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Evidence for Histidine in the Triethyltin-Binding Site of Rat Haemoglobin

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One molecule of rat haemoglobin binds two molecules of triethyltin. The binding sites are located on the globin and there is co-operativity between the sites such that the intrinsic affinity constant at pH 8·0 increases from $3{\cdot}5 \times 10^5 M^{-1}$ for the binding of the first triethyltin molecule to $5{\cdot}0 \times 10^5 M^{-1}$ for the binding of the second. Evidence is presented, from pH studies and the kinetics of inhibition due to photo-oxidation, that each binding site contains two histidine residues.

Mitochondrial oxidative phosphorylation is 50% inhibited *in vitro* by concentrations of triethyltin as low as 0·3 μM (Aldridge, 1958; Aldridge & Threlfall, 1961; Moore & Brody, 1961; Sone & Hagihara, 1964). This indicates that triethyltin may have an affinity for a site or sites in the mitochondrial oxidative-phosphorylation system of the order of $10^6 M^{-1}$. Triethyltin is chemically inert in aqueous systems, having little affinity for the thiol groups of GSH and 2,3-dimercaptopropanol (Aldridge & Cremer, 1955), for EDTA (Aldridge & Cremer, 1957), or for a variety of other compounds (Aldridge & Street, 1964). Thus the biochemical effects of triethyltin have yet to be explained in terms of any chemical reactivity.

Investigations have been carried out in an attempt to understand the way in which this simple organometal compound interacts with biological materials. A study of the interaction of triethyl-[^{113}Sn]tin with tissue and tissue components showed that it binds to many tissue fractions and to rat haemoglobin (Rose & Aldridge, 1968). It was also shown that triethyltin has little affinity for other mammalian haemoglobins and a variety of pure proteins. This paper reports investigations into the chemical nature of the interaction between triethyltin and rat haemoglobin.

MATERIALS AND METHODS

Special chemicals. Triethyl[^{113}Sn]tin and trimethyl-[^{113}Sn]tin were purchased from The Radiochemical Centre, Amersham, Bucks., in the form of chloride salts. The specific radioactivities were 6 mc/m-mole and 4 mc/m-mole respectively. Stock 0·2 M solutions in ethanol were diluted with water to give 2 mM solutions for protein-binding studies.

Rat haemoglobin was prepared by lysing with water erythrocytes that had been washed three times with 0·9% NaCl. The lysate was centrifuged for 5 min. to remove stroma and unbroken cells. Crystals of oxyhaemoglobin settled out when the lysate was allowed to stand (Aldridge, 1951). These crystals were stored at −25° and used in the carbon monoxide form. The concentration of carbon monoxide–haemoglobin solutions was estimated spectrophotometrically at 540 mμ by using a molar extinction coefficient of $5{\cdot}96 \times 10^4$ cm.$^{-1}$ (Snow, 1962).

Equilibrium dialysis. Visking tubing (Vicase Development Co., London, W.C. 2) was repeatedly boiled in water until no further yellow material was extracted. Solutions of haemoglobin (3 or 5 ml.) were dialysed in sacs of Visking tubing against buffer (5 ml.) containing triethyl[^{113}Sn]tin. The half-time for free triethyltin to diffuse across the membrane was found to vary from 100 min. to 40 min. with a batch of Visking tubing, and dialysis was therefore carried out overnight (15 hr.) at 5°, with shaking, to ensure equilibration. The recovery of triethyl[^{113}Sn]tin in dialysis experiments was always 95–100%.

Measurement of ^{113}Sn. Triethyl[^{113}Sn]tin was monitored as described by Rose & Aldridge (1968).

Buffers. Generally 0·1 M-tris–HCl, pH 8·0 at either 5° or 20°, was used; NaCN at a final concentration of 1 μg./ml. was included in some experiments, especially when reactive thiol groups were to be determined. In experiments where a single buffer covering the range pH 5–9 was required, tris acid maleate–NaOH was used (Long, 1961).

Procedure for photo-oxidation. Samples of haemoglobin (10 ml. of approx. 80 μM) in 0·1 M-tris–HCl buffer, pH 8·0 at 20°, containing either 0·01% of methylene blue (British Drug Houses Ltd., Poole, Dorset; laboratory reagent, biologically tested) or 0·03% of rose bengal, were placed in a 50 ml. beaker surrounded by aluminium foil as a reflector. The beaker was placed in a shaking bath containing a mixture of ice and water, and a 150 w spotlight (Philips Comptalux Flood) was mounted 12 cm. above the surface of the haemoglobin. A Perspex shield was placed between the haemoglobin and the lamp to deflect the heat, and cold air was blown across the top of the shield. After irradiation for a given time (5–60 min.), the haemoglobin was divided into two portions: one for binding analysis, and one for globin preparation before protein hydrolysis. Methylene blue was removed from one portion of the haemoglobin solution (4 ml.) by passage down a Sephadex G-25 column

(Pharmacia, Uppsala, Sweden,) equilibrated in 0·1 M-tris–HCl buffer, pH 8·0. The ability of this haemoglobin to bind triethyltin was then tested by equilibrium dialysis, after dilution to a concentration of 20 μM. The concentration was calculated by assuming 100% recovery from the Sephadex column, since after prolonged photo-oxidation the extinction at 540 mμ was no longer an accurate measure of the concentration of protein, owing to the formation of ferrihaemoglobin.

Preparation of globin. Methylene blue and buffer salts were removed from a portion (5 ml.) of the photo-oxidized haemoglobin by passage down a column of Sephadex G-25 equilibrated in water. The most concentrated fraction of the haemoglobin effluent (4 ml.) was dripped, with vigorous stirring, into 40 ml. of acid–acetone (made by mixing 100 ml. of acetone and 0·3 ml. of 2 N-HCl) cooled in a bath of solid CO_2 and methylated spirits at −80° (Rossi-Fanelli, Antonini & Caputo, 1958). The solution was allowed to warm up to room temperature with continuous stirring, and the white globin precipitate was collected by centrifugation. The globin was washed at room temperature once with acid–acetone, twice with ethanol and once with diethyl ether, and dried under vacuum.

Determination of amino acids. Samples of globin (4–6 mg.) were hydrolysed with twice-glass-redistilled 6 N-HCl (0·2 ml.) at 108° for 24 hr. in sealed tubes under N_2. The HCl was removed under vacuum and the residue was taken up in 0·1 N-HCl. A sample of this solution, equivalent to 0·3–0·4 mg. of original globin, was then analysed on an automatic amino acid analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey) by using a standard 21 hr. gradient with buffers of pH 2·88, 3·80 and 5·00. Reactive thiol groups remaining after photo-oxidation were determined on haemoglobin in the presence of the dyes with *p*-chloromercuribenzoate (Boyer, 1954) by the method of Ray & Koshland (1962). Cysteine was determined in the globin hydrolysate by the method of Gaitonde (1967). Tryptophan and tyrosine were determined spectrophotometrically on the globin as described by Beaven & Holiday (1952). Methionine was measured by using the amino acid analyser after basic hydrolysis (Ray & Koshland, 1962), since the photo-oxidation products of methionine are reconverted into methionine by acid hydrolysis.

RESULTS AND DISCUSSION

Analysis of binding. The Scatchard equation (1):

$$\bar{v}/A = K(n-\bar{v}) \qquad (1)$$

(where $\bar{v}$ is the concentration of bound ligand/mole of protein, n is the concentration of total binding sites/mole of protein, A is the concentration of free ligand and K is the intrinsic association constant) predicts that, with one class of binding sites with the same intrinsic affinity constant, a plot of $\bar{v}/A$ against $\bar{v}$ should be linear with a slope $-K$ and an intercept of n when $\bar{v}/A = 0$ (Scatchard, 1949; Edsall & Wyman, 1958).

The results of such analyses of the binding of triethyl[^{113}Sn]tin and trimethyl[^{113}Sn]tin to rat carbon monoxide–haemoglobin are not linear (Figs. 1c and 2). The type of non-linear behaviour

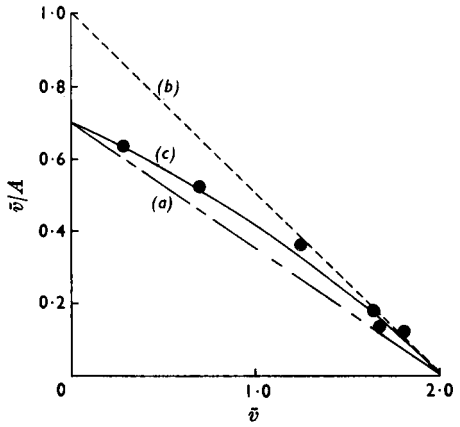


Fig. 1. Scatchard analysis of the binding of triethyltin to rat carbon monoxide–haemoglobin at pH 8·0. $\bar{v}$, Concentration of bound triethyltin (moles/mole of carbon monoxide–haemoglobin); A, concentration of free triethyltin (μM). The curve (*c*) and the lines (*a*) and (*b*) are calculated (see the text); $\bar{v}$ and A were measured by equilibrium dialysis as described in the Materials and Methods section. The concentration of haemoglobin was 20 μM, and the concentrations of triethyltin were 2·5–25 μM.

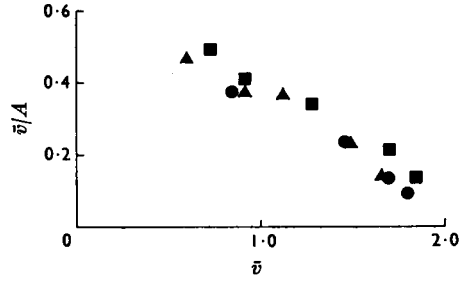


Fig. 2. Binding of trimethyltin to rat haemoglobin at pH 8·0. $\bar{v}$, Concentration of bound trimethyltin (moles/mole of haemoglobin); A, concentration of free trimethyltin (μM); ●, carbon monoxide–haemoglobin, calculated $K_{ass.}$ $2·8 \times 10^5$ M^{-1}; ■, oxyhaemoglobin, calculated $K_{ass.}$ $3·2 \times 10^5$ M^{-1}; ▲, ferrihaemoglobin, calculated $K_{ass.}$ $2·9 \times 10^5$ M^{-1}. $\bar{v}$ and A were measured by equilibrium dialysis as described in the Materials and Methods section. The concentration of haemoglobin was 20 μM, and the concentrations of trimethyltin were 6–40 μM.

observed is not consistent with there being more than one class of binding sites, since with more than one class of sites the slope of the plot decreases with increasing values of $\bar{v}$ (Edsall & Wyman, 1958). An increasing slope with increasing values of $\bar{v}$ is predicted if it is assumed that there are two sites for triethyltin per molecule of haemoglobin and there

is interaction between them such that the binding of triethyltin to one site on a molecule of haemoglobin increases the intrinsic affinity constant of the remaining site. The interaction of triethyltin with haemoglobin can be written in general terms as:

$$Hb + L \rightleftharpoons HbL; \quad K_1 = \frac{[HbL]}{[Hb][L]} \qquad (2)$$

$$HbL + L \rightleftharpoons HbL_2; \quad K_2 = \frac{[HbL_2]}{[HbL][L]} \qquad (3)$$

where Hb is free haemoglobin, L is free triethyltin, HbL is haemoglobin with one site occupied, HbL_2 is haemoglobin with both sites occupied, and K_1 and K_2 are the macroscopic constants for the two steps and are related to the intrinsic or microscopic affinity constant K (Scatchard affinity constant) by the following relationships (Edsall & Wyman, 1958):

$$K_1 = 2K \qquad (4)$$

$$K_2 = K/2 \qquad (5)$$

Bound triethyltin measured experimentally, B, will be given by the expression:

$$B = HbL + 2HbL_2$$

and by using eqns. (2) and (3) it can be shown that:

$$B = \frac{K_1[Hb]_0[L](1 + 2K_2[L])}{1 + K_1[L](1 + K_2[L])} \qquad (6)$$

where $[Hb]_0$ is the total concentration of all forms of haemoglobin. Thus:

$$\bar{v} = B/[Hb]_0 = \frac{K_1[L](1 + 2K_2[L])}{1 + K_1[L](1 + K_2[L])} \qquad (7)$$

Given values for $[Hb]_0$, K and [L], by using relationships (4) and (5), this expression for $\bar{v}$ can be used to construct theoretical Scatchard lines. If $[Hb]_0 = 20\,\mu M$ and the intrinsic affinity constant, K, is taken to be $3{\cdot}5 \times 10^5\,M^{-1}$, then $K_1 = 7{\cdot}0 \times 10^5\,M^{-1}$, $K_2 = 1{\cdot}75 \times 10^5\,M^{-1}$ and a straight line (Fig. 1*a*) is obtained. By taking $K = 5{\cdot}0 \times 10^5\,M^{-1}$, then $K_1 = 1 \times 10^6\,M^{-1}$, $K_2 = 2{\cdot}5 \times 10^5\,M^{-1}$, and a straight line of steeper slope is obtained, having the same intercept for n (Fig. 1*b*). If, however, it is assumed that K initially has the value $3{\cdot}5 \times 10^5\,M^{-1}$ then $K_1 = 7{\cdot}0 \times 10^5\,M^{-1}$. If, owing to co-operative interaction, the remaining site has an increased intrinsic affinity constant of $5{\cdot}0 \times 10^5\,M^{-1}$, then $K_2 = 2{\cdot}5 \times 10^5\,M^{-1}$ and a curve is obtained (Fig. 1*c*) that fits the experimental results reasonably well. An increase in K can therefore account for the non-linearity of the plot.

Since the co-operative effect is small, it was neglected for the purposes of routine binding analysis and the results were analysed by the method of least squares to give a regression line. Such an analysis of the results in Fig. 1 gave $K = 3{\cdot}5 \times 10^5\,M^{-1}$ and $n = 2{\cdot}16$ (instead of the correct value of 2·0).

Binding of trimethyltin to rat haemoglobin. Trimethyltin binds to carbon monoxide–haemoglobin with a smaller affinity constant ($2{\cdot}8 \times 10^5\,M^{-1}$; Fig. 2) than triethyltin. Oxy- and ferrihaemoglobin bind trimethyltin equally as well as the carbon monoxide derivative (Fig. 2), indicating that the state of the haem moiety has little effect on the binding. This result, together with the selectivity exhibited by triethyltin towards rat haemoglobin rather than other mammalian haemoglobins, and the presence of two binding sites/molecule rather than four, suggests that the binding sites are located on the globin.

Effect of ionic strength on the binding of triethyltin to haemoglobin. Various concentrations of sodium chloride, tris, sulphate and arsenate had no effect on the amount of triethyltin bound by haemoglobin, but increased concentrations of phosphate appeared to inhibit the binding (Fig. 3). Phosphate was subsequently shown to be a competitive inhibitor in the system (Fig. 4). An explanation of this was sought, and distribution studies of triethyl[^{113}Sn]tin between chloroform and

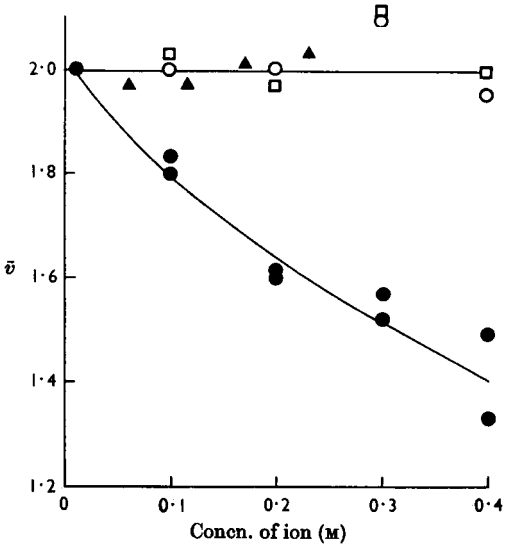


Fig. 3. Effect of ionic strength on the binding of triethyltin to rat haemoglobin. $\bar{v}$, Concentration of triethyltin (moles bound/mole of haemoglobin). The ions whose concentration was varied were: ●, phosphate, pH 7·4; ▲, tris, pH 8·0; ○, sulphate, in 0·2 M-tris–HCl, pH 8·0; □, arsenate, in 0·2 M-tris–HCl, pH 8·0. $\bar{v}$ was measured by equilibrium dialysis as described in the Materials and Methods section. The concentration of haemoglobin was 10 μM, and the concentration of triethyltin was 40 μM.

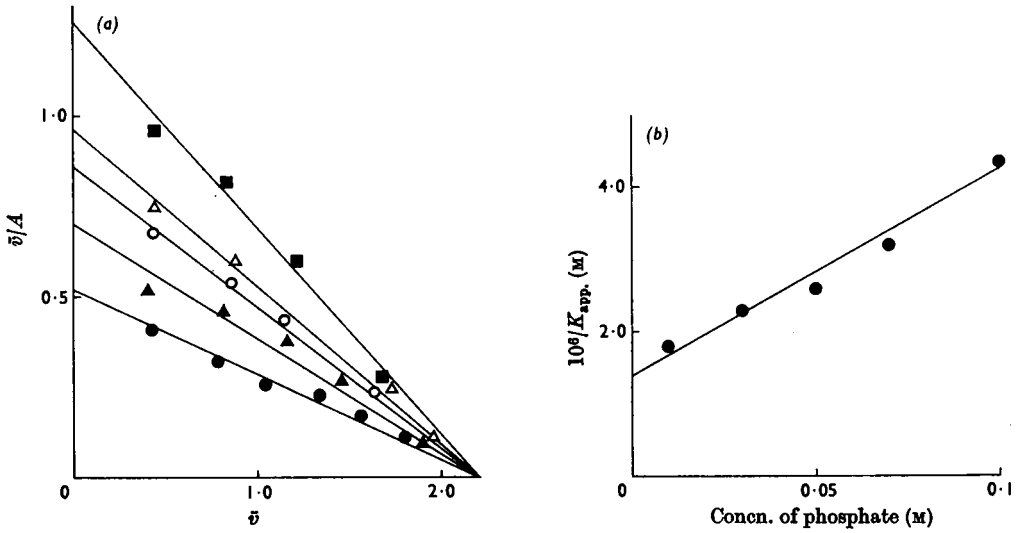


Fig. 4. Competitive inhibition of triethyltin binding to rat haemoglobin by phosphate buffers, pH 7·5. (*a*) Scatchard analyses: ●, 0·10 M-phosphate; ▲, 0·07 M-phosphate; ○, 0·05 M-phosphate; △, 0·03 M-phosphate; ■, 0·01 M-phosphate. In (*b*): ●, concn. of phosphate. $\bar{v}$ and A were defined and measured as described in Fig. 1. The concentration of haemoglobin was 20 μM, and the concentrations of triethyltin were 5–70 μM. Straight lines were fitted to the results by the method of least squares.

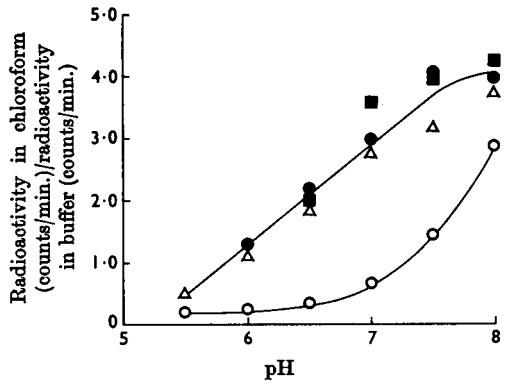
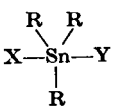


Fig. 5. Distribution of triethyltin between chloroform and buffers of various pH values. The buffers were: ○, 0·2 M-phosphate; ●, 0·1 M-tris–H_2SO_4; △, 0·1 M-imidazole; ■, 0·1 M-pyrophosphate. Triethyl[^{118}Sn]tin (0·1 ml. of a 2 mM solution) was added to 3 ml. of chloroform and 3 ml. of buffer. After being shaken for 1 min. in a mechanical shaker, the phases were separated by centrifugation.

various buffers demonstrated that with phosphate the organotin concentration in chloroform is decreased, presumably through complex-formation with buffer phosphate ions (Fig. 5). The competitive behaviour of phosphate probably arises from this effect.

The lack of effect of high ionic strength on the binding suggests that the binding forces involved are not primarily ionic in character.

Effect of urea on binding. Pretreatment of haemoglobin for 24 hr. at room temperature with urea, buffered at pH 8·0 with tris, inhibited the binding of triethyltin, complete inhibition being obtained after treatment with 7–8 M-urea. This concentration of urea would be expected to denature the structure extensively (Simko & Kauzmann, 1962), and this suggests that the binding site for triethyltin is highly dependent on the three-dimensional structure of the protein and may consist of more than one residue. There is much evidence that organotin compounds in general form penta-co-ordinate and hexa-co-ordinate complexes (Pollar, 1965). Trialkyltin compounds predominantly form penta-co-ordinate complexes with only two donors. It is therefore possible that in the haemoglobin–triethyltin or –trimethyltin complexes the tin is penta-co-ordinate, and the system may have a structure of the type (I), where R is



(I)

ethyl or methyl, and X and Y represent amino acid residues in the protein.

Effect of pH *on binding.* The affinity of haemoglobin for triethylin and trimethyltin was markedly affected by pH (Fig. 6). The results for both compounds fit the same theoretical dissociation curve for a single group with pK 7·1. Triethyltin and trimethyltin act as weak acids according to the equilibrium:

$$R_3Sn^+(H_2O)_m \rightleftharpoons R_3SnOH(H_2O)_{m-1} + H^+$$

and have different pK values, 6·8 and 6·6 respectively (Tobias, 1966). These pK values are not reflected in the results of Fig. 6, and it is therefore probable that the binding is dependent on a group in the protein, of pK 7·1, required in the unprotonated form at the binding site, and is independent of the protonic equilibria of the organotin hydrated cations themselves. It is not possible to identify amino acid residues in proteins solely from their pK values owing to the large perturbations from normal values sometimes observed. However, a pK of 7·1 could be due to an imidazole group of histidine (Bucci, Fronticelli & Ragatz, 1968) or to an N-terminal α-amino group (Tanford & Nozaki, 1966).

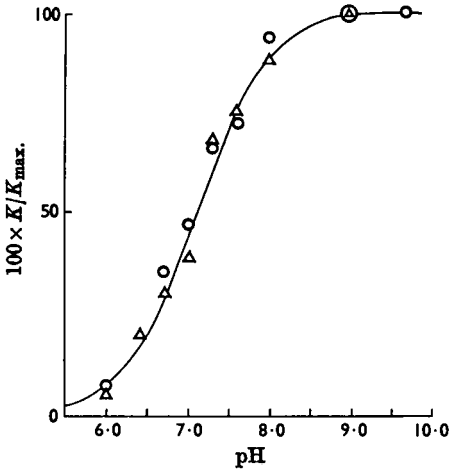


Fig. 6. Effect of pH on the binding of triethyltin and trimethyltin to rat carbon monoxide–haemoglobin. ○, Triethyltin; △, trimethyltin. Each point was obtained from a complete Scatchard binding analysis at the appropriate pH, as described in the Materials and Methods section. The curve is calculated for the dissociation of a single group with pK 7·1.

Effect of photo-oxidation on binding. The kinetics of dye-catalysed photo-oxidation were used as a technique for the investigation of the mechanism of enzyme action by Ray & Koshland (1962). These authors discussed extensively the parameters of the technique as well as the general theory behind the use of the kinetics of amino acid modification for

Table 1. *Amino acid analyses of rat haemoglobin after photo-oxidation with methylene blue*

Amino acid values calculated from the analyser were normalized to the value of tyrosine determined spectrophotometrically on intact globin (see the Materials and Methods section).

	Amino acid composition (moles of amino acid/62000 g. of globin)							
Time after photo-oxidation (min.) ...	Control	5	10	15	20	30	40	60†
Aspartate	51·3	47·2	47·4	50·7	50·5	48·2	53·7	46·5
Threonine	21·6	20·9	21·9	25·9	23·1	23·2	23·4	21·9
Serine	30·7	26·7	26·5	31·6	28·8	29·5	28·1	28·6
Glutamate	28·7	24·9	25·0	28·2	26·1	27·8	27·9	27·1
Proline	17·5	16·7	19·0	21·0	17·4	19·7	22·2	18·3
Glycine	41·9	39·0	39·0	41·8	40·4	40·6	42·4	39·5
Alanine	56·6	55·3	52·2	58·5	54·6	56·3	56·4	55·0
Valine	37·5	36·2	34·3	38·5	35·6	37·4	37·4	36·8
Cysteine	8·1	7·4	—	7·3	7·2	6·9	6·7	6·8
Methionine	6·0*	4·7	—	4·5	4·2	4·2	3·9	2·8
Isoleucine	11·9	10·3	9·9	11·4	10·8	10·8	11·1	10·2
Leucine	54·7	52·6	52·0	55·3	54·0	52·3	54·8	51·0
Tyrosine	10·0	10·0	9·9	10·0	10·0	9·9	9·3	9·4
Tryptophan	5·9	5·6	5·6	5·7	5·6	5·8	5·9	5·3
Phenylalanine	25·4	24·4	23·9	25·3	23·5	24·9	24·5	24·2
Lysine	41·7	42·0	41·2	41·7	40·3	39·9	40·8	40·2
Histidine	34·2	30·0	28·1	27·0	23·9	20·1	18·2	11·9
Arginine	12·0	10·5	9·9	11·5	10·6	11·3	11·1	9·9
Reactive thiol	6·0	5·5	5·0	—	4·0	—	2·7	—

* By acid hydrolysis.

† Results from Expt. (2) (Table 2).

Table 2. *First-order rate constants for loss of binding and affected amino acids*

All the experiments (nos. 1–5) were carried out under identical conditions (see the Materials and Methods section) with methylene blue, but with different preparations of haemoglobin. Each rate constant is derived from four to six separate determinations as described in the Materials and Methods section.

	k (min.$^{-1}$)				
Expt. no. ...	(1)	(2)	(3)	(4)	(5)
Loss of binding	0·028	0·031	0·031	—	—
Loss of histidine	0·015	0·015	—	—	—
Loss of methionine	0·005	0·010	—	—	—
Loss of thiol	—	—	0·021	0·025	0·019
Loss of cysteine to cysteic acid	0·003	0·004	—	—	—

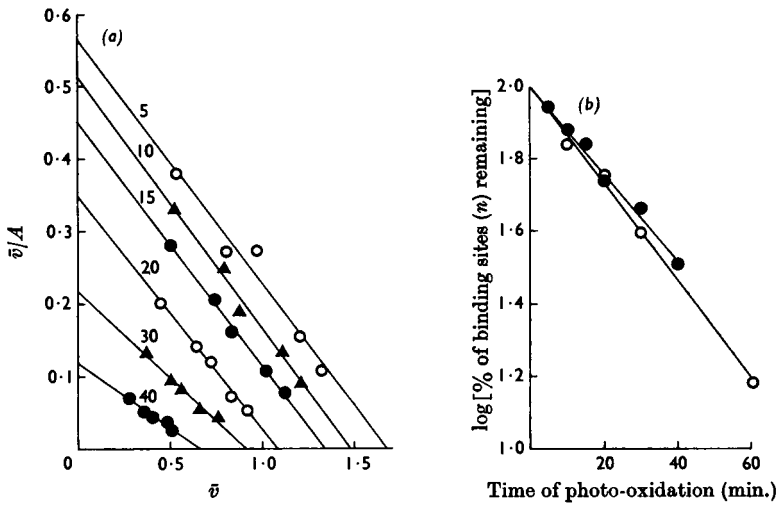


Fig. 7. Effect of photo-oxidation with methylene blue on the binding of triethyltin to rat haemoglobin. $\bar{v}$ and A were defined and measured as described in Fig. 1. (*a*) Scatchard analyses: the numbers on the lines give the number of minutes for which the haemoglobin was photo-oxidized, as described in the Materials and Methods section. (*b*) Loss of binding sites: ●, Expt. 1, $k=0{\cdot}028$ min.$^{-1}$; ○, Expt. 2, $k=0{\cdot}031$ min.$^{-1}$. Straight lines were fitted to the results by the method of least squares.

identifying those residues involved in enzymic activity (Ray & Koshland, 1961). The only amino acids susceptible to photo-oxidation are tyrosine, tryptophan, histidine, methionine and cysteine (Weil, Gordon & Buchert, 1951).

Only histidine, methionine and thiol groups were lost (Table 1) when rat haemoglobin was photo-oxidized under the mild conditions used. The number of triethyltin-binding sites/molecule of haemoglobin (n) was decreased by photo-oxidation and the affinity of the remaining sites (measured by the slopes of the Scatchard analyses) was not grossly affected (Figs. 7*a* and 8*a*). The loss of binding sites was first-order (Figs. 7*b* and 8*b*) even, in one experiment, as far as 85% destruction (Fig. 7*b*). This suggested that loss of binding is not due to non-specific denaturation of the protein, but is due to destruction of amino acids at the binding sites.

The losses of histidine, thiol and methionine were also first-order (Fig. 9) but the rate of loss of methionine in two experiments varied by a factor of 2 (Table 2). In experiment (2), the affinity of photo-oxidized haemoglobin for triethyltin decreased with the time of exposure to light, the affinity after 20min. exposure being 60% of the initial value. It may well be that the differing rates of loss of methionine in experiments (1) and (2) can be correlated with this difference in stability towards photo-oxidation. However, the measured rates of loss of binding in these experiments were approximately the same (Table 2).

It can be shown that if two residues, X and Y,

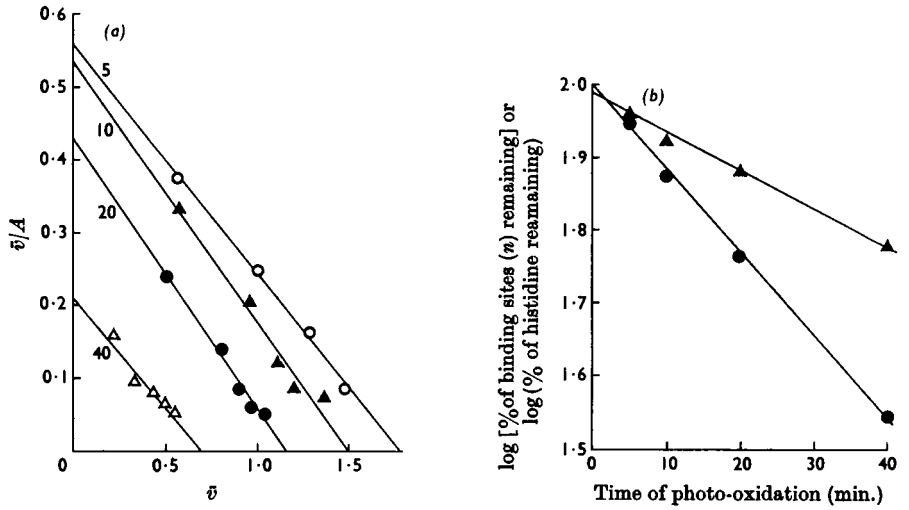


Fig. 8. The effect of photo-oxidation with rose bengal on the binding of triethyltin to rat haemoglobin. $\bar{v}$ and A were defined and measured as described in Fig. 1. (*a*) Scatchard analyses: the numbers on the lines give the number of minutes for which the haemoglobin was photo-oxidized, as described in the Materials and Methods section. (*b*) Loss of binding sites and histidine: ▲, histidine, $k_{\text{histidine}}=0{\cdot}012\,\text{min.}^{-1}$; ●, binding sites, $k_{\text{binding}}=0{\cdot}026\,\text{min.}^{-1}$. Straight lines were fitted to the results by the method of least squares.

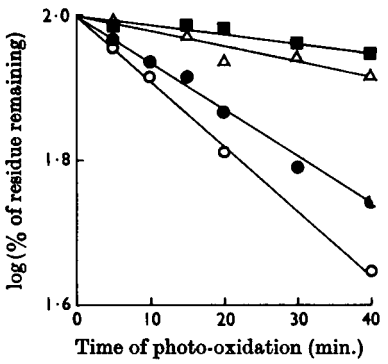


Fig. 9. Loss of affected amino acid residues after photo-oxidation with methylene blue. ■, Oxidation of thiol to cysteic acid, $k=0{\cdot}003\,\text{min.}^{-1}$; △, loss of methionine, $k=0{\cdot}005\,\text{min.}^{-1}$; ●, loss of histidine, $k=0{\cdot}015\,\text{min.}^{-1}$; ○, loss of reactive thiol, $k=0{\cdot}021\,\text{min.}^{-1}$. Straight lines were fitted to the results by the method of least squares.

Table 3. *Effect of dithiothreitol on reactive thiol groups in photo-oxidized haemoglobin*

	No. of reactive thiol groups/molecule
Control haemoglobin	6·4
10 min.-photo-oxidized haemoglobin	4·1
10 min.-photo-oxidized dithiothreitol-treated haemoglobin	5·9

are involved at a binding site such that modification of either X or Y destroys the ability of the site to bind, then:

$$k_{\text{binding}} = k_X + k_Y$$

where k_{binding} is the first-order rate constant for loss of binding, and k_X and k_Y are the first-order rate constants for the modification of residues X and Y respectively (Ray & Koshland, 1961). Since only histidine, thiol and methionine are destroyed, some combination of these groups must be present at the triethyltin-binding site. Taking the means of the rate constants measured (Table 2) the most feasible combinations that fit the above relationship are one methionine residue and one reactive thiol group ($0{\cdot}0075+0{\cdot}022=0{\cdot}0295$), or two histidine residues ($0{\cdot}015+0{\cdot}015=0{\cdot}03$).

Treatment of photo-oxidized haemoglobin with dithiothreitol resulted in substantial restoration of reactive thiol groups that had been oxidized to disulphide (Table 3). Binding sites were not restored, however (Fig. 10), as would have been expected (according to the argument that follows) if a methionine–reactive thiol pair were involved in the binding. The inactive species present after photo-oxidation would be (*a*) M*–SH, (*b*) M–SH* and (*c*) M*–SH* (where M indicates methionine and * indicates oxidation). If methionine and thiol were

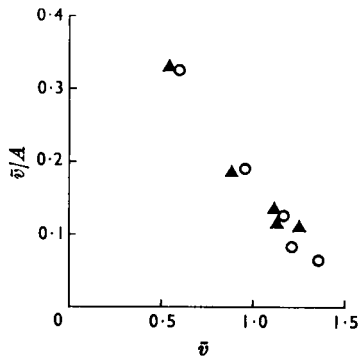


Fig. 10. Binding of triethyltin to photo-oxidized haemoglobin after treatment with dithiothreitol. ○, Haemoglobin photo-oxidized for 10min. in the presence of methylene blue; computed intercept (n) 1·50. ▲, After restoration of reactive thiol with dithiothreitol; computed intercept (n) 1·53.

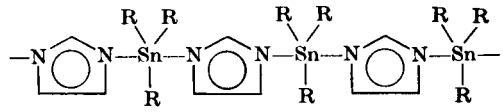
lost at the same rate, the proportions of the species (a), (b) and (c) present would be 1:1:0·5. Regeneration of oxidized thiol would lead to species (b) becoming active, i.e. to 40% regeneration of inactive binding sites. Since reactive thiol is lost at least twice as fast as methionine, even greater regeneration would be expected. The results of Fig. 10 clearly show that regeneration of binding sites does not occur when reactive thiol is 80% regenerated. The combination of one methionine residue and one reactive thiol group at the triethyltin-binding site is therefore not possible. Thus the results can be interpreted to indicate that there are two histidine residues at each binding site. However, it is also possible that each binding site contains only one histidine residue and another residue not affected by photo-oxidation. Each 'binding-site histidine residue' would then have to be destroyed at the same rate as binding is lost ($k = 0{\cdot}03\,\text{min.}^{-1}$), the remaining 32 histidine residues in the molecule being destroyed more slowly, at the rate observed ($0{\cdot}015\,\text{min.}^{-1}$). As a corollary of this interpretation, the relationship:

$$2k_{\text{histidine}} = k_{\text{binding}}$$

must be coincidental. The techniques employed are not accurate enough to be able to detect the existence of two such unique histidine residues among the bulk of slower-reacting histidine residues.

However, photo-oxidation was carried out with both methylene blue (Fig. 7) and rose bengal (Fig. 8), a cationic and an anionic dye respectively. With both dyes, loss of binding and histidine were first-order and the relationship:

$$k_{\text{binding}} = 2k_{\text{histidine}}$$



(II)

was found. It seems highly improbable that this would be so if each binding site contained only one histidine residue, which behaved differently from the remaining histidine residues in the molecule.

The most reasonable interpretation of these results is that two histidine residues are directly involved at each triethyltin-binding site in rat haemoglobin.

The pH-dependence of triethyltin binding suggests that there is a single group at each binding site with a pK of 7·1 (Fig. 6). This may well be a histidine residue. If the conclusion that there are two histidines residues per binding site is correct, the pK of the second histidine must be perturbed some 2 pH units below 7·0 in order that it does not appear in the pH-dependence of binding. This can occur if the two histidine residues at the binding site are close enough to form a hydrogen bond. Under these circumstances the pK values would be forced apart, one being raised and one lowered (cf. the pK values of the carboxyl groups of fumaric acid and maleic acid). Thus if the normal pK of histidine is taken to be 6·0, the pK values of the two histidine residues at the triethyltin-binding site could be 7·0 and 5·0. The pH-dependence of binding would therefore only begin to depart significantly from that of a single group at pH 6·0 and below, when the affinity of binding is already 90% inhibited.

A triethyltin-binding site consisting of two histidine residues is further supported by the stability of polymeric trialkyltin–imidazole complexes in inert non-aqueous solvents, such as toluene (Luijten, Janssen & Van der Kerk, 1962; Van der Kerk, Luijten & Janssen, 1962; Janssen, Luijten & Van der Kerk, 1964). These complexes are thought to have the structure (II).

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