, mainly to sites between the $\alpha$ chains which are far removed from the binding site of the natural allosteric effector 2,3-diphosphoglycerate. They must dissociate before the binding of heme ligands changes the quaternary structure to the oxy form where the central cavity is too narrow to accommodate them. In consequence these compounds lower the oxygen affinity by the equivalent of their binding energy to the quaternary deoxy structure. Our results imply that drugs or peptides may influence the allosteric equi-

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librium of a protein receptor in the same direction as the natural effector even if they are chemically unrelated to it, because proteins may offer a variety of effector binding sites not used in nature.

2. Bezafibrate is bound by van der Waals interaction with at least 9 residues of the globin, most of which belong to one $\alpha\beta$ dimer. Several of these interactions are found to be between dipolar or mutually polarizable groups.

3. Succinyl-L-tryptophan-L-tryptophan is bound to the central cavity and to an intermolecular site, mainly by hydrogen bonds with polar groups of the globin. van der Waals interactions link indole side chains of the same and of neighboring molecules; some of these interactions also appear to be weakly polar, because they take place between opposite poles of mutually polarizable groups.

4. Ethacrynic acid forms covalent bonds with the reactive pair of cysteines and with two of the 10 exposed pairs of histidines. The fractional charges of its dipolar *o*-dichlorobenzene moiety are compensated by interactions with globin side chains of opposite charge. Its carbonyl oxygen forms hydrogen bonds with NH groups of the globin chain and its carboxylate is either solvated by water or linked to a lysine of a neighboring molecule.

5. The binding site of *p*-bromobenzyloxyacetic acid lies in the interior of the $\alpha$ chain, in a position hitherto believed to be filled by close-packed side chains of the globin.

6. The stereochemistry of binding of all these compounds is determined by the available van der Waals space and, within that space, by interactions of a wide range of polarity, from strong hydrogen bonds between ionized groups of opposite charge to weak interactions between aromatic quadrupoles, and nonpolar interactions between aliphatic hydrocarbons. Pauling enunciated two principles relevant to this work. The first was that "the significance of the hydrogen bond for physiology is greater than that of any other structural feature" and the second that the free energy of interaction between a hapten and an antigen will be proportional to the sum of the products of the polarizabilities of the atoms in contact.[47,48] Our results suggest as a third principle that the detailed stereochemistry of binding is likely to be governed by a tendency to maximize the sum of the energies of electrostatic interactions, for example, by aligning the drug relative to the protein so that the mutual polarizabilities of neighboring groups are maximized. A major portion of the binding energy of our compounds is probably due to the hydrophobic effect, caused by the liberation of partially immobilized water molecules.[49]

7. Binding of drugs or aromatic peptides induces small distortions in tertiary and quaternary structure which affect the equilibrium between monomers and polymers of deoxyhemoglobin S in ways which are not yet fully understood.

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**Registry No.** Beazafibrate, 41859-67-0; oxygen, 7782-44-7; succinyl-L-tryptophan-L-tryptophan, 73205-75-1.