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The anion-transport inhibitor H_2DIDS cross-links hemoglobin interdimerically and enhances oxygen unloading *

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Human hemoglobin treated with equal concentrations of the anion-transport inhibitor H_2DIDS produces a right shift in the oxygen dissociation curve. Concomitantly, the Hill coefficient is reduced from $n = 2.7$ to 2.1. When higher concentrations of H_2DIDS are applied (H_2DIDS: hemoglobin = 5:0.5 mM), the Hill coefficient decreases further to 1.5 and the oxygen dissociation curve of hemoglobin is shifted slightly to the left of the control. Similar results were also obtained with DIDS instead of H_2DIDS. SDS-PAGE shows that H_2DIDS cross-links hemoglobin monomers mainly into dimers. Cross-linking is more effective under anaerobic conditions. With tritiated H_2DIDS the larger part of the radioactivity is found in the dimer position of hemoglobin. Separation of the α and β units of hemoglobin reacted with tritiated H_2DIDS demonstrated a stoichiometry of 2.2 and 2.4 molecules H_2DIDS per molecule α and β unit hemoglobin, leading to about 8–9 H_2DIDS molecules per native hemoglobin. The right shift produced in the hemoglobin oxygen dissociation curve and the cross-linking of monomers into dimers, especially under anaerobic condition, suggest that H_2DIDS can also react with those amino groups of hemoglobin which are involved in 2,3-DPG binding. A comparison of H_2DIDS, DIDS and 2,3-DPG at three different concentrations close to the hemoglobin concentration revealed a concentration dependent right shift in the oxygen dissociation curve with the order of potency 2,3-DPG > H_2DIDS > DIDS. The Hill coefficients (n) at the three concentrations of 2,3-DPG demonstrated no changes, but H_2DIDS and DIDS reduced in a concentration-dependent manner the cooperativity of hemoglobin. Again, H_2DIDS is more potent than DIDS, especially at the low concentration. These anion-transport inhibitors provide novel approaches to the exploration of hemoglobin function.

Introduction

The rapid exchange of HCO_3^- for Cl^- across the red blood cell membrane is mediated by the anion transport protein band 3 (reviewed in Ref. 1). This protein was labelled selectively by the tritiated anion transport inhibitor H_2DIDS. In intact red blood cells, H_2DIDS almost exclusively binds covalently to band 3 with a stoichiometry of 1 : 1 [2,3], thereby competitively inhibiting the high capacity exchange of anion. It has further been shown that changes in the chloride concentration

* Dedicated to Dr. Erhard Wolf, Wiesbaden, on the occasion of his 60th birthday.

Abbreviations: 2,3-DPG or DPG, 2,3-diphosphoglycerate; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; DNDS, 4,4′dinitrostilbene-2,2′disulfonate; Compound I, 4,4′-diformyldibenzyl-2- oxyacetic acid; Compound III, 4,4′-dihydroxysulfonatedibenzyl-2-oxyacetic acid; PLP, pyridoxal phosphate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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inside the red cell strongly affect the ability of external H_2DIDS to inhibit chloride exchange [4]. This transmembranous effect of internal chloride on external H_2DIDS potency provides evidence that band 3 is an allosteric protein in which the chloride gradient can be used to change the orientation of the transport protein.

Another allosteric protein in red cells is the hemoglobin molecule, which can exist in an equilibrium between two quarternary protein conformations – the oxy state with a high and the deoxy state with a low oxygen affinity [5]. 2,3-DPG binds to the deoxy state, thereby lowering the oxygen affinity of hemoglobin [6]. The specific site in the deoxygenated form of hemoglobin at which 2,3-DPG interacts is known [7] and compounds designed to model the polyanion 2,3-DPG have been constructed [8–10]. The structural similarities to H_2DIDS of biphenyl-4,4'-dialdehyde and those derivatives which lower the oxygen affinity of hemoglobin are striking. Encouraged by these facts, we studied the effect of H_2DIDS on the function of isolated human hemoglobin. In a short report [11] we demonstrated previously that H_2DIDS cross-links hemoglobin and enhances oxygen unloading.

Material and Methods

Preparation of hemoglobin

Freshly drawn human red blood cells with heparin as anticoagulant were washed three times with ice-cold 150 mM NaCl, 20 mM Hepes solution (adjusted to pH 7.6 at room temperature) or with 120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.27 mM $CaCl_2$ and 25 mM $NaHCO_3$ (pH 7.4, Krebs-Ringer bicarbonate buffer). For hemolysis the packed red cells were frozen and thawed three times with liquid nitrogen. The hemolysate was centrifuged for 15 min at $48000 \times g$ in order to sediment membranes. The membrane-free supernatant was dialysed overnight in a cold room (about 4°C) against Krebs-Ringer bicarbonate buffer for the determination of hemoglobin-oxygen equilibrium curves (Figs. 2a, 2b, 3, and Table I) and against 150 mM NaCl and 20 mM Hepes (pH 7.6) for the reaction with H_2DIDS. The concentration of hemoglobin was determined at the isobestic point for oxy- and methemoglobin at 527 nm [12], and for storage the hemoglobin solution was frozen at −80°C.

Determination of hemoglobin oxygen equilibrium curves

A contiuous hemoglobin-oxygen curve [13] was determined from 2 µl hemoglobin or red blood cell samples at 37°C with an Aminco HEM-O-SCAN oxygen dissociation analyzer (American Instrument Company, Silver Spring, MD, U.S.A.). The red blood cell and the hemoglobin samples were recorded in Krebs-Ringer bicarbonate buffer fixed at 40 mmHg p_{CO_2}. In Table I P_{50} values and the Hill coefficient n (analyzed between 15 and 85% saturation) of freshly prepared red blood cells, hemolysate and DPG stripped hemoglobin by overnight dialysis are given. The latter preparation was used to test the effects of H_2DIDS on O_2 binding.

H_2DIDS labelling of hemoglobin

Reaction of 1.5 mM hemoglobin with H_2DIDS in 150 mM NaCl and 20 mM Hepes buffer at pH 7.4 and 37°C was carried out under air or N_2 atmosphere for the given time periods (Fig. 4). The H_2DIDS solution was always freshly prepared. For reaction of hemoglobin with ^{3}H-labelled H_2DIDS, 100 µM hemoglobin in 150 mM NaCl and 20 mM Hepes buffer pH 7.4 at 37°C were incubated with 165 µM ^{3}H-labelled H_2DIDS under N_2 atmosphere for 60 min.

SDS-Polyacrylamide Gel electrophoresis (PAGE)

The buffer system of Laemmli [14], with a 4% stacking gel and a 13.5% gel was used.

Fluorography was carried out with 'Amplify' as fluorescent agent according to the recommendation of the manufacturer, Amersham.

Separation of hemoglobin chains

Soluble starch granules, prepared after the procedure of Wieland et al. [15], were resuspended in a 0.01 M Tris-HCl buffer (pH 7.8), containing 0.001 M EDTA, and 6 M urea, and filled into a semicircular glass trough of 1.5 cm maximal diameter and 35 cm lenght. A current of 50 mA at 250 mV/cm was applied via thick paper wicks, and the electrophoreses were run in the cold room overnight. The bands were visualized by a filter

paper blot with Coomassie blue staining, cut out, and dialysed after elution with water. An intermediary band, corresponding to the α-β-interchain cross-linked material, was observed, and cut out separately. The resulting protein solution was lyophilized and redissolved in a minimal buffer volume for protein determination and counting. Protein was determined by the procedure of Lowry et al. [16] with bovine albumin serum a standard.

Materials

Hepes was obtained from Serva (Heidelberg, F.R.G.). Labelled H_2DIDS was prepared as described previously [17]. DNDS as sodium salt was purchased by Bayer (Leverkusen, F.R.G.). For fluorography Kodak XOMAT AR film was used. Gas mixtures contained 5.6% CO_2, 25% O_2, balance N_2 and 5.6% CO_2, balance N_2 (American Instrument Company, Silver Spring, MD U.S.A.). All other reagents were of analytical grade.

Results and Discussion

Fig. 1 shows the structural similarities of 2,3-DPG, compound I and compound III with H_2DIDS and DNDS. Compounds I and III were designed to fit the basic region between the two β-chains of deoxyhemoglobin (terminal amino group of valine-1 and the basic amino residues histidine-2, lysine-82 and histidine-143) instead of the natural effector 2,3-DPG [8,9]. Both model compounds gave the expected right shift of the oxygen dissociation curve of human adult hemoglobin, and NMR measurements supported the predicted modes of binding [18]. In addition, investigations with 2,3-DPG, compound I, and compound III in six hemoglobins that differed at the 2,3-DPG receptor site showed that the size of the shifts of the dissociation curves is in the sequence expected from the postulated numbers of interactions made by each compound with each hemoglobin type [9]. This is in agreement with the assumption of a common receptor site for the three mentioned substances.

In this investigation we tested whether the inhibitors of the anion exchange in erythrocyte membranes, namely H_2DIDS and DNDS, could also have similar effects on hemoglobin to those of compounds I and III. Fig. 2a demonstrates that

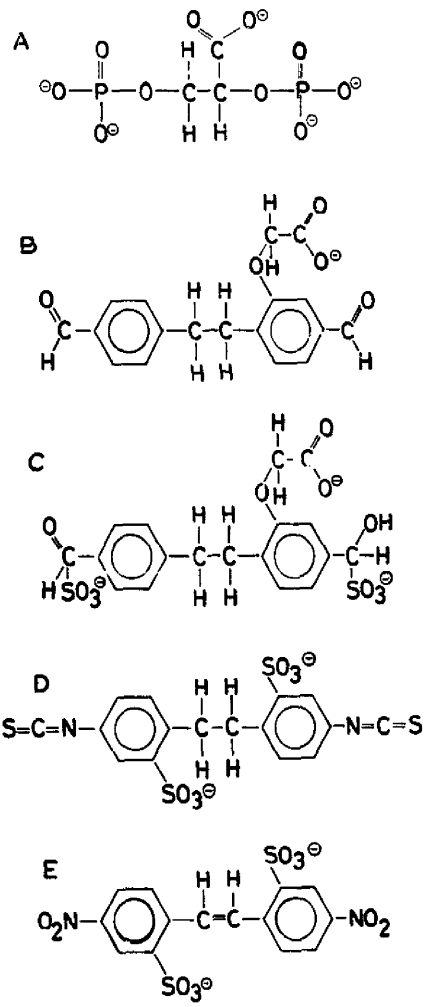

Fig. 1. Stuctural analogues of 2,3-diphosphoglycerate (2,3-DPG). A, DPG; B, 4,4′-diformyldibenzyl-2-oxyacetic acid (Compound I); C, 4,4′-dihydroxysulfonatedibenzyl-2-oxyacetic acid (Compound III); D, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS); E, 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS).

H_2DIDS applied at twice the molar cencentration of dialysed hemoglobin produces a considerable right shift in oxygen dissociation curve. The oxygen affinity of hemoglobin can be characterized by its P_{50} value. This value is shifted from 15 mmHg for the control to 20 mmHg. The shift is about half of that expected with 2,3-DPG. Fig. 2b depicts the

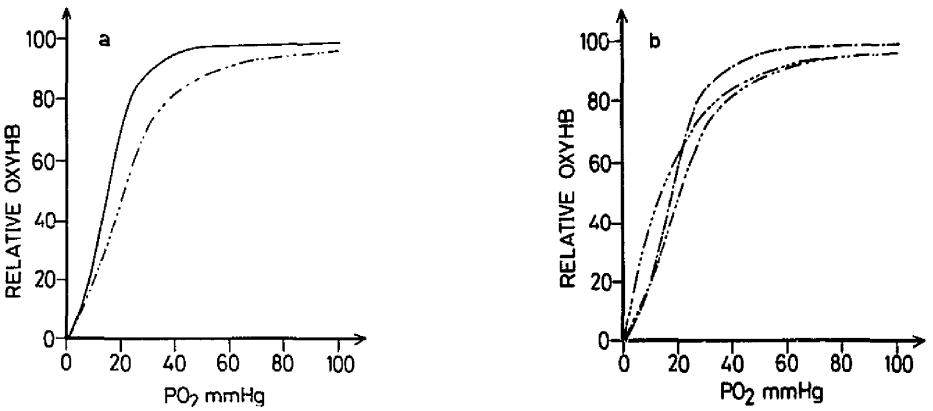

Fig. 2. (a) The effect of 1.7 mM H_2DIDS (– · · –) on the oxygen dissociation curve of dialysed hemoglobin at 0.83 mM concentration. ———, control without H_2DIDS. Samples were run in Krebs-Ringer bicarbonate buffer (pH 7.4) and 37 °C. (b) The effect of 0.5 mM (– · –), 1.7 mM (– · · –), and 5 mM H_2DIDS (– · · · –) on the oxygen dissociation curve of dialysed hemoglobin at concentrations of 0.95, 0.83 and 0.5 mM, respectively.

behaviour of the oxygen dissociation curve at three different H_2DIDS concentrations. At the high concentration of H_2DIDS, 5 mM, the dissociation curve changes from sigmoidal to the hyperbolic form. This effect is suggestive of a decrease in cooperativity of hemoglobin by H_2DIDS interaction. The cooperativity was, therefore, analysed by a Hill plot [19]. The Hill coefficient n was calculated at the midpoint of the oxygen binding curve (Table I). The first column of Table I shows the P_{50} values, and in the second the Hill coefficients are listed with the correlation coefficients r for the linearized oxygen dissociation curve. At 0.5 mM H_2DIDS, there is only a slight decrease from about $n = 2.7$ to 2.5 At 1.7 mM H_2DIDS a diminution to $n = 2.1$ is calculated and at 5 mM H_2DIDS the cooperativity is drastically diminished to $n = 1.5$. Thus, in contrast to the natural effector 2,3-DPG, the anion-exchange inhibitor H_2DIDS reduces the cooperativity of hemoglobin.

TABLE I

Samples		P_{50} (mmHg)	Hill coefficient (n)	Correlation coefficient (r)
Human blood		26.9	2.69	0.997
Hemolysate		21.0	2.94	0.994
Hemolysate dialysed		15.1	2.74	0.995
		15.1	2.60	0.993
Hemolysate dialysed, 0.5 mM H_2DIDS,	22 min	17.5	2.61	0.994
	45 min	16.8	2.46	0.995
Hemolysate dialysed, 1.7 mM H_2DIDS,	20 min	18.0	2.13	0.993
	40 min	19.8	2.06	0.992
	67 min	20.9	2.14	0.995
Hemolysate dialysed, 5.0 mM H_2DIDS,	28 min	13.0	1.50	0.997
	50 min	14.0	1.46	0.998
Hemolysate dialysed, 1.7 mM DNDS,	15 min	16.8	2.75	0.995
	62 min	16.1	2.49	0.992
Hemolysate dialysed, 5.0 mM DNDS,	18 min	16.8	2.11	0.989
	58 min	16.0	2.17	0.992

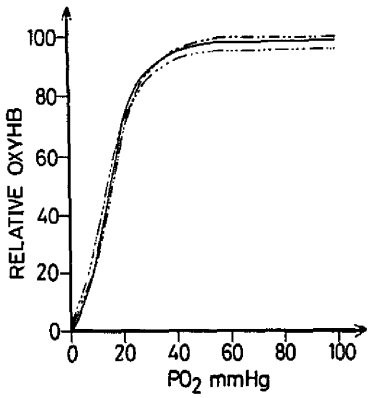

Fig. 3. The effect of 1.7 mM (– · · –) and 5 mM DNDS (– · · · –) on the oxygen dissociation curve of dialysed hemoglobin at concentrations 0.83 and 0.5 mM, respectively. ———, control without DNDS. Conditions were as described in Fig. 2.

The effects of DNDS on the dissociation curve of dialysed hemoglobin are comparatively low (Fig. 3). The P_{50} values at 1.7 and 5 mM DNDS are only shifted slightly to the right. Only at 5 mM DNDS is the cooperativity of hemoglobin reduced significantly from 2.7 of control to 2.1 (Table I). Because of these comparatively small effects of DNDS on oxygen dissociation of hemoglobin and the fact that H_2DIDS in contrast to DNDS binds covalently to proteins, we investigated the effects of H_2DIDS more extensively.

In Fig. 4 it is shown by SDS-PAGE that the bifunctional H_2DIDS molecule can cross-link hemoglobin with the two thiocyanate groups. Hemoglobin was incubated at 37° C for the time periods given in Fig. 4 with 2 mM H_2DIDS under O_2 or N_2 atmosphere. The controls without H_2DIDS (lane 1 and 2) demonstrate mainly a high amount of hemoglobin not resolved as double band around 16 kDa. The lower part reflects most likely the smaller α units and the upper part the heavier β units of the hemoglobin monomers. If only small amounts of hemoglobin are applied, the α and the β units appear clearly separated. At the 32 kDa position a faint dimer band is seen and also a

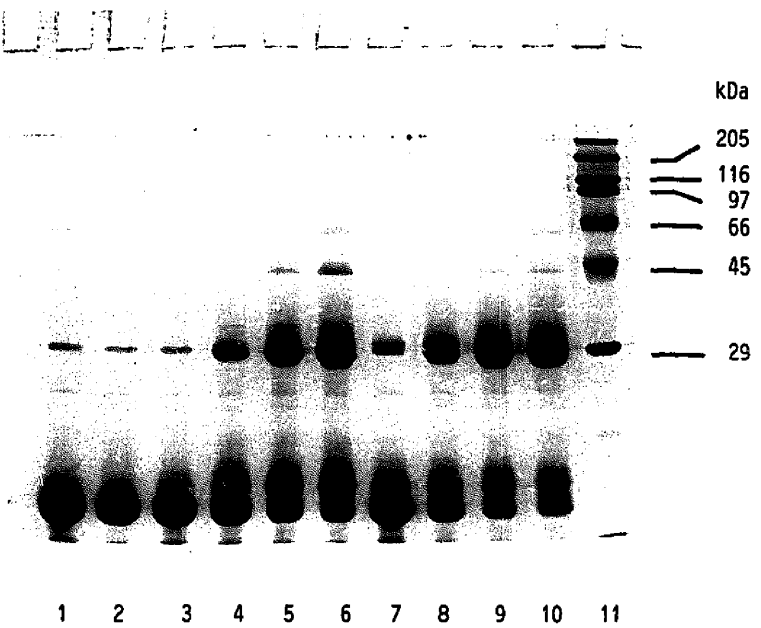

Fig. 4. SDS-PAGE of human hemoglobin. 1.5 mM hemoglobin incubated with 2 mM H_2DIDS under O_2 or N_2 atmosphere at 37° C in 150 mM NaCl and 20 mM Hepes buffer (pH 7.4). Lanes 1,2: incubation without H_2DIDS for 0 and 60 min under O_2 atmosphere. Lanes 3–6: treatment with H_2DIDS under O_2 atmosphere for 2, 10, 30 and 60 min. Lanes 7–10: treatment with H_2DIDS under N_2 atmosphere for 2, 10, 30 and 60 min. Lane 11: marker proteins.

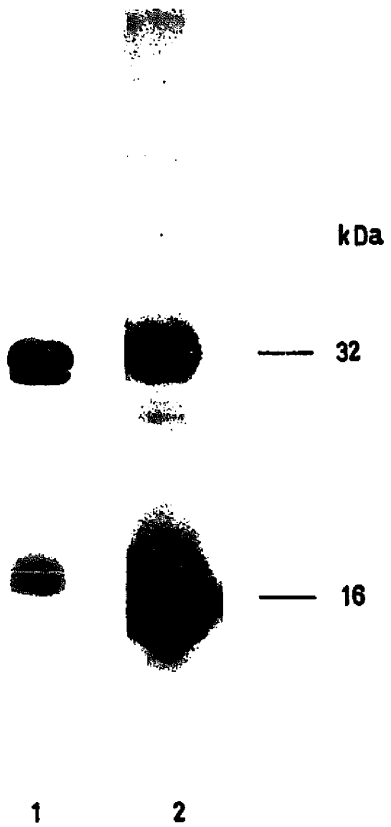

Fig. 5. SDS-PAGE of human hemoglobin. 100 μM hemoglobin treated with 165 μM ^{3}H-labelled H_2DIDS ($2.65 \cdot 10^{14}$ cpm/mol) under N_2 atmosphere for 60 min at 37 °C in 150 mM NaCl and 20 mM Hepes buffer (pH 7.4). Lane 1: fluorography. Lane 2: Coomassie blue stain of sample in lane 1.

slight indication for a tetramer corresponding to 64 kDa. After incubation with 2 mM H_2DIDS under aerobic conditions for a short period of 2 minutes (lane 3), subsequent dimer formation is not yet visible. After 10, 30 and 60 min of incubation (lanes 4, 5 and 6), however, an increasing amount of dimer formation is observable at the expense of monomers. Additional split bands of about 17 kDa to 18 kdA are seen which could result from reaction of the hemoglobin monomers with 2 to 4 H_2DIDS molecules, thus increasing the molecular weight by 1 to 2 kDa (see also Fig. 5). After 30 and 60 min incubation (lanes 5 and 6) a trimer band is also pronounced.

When hemoglobin is incubated with H_2DIDS under nitrogen, after 2 min dimer formation initiates at the expense monomers. This reaction obviously proceeds faster under nitrogen (Fig. 4, lanes 7–10) than under oxygen (Fig. 4, lanes 3–6). The more efficient reaction of H_2DIDS under anaerobic conditions can be explained by the functional effect of H_2DIDS on the hemoglobin oxygen-dissociation curve (Fig. 2), as under anerobic conditions the cleft between the β chains is opened and, thus, the reaction of H_2DIDS with the exposed amino groups is more efficient.

When hemoglobin is reacted with tritiated H_2DIDS under anaerobic conditions, the majority of the radioactivity is found in the dimer position (Fig. 5, lane 1). Also slightly above the monomer bands at 16 kDa (lane 2, protein stain) radioactive H_2DIDS is seen bound to monomers. The cross-linking effect of H_2DIDS (Figs. 4 and 5) and the right shift of the oxygen-dissociation curve of hemoglobin (Figs. 2a and b, Table I) suggest that H_2DIDS reacts, in addition to other sites, with those amino groups involved in the physiological 2,3-DPG binding.

Finally, we reacted hemoglobin under anaerobic conditions with highly tritiated H_2DIDS, and separated the α and β units. Both units contained about the same amount of H_2DIDS. From the specific activity of the labelled H_2DIDS a substitution of 2.2 residues was calculated for the α unit and 2.4 residues for the β unit. The hemoglobin tetramer contains about 8 to 9 H_2DIDS molecules. From the distribution of radioactivity between the monomer and dimer bands (Fig. 5), it may be concluded that 50 to 60% of the cross-links occur within one subunit. The α-β-cross-link, detected after separation of the subunits in starch gel electrophoresis, amounted to only 15% of the total radioactivity. It seems probable, therefore, that the α-α-, α-β, and β-β-interchain cross-links each contribute 15% to the total. Thus, there is no preferential reaction of H_2DIDS with the β units, as has been shown for 2-nor-2-formylpyridoxal 5'-phosphate [20, 21], which cross-linked only the β units of hemoglobin, presumably at the lysine-82 position. Pyridoxal phosphate itself has been known since 1969 [22] to serve as an analog for 2,3-DPG. Furthermore, pyridoxal phosphate can be attached covalently to the N-terminal amino

group of the β chains, leading to a hemoglobin with a permanently lowered oxygen affinity [23].

During preparation of the present manuscript, Kavanaugh et al. [24] described affinity labeling of hemoglobin with DIDS which probably covalently cross-links hemoglobin in the 2,3-DPG binding site. In contrast to our results with H_2DIDS, cross-linking by DIDS occurred only with the β units of hemoglobin with a stoichiometry of 1 or 2 DIDS molecules. With the incorporation of one molecule of DIDS between the β-unit amino termini, a right shift of the oxygen dissociation curve of hemoglobin was observed [24].

These differences in hemoglobin cross-linking with DIDS and H_2DIDS can be ascribed to the separation and purification procedure used by Kavanaugh et al. [24]. From the DIDS-hemoglobin reaction mixtures with only one DIDS concentration two hemoglobin-DIDS derivatives from the several peaks observed were each isolated and tested [24].

To determine whether the results obtained under such limited conditions could be more generalizable, we compared at several different concentrations the effects of H_2DIDS, DIDS and 2,3-DPG on the oxygen dissociation curve of hemoglobin (Fig. 6). The upper panel of Fig. 6 shows by the P_{50} values that all three compounds can shift the oxygen dissociation curve in a concentration-dependent manner to the right. The three different concentrations were selected to be close to the hemoglobin concentration of 1.5 mM. The order of potency observed is as follows: 2,3-DPG > H_2DIDS > DIDS (Fig. 6, upper panel). Similar to H_2DIDS, if a DIDS concentration is used higher than the hemoglobin concentration, for example if 1 mM hemoglobin is incubated with 7.5 mM DIDS, the dissociation curve is shifted back to the left to a P_{50} value of 8.3 mmHg. Thus, the qualitative aspects are similar of H_2DIDS and DIDS on the hemoglobin dissociation curve without further purification of the hemoglobin reaction mixture.

The lower panel in Fig. 6 shows the Hill coefficient (n) at the three concentrations of 2,3-DPG, H_2DIDS and DIDS. In contrast to H_2DIDS and DIDS, 2,3-DPG does not change the Hill coefficient but H_2DIDS and DIDS decrease significantly in a concentration-dependent manner the

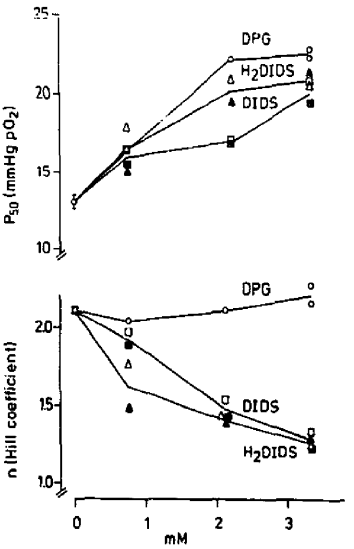

Fig. 6. Comparison of the effects of H_2DIDS, DIDS and 2,3-DPG on P_{50} values (upper panel) and the Hill coefficient n (lower panel) of oxygen dissociation curves of hemoglobin at 1.5 mM concentration. The 2,3-DPG stripped hemoglobin used in this experiment had been kept stored for half a year at −28°C. Samples were run in 150 mM NaCl and 20 mM Hepes buffer (pH 7.4) at 37°C with pure N_2 and a gas mixture of 20.5% O_2 and 79.5% N_2, respectively. $x = 0$, mean of four control values ± S.D.; ○——○, 2,3-DPG; △, H_2DIDS after 50 min at 37°C; ▲, H_2DIDS after one day at 4°C; □, DIDS after 50 min at 37°C; ■, DIDS after one day at 4°C.

cooperativity. Again the potency of H_2DIDS, especially at the low concentrations, exceeds that of DIDS.

A protective effect of 2,3-DPG against H_2DIDS appeared only at the lowest concentration of H_2DIDS investigated. If 0.75 mM 2,3-DPG were added to the hemoglobin and then 0.75 mM H_2DIDS, the Hill coefficient decreased to only 1.95 instead of to 1.55 for the control without 2,3-DPG protection. It is difficult to test the competition of H_2DIDS or DIDS on the one hand and 2,3-DPG on the other, since H_2DIDS and DIDS are known to react covalently [2,3], whereas 2,3-DPG is a fully reversible effector [6].

In contrast to conditions used by Kavanaugh et al. [24], our conditions are much closer to those that prevail normally. We investigated effects of each inhibitor at several different concentrations rather than just one, which might possibly provide

misleading or non representative results, and indeed our results disclose a concentration-dependence of the effects of H_2DIDS and DIDS (Fig. 6). Nevertheless, our results without further purification of the H_2DIDS-hemoglobin mixtures agree in some respects with those of Kavanaugh et al. [24], suggesting that our results, as well as theirs, do indeed closely reflect how these compounds interact with hemoglobin.

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References

1 Knauf, Ph. A. (1979) in Current Topics in Membranes and Transport, Vol. 12, pp. 251–348, Academic Press, New York.
2 Ship, S., Shami, Y., Breuer, W. and Rothstein, A. (1977) J. Membr. Biol. 33, 311–323.
3 Shami, Y., Rothstein, A. and Knauf, Ph.A. (1978) Biochim. Biophys. Acta 508, 357–363.
4 Furuya, W., Tarshis, T., Law, F.-Y. and Knauf, Ph.A. (1984) J. Gen. Physiol. 83, 657–681.
5 Perutz, M.F. (1970) Nature (London) 228, 726–734.
6 Benesch, R. and Benesch, R.E. (1967) Biochem. Biophys. Res. Commun. 26, 162–167.
7 Arnone, A. (1972) Nature (London) 237, 146–149.
8 Breddell, C.R., Goodford, P.J. Norrington, F.E., Wilkinson, S. and Wootton, R. (1976) Br. J. Pharmac. 57, 201–209.
9 Breddell, C.R., Goodford, P.J., Stammers, D.K. and Wootton, R. (1979) Br. J. Pharmac. 65, 535–543.
10 Goodford, P.J. (1981) in Towards Understanding Receptors, pp. 205–216, Elsevier/North Holland Biomedical Press, Amsterdam.
11 Fuhrmann, G.F. and Kreutzfeldt, Ch. (1985) Naunyn-Schmiedebergs Arch. Pharmacol. 329, R 43.
12 Grey, J.E. and Lauf, P.K. (1980) Membr. Biochem. 3, 21–35.
13 Nelson, M.G., Savage, G., G.A., Cooke, P.J. and Lappin, T.R.J. (1981) Am. J. Clin. Pathol. 75, 395–399.
14 Laemmli, U.K. (1970) Nature (London) 227, 680–685.
15 Wieland, Th., Pfleiderer, G. and Rettig, H.L. (1958) Angew. Chem. 70, 341–343.
16 Lowry, O.H. Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
17 Zaki, L., Fasold, H., Schumann, B. and Passow, H. (1975) J. Cell Physiol. 86, 471–494.
18 Brown, F.F. and Goodford, P.J. (1977) Br. J. Pharmac. 60, 337–341.
19 Hill, A.V. (1910) J. Physiol. (London) 40, IV–VII.
20 Benesch, R., Benesch, R.E. Yung, S. and Edalji, R. (1975) Biochem. Biophys. Res. Commun. 63, 1123–1129.
21 Benesch, R., Triner, L., Benesch, R.E., Kwong, S. and Verosky, M. (1984) Proc. Natl. Acad. Sci. USA 81, 2941–2943.
22 Benesch, R.E., Benesch, R. and Yu, C.I. (1969) Fed. Proc. 28, 604.
23 Benesch, R.E., Benesch, R., Renthal, R.D. and Maeda, NH. (1972) Biochemistry 11, 3576–3582.
24 Kavanaugh, M.P., Shih, D.T.-B. and Jones, R.T. (1988) Biochemistry 27, 1804–1808.