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A New Class of Chromophoric Organomercurials and their Reactions with D-Glyceraldehyde 3-Phosphate Dehydrogenase

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The syntheses of the following organomercurials are described: 2-chloromercuri-4-nitrophenol, 2-chloromercuri-4,6-dinitrophenol, 4-chloromercuri-2-nitrophenol and 2,6-dichloromercuri-4-nitrophenol. All four organomercurials show large spectral changes in the visible spectrum when thiols displace a more weakly bound ligand such as EDTA from the mercury atom. These spectral changes are primarily associated with pK perturbation of the nitrophenols. The mercurials are therefore chromophoric probes for thiol groups in proteins and other thiols of biological interest. The enzyme D-glyceraldehyde 3-phosphate dehydrogenase from lobster muscle is used as a model system in which the properties of the organomercurials may be illustrated. In particular it is shown how D-glyceraldehyde 3-phosphate dehydrogenase carboxymethylated at the active site may be mercurated at a specific site. This mercurial derivative may be crystallized and shown to be isomorphous with the parent enzyme. The mercurials also act as 'reporter groups' by monitoring phosphate or pyrophosphate binding to the enzyme. The mercurials may also be used to estimate cations by an EDTA displacement method.

With the recent advances in the study of biological systems at the molecular level there is an increasing requirement for reagents that will react with specific sites on biological macromolecules. It is an advantage if a measurable signal occurs concomitant with the reaction and if the magnitude of the signal is sensitive to local environment.

Derivatives of mono- or di-nitrophenols are satisfactory as probes since they contain ionizing hydroxyl groups with pK values in the pH range of interest in biological studies, and the phenolate anions have chromophores in the visible spectrum clear of nucleotide and protein absorption bands (Horton & Koshland, 1965; Halford, Bennett, Trentham & Gutfreund, 1969). Mercuration of the nitrophenols is a potentially promising method of incorporating the chromophore into a biological molecule since organomercurials are specific for thiols, which occur to a limited extent in many proteins. The reason that such reagents have not been more widely used in the past is twofold. First, without p.m.r.* spectroscopy, analysis of the site of mercuration in the nitrophenyl ring is not necessarily simple (Raiziss & Proskouriakoff, 1922; Ohno, 1956*a*,*b*; Karoua & Nesmeyanov, 1967). Secondly, significant spectral changes resulting from the reaction of mercurial nitrophenols with thiols were not noted (Boyer, 1954; Åkerfeldt, 1959). However, as shown below, if ligands are displaced from the organomercurial when it reacts with a thiol, then chromophoric changes are more readily observed.

The organomercurials described here are readily synthesized and easy to handle. They have the following potential uses: (1) as chromophoric probes for thiol groups in proteins and other thiols; (2) in kinetic studies of the reactivity of thiol groups; (3) as 'Reporter groups' (Burr & Koshland, 1964), i.e. indicators of perturbations in the biological systems to which they are attached; (4) in the tertiary structure determination of biological macromolecules; (5) in the estimation of cations.

We describe here the application of these mercurials to studies of the thiol groups of lobster muscle GPDH and, in particular, illustrate their use in the preparation of crystalline mercurial derivatives of proteins. Such protein derivatives in which the degree of heavy-atom substitution is chemically well defined are required for tertiary-structure determination by X-ray diffraction analysis, particularly as the size of biological molecule under

* Abbreviations: p.m.r., proton magnetic resonance; GPDH, D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); CM-GPDH, GPDH carboxymethylated at the active-site cysteine-148 (Davidson, Sajgò, Noller & Harris, 1967).

investigation increases (Green, Ingram & Perutz, 1954; Blake, 1968).

MATERIALS AND METHODS

GPDH from lobster tail muscle was prepared and assayed as described by Trentham (1968).

Preparation of CM-GPDH. Crystalline GPDH (50mg./ml.), dissolved in EDTA (1mM) at pH7, was treated with 1molar equivalent of 2-mercaptoethanol/active site, to ensure that the enzyme was fully active. The enzyme was eluted from columns of Sephadex G-25 (fine grade). The eluting solvent contained NaCl (0·15M), EDTA (1mM) and triethanolamine (0·1M) adjusted to pH7 with HCl. The enzyme concentration was determined from extinction measurements at 260, 276 and 290nm., corrections being made for the small amount of NAD^+ removed by Sephadex filtration. This correction term could be deduced since the E_{276}/E_{260} ratio of GPDH with its full complement of 4moles of NAD^+/mole of tetramer is 1·01 and the E_{276}/E_{260} ratio of NAD^+ is 0·4. $E^{1\%}_{290}$ of GPDH is 5·55 and is relatively independent of the amount of NAD^+ bound. The eluted enzyme solution was treated with NAD^+ (1mM) and 1molar equivalent of iodoacetic acid/active site and left overnight at 5°. The carboxymethylated enzyme was analysed by assaying samples diluted into a solution of EDTA (1mM), 2-mercaptoethanol (1mM) and inorganic phosphate (50mM) at pH6·9. The residual activity was less than 1% of the native enzyme activity.

Preparation of the mercurial derivative of CM-GPDH. The stoicheiometry of the reaction between 2-chloromercuri-4-nitrophenol and CM-GPDH was determined as described (Fig. 3). The concentrated CM-GPDH solution was adjusted to pH5·8 and maintained in an ice bath. Phosphate (10mM) and NAD^+ (1mM) were added. The organomercurial was added at a rate of 5μl./min. to the constantly stirred solution by using the titrator described below.

Crystallization of GPDH derivatives. The modified enzyme at a final concentration of 5mg./ml. was crystallized at pH6 from $(NH_4)_2SO_4$ solutions containing NAD^+ (1mM) and phosphate (10mM) (Trentham, 1968).

Reagents. DL-Glyceraldehyde 3-phosphate (as the barium salt of the diethyl acetal) and NAD^+ were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Glyceraldehyde 3-phosphate was prepared and assayed as described by Trentham (1968). Water was glass-distilled. All other reagents were A.R. grade where possible and were used without further purification.

Spectroscopic measurements. The p.m.r. spectra were recorded with a Varian HA100 spectrophotometer locked on the tetramethylsilane signal. τ values are quoted with reference to the internal standard, tetramethylsilane. The organomercurials were dissolved in deuterated acetone.

Visible and u.v. spectra were recorded on a Unicam SP.800 scanning spectrophotometer. Non-scanning visible- and u.v.-absorption measurements including enzyme assays were made in the 1cm. thermostatically controlled cuvette of a Uvispek H700 spectrophotometer (Hilger and Watts Ltd.) provided with a Gilford recording attachment and a Servoscribe RE511 recorder (Kelvin Electronics Co.).

The binding studies (Fig. 3) were carried out in an apparatus designed by Dr J. J. Holbrook. The solution of the organomercurial in a motor-driven Agla syringe (Burroughs Wellcome) was added at a constant flow rate to a 5ml. cuvette with a 1cm. light-path. The cuvette was housed in the thermostatically controlled cell compartment of the Hilger and Watts spectrophotometer. The solution was stirred continuously by using an overhead stirrer. The inlets for the stirrer rod and added solution were light-sealed.

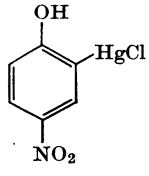
The stopped-flow apparatus used was similar to that described by Gutfreund (1965). The interchangeable mixer and observation cell had eight mixing jets and a 1cm. light-path. The solution in the observation chamber was 3msec. old when flow stopped. Monochromatic light was obtained from a quartz tungsten–iodine lamp and a Bausch and Lomb grating monochromator. Transmission of light was recorded on a Tektronix storage oscilloscope, which amplified the output of an EMI 9592B photomultiplier. Reactions were studied at room temperature (21±2°).

pH values were determined by using a Vibron Electrometer (model 33B-2) with associated pH-measuring unit C33B and using a Yena pH7 microdual electrode 9259/81 (Electronic Instruments Ltd.). pH values of ice-cooled solutions were standardized against phosphate and pyrophosphate buffers of known pH value at 0°.

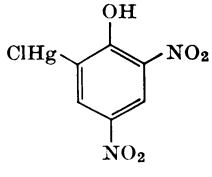
Microanalyses. These were performed by Dr Franz Pascher (Mikroanalytisches Laboratorium, Bonn, Germany).

RESULTS

Synthesis and structural determination of the organomercurials. Four organomercurials have been synthesized: 2-chloromercuri-4-nitrophenol (I), 2-chloromercuri-4,6-dinitrophenol (II), 4-chloromercuri-2-nitrophenol (III), and 2,6-dichloromercuri-4-nitrophenol (IV). The compounds were all synthesized by the mercuration of the parent



(I)



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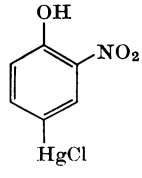

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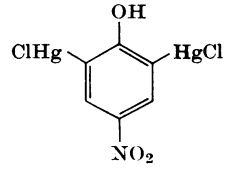


(IV)

nitrophenol. The preparation of 2-chloromercuri-4-nitrophenol (I) is described in detail.

Preparation of 2-chloromercuri-4-nitrophenol. A well-stirred solution of 4-nitrophenol (10g., 72m-moles) in 210ml. of aqueous potassium hydroxide (0·37M) at 70° was treated dropwise with mercuric acetate (23g., 72m-moles) dissolved in 200ml. of acetic acid (80mM). A yellow precipitate formed. When the addition was complete (after 30min.), the precipitate was filtered off from the hot solution and washed successively with dilute acetic acid, water, methanol and diethyl ether. The pale-yellow solid (17g) was recrystallized as the acetate of the organomercurial from acetic acid. The acetate (16g.) was dissolved in 500ml. of potassium hydroxide (1M) and treated with concentrated hydrochloric acid until all the mercurial had precipitated. The 2-chloromercuri-4-nitrophenol was filtered off, washed with water and dried for 24hr. at 75°. The bright-yellow solid (12g.) was recrystallized from a methanol–water solvent (a few drops of water where necessary to assist the methanol to dissolve the mercurial). The 2-chloromercuri-4-nitrophenol (11g.) was obtained in 40% overall yield from the parent phenol.

The syntheses of 2-chloromercuri-4,6-dinitrophenol (II) and 4-chloromercuri-2-nitrophenol (III) were similar. The 2,6-dichloromercuri-4-nitrophenol (IV) was prepared by treating 4-nitrophenol with 2 molar equivalents of mercuric acetate. The phenol and mercuric acetate were stirred for 3hr. at 70° before recovery of the product.

The structures of the chloromercurials were established by their elemental analyses and their u.v., visible and p.m.r. spectra. The site of mercuration in the aromatic nucleus was evident from the p.m.r. spectra and from the property of 2-nitrophenol to direct *ortho* and *para* and 4-nitrophenol *ortho* to the hydroxyl group.

2-Chloromercuri-4-nitrophenol had: m.p. 238° (decomp.); mol.wt. 374; $\lambda_{max.}$ 405nm. (ϵ $1{\cdot}74 \times 10^4$) in potassium hydroxide (0·1M); p.m.r. spectrum, a doublet, τ 1·55 (J 1·5Hz), assigned to $H_{(3)}$, a quartet, τ 1·96 (J 4·5Hz), and fine splitting (J 1·5Hz), assigned to $H_{(5)}$, and a doublet, τ 2·95 (J 4·5Hz), assigned to $H_{(6)}$ (Found: C, 19·1; H, 1·2; Cl, 8·9; N, 3·7; Hg, 52·2. Calc. for $C_6H_4ClHgNO_3$: C, 19·3; H, 1·1; Cl, 9·5; Hg, 53·6; N, 3·7%).

2-Chloromercuri-4,6-dinitrophenol had: m.p. 185°; mol-wt. 419; $\lambda_{max.}$ 371nm. (ϵ $1{\cdot}57 \times 10^4$) in potassium hydroxide (0·1M) and λ410nm. (shoulder) (ϵ $1{\cdot}13 \times 10^4$) in potassium hydroxide (0·1M); p.m.r. spectrum, a singlet, τ 1·18, assigned to $H_{(3)}$ and $H_{(5)}$ (Found: C, 17·3; H, 0·8; Cl, 8·5; Hg, 47·8; N, 6·8. Calc. for $C_6H_3ClHgN_2O_5$: C, 17·2; H, 0·7; Cl, 8·5; Hg, 47·9; N, 6·7%).

4-Chloromercuri-2-nitrophenol had: m.p. 220°; mol.wt. 374; $\lambda_{max.}$ 416nm. (ϵ $4{\cdot}1 \times 10^3$) in potassium hydroxide (0·1M); p.m.r. spectrum, a singlet, τ 1·63, assigned to $H_{(3)}$, a doublet, τ 2·15 (J 4·5Hz), assigned to $H_{(5)}$, and a doublet, τ 2·84 (J 4·5Hz), assigned to $H_{(6)}$ (Found: C, 19·3; H, 1·1; Cl, 9·3; Hg, 53·7; N, 3·6; Calc. for $C_6H_4ClHgNO_3$: C, 19·3; H, 1·1; Cl, 9·5; Hg, 53·6; N, 3·7%).

2,6-Dichloromercuri-4-nitrophenol had: m.p. >320°; mol.wt. 609; $\lambda_{max.}$ 410nm. (ϵ $1{\cdot}74 \times 10^4$) in potassium hydroxide (0·1M); p.m.r. spectrum, a singlet, τ 1·74, assigned to $H_{(3)}$ and $H_{(5)}$ (Found: C, 12·3; H, 0·7; Cl, 11·5; Hg, 63·5; N, 2·1. Calc. for $C_6H_3Cl_2Hg_2NO_3$: C, 11·8; H, 0·5; Cl, 11·7; Hg, 65·9; N, 2·3%).

In the p.m.r. spectra peaks associated with the hydroxyl protons were not observed except with 4-chloromercuri-2-nitrophenol. In this case a singlet peak, τ 0·36, which disappeared when D_2O was added to the acetone solution, was assigned to the hydroxyl proton.

Properties of the organomercurials. Nitrophenolate ions have visible-absorption bands and the extinction of the chloromercurinitrophenols at any pH is determined by the extent of ionization of the phenol. The perturbation of the pK of the nitrophenol and the resultant chromophoric change provide the main basis for the uses of the organomercurials outlined below. For this reason these properties are discussed in some detail.

Organomercurials bind very tightly to thiols. The reaction of a thiol and a mercurinitrophenol might be expected to introduce a pK change in the phenol and thus a chromophoric change at certain

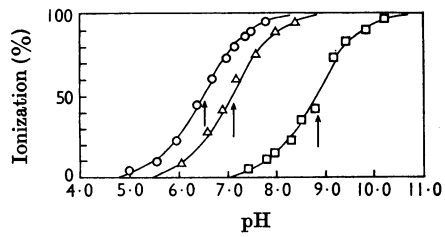


Fig. 1. Ionization constants of 2-chloromercuri-4-nitrophenol. The variation of the phenol ionization with pH was measured in the presence of various ligands. The percentage ionization of the phenol was deduced from the ratio of the extinction at the measured pH to the plateau extinction at high pH values. The extinction was measured at 410nm. at 25° with a cuvette with a 1cm. light-path. The extinction of the un-ionized phenol at 410nm. is negligible. ○, 2-Chloromercuri-4-nitrophenol (38·7μM); △, as ○ plus thioglycollic acid (50μM); □, as ○ plus EDTA (5mM). The solutions contained either sodium citrate (0·1M) or triethanolamine hydrochloride (0·1M) adjusted to the measured pH with HCl or NaOH. The solid lines are theoretical curves for species with pK values of 6·5, 7·1 and 8·85, shown by arrows.

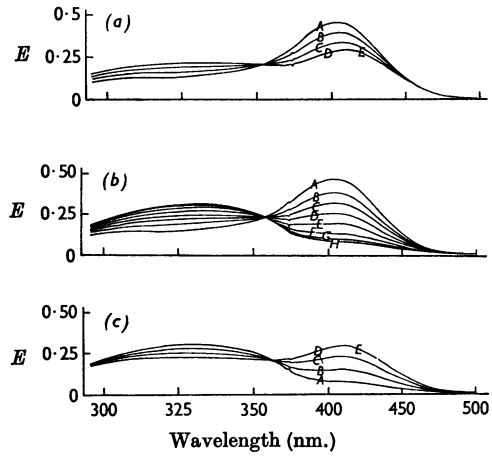


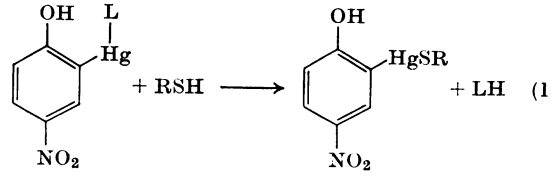
Fig. 2. Absorption spectra of 2-chloromercuri-4-nitrophenol showing the effect of added ligands. The solutions at 25° in a cuvette with a 1 cm. light-path contained 2-chloromercuri-4-nitrophenol (38·7 μM) and triethanolamine hydrochloride (0·2 M) adjusted to pH 7·0 with NaOH. In addition, the solution in traces (*c*) contained EDTA (1·87 mM). The families of traces (*a*) and (*c*) contained successive increments (14·4 μM) of thioglycollic acid from zero concentration (curve *A*) to 57·6 μM (curve *E*). The family of traces (*b*) contained successive increments of EDTA: curve *A*, nil; curve *B*, 33 μM; curve *C*, 67 μM; curve *D*, 133 μM; curve *E*, 267 μM; curve *F*, 600 μM; curve *G*, 1·27 mM; curve *H*, 1·87 mM.

Table 1. p*K* *values of phenolic hydroxyl groups of organomercurials*

The p*K* values were determined spectrophotometrically at 25° (Fig. 1), and were measured in aqueous solution or in the presence of thioglycollic acid (just in molar excess of the mercurial) or in the presence of EDTA [5 mM for mercurials (I), (III) and (IV) and up to 200 mM for mercurial (II)]. The solutions also contained sodium citrate (0·1 M) or triethanolamine hydrochloride (0·1 M) adjusted to the measured pH with HCl or NaOH. A high concentration of EDTA was required to saturate 2-chloromercuri-4,6-dinitrophenol since the binding of EDTA to mercurials becomes much weaker at low pH.

		p*K*		
Added ligand	...	None	Thioglycollic acid	EDTA
2,Chloromercuri-4-nitrophenol	(I)	6·5	7·1	8·85
2-Chloromercuri-4,6-dinitrophenol	(II)	4·0	5·0	6·3
4-Chloromercuri-2-nitrophenol	(III)	6·8	7·3	7·9
2,6-Dichloromercuri-4-nitrophenol	(IV)	6·3	7·05	10·1

pH values. This is the case to a certain extent (Figs. 1 and 2). However, a much more dramatic p*K* change is observed if the thiol mercurial reaction occurs concomitant with the displacement of a weakly bound ligand that also appears to interact with the hydroxyl group of the mercurinitrophenol. (Figs. 1 and 2 and Table 1). This reaction is illustrated in eqn. (1):



(1)

where L represents a ligand and RSH a thiol. EDTA showed the largest effect of the potential ligands tested (namely EDTA, ethylenedioxybis-(ethylamine)tetra-acetic acid, cyanide, citrate, ethylenediamine and triethanolamine). EDTA has four carboxylate anions. Some of these anions probably interact with the hydroxyl group and prevent its ionization.

Since crystallization of mercurial derivatives of GPDH was carried out in 80% ammonium sulphate solution in the pH range 5·5–6·5, it was important to test whether or not the large concentration of NH_4^+ ion might displace a sulphur ligand from the mercurial. A chromophoric change was observed when thioglycollic acid was titrated into organomercurial (II) (50 μM) in 50% ammonium sulphate and EDTA (0·1 M) at pH 5·5. A 1:1 complex between the thiol and organomercurial formed, and the dissociation constant associated with the complex-formation was too small to measure ($< 10^{-6}$ M). Hence, in crystallization of mercurial enzyme derivatives, the mercurial will not be displaced from the enzyme by the solvent.

The rate of mercurial migration between thiols was studied since this is an important aspect of preparing crystalline organomercurial protein derivatives for X-ray diffraction analysis. The extinction of 2,6-dichloromercuri-4-nitrophenol treated with 1 molar equivalent of thioglycollic acid is less than half the extinction of the organomercurial with no thiol plus the extinction of the organomercurial with two thiols bound. When a complex of organomercurial (IV) with two molar equivalents of thioglycollic acid is mixed with an equal concentration of thiol-free organomercurial (IV) in EDTA (1 mM) at pH 8·0, there is a decrease in extinction, which occurs within the time resolution of the addition (5 sec.). Therefore, unless a thiol group is so shielded by protein structure that it would require high activation energy to unmask it, a mercurial bound to a protein will rapidly equilibrate with the thiols around the molecule to

yield the thermodynamically stable product. This consideration is particularly important if, during crystallization of a protein after mercuration of a specific thiol, a fraction of the protein denatures exposing further thiols.

The pK perturbations of the phenol are greatest when the mercury atom is *ortho* to the hydroxyl group. Of the monomercurials, the largest spectral changes ($\sim 10^4$ at λ410nm.) in the pH range 3·3–6·5 are observed with 2-chloromercuri-4,6-dinitrophenol, and in the pH range 6·5–9 with 2-chloromercuri-4-nitrophenol. The pK values of the organomercurials associated with various ligands are summarized in Table 1.

The pK value at 25° of 2-chloromercuri-4-nitrophenol treated with excess of thioglycollic acid was 8·2 when measured in acetone–water (1:1, v/v) compared with 7·1 in water. When 2-mercaptoethanol was the added thiol the pK values were 7·9 and 6·8 respectively, and 8·0 and 6·5 in the absence of thiol. The solvent contained 0·1 M-triethanolamine hydrochloride adjusted to the measured pH with sodium hydroxide. The measured pH value of an aqueous triethanolamine buffer solution was about 0·2 larger than a similar buffer in acetone–water (1:1, v/v). No corrections to the pK values were made for this. ϵ_{410} of 2-chloromercuri-4-nitrophenol in acetone–water (1:1, v/v) containing 0·1 M-sodium hydroxide was 20% greater than ϵ_{410} in 0·1 M-sodium hydroxide.

The anions of all the organomercurials are water-soluble, though the more substituted phenols (II and IV) are only sparingly so (1mg./ml.).

The organomercurials are all stable in neutral and alkaline aqueous solution. However, they are labile in the presence of reducing agents such as dithionite.

The potential uses of the organomercurials are illustrated by studies of their reactions with GPDH and CM-GPDH.

Chromophoric probes for thiol groups in proteins and other thiols. The spectra (Fig. 2) indicate that an extinction change occurs when a thiol group reacts with 2-chloromercuri-4-nitrophenol. Hence the mercurial is a probe for thiol groups on proteins and other biological macromolecules. This is illustrated by the titration of 2-chloromercuri-4-nitrophenol with the enzyme GPDH (curve *A* in Fig. 3), and with the same enzyme in which the active-centre 'essential' thiol group has been selectively carboxymethylated (curve *B* in Fig. 3). The extent of mercuration of the enzyme may be determined: 2·02 ± 0·1 mercurials bind/subunit of native GPDH and 0·97 ± 0·05 mercurials bind/subunit of CM-GPDH. Loss of enzyme activity closely follows the titration of native GPDH with 1mole of 2-chloromercuri-4-nitrophenol/mole of enzyme subunit.

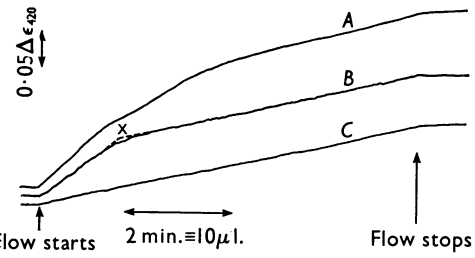


Fig. 3. Spectrophotometric record of a titration of 2-chloromercuri-4-nitrophenol at constant rate against: curve *A*, native GPDH; curve *B*, CM-GPDH; curve *C*, buffer solution. Traces were recorded at 410nm. at 4°. The observation chamber had a 1cm. light-path. The reaction cell contained: curve *A*, GPDH (48·2nmoles of sites); curve *B*, CM-GPDH (48·2nmoles of sites); curve *C*, blank. The solvent (5·0ml.) contained NAD^+ (50μM), EDTA (1·0mM), NaCl (10mM) and triethanolamine (0·1 M) adjusted at 20° to pH 7·9 with HCl. 2-Chloromercuri-4-nitrophenol (6·4mM) was added to the stirred solution at a constant flow rate of 5μl./min. as shown. The stoicheiometry of the reaction between CM-GPDH and 2-chloromercuri-4-nitrophenol (curve *B*) is determined as follows. A tangent is drawn to the initial slope after mercurial addition starts to intercept at X, the tangent to the slope of the trace after the mercurial site is saturated. The time from the start of flow to X is measured on the abscissa and hence the amount of mercurial equivalent to the enzyme may be measured. The extinction coefficient of the mercurial bound to the enzyme may be deduced from the slope of the tangent drawn from the point where flow starts to X, since this is a measure of the extinction change for a known concentration of mercurial. Extension of this procedure allows the determination of the stoicheiometry and extinction coefficients of mercurial derivatives of enzymes with multiple mercurial-binding sites as in curve *A*.

It is apparent from curve *A* that the extinction of the nitrophenol group differs on the two thiols. This is a reflection of the different local environment around each nitrophenol. The extinction differences therefore provide a probe to study the local environment of the thiol groups.

The stoicheiometry of the reaction of a mercurial and a protein may be measured in two ways. (1) From results such as those collected from the ionization and spectroscopic measurements and the titration curves (Figs. 1, 2 and 3) the concentration of mercurial on the protein may be determined from its extinction measured at 410nm. under the specific conditions. (2) Advantage can be taken of the fact that excess of a thiol (e.g. thioglycollic acid or 2-mercaptoethanol) will displace the mercurial from the protein. The extinction of the solution of the thiol–mercurinitrophenol complex of known extinction coefficient may then be measured. The mercurial content of the crystalline derivative of CM-GPDH described below was measured in this

way. If the protein concentration is measured spectroscopically at 280nm. then corrections should be made for mercurial absorption. 2-Chloromercuri-4-nitrophenol and its phenolate form have an isosbestic point at 280nm.; E_{280} is $3{\cdot}6 \times 10^3$. E_{280} of the neutral form in the presence of thioglycollic acid is $3{\cdot}6 \times 10^3$, and of the phenolate form $4{\cdot}6 \times 10^3$.

Kinetic studies of reactivity of thiol groups. The thiol groups on the surface of a biological macromolecule have different reactivities. The reactivity depends on such factors as the steric interaction of neighbouring groups, the local solvent environment created by these groups and the role of neighbouring acid or base catalysis. This reactivity may be investigated by measuring the rate of the reaction of the thiol groups with the chromophoric organomercurials. Thus, whereas two thiol groups on GPDH react with rate constants of 220sec.$^{-1}$ and 2·2sec.$^{-1}$ under specified conditions (Fig. 4*a*), the thiol group of CM-GPDH reacts with a rate constant of 2·0sec.$^{-1}$ (Fig. 4*b*).

'*Reporter groups*'. If a molecule that is bound to a biological macromolecule has the property of signalling changes that occur in another part of the macromolecule, it is termed a 'reporter group' (Burr & Koshland, 1964). The organomercurials have this property because chromophoric changes are associated with the ionization state of the nitrophenols and the extent of ionization is dependent on the environment. Since the thiol-bound mercurinitrophenols have pK values about 7 (Table 1), the maximum chromophoric signals after a pK perturbation will occur in the most suitable pH range for biological studies. For example, a conformation change of a protein may induce a local solvent environment change around the organomercurial,

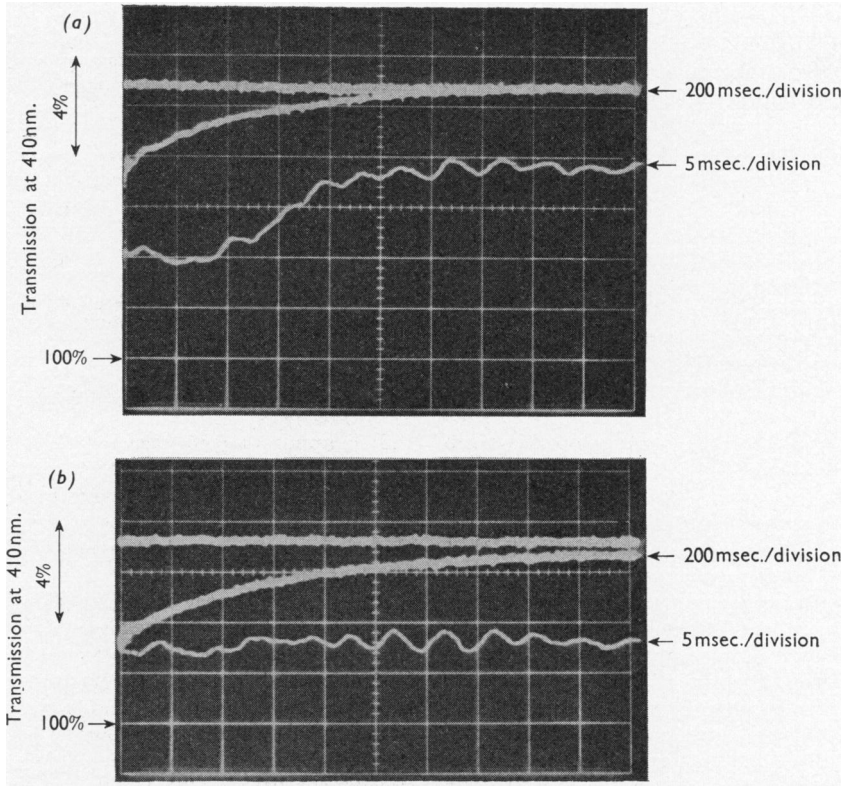


Fig. 4. (*a*) Reaction of 2-chloromercuri-4-nitrophenol and GPDH recorded spectrophotometrically at 410nm. by using the stopped-flow apparatus. The reaction mixture contained GPDH (3·01 μM in sites), 2-chloromercuri-4-nitrophenol (65 μM), EDTA (1·0mM), NAD^+ (20 μM) and triethanolamine (0·1M) adjusted to pH7·9 with HCl. The reaction was initiated by rapid mixing of the mercurial with GPDH. The rate of the bottom trace is 5msec./division and of the upper two traces 200msec./division. The top trace was triggered about 5sec. after initiating the reaction and indicates end-point stability. The plateau at the start of the bottom trace represents the constant extinction of the solution during rapid flow of the solutions. The flow stopped about 10msec. after the oscilloscope trace was triggered. (*b*) As (*a*) except that CM-GPDH replaced GPDH.

thus perturbing its pK. The pK change will be signalled by a chromophoric change. Since the proton transfers associated with a pK change are extremely rapid, the kinetics of the slower processes, such as the conformation change, may be monitored.

These points are illustrated by pK measurements of the nitrophenol residue of CM-GPDH and the native enzyme, each treated with 0·8mole of 2-chloromercuri-4-nitrophenol/mole of subunit (Table 2). The pK of the nitrophenol residue near the active site is perturbed by pyrophosphate and phosphate. An analysis indicates that there are at least two pyrophosphate binding sites near the active site (K_s $< 10^{-6}$M and 8×10^{-5}M) (Fig. 5). Only a weakly bound phosphate group (K_s 6×10^{-3}M) is associated with a spectral change. The perturbation of the pK by pyrophosphate and phosphate is unlikely to be caused by mercurial migration, since the same perturbation occurs when 2moles of mercurial (I) are bound/mole of subunit of native enzyme.

Table 2. pK *values of 2-chloromercuri-4-nitrophenol bound to GPDH, CM-GPDH and thioglycollic acid*

The pK values were determined spectrophotometrically at 4°. The enzyme subunit or thioglycollic acid concentration was 50μM and organomercurial concentration was 40μM. Solution A contained EDTA (1mM), pyrophosphate (0·1M) and phosphate (0·1M) adjusted to I 0·6 with NaCl. Solution B contained EDTA (1mM) and ethylenediamine (0·2M) adjusted to I 0·6 with NaCl. The enzyme solutions contained NAD^+ (1mM).

	pK	
	Solution A	Solution B
GPDH	8·55	7·8
CM-GPDH	8·05	8·05
Thioglycollic acid	7·7	7·7

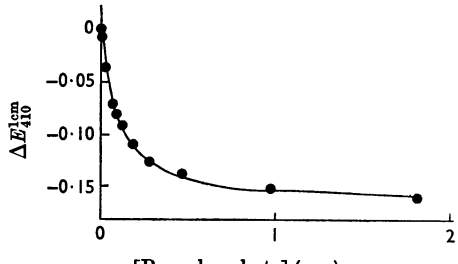


Fig. 5. Spectrophotometric titration at 4° of pyrophosphate into native GPDH (50μM) treated with 2-chloromercuri-4-nitrophenol (40μM). The solutions also contained triethanolamine hydrochloride adjusted to pH8·6 with aq. NH_3, EDTA (1mM) and NAD^+ (1mM).

Tertiary-structure determination of biological macromolecules. To determine the tertiary structure of a large biological molecule such as an enzyme by X-ray diffraction analysis, current methods require the preparation of heavy-atom derivatives of the molecule isomorphous with a parent molecule (Green, Ingram & Perutz, 1954; Blake, 1968). This has proved difficult in the past because of the lack of satisfactory methods of achieving the heavy-atom incorporation in stoicheiometric amounts under well-defined conditions. It is now clear (Fig. 3) that 2-chloromercuri-4-nitrophenol may be incorporated into GPDH under defined conditions. Moreover, analysis of the heavy-atom content of samples of crystalline enzymes is achieved with all four mercurials since their absorption bands ($\lambda_{max.}$ 410nm.) are separate from protein absorption bands.

Large crystals of CM-GPDH containing 1mole of 2-chloromercuri-4-nitrophenol/mole of subunit have been grown. The 7°-precession photographs show that these crystals are isomorphous with CM-GPDH crystals (Plate 1).

Estimation of cations. 2-Chloromercuri-4-nitrophenol may be used as a chromophoric reagent for the colorimetric titration of certain cations. Use is made of the tighter binding of a ligand to the cation than to the mercurial, coupled with the extinction change associated with the binding of the ligand to the mercurial. For example, Ca^{2+} may be estimated, by the reaction given in eqn. (2), from the results shown in Fig. 6.

DISCUSSION

The spectral data show that the mercurinitrophenols do provide excellent probes for thiol groups in biological molecules. The application that is described here relates to the specific problem of obtaining a crystalline heavy-atom derivative of GPDH isomorphous with the native enzyme (Watson & Banaszak, 1964; Wassarman & Watson, 1968), so other properties of the compounds have been treated in less detail. Factors that change pK values of the nitrophenol residues appear to be the introduction of anions in the vicinity of the hydroxyl group and alteration of the solvent. Both of these factors are likely to change in the type of reactions that have been described, so chromophoric changes may be monitored.

The kinetic and enzyme-activity studies suggest that the more slowly reacting thiol group of the native enzyme is similar to that of CM-GPDH and is unaffected by mercuration of the more reactive thiol. Titration studies indicate that one EDTA molecule is associated with each molecule of organomercurial before reaction with a thiol (C. H. McMurray & D. R. Trentham, unpublished

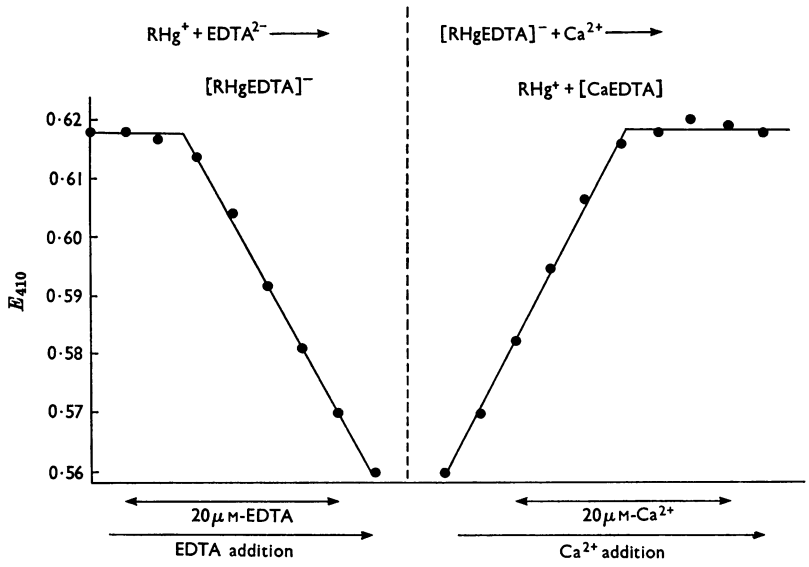
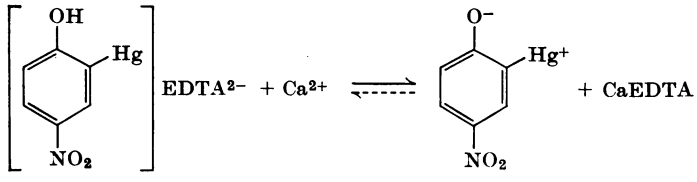


Fig. 6. Spectrophotometric titration of Ca^{2+} against EDTA. The left-hand titration curve shows the extinction changes of a solution of 2-chloromercuri-4-nitrophenol (39 μM) and triethanolamine hydrochloride (0·1 M) adjusted to pH 7·55 with NaOH when treated with 1 μl. portions of EDTA (10·0 mM). The plateau at the start of the titration is because initially the EDTA binds cation impurities in the solution (such as Ca^{2+} in the water) preferentially to the mercurial. The right-hand titration curve shows the extinction changes of the same solution containing the added EDTA when treated with 1 μl. portions of $CaCl_2$ (10·0 mM). The plateau at the end of the titration occurs when the Ca^{2+} ions have completely removed EDTA from the mercurial.



(2)

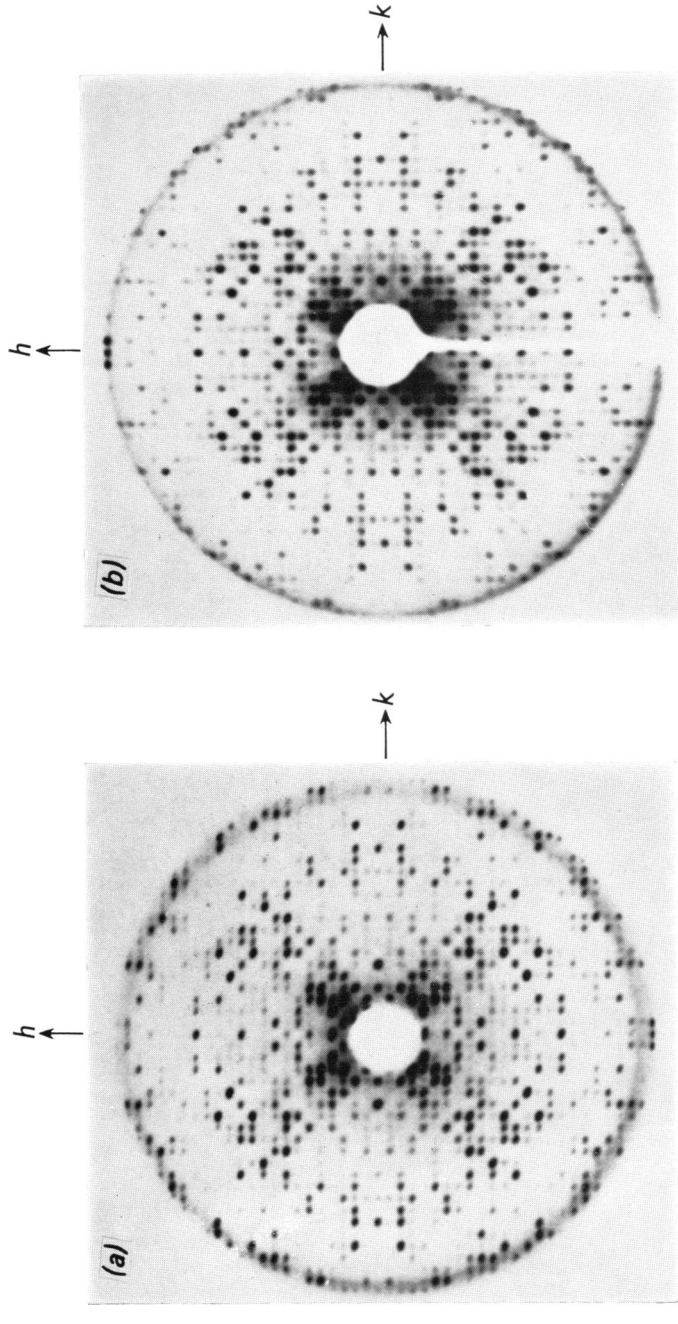
work). Further work will require detailed quantitative studies of thiol reactivity with the organomercurials by using simple model thiols.

It has been noted by Velick & Hayes (1953) that phosphate binds to GPDH at certain sites preferentially to the active site. The phosphate-binding site with a dissociation constant of 6×10^{-3} M may be the active-centre site. Such a dissociation constant is comparable with the constant previously observed that related to the phosphate perturbation of the 365 nm. absorption band associated with the NAD^+–GPDH complex (Trentham, 1968). There is no evidence from our results that phosphate would not also bind tightly at the tight pyrophosphate-binding site. In such a case presumably the smaller phosphate molecule would not be long enough to interact with the nitrophenol. Interpretation of the phosphate- and pyrophosphate-binding studies should be treated with reservation, since pyrophosphate cannot substitute as a substrate for phosphate, nor does it appear to be an effective competitive inhibitor.

2,6-Dichloromercuri-4-nitrophenol is a valuable reagent in that it may provide a second heavy-atom

EXPLANATION OF PLATE 1

Two X-ray diffraction photographs of lobster GPDH. (*a*) *hko* reciprocal lattice section of the native enzyme carboxymethylated at cysteine-148. (*b*) *hko* reciprocal lattice section of the 2-chloromercuri-4-nitrophenol derivative of the enzyme carboxymethylated at cysteine-148. Both photographs contain reflexions that correspond to a resolution at 6·3 Å.


k
h
(b)
k
h
(a)

derivative in addition to the one provided by the monomercurials. For example, large crystals of the dimercurial derivatives of triose phosphate isomerase (C. I. Pogson, personal communication) and GPDH have been prepared.

In conclusion it may be noted that the organomercurials described combine the properties of other reversible thiol agents currently available, notably 5,5′-dithiobis-(2-nitrobenzoic acid) and *p*-chloromercuribenzoic acid. However, they have the advantage over these materials in that they reflect further properties of the biological macromolecule, and they are proving of value in providing chemically well characterized heavy-atom derivatives of enzymes for the X-ray crystallographer.

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REFERENCES

Åkerfeldt, S. (1959). *Acta chem. scand.* **13**, 627.
Blake, G. C. F. (1968). *Advanc. Protein Chem.* **23**, 59.
Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.
Burr, M. E. & Koshland, D. E. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 1017.
Davidson, B. E., Sajgò, M., Noller, H. F. & Harris, J. I. (1967). *Nature, Lond.*, **216**, 1181.
Green, D. W., Ingram, V. M. & Perutz, M. F. (1954). *Proc. Roy. Soc.* A, **225**, 287.
Gutfreund, H. (1965). *An Introduction to the Study of Enzymes*, p. 123. Oxford and Edinburgh: Blackwell Scientific Publications.
Halford, S. E., Bennett, N. G., Trentham, D. R. & Gutfreund, H. (1969). *Biochem. J.* **114**, 243.
Harris, J. I. & Perham, R. N. (1968). *Nature, Lond.*, **219**, 1025.
Horton, H. R. & Koshland, D. E. (1965). *J. Amer. chem. Soc.* **87**, 1126.
Karoua, L. G. & Nesmeyanov, A. N. (1967). In *Methods of Elemento-organic Chemistry*, vol. 4, p. 84. Ed. by Makarova, L. G. & Nesmeyanov, A. N. Amsterdam: North Holland Publishing Co.
Ohno, T. (1956*a*). *J. Pharm. Soc., Japan*, **76**, 713.
Ohno, T. (1956*b*). *J. Pharm. Soc., Japan*, **76**, 718.
Raiziss, G. W. & Proskouriakoff, A. (1922). *J. Amer. chem. Soc.* **44**, 787.
Trentham, D. R. (1968). *Biochem. J.* **109**, 603.
Velick, S. F. & Hayes, J. E. (1953). *J. biol. Chem.* **203**, 545.
Wassarman, P. M. & Watson, H. C. (1968). *Abstr. 5th Meet. Fed. Europ. biochem. Soc., Prague*, A 769, p. 193.
Watson, H. C. & Banaszak, L. J. (1964). *Nature, Lond.*, **204**, 918.