The Interaction of Triethyltin with a Component of Guinea-Pig Liver Supernatant

EVIDENCE FOR HISTIDINE IN THE BINDING SITES

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A protein fraction was isolated from guinea-pig liver that binds triethyltin with an affinity of approx. $2 \times 10^6 \text{ M}^{-1}$ at pH 8.0. It was shown that the protein responsible for binding 70% of the triethyltin found in guinea-pig liver after injection of radioactively labelled triethyltin is at most a few per cent of the total liver protein. Evidence is presented from the kinetics of loss of binding and loss of certain amino acids on photo-oxidation with either Methylene Blue or Rose Bengal that each binding site consists of two histidine residues.

Mitochondrial oxidative phosphorylation is inhibited *in vitro* by very low concentrations (0.1–1.0 μM) of triethyltin (Aldridge, 1958; Aldridge & Threlfall, 1961; Moore & Brody, 1961; Sone & Hagihara, 1964). Studies of the interaction of triethyltin with rat liver mitochondria under conditions identical with those used for inhibition studies have led to the discovery of a class of binding sites in mitochondria (0.8 nmol/mg of mitochondrial protein; affinity constant $5 \times 10^5 \text{ M}^{-1}$) to which triethyltin binds when it inhibits oxidative phosphorylation (Aldridge & Street, 1970). A study aimed at the identification of the molecular groupings involved at these high-affinity mitochondrial binding sites is difficult owing to the insolubility of the system and the presence of a large number of binding sites of very much lower affinity (Aldridge & Street, 1970).

The investigation of the interaction of triethyltin with tissues and tissue components has indicated similar high-affinity binding to rat haemoglobin and to a component or components in the 105000g supernatant obtained from liver homogenates (Rose & Aldridge, 1968). DNA, RNA, a wide variety of low-molecular-weight compounds and a range of purified and semi-purified proteins, including haemoglobin of species other than rat, show no measurable affinity for triethyltin (Aldridge & Cremer, 1955; Aldridge & Cremer, 1957; Aldridge & Street, 1964; Rose & Aldridge, 1968; Aldridge & Rose, 1969a). Because of this very high specificity, information about the nature of the molecular groupings responsible for binding triethyltin with an affinity of 10^5 – 10^6 M^{-1} to a macromolecule may

well be applicable to the high-affinity binding in mitochondria. Rat haemoglobin and the component responsible for binding triethyltin in liver supernatant present fewer technical problems for such studies than mitochondria and have consequently been used as model systems for the study of the properties of triethyltin binding.

Rat haemoglobin binds 2 molecules of triethyltin/tetramer with an affinity of approx. $5 \times 10^5 \text{ M}^{-1}$ and evidence has been produced that each of these sites consists of two histidine residues (Rose, 1969). The investigation of the binding of triethyltin to guinea-pig liver supernatant is described in the present paper. The results of binding studies together with results on the relationship between binding of triethyltin to mitochondria and inhibition of oxidative phosphorylation have resulted in a new hypothesis for the mechanism of oxidative phosphorylation (Aldridge & Rose, 1969b).

MATERIALS AND METHODS

Reagents. Triethyl[^{113}Sn]tin chloride was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.; the specific radioactivity when received was in the range 6–8 mCi/mmol. Stock 0.2 M solutions in ethanol were diluted with water to give 2 mM solutions for binding studies.

The following reagents were obtained from the sources stated: Methylene Blue (laboratory grade) from BDH Chemicals Ltd., Poole, Dorset, U.K.; Rose Bengal from G. T. Gurr Ltd., London S.W.6, U.K.; 5-amino-1,2,3,4-tetrazole monohydrate (practical grade) from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other reagents were of A.R. grade.

Preparation of liver supernatant fraction. Liver (25–35 g) from a male guinea pig (600–800 g body wt.) was homogenized with ten up-down strokes in 30 ml of ice-cold 0.3 M-sucrose in a homogenizer (Aldridge, Emery & Street, 1960) with a total clearance of 0.254 mm. The homogenate was centrifuged at 105 000 g_{av} for 60 min at 0°C to remove particulate matter and then the supernatant was poured off.

Fractionation of liver supernatant. The supernatant was fractionated at 0°C by adding solid $(NH_4)_2SO_4$ to give 50% saturation (Di Jeso, 1968) and after 20 min the precipitate was collected by centrifugation at 0°C. This pellet was discarded and more $(NH_4)_2SO_4$ added to the solution to bring it to 85% saturation. After 20 min the pellet was collected by centrifugation and stored at –20°C.

Fractionation of ammonium sulphate-precipitated pellet on CM-cellulose. This $(NH_4)_2SO_4$ -precipitated pellet was further fractionated on a column of CM-cellulose (Whatman CM 23; H. Reeve Angel and Co. Ltd., London E.C.4, U.K.). The pellet was dissolved in the minimal volume of 0.01 M-tris-HCl buffer, pH 7.1, at 20°C containing 1 μ g of NaCN/ml to give a protein concentration of approx. 45 mg/ml. The $(NH_4)_2SO_4$ present was removed by passage of the solution down a column of Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in the same buffer used to dissolve the pellet. The eluate from this column was passed on to a column (12 cm $\times$ 2 cm) of CM-cellulose equilibrated with 0.01 M-tris-HCl buffer, pH 7.1, and the material passing straight through was collected. Sufficient solid NaCl was then added to give a final concentration of approx. 0.05 M, the solution was cooled to 5°C and the pH adjusted to 8.0 with 1.0 M-tris. The NaCl was added since incubation at 5°C at low ionic strength ($I < 0.01$) was shown to bring about a lowering of the affinity of binding. The protein concentration at this stage was about 30–35 mg/ml. This fraction is called the CM-fraction throughout this paper.

Photo-oxidation. Four guinea-pig livers were fractionated to the $(NH_4)_2SO_4$ -precipitated pellet stage. These pellets were then pooled and fractionated as described above to give 31 ml of CM-fraction. Portions (5 ml) of this solution were then photo-oxidized in the presence of a final concentration of 0.01% Methylene Blue or 0.03% Rose Bengal for 2–40 min as described by Rose (1969). After photo-oxidation the protein solution was divided into two portions. One portion (4 ml) was diluted to 11.0 ml with 0.1 M tris-HCl buffer, pH 8.0, at 5°C, and NaCN was added to give a final concentration of 2 μ g/ml. The ability of this protein to bind triethyltin after different times of photo-oxidation was then measured by equilibrium dialysis as described by Rose (1969). Those amino acids susceptible to photo-oxidation were then determined in the remaining portion (1.0 ml); a small volume (0.02 ml) was removed for thiol determination and the remainder was precipitated with 4 ml of 12% (w/v) $HClO_4$. This precipitate was washed twice with boiling ethanol and twice with diethyl ether before being dried under vacuum.

Amino acid analysis. (a) Cysteine. A 0.02 ml portion of protein solution was added to 3.0 ml of 0.1 M-tris-HCl buffer, pH 7.3, at 20°C. *p*-Chloromercuribenzoate (0.2 ml of a 1.0 mM solution) was then added and the increase in E_{250} followed for 90 min (Boyer, 1954). The number of reactive thiol groups was then calculated as described by Ray & Koshland (1962).

(b) Histidine. Approx. 10 mg of protein powder was dissolved in 0.5 ml of 1.0 M-NaOH and the solution diluted to 5.0 ml with water. Duplicate samples (0.3 ml) were added to 2.7 ml of 0.67 M- Na_2CO_3 -NaHCO₃ buffer, pH 8.8. To each was then added 0.3 ml of 5-diazonium 1,2,3,4-tetrazole reagent (prepared by diazotization of 1 g of 5-amino-1,2,3,4-tetrazole dissolved in 23 ml of 1.6 M-HCl with 0.7 g of NaNO₂ in 10 ml of water at 0°C and adjustment to pH 5.0 with saturated KOH). After 10 min a further 0.3 ml of diazo reagent was added. After each addition the pH was adjusted back to 8.8 and after the second addition the volume was made up to 4.0 ml. At 90 min after the second addition of diazo reagent E_{480} and E_{600} were measured and the histidine content was calculated as described by Sokolovsky & Vallee (1966).

(c) Tyrosine and tryptophan. These were determined spectrophotometrically (Beaven & Holiday, 1952) in the NaOH solution used for the histidine assay.

(d) Methionine. This was measured on a Technicon AutoAnalyzer after alkaline hydrolysis of the protein powder (Jori, Galiazzo, Marzotto & Scoffone, 1968).

Protein determination. Protein was determined by using the biuret method of Robinson & Hogden (1940) as modified by Aldridge (1957).

Radioactivity measurement. ^{113}Sn radioactivity was measured with a Packard Autogamma counter as described by Rose & Aldridge (1968).

Binding analysis. Bound and free triethyltin were measured after equilibrium dialysis as described by Rose (1969). The results of these measurements were analysed according to the Scatchard (1949) equation, the slope of the line giving the affinity constant, K , and the intercept on the abscissa giving the number of sites, n . The binding of triethyltin to photo-oxidized protein was measured in the presence of the dyes since the addition of the dyes to the protein without photo-oxidation was shown to have no significant effect on the binding parameters.

RESULTS

Binding of triethyltin to guinea-pig liver 105 000 g supernatant. Analysis of the binding of triethyltin to guinea-pig liver supernatant indicates the presence of two classes of binding sites (Fig. 1), one class binding 2–3 nmol of triethyltin/mg of protein with an affinity of approx. $4 \times 10^6 M^{-1}$ and the other binding 3–6 nmol of triethyltin/mg of protein with an affinity of approx. $10^4 M^{-1}$. The affinity of this second class of sites is so much lower than that of the first class that accurate measurement is difficult. As the binding component is purified these low-affinity sites remain (Fig. 1), but over the range of triethyltin concentrations used to measure the tight binding do not cause the plot to deviate from linearity (Figs. 2 and 3).

More than 90% of the tight-binding site (K approx. $4 \times 10^6 M^{-1}$) can be recovered in the supernatant remaining after precipitation of about 60% of the protein with ammonium sulphate (50% saturation), and a large proportion of this can be recovered in the pellet obtained by increasing the

Fig. 1. Binding of triethyltin to guinea-pig liver 105000g supernatant and derived fractions. $\bar{v}$ is the concentration of bound triethyltin (nmol/mg of protein); A is the concentration of free triethyltin (μM). The triethyltin concentration was varied from 6 to 40 μM . ●, 105000g supernatant (total protein in fraction 635mg); ▲, supernatant remaining after 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ (total protein in fraction 201mg); ■, protein fraction precipitated from 50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ supernatant by 85% saturation with $(\text{NH}_4)_2\text{SO}_4$ [not prepared from 50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ supernatant shown in this figure]. For derivation of fractions see the Materials and Methods section.

Fig. 2. Effect of photo-oxidation on binding of triethyltin to CM-fraction. $\bar{v}$ and A are as defined in Fig. 1. Photo-oxidation was carried out as described in the Materials and Methods section with Methylene Blue. The numbers on each line represent the time (min) of exposure of the sample to photo-oxidation. The lines are the calculated best fit to the results by the method of least squares. The triethyltin concentration was varied from 1.25 to 15 μM .

ammonium sulphate content to 85% saturation (Fig. 1 and Table 1). This pellet is of a red colour, due in part to the presence of haemoglobin. On passage of the solubilized and desalted ammonium sulphate pellet through CM-cellulose the haemoglobin is removed along with about 30% of the total remaining protein (Table 1), to yield the CM-fraction, which is a pale-yellow solution. This has the absorption spectrum of protein with an E_{280}/E_{260} ratio of 1.3, indicating less than 2% contamination with nucleic acid (Warburg & Christian, 1941). The CM-fraction binds 6–9nmol of triethyltin/mg of protein with an affinity in the range $1\text{--}2 \times 10^6 \text{ M}^{-1}$.

It can be calculated that nearly 70% of the triethyltin in guinea-pig liver, after an intraperitoneal injection of approx. 20 μmol of triethyltin/kg body wt., is present in the 105000g supernatant fraction (Rose & Aldridge, 1968), which contains about 25% of the total liver protein. The CM-fraction is about 20% of the 105000g supernatant protein, so that nearly 70% of the triethyltin bound in the liver after injection is associated with a protein fraction that comprises about 5% of the total liver protein. On disc gel electrophoresis the CM-

Fig. 3. Effect of heat treatment on binding of triethyltin to CM-fraction. $\bar{v}$ and A are as defined in Fig. 1. Samples of CM-fraction (5ml) were heated in a test tube immersed in a water bath at 60°C for the times indicated. The lines are the calculated best fit to the results by the method of least squares. The triethyltin concentration was varied from 1.25 to 15 μM . ○, Control (unheated); ▲, heated for 4 min; ■, heated for 10 min.

fraction is found to contain three major components and four to six minor components. The triethyltin binding appears to be associated with the smallest

Table 1. *Recovery of protein and binding sites on fractionation of guinea-pig liver supernatant*

Values given are means $\pm$ S.E.M. with numbers of determinations in parentheses.

	Total protein in fraction (% of supernatant)	Total tight-binding sites in fraction (% of supernatant)
Supernatant	100	100
(NH ₄) ₂ SO ₄ -pptd.	31.5 $\pm$ 3.6 (3)	89.0 $\pm$ 6.0 (3)
pellet		
CM-fraction	21.5 $\pm$ 0.7 (3)	72.0 $\pm$ 1.9 (3)

of the major components, suggesting that the protein responsible for the binding in the 105000g supernatant can only be present as a few per cent of the total liver protein. This indicates that triethyltin does not bind to most of the liver protein and reinforces the view that it is a very specific reagent and not a general protein-binding reagent.

Effect of photo-oxidation on triethyltin binding by CM-fraction. Binding of triethyltin by the CM-fraction was very sensitive to photo-oxidation with Methylene Blue (Fig. 2) or Rose Bengal as photosensitizer. The affinity of binding was not affected (K for controls, $1.41 \pm 0.12 \times 10^6 \text{ M}^{-1}$; K for 30 min photo-oxidized, $1.68 \pm 0.22 \times 10^6 \text{ M}^{-1}$), but the binding capacity was readily lost in a progressive manner. This form of inhibition contrasts with that obtained by denaturation procedures such as heating (Fig. 3) and urea treatment, in which loss of affinity occurs but not loss of binding sites. This suggests that photo-oxidation is destroying the structure at the binding sites and is not causing changes in the general three-dimensional structure that lead to loss of affinity.

Semi-logarithmic plots of binding-site loss with time are not linear (Fig. 4) but may be analysed into two first-order components. Curves were fitted to the results by the method of successive approximation and some of the derived constants were checked by computer fitting of the results to the sum of two exponentials by using a program based on algorithms due to J. A. Nelder & D. W. Marquardt and developed by R. Jagoe of the Medical Research Council Computer Unit, 242 Pentonville Road, London N.1., U.K. The same type of plot is obtained with either Methylene Blue or Rose Bengal as photosensitizer. The only amino acids susceptible to photo-oxidation are histidine, cysteine, methionine, tryptophan and tyrosine (Weil, Gordon & Buchert, 1951). Of these, all but tyrosine were lost from the CM-fraction under the conditions employed for photo-oxidation with either dye. Tryptophan loss was variable; in some experiments a measurable loss was observed whereas in others no loss was detected (Table 2). Semi-logarithmic plots of methionine and thiol loss were always first-order whereas those of histidine loss

Fig. 4. Semi-logarithmic plot of loss of triethyltin-binding sites from CM-fraction on photo-oxidation with Methylene Blue. The curve was fitted to the results by the method of successive approximations and has the form:

$$n/n_0 = 0.563 e^{-0.3t} + 0.437 e^{-0.0184t}$$

where n is the number of binding sites remaining at time t and n_0 is the initial number of binding sites.

were usually curved (Fig. 5). Curved semi-logarithmic plots for loss of histidine were analysed into two first-order components in the same way as described above for the plots of binding loss.

Correlations between loss of binding and loss of amino acids on photo-oxidation. If an enzyme requires amino acid residues X and Y at its catalytic site for activity such that modification of either residue results in complete inactivation of the enzyme, then it follows that if X and Y are destroyed in first-order reactions loss of activity is also a first-order reaction and:

$$k_A = k_X + k_Y \quad (1)$$

where k_A is the first-order rate constant for loss of activity and k_X and k_Y are the first-order rate constants for loss of the amino acids X and Y respectively (Ray & Koshland, 1961, 1962).

The first-order rate constants for loss of binding sites, histidine, thiol, methionine and tryptophan

Table 2. First-order rate constants for loss of binding sites and loss of amino acids from CM-fraction on photo-oxidation

Each experiment number represents a different preparation of CM-fraction. Expts. 1–13 were carried out with Methylene Blue as photo-sensitizer and Expts. 14–19 with Rose Bengal. All rate constants are expressed in min^{-1} . —, Measurement not made; 0, no loss measurable.

Expt. no.	Binding		Histidine		Thiol	Methionine	Tryptophan
	k_1	k_2	k_1	k_2	k	k	k
1	0.30	0.021	0.20	0.012	0.006	—	0
2	0.25	0.016	0.27	0.0078	0.005	0.013	0.004
3	0.28	0.023	0.14	0.0115	—	0.018	—
4	0.46	0.0138	0.23	0.008	0.009	—	—
5	0.21	0.0115	0.115	0.0058	0.006	—	—
6	0.21	0.0076	0.11	0.0055	0.01	—	0.0035
7	0.26	0.033	0.12	0.0069	0.01	—	—
8	0.30	0.0184	0.138	0.0077	0.009	—	0.009
9	0.22	0.015	*	0.007	—	—	—
10	0.38	0.054	*	0.019	0.007	0.024	0.008
11	0.22	0.029	*	0.013	0.006	0.009	0
12	0.33	0.026	0.24	0.012	0.01	0.018	0
13	0.22	0.030	*	0.016	0.007	0.016	0.005
14	0.24	0.022	0.12	0.01	0.024	0.025	0.005
15	0.35	0.022	0.14	0.01	0.019	—	0.01
16	0.29	0.025	0.14	0.012	0.019	0.023	0
17	0.23	0.038	*	0.021	0.015	0.026	0.006
18	0.23	0.04	*	0.018	0.016	0.016	0.008
19	0.23	0.03	0.18	0.017	0.014	0.018	0

* Fast component not detected.

Fig. 5. Semi-logarithmic plots of loss of affected amino acids from CM-fraction on photo-oxidation with Methylene Blue. The straight lines are the calculated best fit to the results by the method of least squares and the curve was fitted to the results by the method of successive approximations and has the form:

$$\text{His}/\text{His}_0 = 0.084 e^{-0.138t} + 0.916 e^{-0.0077t}$$

where His is the histidine content at time t and His_0 the initial histidine content. $\blacktriangle$, Loss of thiol; $\bullet$, loss of histidine; $\blacksquare$, loss of methionine.

vary from one preparation of CM-fraction to another (Table 2), and there are two first-order rate constants (k_1 and k_2) for loss of binding to be accounted

for in terms of loss of amino acids. The fact that there are two constants is indicative of the existence of two types of triethyltin-binding sites differing in their sensitivity to photo-oxidation.

The value of k_2 (binding) varies over the range 0.0076 – 0.054 min^{-1} (Table 2) in different preparations. A clear relationship emerges between that binding loss characterized by the constant k_2 (binding) and loss of histidine when k_2 (binding) is plotted against k_2 (histidine) (Fig. 6c). No such relationship can be detected between k_2 (binding) and the rate constants for loss of any of the other amino acids (Fig. 6), indicating that only histidine loss is involved in binding loss. The slope of the theoretical regression line relating k_2 (binding) to k_2 (histidine) in Fig. 6 is 2.0. The fit of the results to this suggests that binding loss is occurring twice as fast as histidine loss; this would be expected to occur if each binding site consisted of a pair of histidine residues, loss of either one leading to complete loss of binding at that site.

The only amino acid that is lost fast enough to account for the fast component of binding loss [k_1 (binding)] is histidine (Table 2). The values obtained for k_1 (histidine) can only be approximate since only 10% or less of the total histidine is involved, and, unless this fraction is lost at least ten times as fast as the remainder, accurate measurement is very difficult. Despite the scatter, the results relating k_1 (binding) to k_1 (histidine) fall

Fig. 6. Relationship between k_1 and k_2 (binding) and the first-order rate constants for loss of affected amino acids. The results plotted are from Table 2. $\circ$, Methylene Blue experiments; $\bullet$, Rose Bengal experiments.

closest to the theoretical line giving a rate of loss of binding sites twice as fast as loss of histidine residues (Fig. 6d). This suggests that each binding site characterized by the constant k_1 (binding) consists of two histidine residues by the same argument as described above for k_2 (binding) and k_2 (histidine).

Thus both rate constants [k_1 (binding) and k_2 (binding)] for loss of triethyltin binding on photo-oxidation can be accounted for in terms of the rate constants for loss of histidine, and the results can be interpreted to indicate that each binding site in the CM-fraction characterized by an affinity constant of $1\text{--}2 \times 10^6 \text{ M}^{-1}$ consists of a pair of histidine residues.

DISCUSSION

Correlation of binding loss and loss of amino acids.

The correlations between loss of triethyltin-binding sites and loss of histidine on photo-oxidation of the CM-fraction arise from measurements of total histidine, total methionine, total tryptophan and total reactive thiol present in the mixture of proteins that comprise the CM-fraction. The rate constants obtained are therefore statistical averages. Single residues that have a greater or lesser sensitivity towards photo-oxidation may exist within the bulk residues. Such anomalous residues would be undetectable unless the methods for assay of amino acids were very sensitive or the difference in reactivity towards photo-oxidation of the single residue and the rest was very large. However, if

such residues exist in the CM-fraction and are responsible for the observed loss of binding, then the correlations obtained (Table 2 and Fig. 6) are entirely coincidental. This seems an unlikely interpretation especially since the correlations hold despite the use of two very different dyes for photo-oxidation and despite the variation in the rate of loss of binding observed in different preparations (Fig. 6). That the binding sites consist of paired histidine residues is therefore the most reasonable interpretation of the results presented.

Heterogeneity of binding sites. Analyses of the binding of triethyltin to CM-fractions (Figs. 2 and 3) indicate that there is a uniform class of binding sites of high affinity characterized by an affinity constant in the region of $1\text{--}2 \times 10^6 \text{ M}^{-1}$. The response of these sites to photo-oxidation, however, indicates that these high-affinity sites are not uniform and can be divided into two approximately equal classes differing in their sensitivity to photo-oxidation. The possibility therefore arises that the CM-fraction contains at least two proteins that are capable of binding triethyltin. Chromatography on Sephadex G-100 and G-200 of fractions from the 105000g supernatant labelled by the addition of a very small quantity of triethyl[^{113}Sn]tin indicated the presence of only one peak of radioactivity eluting with the low-molecular-weight proteins and before the low-molecular-weight nucleic acids. Similarly, chromatography on DEAE- or CM-cellulose, using stepwise elution with buffers of increasing ionic strength, only gave a single peak of radioactivity that was shown to be bound to protein by passage down Sephadex G-25. Thus both these types of separative procedures failed to reveal the presence of more than one binding protein. It therefore seems likely that the division of the binding sites into two approximately equal classes differing in their sensitivity towards photo-oxidation is due to the presence of two different micro-environments for the sites in one protein, allowing equal access to triethyltin (and thus having the same affinity constant) but giving differential access to the photo-sensitizing dyes.

Response of amino acid residues to photo-oxidation.

There are few reports in the literature of the measurement of rates of loss of amino acids on photo-oxidation. The absolute rates observed are very dependent on the apparatus and the conditions employed (Ray, 1967) and are therefore not directly comparable. What is comparable is the qualitative response of the various amino acids to photo-oxidation. A linear first-order response of amino acids is often found and a not uncommon finding is a biphasic response of histidine. Ray & Koshland (1962) reported that the 13 histidine residues of phosphoglucomutase (EC 2.7.5.1) behaved as two classes, with seven accessible residues lost with a

first-order rate constant of 0.65min^{-1} and six less accessible residues lost with a first-order rate constant of 0.048min^{-1} . Moreover a decrease of enzyme activity was associated with loss of only one of the seven accessible residues. Martinez-Carrion, Turano, Riva & Fasella (1967) found a similar response of histidine residues in soluble aspartate aminotransferase, where two of the residues were lost with a first-order rate constant of 0.0461min^{-1} and six with a first-order rate constant of 0.0045min^{-1} . Here again activity loss was associated with one of the two more reactive residues. In the CM-fraction only 10% or less of the histidine residues appear to be more readily photo-oxidized than the rest at a rate about ten times that of the rest and for this reason they were not always detectable (Table 2).

Nature of high-affinity triethyltin-binding sites. Evidence has been obtained that, when triethyltin binds to rat haemoglobin with an affinity of $5 \times 10^5\text{M}^{-1}$ (Rose, 1969) and to a protein present in guinea-pig liver supernatant fraction with an affinity of approx. $2 \times 10^6\text{M}^{-1}$, the binding sites consist of pairs of histidine residues. The geometry of the histidine residues relative to each other must be important since pancreatic ribonuclease (EC 2.7.7.16), an enzyme considered to have two histidine residues at its active site (Mathias, Deavin & Rabin, 1964; Wyckoff, Hardman, Allewell, Inagami, Johnson & Richards, 1967; Kartha, Bello & Harker, 1967) does not bind triethyltin (Rose & Aldridge, 1968). The type of geometry required may be similar to that deduced for trialkyltin-imidazole complexes in toluene (Janssen, Luijten & Van der Kerk, 1964). In this system linear polymers are formed with trialkyltin molecules bridging imidazole molecules (Fig. 7).

Implications with respect to mitochondrial triethyltin-binding sites. The interaction of triethyltin with an affinity constant of 10^5 – 10^6M^{-1} with proteins is very specific. This is shown by the lack of binding to haemoglobin of species other than rat, though these presumably have similar tertiary structures, and the finding that 70% of the triethyltin present in guinea-pig liver after injection of the compound is associated with only a few per cent of the total liver protein. It is therefore likely that the mitochondrial binding sites that are characterized by an affinity constant of $5 \times 10^5\text{M}^{-1}$ (Aldridge & Street, 1970) have a similar structure to those

found in rat haemoglobin and guinea-pig liver supernatant.

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Fig. 7. Proposed structure for trialkyltin-imidazole polymers.