Triethyltin Binding to Cat Haemoglobin

EVIDENCE FOR TWO CHEMICALLY DISTINCT SITES AND A ROLE FOR BOTH HISTIDINE AND CYSTEINE RESIDUES

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Triethyltin binding to cat haemoglobin was measured after pretreatment of the protein with diethyl pyrocarbonate at pH 6.0, iodoacetamide or phenylmercuric acetate or by photo-oxidation in the presence of Methylene Blue. The pentaco-ordinate nature of the binding of triethyltin to cat haemoglobin is confirmed by the inability of intramolecularly pentaco-ordinate tin compounds to compete. Consideration of the symmetry of the haemoglobin molecule in the light of the above results suggests that a unique arrangement of histidine and cysteine residues is required for the binding of triethyltin. The effects of treatment with diethyl pyrocarbonate of other preparations which bind triethyltin (rat liver supernatant, a fraction from rat liver mitochondria and rat brain myelin) were determined and shown to be complex.

Triethyltin binds to few proteins (Elliott & Aldridge, 1977). Triethyltin ($1\text{--}10\ \mu\text{M}$) inhibits energy conservation and this is due to binding to the adenosine triphosphatase system in mitochondria (Aldridge, 1977; Cain & Griffiths, 1977). Glutathione-S-aryltransferases (EC 2.5.1.18) are also inhibited by $0.1\text{--}1\ \mu\text{M}$ -triethyltin (Henry & Byington, 1976; E. Tipping, B. Ketterer, L. Christodoulides, W. N. Aldridge, B. M. Elliott & J. W. Bridges, unpublished work). Since the chemistry of neither of these enzyme systems is fully understood, knowledge of the chemical nature of the binding of triethyltin to other model, and better-characterized, proteins will provide testable hypotheses for other macromolecules. With this in mind, a detailed examination has been made of the binding of triethyltin to rat and cat haemoglobin (Rose & Aldridge, 1968; Rose, 1969; Elliott & Aldridge, 1977).

Rat and cat haemoglobins bind two molecules of triethyltin to each tetramer and it was postulated that each triethyltin binds pentaco-ordinately with two histidines (Rose, 1969). Treatment with diethyl pyrocarbonate removes only one binding site (Elliott & Aldridge, 1977), thus raising the possibility that the chemical nature of the sites may be different. The

effect of other reagents and experimental conditions on the binding of triethyltin to cat haemoglobin has been further investigated and a new hypothesis on the nature of the amino acid residues involved in binding is presented.

Materials and Methods

Materials

Triethyltin sulphate, triethyl[^{113}Sn]tin chloride, diethyl pyrocarbonate and cat haemoglobin were obtained as described by Elliott & Aldridge (1977). The following were obtained as gifts: phenylarsine oxide from the late A. H. Ford-Moore, diethylphenyltin bromide, $\{2\text{--}[(\text{dimethylamino})\text{methyl}]\text{phenyl}\}$ diethyltin bromide, $\text{di-}\{2\text{--}[(\text{dimethylamino})\text{methyl}]\text{phenyl}\}$ ethyltin bromide and $\{2,6\text{-bis}[(\text{dimethylamino})\text{methyl}]\text{phenyl}\}$ diethyltin bromide (Fig. 5, I, II, III and IV respectively) from Dr. J. G. Noltes, Institute for Organic Chemistry TNO, Utrecht, The Netherlands. Phenylmercuric acetate was purchased from Hopkin and Williams, Chadwell Heath, Essex, U.K., and all other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A., or BDH Chemicals, Poole, Dorset, U.K.

Methods

Measurement of cat haemoglobin and assessment of the purity of the diethyl pyrocarbonate were as described by Elliott & Aldridge (1977). Protein was estimated by a modification of the method of Lowry

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et al. (1951) as described by Schacterle & Pollack (1973), with bovine serum albumin as standard.

Preparation of rat brain myelin and liver fractions. Myelin from whole rat brain was prepared as described by Lock & Aldridge (1975), but by centrifuging a 5% (w/v) homogenate at $57000g_{av}$. (22 500 rev./min in a SW 25 swing-out rotor in a Beckman-Spinco model L centrifuge) over the discontinuous sucrose density gradient. The Triton X-100 insoluble fraction from rat liver mitochondria was prepared by incubating mitochondria in 2% (w/v) Triton X-100 for 30 min at 37°C as described by Aldridge & Street (1970). An unpurified fraction of rat liver supernatant was prepared as described by Rose & Lock (1970) for guinea-pig liver. All the fractions were finally dissolved or suspended in 0.1 M-KH₂PO₄ adjusted to pH 6.0 or 7.3 with NaOH.

Measurement of triethyltin binding. Measurement of triethyltin binding to cat haemoglobin (20 µM) and rat liver supernatant (2–4 mg of protein/ml) was performed by equilibrium dialysis (Rose, 1969), by using triethyltin concentrations of 5–100 µM and 1.5–100 µM respectively, and the results were used to construct Scatchard (1949) plots. For the Triton X-100-insoluble fraction from rat liver mitochondria and myelin from the rat brain, incubation with diethyl pyrocarbonate and binding studies were performed in the same 50 ml polypropylene centrifuge tubes. Fractions (1–2 mg of protein, 0.8–1.0 ml) were added to 0.1 M-KH₂PO₄ buffer at pH 6 in the centrifuge tube and incubation with diethyl pyrocarbonate (added in 0.25 ml of ethanol) or ethanol was carried out at room temperature (18–20°C) with occasional shaking, for 20 min. The pH was then raised to 7.3 with NaOH and the triethyl[¹¹³Sn]tin added (in 0.1 M-KH₂PO₄, pH 7.3). The final volume was 10 ml for the mitochondrial and 5 ml for the myelin fractions. After 15 min incubation the tubes were centrifuged at $39000g_{av}$, for 25 min at room temperature. The supernatant was poured off and a sample taken for radioactivity counting. The tube was carefully drained and wiped free of surface liquid and the whole of the pellet removed for counting.

Chemical modification of proteins. Cat haemoglobin and rat liver supernatant were treated with diethyl pyrocarbonate as described by Elliott & Aldridge (1977) for cat haemoglobin. Photo-oxidation of cat haemoglobin in the presence of Methylene Blue was as performed by Rose (1969). Iodoacetamide was dissolved in water and added to cat haemoglobin (30 ml, 20 µM) in 0.1 M-KH₂PO₄, pH 6.0, to give concentrations of 5–50 mM. After incubation at 18–20°C for 1 h the pH was adjusted to 7.3 and triethyltin binding was measured by equilibrium dialysis. Phenylmercuric acetate was prepared in dimethylformamide and added to cat haemoglobin (30 ml, 20 µM) in 0.1 M-KH₂PO₄, pH 7.3. The solution was left for 30 min before measurement of the tri-

ethyltin binding. The dimethylformamide was less than 2% (v/v) of the haemoglobin solution.

Preparation of cat haemoglobin subunits. Cat haemoglobin αβ dimers were prepared by treatment of cat haemoglobin in 0.1 M-KH₂PO₄ in the presence of 1.5 M-guanidine hydrochloride (Kawahara *et al.*, 1965; Guidotti, 1967) at the required pH. Treatment with diethyl pyrocarbonate was performed as described above.

Recording of the ¹¹⁹Sn Mössbauer spectrum of the cat haemoglobin-triethyltin complex. The Mössbauer spectrum for the cat haemoglobin-triethyltin complex was recorded in 0.09 M-Tris/HCl buffer, pH 7.8 at 20°C, at 77 K relative to a CaSnO₃ source at the Physico-Chemical Measurements Unit, AERE, Harwell, U.K., over a period of 2 days. The concentration of cat haemoglobin was 2.7 mM and of triethyltin 5.0 mM. A computer fit to the spectrum obtained, together with the derived values of the isomer shift and quadrupole splitting, was also provided by AERE Harwell.

Calculation of affinity constants. The affinity constants for the binding of triethyltin to cat haemoglobin after modification of the protein have been calculated directly from the straight-line Scatchard (1949) plots, which in the presence of competing ligands are complex. When the ratio of the total concentrations of measured and competing ligands are constant the following equation holds:

$$\frac{B}{F} = NK_{L_1} - B \left(K_{L_1} + K_{L_2} \frac{[L_2]}{[L_1]} \right)$$

L_1 and L_2 are the measured and competing ligand respectively; B and F are the concentrations of bound and free ligand L_1 ; K_{L_1} and K_{L_2} are the affinity constants for L_1 and L_2 respectively and N is the total number or concentration of binding sites.

The slope of the line relating B/F and B is $-(K_{L_1} + K_{L_2}[L_2]/[L_1])$, which is non linear except for the condition $K_{L_1} = K_{L_2}$. The affinity constants for the competing ligands (diethylphenyltin, Fig. 6; phenylarsenious acid, Figs. 7 and 9) have been calculated from each experimental point as described by Tipping *et al.* (1976). The mean affinity constant has then been used to calculate the expected relationship between B/F and B and is shown as the solid lines in the above Figures.

Results

Binding of triethyltin to chemically modified cat haemoglobin

Photo-oxidation with Methylene Blue. Rose (1969) showed that the photo-oxidation of rat haemoglobin in the presence of Methylene Blue resulted in the destruction of histidine, cysteine and methionine residues only, with loss of both binding sites for

Fig. 1. Effect of photo-oxidation on the binding of triethyltin to cat haemoglobin

Haemoglobin with 0.01% (w/v) Methylene Blue was exposed to light for 45 min (■), 90 min (□) and 135 min (▼). Control experiments: no treatment (●); exposure to light only (135 min) (○); exposure to dye only (135 min) (◐). Inset: the rate of loss of binding sites for these and other experiments.

triethyltin being progressive with increasing time of illumination. Similar treatment of cat haemoglobin also resulted in the progressive loss of both triethyltin-binding sites (Fig. 1), which obeyed first-order kinetics over 140 min illumination and led to the loss of 85% of the binding. The affinity of the remaining sites for triethyltin was similar to that of the untreated haemoglobin.

Treatment with iodoacetamide. Iodoacetamide is generally regarded as a reagent for modifying thiol groups in proteins, although histidine, methionine and lysine residues may also be affected (Webb, 1966a; Gundlach *et al.*, 1959). Treatment of cat haemoglobin with iodoacetamide (5–50 mM at pH 6–7) decreased the number of triethyltin-binding sites to 0.6 mol/mol of haemoglobin (Fig. 2). The rate of loss of both binding sites was first order with respect to iodoacetamide concentration and the affinity of those binding sites remaining after treatment was the same as in the untreated haemoglobin. These findings indicate that the loss of both sites is probably due to alkylation of a single class of site.

Treatment with phenylmercuric acetate. The alkyl and aryl mercurials are one of the more specific classes of chemical reagent for thiol groups in proteins (Webb, 1966b). Cat haemoglobin has eight thiol groups in each tetramer titratable with *p*-chloromercuribenzoate (Taketa *et al.*, 1968); the effect of preincubation of cat haemoglobin with increasing concentrations of phenylmercuric acetate on the

Fig. 2. Triethyltin binding to cat haemoglobin pretreated with iodoacetamide

●, Control. Treatment was with iodoacetamide at concentrations of 5 mM (■), 20 mM (○), 50 mM (□) or for 2 h at 50 mM (▼) (see under 'Methods').

Fig. 3. Triethyltin binding to cat haemoglobin pretreated with phenylmercuric acetate

●, Control. Treatment with phenylmercuric acetate (molar excess over haemoglobin): 3:1 (■); 5:1 (○); for 8:1 no binding was found.

triethyltin binding is shown in Fig. 3. Binding was progressively decreased until, when present at an 8-fold molar excess over the haemoglobin, it was absent.

Treatment with diethyl pyrocarbonate at pH 8.0. At pH values greater than 7, diethyl pyrocarbonate is able to react with cysteine, lysine, tyrosine and arginine residues, the reactivity being greater at pH values at or above the *pK* value of the particular amino acid residue (Muhlrad *et al.*, 1967). Treatment of cat haemoglobin with diethyl pyrocarbonate at

Fig. 4. Triethyltin binding to cat haemoglobin pretreated with diethyl pyrocarbonate at pH 8.0

Haemoglobin ($20\mu\text{M}$) was incubated with diethyl pyrocarbonate (5mM) at pH 8.0 for 2 (▼), 15 (■) and 20 (●) min. The broken line represents a typical plot for the binding of triethyltin to untreated haemoglobin. Derived affinity constant for the treated haemoglobin (20min) = $0.7 \times 10^4 \text{M}^{-1}$.

pH 8.0 for 15 min resulted in an increase in the number of triethyltin-binding sites per molecule of haemoglobin from 2.0 to 3.0, but with a lower affinity (0.7×10^4 – $2.5 \times 10^4 \text{M}^{-1}$) compared with untreated haemoglobin (3.5×10^4 – $4.0 \times 10^4 \text{M}^{-1}$) (Fig. 4). Treatment with diethyl pyrocarbonate for a shorter time period showed that triethyltin binding was initially decreased, but that treatment for longer times resulted in an increase in the number of binding sites to 3 (Fig. 4). All of these sites can be removed by treatment with iodoacetamide (10mM for 1 h at 18 – 20°C). The formation of a precipitate of modified haemoglobin similar to that obtained when rat haemoglobin was treated with diethyl pyrocarbonate at pH 6.0 for times in excess of 1 h (Elliott & Aldridge, 1977) was observed, although to a lesser extent. The binding data obtained with the partially precipitated haemoglobin shown in Fig. 4 suggest that two new triethyltin-binding sites are created after the removal of one of the two sites found on untreated haemo-

Fig. 5. Formulae of some intramolecularly pentaco-ordinated tin compounds based on diethylphenyltin bromide (see under 'Materials')

Table 1. Effect of treatment of cat haemoglobin with photo-oxidation or various reagents on the binding of triethyltin

Treatment	Residues affected	Binding sites lost	Equivalence of binding sites
Photo-oxidation	His, Cys, Met	2	Loss of sites is first order with time to 83% loss
Iodoacetamide	His, Cys, Met (Lys)	2	Loss of sites is first order with concentration to 60% loss
Phenylmercuric acetate	Cys	2	Loss of sites linear with concentration to 100% loss
Diethyl pyrocarbonate (pH 6.0)	His (Tyr)	1	—

Fig. 6. Competition of diethylphenyltin and intramolecularly pentaco-ordinated tin compounds with triethyltin for cat haemoglobin

The compounds used were those in Fig. 5 and were present at equimolar concentrations to the triethyltin: ●, control; ▼, (I); ○, (II); □, (III); ■, (IV). The compounds were dissolved in redistilled dimethylformamide (<2%, v/v, of the final solution) and exposed to the haemoglobin with the triethyltin. The derived affinity constant for (I) ($\pm$ s.d., four determinations; see under 'Methods'), is $2.7 \times 10^5 \pm 1.8 \times 10^5 \text{ M}^{-1}$ and the curve is that calculated for this constant.

Fig. 7. Binding of triethyltin to cat haemoglobin in the presence of phenylarsenious acid

(a) Untreated haemoglobin. ●, Control; ■, phenylarsenious acid added in 5:1 molar excess over triethyltin. (b) Haemoglobin treated with diethyl pyrocarbonate at pH 6.0 for 15 min. ●, Control; □, phenylarsenious acid added (5:1). The phenylarsenious acid was freshly prepared as described by Aldridge & Cremer (1955) and was exposed to the haemoglobin with the triethyltin. The derived affinity constants for phenylarsenious acid ($\pm$ s.d., five determinations; see under 'Methods') are: (a) $1.9 \times 10^4 \pm 0.5 \times 10^4$, and (b) $5.7 \times 10^3 \pm 2.1 \times 10^3 \text{ M}^{-1}$, and the solid lines are those calculated for these constants.

globin. After the incubation with diethyl pyrocarbonate for 2 and 15 min, the affinity of the remaining site was much decreased.

A summary of the data obtained from the action of various chemical reagents on cat haemoglobin able to cause loss of triethyltin binding is shown in Table 1. Reagents able to modify cysteine alone, or cysteine and histidine residues, are able to destroy both triethyltin-binding sites, whereas the modification of histidine residues alone leads to the loss of only one site. It follows that both cysteine and histidine residues are probably involved in the binding of triethyltin to cat haemoglobin.

Binding studies in the presence of intramolecularly pentaco-ordinate tin compounds. The compounds shown in Fig. 5 were examined for their ability to affect the binding of triethyltin to cat haemoglobin (Fig. 6). Only diethylphenyltin bromide ($K = 2.7 \times 10^5 \text{ M}^{-1}$) was able to alter the binding of triethyltin to cat haemoglobin, indicating competition at the binding sites; the intramolecularly pentaco-ordinate compounds were all inactive.

¹¹⁹Sn Mössbauer spectrum of the cat haemoglobin-triethyltin complex. The Mössbauer spectrum of the cat haemoglobin-(triethyltin)₂ complex was recorded

as described under 'Methods'. The spectrum was a simple doublet, indicating a single class of binding site; the values obtained for the isomer shift (1.48 mm/s) and the quadrupole splitting (1.74 mm/s) are consistent with a pentaco-ordinate tin atom, where the two donor ligands are at 90° to each other (Zuckerman, 1970; Ho & Zuckerman, 1973; Ruddick, 1976).

Binding studies in the presence of phenylarsenious acid and diethyltin. It is well known that phenylarsenious acid and diethyltin have an affinity for dithiol groups (Peters, 1948; Aldridge & Cremer, 1955). Fig. 7 shows that phenylarsenious acid can affect the triethyltin binding to both sites in untreated cat haemoglobin and to the one site remaining in cat haemoglobin after treatment with diethyl pyro-

Fig. 8. Triethyltin binding to cat haemoglobin $\alpha\beta$ subunits after treatment with diethyl pyrocarbonate

Haemoglobin in buffer containing 1.5M-guanidine hydrochloride was treated with diethyl pyrocarbonate (5mM) at pH 6.0 for 3 min ($\circ$), 15 min ($\bullet$), 15 min, and then after addition of further 5mM-diethyl pyrocarbonate was incubated for another 15 min (Δ). Treatment with diethyl pyrocarbonate at pH 8.0: ∇ , for 2 min; ∇ , for 7 min. $\blacksquare$, Control subunits (see under 'Methods'). Derived affinity constants for triethyltin: control $9.5 \times 10^4 \text{ M}^{-1}$, diethyl pyrocarbonate (pH 6.0) 3×10^4 – $4 \times 10^4 \text{ M}^{-1}$, diethyl pyrocarbonate (pH 8.0) $2 \times 10^4 \text{ M}^{-1}$.

carbonate at pH 6.0 (with a derived affinity constant for phenylarsenious acid of 2×10^4 and $6 \times 10^3 \text{ M}^{-1}$ respectively). Experiments with diethyltin and triethyltin indicated that the affinity constant for diethyltin was approximately $1 \times 10^2 \text{ M}^{-1}$ or less. Although the concentrations of phenylarsenious acid and diethyltin that inhibit the disulphide-requiring enzyme systems are much less than those indicated by the above affinity constants, phenylarsenious acid is much more active than diethyltin (20–30 times; Aldridge & Cremer, 1955).

Triethyltin binding to subunits of cat haemoglobin

A common feature of mammalian haemoglobins is their subunit structure. Under normal conditions of physiological pH and low to medium ionic strength, two α - and two β -chains are arranged tetrahedrally

Fig. 9. Binding of triethyltin to cat haemoglobin $\alpha\beta$ subunits in the presence of phenylarsenious acid

Binding to cat haemoglobin measured in buffer containing 1.5M-guanidine hydrochloride alone ($\blacksquare$), and in the presence of phenylarsenious acid (5:1 molar excess over triethyltin) ($\bullet$). The derived affinity constant ($\pm$ s.d., five determinations) for phenylarsenious acid (see under 'Methods') is $1.5 \times 10^4 \pm 0.6 \times 10^4 \text{ M}^{-1}$. The curve is that calculated for this constant.

to form a haemoglobin molecule possessing a twofold axis of symmetry (Perutz, 1965). Therefore any binding site for triethyltin within any subunit of the haemoglobin molecule must have either an identical counterpart on the opposite side of the molecule, across the axis of symmetry or the triethyltin must bind across the axis of symmetry. The observation of an apparent inequality of the two triethyltin-binding sites after treatment of cat haemoglobin with diethyl pyrocarbonate at pH 6.0 (Elliott & Aldridge, 1977) indicated the need to examine the binding of triethyltin to cat haemoglobin subunits: 2mol of triethyltin bind to 1mol of cat haemoglobin $\alpha\beta$ -dimers with an affinity constant more than that for the tetramer (Fig. 8). It therefore appears that there is an identical binding site for triethyltin on each $\alpha\beta$ -dimer. The effect of pretreatment of the cat haemoglobin subunits with diethyl pyrocarbonate at pH 6.0 and 8.0, on the binding of triethyltin, is also shown in Fig. 8. In these experiments the diethyl pyrocarbonate caused an extensive precipitation of the haemoglobin, increasing with the time of incubation. After treatment at pH 8 for 7 min (Fig. 8), the haemoglobin was almost all precipitated at the end of the dialysis, but a slight increase in the number of binding sites was observed. Treatment at pH 6.0 for up to 15 min resulted in a decrease in the number of binding sites by approximately one-half. After treatment for a longer time more binding was found. Phenylarsenious acid was found to compete with triethyltin for the untreated haemoglobin subunits (Fig. 9).

Fig. 10. Triethyltin binding to: (a) rat liver supernatant, (b)–(e) the Triton X-100-insoluble fraction from rat liver mitochondria and (f) rat brain myelin after pretreatment of the protein with diethyl pyrocarbonate at pH 6.0

B , Bound triethyltin (nmol/mg of protein), F , free triethyltin (μM). The fractions were incubated with diethyl pyrocarbonate (pH 6.0, 20–30 min) at concentrations: (a) untreated ($\blacktriangle$), 50 mM ($\bullet$); (b) untreated ($\blacktriangle$); (c) 25 mM (∇); (d) 50 mM ($\blacksquare$); (e) 100 mM ($\bullet$), two consecutive additions of 100 mM (∇); (f) untreated ($\blacktriangle$), 2.5 mM ($\circ$), 25 mM (∇). Binding was measured as described under 'Methods'.

Binding of triethyltin to other protein fractions after treatment with diethyl pyrocarbonate

Three other proteins to which triethyltin has been shown to bind, rat brain myelin (Lock & Aldridge, 1975), rat liver supernatant (Rose & Lock, 1970) and a fraction from rat liver mitochondria (Aldridge & Street, 1970), were treated with diethyl pyrocarbonate. The results, shown in Fig. 10, reflect the complex

nature of the biological material involved. After treatment of rat liver supernatant with diethyl pyrocarbonate, high-affinity triethyltin binding was almost absent (Fig. 10a). For the mitochondrial protein and myelin (Figs. 10b–10f), however, the data are not sufficiently accurate for detailed conclusions to be drawn. It appears, however, that with the mitochondrial protein only part of the high-affinity binding is removed even after extensive treatment with diethyl

pyrocarbonate, whereas with the myelin the affinity for triethyltin of the high-affinity class of sites is increased. It therefore seems unlikely that the high-affinity binding sites in these different preparations are chemically identical.

Discussion

The binding of triethyltin to cat haemoglobin pretreated with various selective chemical reagents (Table 1), and the apparent competition observed with both phenylarsenious acid (Fig. 7) and diethyltin, indicates a requirement for both histidine and cysteine residues in the binding sites. In reaching this conclusion we have regarded changes in stoichiometry without substantial changes in affinity constant as due to reaction at the binding sites rather than due to alterations in conformation (cf. Figs. 1, 2 and 3, and Fig. 3 in Elliott & Aldridge, 1977). The apparent inequality of the two sites found on untreated cat haemoglobin, after treatment with diethyl pyrocarbonate at pH 6.0 has been investigated by examining the binding of triethyltin to cat haemoglobin $\alpha\beta$ subunits. In a solution containing $\alpha\beta$ subunits of cat haemoglobin there cannot be an odd number of triethyltin-binding sites. If the sites are within either the α or the β subunits or even across the $\alpha\beta$ interface, an even number of sites must result. The data obtained (Fig. 8) may then be explained if reaction of the $\alpha\beta$ subunits with diethyl pyrocarbonate at pH 6.0 also uncovers the two sites normally exposed on the tetramer by treatment at pH 8.0. The number of sites seen at a given time (Fig. 8) will then be a composite of the control sites (found on untreated haemoglobin)

going from 2.0 to 0, and the sites normally exposed by treatment of the tetramer at pH 8.0 going from 0 to 2.0. It is therefore not possible to prove unequivocally that the $\alpha\beta$ subunits from cat haemoglobin do not contain the diethyl pyrocarbonate (pH 6.0)-insensitive site found on the tetramer.

The inability of the intramolecularly pentacoordinated tin compounds to influence the binding of triethyltin to cat haemoglobin, together with the interpretation of the Mössbauer spectrum obtained of the cat haemoglobin-triethyltin complex, supports the idea of the triethyltin being made pentacoordinate due to binding to two amino acid residues on the protein, as originally proposed by Rose (1969).

The evidence for the involvement of both histidine and cysteine residues in the triethyltin-binding sites together with the symmetry of the haemoglobin molecule has led to the proposal shown in Fig. 11. Attachments of triethyltin to the two residues of histidine and cysteine may be in two different ways. For example, each of the two molecules of triethyltin may bind to one histidine and one cysteine residue in each site. The other attachment, involving the two cysteine residues, allows the binding of only one triethyltin molecule per molecule of haemoglobin. It is an integral part of this hypothesis that, in untreated cat haemoglobin, the two histidine/cysteine sites are involved in binding in preference to the cysteine/cysteine site, since binding to the two classes of site is mutually exclusive. The lower affinity of the one diethyl pyrocarbonate (pH 6.0)-insensitive site for triethyltin allows for this (affinity before treatment $3.5 \times 10^4 \text{ M}^{-1}$, Elliott & Aldridge, 1977; after treatment $1 \times 10^4 \text{ M}^{-1}$). Once one molecule of tri-

Fig. 11. Diagrammatic representation of proposed binding sites for triethyltin in cat haemoglobin before and after treatment with diethyl pyrocarbonate at pH 6.0

ethyltin has bound to a histidine/cysteine site then the second site must be automatically taken. With all three modes of attachment having an equal affinity for triethyltin we would still expect an observed value of approximately 1.7 mol of triethyltin bound per mol of haemoglobin. Thus the difference of at least 3-fold in the affinity would lead within experimental error to the determined 2 mol of triethyltin per mol of untreated haemoglobin. The two sites in untreated haemoglobin are identical, and it is only after treatment with diethyl pyrocarbonate (pH 6.0), when binding to both of these sites is lost due to modification of the histidine component, that the latent binding site consisting of the two remaining cysteine residues can express itself.

Phenylarsenious acid has an affinity for dithiol groups, e.g. 2,3-dimercaptopropanol (Aldridge & Cremer, 1955) and lipoic acid (Peters, 1948). The affinity of phenylarsenious acid for the triethyltin-binding sites on cat haemoglobin (Figs. 7 and 9) is lower than would be expected if phenylarsenious acid were exerting its effect by interaction with two thiol groups in the correct spatial configuration. Aldridge & Cremer (1955) examined the ability of several arsenic and tin compounds to interact with thiol-containing compounds, utilizing a technique involving the decolouration of a dye, a process requiring free thiol groups. Assuming the rate of decolouration to be directly related to the concentration of free thiol groups present, approximate affinity constants of 5.9×10^3 and $5.7 \times 10^2 \text{ M}^{-1}$ for the interaction of phenylarsenious acid and diethyltin respectively with glutathione may be derived. These values are similar to those obtained from the present competition studies with triethyltin binding to cat haemoglobin, and suggest that the effect of the phenylarsenious acid and diethyltin may be due to an interaction with monothiol components at the binding site. On the basis of the nature of the triethyltin-binding sites postulated in Fig. 11, the above reasoning means that the phenylarsenious acid and diethyltin are unable to interact with the two cysteine residues as a dithiol unit, only as monothiol components. This might be due to the incorrect positioning of the two cysteine residues for the preferred dithiol interaction; if the configuration were such as to allow the triethyltin to pentaco-ordinate with the thiol groups as axial ligands, this might be expected.

Treatment of cat haemoglobin with diethyl pyrocarbonate at pH 8.0 results in the observation of three triethyltin-binding sites per molecule of haemoglobin (Fig. 4). The proposal that these sites are composed of the one diethyl pyrocarbonate (pH 6.0)-insensitive site and two new sites requires that the cysteine residues of the former are largely unreactive to the diethyl pyrocarbonate at pH 8 (Muhlrad *et al.*, 1967). This may be due to their having a different pK value from the free amino acid cysteine. The chemical

nature of the other two sites is unknown, although treatment with iodoacetamide (10 mM for 1 h at 18–20°C) removes all three binding sites. The straight-line nature of the Scatchard plots (Fig. 4) also shows that these three sites bind triethyltin with a similar affinity.

The reduction in the high-affinity class of binding sites for triethyltin after treatment of rat liver supernatant with diethyl pyrocarbonate at pH 6.0 (Fig. 10) suggests that the binding to the protein or proteins in this fraction may also involve histidine residues, possibly in sites similar to those on cat haemoglobin. The results obtained from the treatment of rat brain myelin and the Triton X-100-insoluble fraction from rat liver mitochondria with diethyl pyrocarbonate at pH 6.0 at present, however, cannot be interpreted. It is clear that to investigate further the chemical nature of the triethyltin-binding sites in the liver and myelin fractions, the proteins responsible for binding the triethyltin must be purified further.

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