The Binding of Heavy Metals to Proteins

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1 Introduction

Although heavy metals such as cadmium, mercury, platinum, and lead may have a physiological role, it is their toxicity which has attracted most attention. Thus cadmium from a mine in Japan became a real hazard to the local population giving rise to itai-itai disease (itai means pain); thallium is a rat poison and was used effectively by the Resistance in a factory in Holland against the Nazi management; chronic lead poisoning has been reported not only from occupational exposure but also in children who have eaten flaking paint; mercury caused headline news when mercury-containing waste from a factory manufacturing PVC and acetaldehyde was dumped in the sea in Minimata Bay and eventually found its way into fish which were the staple diet of the local inhabitants; and similar but less advertised tragedies have occurred with platinum and other toxic metals.

Increasing concern over the toxicity of heavy metals in the environment has lead to increased research activity to identify the fate of metal ions in the organism and the dependence of toxicity on dietary deficiencies of metal ions such as zinc and calcium (see reviews by Bremner[1]; Vallee and Ullmer[2]). Proteins are involved in the action of most heavy-metal ions at normal and toxic concentrations, although nucleic acid and lipid interactions are also important. For example, proteins in intranuclear inclusion bodies bind lead in chronic poisoning by this metal[3] and much of any cadmium and mercury absorbed is sequestered by a soluble kidney protein, metallothionen.[4] *In vitro* experiments have given further information concerning heavy-metal–protein interactions. Thus cadmium can mimic zinc in forming insulin hexamers; zinc-free carboxypeptidases with lead, mercury, or cadmium at the active site are effective in ester hydrolysis but cannot hydrolyse peptides,[5] and mercury binds many plasma proteins and gives rise to dimerization of albumin.[6] It is clear that heavy-metal ions may be transported or sequestered in the organism by proteins; they may also bind specifically to certain proteins with a concomitant enhancement, modification, or inhibition of the normal biological activity.

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Early studies of the nature of these different heavy-metal–protein interactions emphasized the importance of covalent binding to thiols of cysteine residues. The stability of the metal–sulphur bond allowed a ready identification of the ligand. However, observations such as the binding of cadmium to insulin, and mercury to the active site of carbonic anhydrase and of carboxypeptidase, which cannot involve cysteine, imply that cystine disulphides, histidine imidazole, methionine sulphur, and aspartic and glutamic carboxylic acids must also play an important role.

The problem of defining these ligands has been partly operational; they are difficult to identify in a large protein. However, the difficulties are partly conceptual. Proteins exist in a complex biochemical environment in living organisms and even in the laboratory are kept in buffered solutions containing many potential small molecular weight ligands, such as acetate, citrate, tris, phosphate, ammonia, and so on. These ligands can complex the metal ions and so modify their reactivity giving rise to many varied potential protein binding species and a complicated pattern of interaction. The pH and ionic strength can also affect the protein ligand and change its affinity for the metal. This complexity is further confused by the multidentate nature of the protein. Most metal ions are bound through several protein ligands in a specific three-dimensional arrangement. Thus the interaction with the protein tends to be largely entropically driven; the geometrical specificity is increased but energetic considerations of the strength of metal–ligand bonds become relatively less important. At the same time chemical analysis of the metal–protein interaction becomes prohibitively difficult; the interaction depends critically on the correct protein conformation whereas chemical analysis usually involves denaturation and degradation of the protein.

This depressing account of the difficulties in the study of heavy-metal–protein interactions ignores one important source of data. Protein crystallographers have long taken advantage of these heavy-metal ions to label proteins for use in *X*-ray analysis by the method of multiple isomorphous replacement.[7–10] In fact the successful use of metal ions in protein structure analyses is a fairly good indication of their toxicity: mercury, platinum, uranyl, thallium, and lead compounds have all found use. Theoretically, if the protein sequence is known, *X*-ray analysis can lead to a complete description of the metal ion binding site in a way that is not feasible otherwise. In reality this has not been the main interest of the study and most of the useful information has remained unpublished, and that which has been put in print lies scattered in many different journals. In this review we have brought together much of this information, published and unpublished, and present here a detailed analysis of protein–heavy-metal interactions. Although many bound metal ions may not affect the active site of the proteins, these interactions may affect their solubility or supramolecular

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organization and so lead to toxic effects. Alternatively, binding sites, especially in plasma proteins, may be important in transport of metal ions within the organism. In any case the nature of the ligands binding metal ions may be little different in situations which have physiological effects and those where the effects are neutral.

2 *X*-Ray Studies of Metals in Protein Crystals

Protein crystals exist as two phases: a solid phase of protein molecules packed in an open lattice and a liquid phase occupying the channels and spaces in this lattice. (For a review, see ref.7.) For instance, rhombohedral crysals of zinc insulin hexamers contain about 30% solvent, but other crystals of globular proteins can comprise as much as 60% solvent. Thus most of the protein is bathed in aqueous solvent in a way which is probably not unlike that found in biology. In insulin the solvent is a 0.2M citrate buffer at pH 6.3 and the lattice is stable with respect to changes of pH in the range of a few pH units. Other protein crystals require a high salt concentration to prevent dissolution but these may often be desalted after prior cross-linking with a bifunctional agent such as glutaraldehyde.

The solvent of crystallization is in equilibrium with the mother liquor surrounding the crystal, and the crystals must be kept covered in liquid to avoid evaporation of the solvent and consequent disordering of the lattice. However, this equilibration of the mother liquor and solvent in the lattice provides a straightforward mechanism for introduction of metal ions. Metal ions may be dialysed into the mother liquor and allowed to diffuse into the crystal. Studies using concentrations from 0.01mM to 1M of heavy-metal salt in a buffered solution have shown that the metal ions may diffuse into the crystal in a matter of minutes, although the reaction with the protein may take days or even weeks. Generally a metal ion concentration of 1mM is satisfactory for specific binding. Higher concentrations to give non-specific association of the metal in the lattice channels, but in a few cases can increase the occupancy of a site of interaction. Occasionally different interactions are given by co-crystallization or even controlled reaction between metal ion and protein prior to crystallization.

The interaction of metal ions with proteins can be monitored by following changes in the *X*-ray diffraction pattern. The method can be extremely sensitive and with modern methods of intensity measurement changes due to about 10 electrons in a protein of molecular weight 20 000 or more can be identified. However, some caution is required. Occasionally metal ion binding leads to lack of isomorphism. This manifests itself either in changes in dimensions of the crystal unit cell and consequently in the geometry of the diffraction pattern, or in large changes in the diffraction pattern at higher angles only. For the *X*-ray analysis to be successful the dimensions and symmetry of the diffraction patterns should not differ between the native and heavy atom derivative, and the changes in intensities should be distributed fairly evenly throughout the diffraction pattern. Intensity changes at low angle only usually result from non-specific binding and concentration of the metal ions in the lattice of the crystal.

The positions of the heavy atoms in the cell can be determined without solving the phase problem for the protein crystals, but this is a rather academic point as far as this discussion is concerned as it is also necessary to find the structure of the protein to define the nature of the protein–metal interaction. In fact, high-resolution analyses of over fifty proteins have now been reported,[7] and where protein sequences are available the metal co-ordination can be described. Generally the heavy-metal position will be more precisely defined than that of the protein ligands, and it is often difficult to fix the precise orientation of the liganding side-chain as protein maps, with the exception of two or three carefully refined structures, are not at atomic resolution. For most of the data tabulated here detailed metal co-ordination geometries are not available; but even identification of the ligands is a very important step forward.

3 The Metal Ions, Potential Ligands, and Factors Affecting the Stability of Metal–Protein Complexes

The ions of interest in this discussion include not only ions such as barium, uranyl, and rare earths, which are hard ions and usually bind preferentially to hard ligands such as water, fluoride, carboxylate, or alcohol hydroxyl, but also the B metals such as platinum, silver, gold, cadmium, and mercury, which bind softer ligands such as iodide, bromide, RS, R_2S, CN^- and $\diagdown\!\!\diagup N$ more strongly. The Tl^+ and Pb^{2+} ions have non-group valencies and larger radii; unlike the other B metal ions they prefer harder ligands such as carboxylates but may also become oxidized to Tl^{III} and Pb^{IV}, which binds soft ligands more strongly.

The potential ligands include not only the amino-acid functional groups of the protein but also the small molecules and ions present in the salt and buffer. Thus soft ligands are histidine imidazole, cysteine thiol, cystine disulphide, bromide, iodide, and cyanide. Terminal glutamate and aspartate carboxylates, hydroxyls of threonine and serine, amides of glutamine and asparagine, water, fluoride, acetate, citrate, and phosphate are harder ligands. The intermediate chloride, ammonia, and amino-groups will also often be present; like the softer ligands, these bind the B metals preferentially.

The reaction of the metal with the protein depends critically on the chemistry of the metal salt or compound. Thermodynamically stable complexes such as $Pt(CN)_4^{2-}$ will not react with the protein through ligand displacement whereas other complexes, such as $PtCl_4^{2-}$ of the same metal, may do so. Kinetic lability may also affect the course of the reaction. Thus platinum(II) substituted with a nitrogen ligand is further substituted slowly *trans* to this ligand, and sulphur atoms give stable complexes because they are not only poor leaving groups but also strong nucleophiles.

The charges of the metal ion and ligand are also important factors in determining the nature and extent of the reaction. Thus platinum complexes can exist as anions, neutral compounds, or cations. Clearly a negative ion such as $PtCl_4^{2-}$ will interact less readily with a negatively charged thiol: but a neutral compound

such as $PtCl_2(NH_3)_2$ will more easily bind. In a similar way a negatively charged thiol or carboxylate will be more nucleophilic than the protonated species. Hydrophobicity also plays an important role. $MeHg^+$ will more easily penetrate the hydrophobic core of the protein than the bivalent cation, Hg^{2+}.

Data on the relative importance of all these factors are available from *X*-ray studies (see Chapter 8 of ref. 7). This interplay of the various characteristics of the metal and protein underlie the interaction of metal ions with proteins not only in crystals but also in biology. Thus MeHgCl is a much better inhibitor of membrane-bound enzymes than inorganic mercury,[11] while the highly hydrophobic Me_2Hg will be concentrated almost entirely in lipids and the mechanisms of its toxicity will reflect both this and its comparative lack of reactivity with nucleophiles.[12]

4 The Protein Chemistry of Hard Cations

Let us first consider the binding of actinide and lanthanide ions. The results with the relevant references are summarized in Table 1.[13–27] The UO_2^{2+} ion binds carboxylate groups of glutamate or aspartate, and occasionally hydroxy side chains of threonine or serine as expected of a hard cation. Table 1 shows that ribonuclease S, rubredoxin, and insulin bind uranyl cations at many sites and there is considerable variation in occupancy. Quite often sites of low occupancy are clustered together. The reactivity of the uranyl cation is modified by complexing it with a hard ligand such as fluoride in $UO_2F_5^{3-}$ or acetate in $UO_2Ac_3^-$. In lysozyme, elastase, and insulin, the binding of the uranylfluoride anion is close to sites occupied by other uranyl salts, but the binding is less extensive. The carboxylate ions almost certainly co-ordinate by displacing the fluoride ligands.

Tervalent lanthanide ions also bind to carboxylate side-chains (Table 1). Samarium in the form of its acetate binds to two glutamate carboxylate groups

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Table 1 *The ligands of uranyl, lanthanide, plumbous and thallous cations and their complexes in proteins. Further details are given in reference 7 and in the references given in the table. Highly occupied sites are indicated by an asterisk*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*[a]	pH	*Site number*	*Binding site*
UO_2Ac_2	Insulin (13)	1 mM	0.05M-acetate 0.01M-$ZnAc_2$	6	1*	B13-Glu, B13′-Glu
					2	Cluster of sites
					3	
					4	
UO_2Ac_2	Cytochrome b_5 (14)	100 mM	4M-AS 0.1M-tris	7.5	1*	Asp-66; Glu-48
					2*	Glu-78
					3	Asn-11
					4	Asp-83, Lys-86; Glu-38, Glu-30
					5	Glu-43, Glu-37
					6	Glu-44
$UO_2(NO_3)_2$ at 37°C	Ferricytochrome c_2 (*Rhodospirillum rubrum*) (15)	10 × protein conc.	3M-AS	5.8	1*	Glu-64
					2*	Thr-63
					3	Lys-97
					4	Gly-37, Lys-112
$UO_2(NO_3)_2$	Bacterial ferredoxin (16)	100 mM	3.3M-AS 0.7M-tris/HCl	7.5	12 sites	Clusters of sites, some common with Yb^{3+} and Sm^{3+}
$UO_2(NO_3)_2$	Rubredoxin (17)	~100 mM	3.5M-AS	4	6 sites	Most highly occupied close to Asp-47. Cluster of 4 sites
$UO_2(NO_3)_2$	Tosyl elastase (18)	5 mM	1—2M-Na_2SO_4 0.01M-NaAc	5.0	1*	Glu-70, Glu-80, Try-82, Val-67, Leu-73 (Mainchain CO)

$UO_2(NO_3)_2$	Prealbumin (19)	10 mM	3.1M-AS	5.0	6 major sites	Between 2 Glu one on each of two subunits
$UO_2(NO_3)_2$	Lysozyme (hen egg white) (20)		0.85M-NaCl	4.7	5 sites	Asp and Glu carboxylates Thr-89
$UO_2(NO_3)_2$	α-Chymotrypsin (21)			3.6		Major site. Glu-21, Asp-153
$UO_2(NO_3)_2$	Concanavalin A (22)	0.1 mM			1*	Asp-80, Asp-83
					2	Not close to any side-chains
$K_3UO_2F_5$	Insulin (13)	1 mM	0.05M-citrate	6.3	1	2 sites close together
			0.01M-$ZnAc_2$		2	Glu-B13, Glu-B13′ (similar to UO_2Ac_2 but lower occupancy)
$K_3UO_2F_5$	Tosyl elastase (18)	30 mM	1.2M-Na_2SO_4 0.01M-NaAc	5.0	1	Glu-70, Glu-80, Tyr-82, Val-67, Leu-73 (mainchain). (same as $UO_2(NO_3)_2$ but lower occupancy)
$K_3UO_2F_5$	Lysozyme (hen egg white) (20)		0.0M-NaCl	4.7	1	Asp and Glu groups; (same
					2	as two largest $UO_2(NO_3)_2$ sites)
$SmAc_3$	Insulin (23)	1 mM	0.05M-NaAc			
$GdAc_3$			0.01M-$ZnAc_2$	6.2	1	Glu-B13, Glu-B13′ (same as
$DyCl_3$						UO_2Ac_2 major site)
$Sm(NO_3)_3$	Bacterial ferrodoxin (16)	40 mM	3.3M-AS	7.5	~10	Many sites clustered and
$PrCl_3$			0.7M-tris/HCl			some common with $UO_2(NO_3)_2$
$LaCl_3$	Thermolysin (24)				1	Ca^{2+} double site
$EuCl_3$			Tris/acetate	5.5	2	Asp-57
					3	Asp-200

(*continued overleaf*)

Table 1—*continued*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*	pH	*Site number*	*Binding site*
$Sm(NO_3)_3$	Concanavalin A (22)	10 mM			1	Glu-87, Asp-136, Asn-131,
					2	Gln-132, Gly-152, Asp-80,
					3	Asn-82, Asp-83
$PbAc_2$	Insulin (apo protein) (13)	10 mM	0.05M-acetate	6.3	1*	B13-Glu, B13-Glu
					2	His-B10
					3	His-B10
					4	*N* terminus B1, A17-Glu
$PbAc_2$	Insulin (13)	100 mM	0.05M-acetate	6.3	4 sites	As at 10 mM but site 4 is higher in occupation
$PbCl_2$	Carboxypeptidase (25)	3 mM	0.01M-citrate	7.5	1*	Glu-270
			0.02M-tris		2*	Citrate, not protein
$Pb(NO_3)_2$	Concanavalin A (26)		0.3M-$NaNO_3$	6.8	1*	Gln-87, Asp-136, Asp-80,
			0.01M-Na maleate		2*	Lys-82, Asp-83
TlF	Subtilisin novo (27)		0.05M-glycine/NaOH 55% acetone	9.1	1	Asp-197
TlAc	Insulin (23)	10 mM	0.05M-NaAc 0.01M-$ZnAc_2$	6.3		B13 Glu, B13 Glu (same as uranyl but lower occupancy)

a) AS = ammonium sulphate; Ac = acetate.

in insulin. Similar substitution is given by gadolinium and dysprosium. In flavodoxin, samarium binds close to an aspartate, and in lysozyme, europium and gadolinium bind between the active site glutamate and aspartate groups. The difference in specificity of the lanthanides is mainly due to the difference of size, and heavier and smaller lanthanides may often bind where others will not. This has been neatly illustrated in the work on thermolysin where lanthanides bind at calcium sites (see section 8).

In proteins Tl^+ and Pb^{2+} also bind in a similar way to the hard cations (Table 1). In insulin the major site for both Pb^{2+} and Tl^+ is at two glutamates in the same way as Sm^{3+} or UO_2^{2+} ions. In carboxypeptidase, Pb^{2+} binds to glutamate but in concanavalin it binds to two sites both of which involve aspartic acids. The uptake of Tl^+ by cells will reflect its similarity to K^+ though toxicity may depend on its unique redox characteristic. The similarity of the reactivity of Pb^{2+} and other hard cations is reflected in the 50% inhibition of the noradrenaline sensitive adenylcyclase of Purkinje cells in the rat cerebellium[28] by both Pb^{2+} (at 3 μM) and La^{3+} (at 2 μM). This has been suggested as the primary site of lead neurotoxicity. In this case Ca^{2+} ions do not prevent the inhibition but Pb^{2+} and Ca^{2+} can compete, as suggested by the finding that voluntary ingestion of lead is linked to subclinical Ca^{2+} deficiency in Rhesus monkeys.[29] The intermediate character of the Pb^{2+} ions compared to uranyl and lanthanide ions is shown by the fact that Pb^{2+} binds to a small extent with the imidazoles which bind zinc in zinc-free insulin and to a terminal amino-group when the lead is at high concentration.

In conclusion, it appears that lanthanides are more selective than uranyl ions. In contrast to uranyl, which often gives multisite binding, samarium (and lead) may give one major site. In insulin the extent and rate of lead binding was found to be very dependent on the concentration of ions and the temperature.[23]

The binding of uranyl and lanthanide ions is sometimes prevented by formation of insoluble phosphates and hydroxides. Chelating agents such as citrate will also bind the metal ions and inhibit the binding; for instance uranyl acetate and samarium acetate show little reaction with insulin in citrate buffer although they bind strongly in acetate or tris buffer. However, they bind readily to proteins in the presence of large concentrations of nitrogen ligands.

5 Protein Chemistry of Soft Ions: Covalent Interactions

B-metals such as platinum, mercury, and gold tend to form covalent complexes with ligands such as thiol, imidazole, and thioethers rather than to harder ligands such as carboxylate or hydroxy-groups. Their chemistry has been widely reviewed.[7,20,30–34]

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A. Mercuric Compounds.—In 1954 Perutz and his colleagues[35] exploited the different reactions of the thiols in the α- and β-chains of haemoglobin towards *p*-chloromercuribenzoate (PCMB). Binding at the α-chain was achieved by first blocking the more reactive β-chain thiols with iodoacetamide and then crystallizing in the presence of mercuric acetate. Binding was also observed with dimercuriacetate (DMA) and 1,4-diacetoxymercuri-2,3-dimethoxybutane (Baker's mercurial), which contain two mercury atoms and were found crystallographically to have their mercury atoms separated by 1.7 and 4.9 Å, respectively. Rossmann and his co-workers in their study of lactate dehydrogenase found that two thiol groups are reactive with PCMB, $HgCl_2$, and DMA (see Table 2) but only one was reactive with the more bulky Baker's mercurial. Different mercurials all bind to the same cysteine thiol in calcium-binding protein. These data are summarized in Table 2.[36–56]

Many of these reagents have a covalent mercury–carbon bond which is not easily broken. The chloride, acetate, or nitrate ligands are not bound strongly, and the mercury cation is particularly reactive towards the negatively-charged and polarizable —S groups. The cysteines will be less reactive at lower pH when the thiol is protonated. Ammonia will complex the mercury but will not displace carbon substituents and is unlikely to change its charge. An excess of chloride decreases the reaction by complexing with the mercury and giving it a net negative charge; this has been observed with calf rennin in 2M-NaCl

The —SMe of methionine is less nucleophilic than the thiol group, $—S^-$, and anyway has no negative charge. This probably explains the fact that it rarely binds mercury reagents. One example is the binding of HgI_3^- to rubredoxin.

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Table 2 *The ligands of mercurials in proteins. The chemical formulae for these mercurials are further detailed in reference* 7

Reagent[a]	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*[a]	pH	*Site number*	*Binding site*
$HgAc_2$	Carbonic anhydrase (36) (apoenzyme)		2.3M-AS	8.5	1	His-93, His-95, His-117, (zinc site)
$HgAC_2$ $HgBr_2$	Calcium binding protein (37)	0.8 mM	4M-phosphate	6.8	1	Cys-18
Hg (succin-imide)$_2$	Thermolysin (24)	5 mM	5% DMS 0.01M-$CaAc_2$ 0.01M-tris/acetate	4	1	His-231
$HgCl_2$; LiCl	Thermolysin (24)	1 mM 20 mM	5% DMS 0.01M-$CaAc_2$ 0.01M-tris acetate	6	1	His-231
$HgCl_2$	Carboxypeptidase A (25)	0.8 mM	0.2M-LiCl 0.02M-tris	7.5	1*	His-69, Glu-72, His-196, zinc site
					2*	His-120
					3*	His-29, Lys-84
					4	His-303
$Hg(NH_3)_2^{2+}$ (HgO in AS)	Myoglobin (38)	Equimolar with protein	3M-AS	6.5	1	His GH-1 close to AG^+ site Asn GH-4 Lys A-14 (same as Zn^{2+})
$HgAc_2$	Glycera haemoglobin (39, 40)			6.8	1	Cys-B30
$HgAc_2$	Concanavalin (41)		2.1M-phosphate	6.0	1	His-127
					2	Met-129, His-127
					3	Asp-118
					4*	Asp-80, Asx-83, Tyr-100, His-205
					5	Lys-135
					6	Tyr-12, His-205

(*continued overleaf*)

Table 2—*continued*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*[a]	pH	*Site number*	*Binding site*
$HgCl_2$	Hen egg white lysozyme chloride (20)		0.85M-NaCl	4.7	1 major	Arg-14, His-15, Asn-93, Lys-96, Arg-128 (same as $PdCl_4^{2-}$, $PtCl_6^{2-}$)
$HgAc_2$	Prealbumin (19)	0.5 mM	3.1M-AS	5.0	4 sites	Cys-10 (one per monomer)
$HgAc_2$	Human lysozyme (42)	50 mM	3M-NaCl 0.02M-NaAc	4.5	1* 2	
$HgAc_2$	Chironomus haemoglobin (43)		3.75M-phosphate	7	1* 2	His-G2, Asn-G7 His-G19
$Hg(CN)_2$	Lamprey haemoglobin (39)	0.5 mM	2M-phosphate 10 μM-NaCN	6.8	1* 2 3	Three sites close to SH of Cys-141 position, *i.e.* side-chain occupies different positions. ($HgCN_4^{2-}$ does not bind)
$HgCl_2$	Lactate dehydrogenase (44)	10 × conc. of protein			1 2	Cys(SH) Cys(SH)
$HgCl_2$	Papain (HgCl Blocked-SH) (45)	mM			1* 2	His-159 Asn-194
$HgCl_2$	Haemoglobin (46)		1.9M-AS	7.0	1* 2*	Cys-104α(SH) Cys-93β(SH)
$Hg(Ac)_2$	Glycera haemoglobin (40)	2 × protein conc. prior to crystal-lization			1	Cys-30 (B39)
EtHgCl	Insulin (47, 48)	Sat.	0.05M-acetate 0.01M-$ZnAc_2$	6.3	1	His-B5
EtHgCl MeHgCl	Calcium binding protein (37)	0.8 mM	4M-phosphate	6.8	1	Cys-18-SH
$MeHgNO_3$	Lactate dehydrogenase (44)					Cys(SH)

$MeHgNO_2$	Adenyl kinase (49)	0.05 mM			1*	Cys-25
					2	Cys-187
					3	His-36
MeHgCl	Concanavalin (41)		2.1M-phosphate	6.0	1*	Met-129, His-127
					2*	His-205
					3*	Tyr-100
					4*	Asp-118
					5	Tyr-100
					6	His-180, Gln-87, Trp-88, Trp-182
PCMBS	Papain (45)	10 mM	Methanol Water	9.3	1	His-81
					2	His-159
PCMBS	Myoglobin (38)	Equimolar with protein	3M-AS	6.5	1	His-G14 (Hg binding) Asn-H8, Lys-FG2, Ser-F7 (Sulphonate binding)
PCMBS	Lysozyme (hen egg white) (20)		0.85M-NaCl	4.7	1	Sulphonate of PCMBS binds Arg-68
PCMB PCMA	Papain (45)	1.5 mM	Methanol Water	9.3	1*	His-81 (Same as PCMBS)
					2	His-159
PCMB	Bovine pancreas basic trypsin inhibitor (50)	Sat.	2.25M-phosphate	10	1*	*C*-terminus
					2	Lys-41, Tyr-10
					3	*N*-terminus
PCMB PCMA	Calcium binding protein (37)	0.8 mM	4M-phosphate	6.8	1*	Cys-18 (SH)
PCMA	Myoglobin (38)	Equimolar with protein	3M-AS	6.5	1*	His-GH-1 Asn-GH-4 Lys-A-14
PCMB	Lactate dehydrogenase (44)	10 × protein conc.			1*	Cys(SH)
					2	Cys(SH)
PCMB	Haemoglobin (46)				1*	Cys-93 β(SH)

(continued overleaf)

Table 2—*continued*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*[a]	pH	*Site number*	*Binding site*
HMSA	Bovine pancreas basic trypsin inhibitor (50)	4 mM	2.25M-phosphate	10	1	Asn-24, Gln-31, Lys-15
					2	*N*-terminus
					3	*N*-terminus
					4	Asn-24, Gln-31, Lys-15
					5	*C*-terminus
MSSS	Bovine pancreas basic trypsin inhibitor (50)	6 mM	2.25M-phosphate	10	1*	*N*-terminus
					2*	Tyr-21, Arg-19
					3	Asn-24, Gln-31, Lys-15
					4	*N*-terminus
					5	Lys-46
HMTS	Bovine pancreas basic trypsin inhibitor (50)	6 mM	2.25M-phosphate	10	1	*N*-terminus
HMTS	Lysozyme (20)		0.85M-NaCl	4.7		Sulphonate binding to Arg-68
Baker's Dimercurial	Haemoglobin (46)		1.9M-AS	7	1	Cys-93 β(SH)
Baker's Dimercurial	Lactate dehydrogenase (44)	10 × protein conc.			1	Cys(SH)
PHMB	Lactate dehydrogenase (44)	4 × protein conc.				Cys(SH)
PHMBS	Malate dehydrogenase (51)	0.1 mM	2.8M-AS 0.1M-NaAc	5	3 sites	Cys(SH)
PMA	α-Chymotrypsin (52)		3.5M-phosphate	4.2	1, 2	*N*-terminus
PhHgAc			2—4% dioxan			S—S Cys-1—27
Thiomersal PhHgAc $PhHgNO_2$	Calcium binding protein (37)	0.8 mM	4M-phosphate	6.8	1	Cys-18(SH)

DMA	Thermolysin (24)	0.001 M	5% DMS 0.01M-$CaAc_2$ 0.01M-tris/acetate	7.5	1	His-231
Thiomersal	Liver alcohol dehydrogenase (53)	10^{-2} mM	tris/HCl 0.05M	8.4	1* 1*	Cys-240 Cys-9
DMA	Lactate dehydrogenase (24)	10 × protein conc.			2	Cys(SH)
DMA	Haemoglobin (46)					β-chain SH
Mersalyl	Ferricytochrome *c* (horse) (54)		4.6M-phosphate	6.2		His-33
Mersalyl	Cytochrome b_5 (14)	0.3 mM	3M-phosphate	7.5		Glu-48 Tyr-27(O) Arg-84
Mersalyl	Subtilisin BPN′ (55)	0.9 mM	2.1M-AS 0.05M-NaAc	5.9	1 2 3	His-64 *N*-terminus His-64
Mersalyl	Chironomus haemoglobin (43)		3.75M-phosphate	7	1* 2*	His-G2, Asn-67, His-G19. Same as $HgAc_2$
Mersalyl	Concanavalin A (22)	0.1 mM			1* 2	His-127, Met-129 His-127
K_2HgI_4	Ferricytochrome *c* (tuna) (56)	0.3 mM	4M-AS	6	1 2	Gln-16 Cys-17 thioether bridge to haem Gly-37, Asn-60
K_2HgI_4	Rubredoxin (17)	High conc.	3.3M-AS	4	1*	Gly-43, Met-1

(*continued overleaf*)

Table 2—*continued*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*[a]	pH	*Site number*	*Binding site*
K_2HgI_4	Papain (45)	5 mM	Methanol water		1	*N*-terminus. (same as His-159)
					2	$IrCl_6^{3-}$ and $PtCl_6^{2-}$
$KHgI_3$	Myoglobin (35)	Same as protein conc.	3.0M-AS	6.5	1*	Next to haem in hydrophobic pocket
					2	Lys-FG2, Gln-F6, Asn-118, Gln-114
K_2HgI_4	Lysozyme (20)		0.85M-NaCl	4.7	1 major	Arg-13, Arg-13′
					2 minor	(2 sites 5.6 Å apart) (same as $IrCl_6^{3-}$, $AuCl_4^-$, PdI_4^{2-})
$K_2Hg(SCN)_4$	Chironomus haemoglobin (43)		3.75M-phosphate	7	1	haem
					2*	His-G19
$K_2Hg(SCN)_4$	Lamprey haemoglobin (39)	0.5 mM	2M-phosphate 10μM-NaCN	6.8	1*	His-73
					2	Cys-141
					3	Cys-141
K_2HgI_4	Glycera haemoglobin (40)	1.5 mM	3.2M-AS phosphate	6.8	3 sites	Cys-B30 (major site)

(*a*) AS ≡ ammonium sulphate; Ac ≡ acetate; Ph ≡ phenyl; Me ≡ methyl; Et ≡ ethyl; PCMB ≡ *p*-chloromercuribenzoate; PCMBS ≡ *p*-chloromercuribenzene sulphonate; PHMB ≡ *p*-hydroxymercuribenzoate; PHMBS ≡ *p*-hydroxymercuribenzene sulphonate; HMSA ≡ 3- or 5-hydroxymercurisalicyclic acid; MSSS ≡ 3-hydroxymercuri-5-sulphosalicyclic acid; PCMA ≡ *p*-chloromercurianiline; Mersalyl ≡ salyrganic acid; DMA ≡ dimercuriacetate; Baker's Dimercurial ≡ 1,4-diacetoxymercuri-2,3-dimethoxybutane; HMTS ≡ 2-hydroxymercuritoluol-4-sulphonic acid. The chemical formulae for these mercurials are further detailed in reference 7.

Sulphur atoms are not only possible ligands for mercurials. In fact, histidines very often bind mercury reagents. Imidazole becomes a very good ligand above pH 6—7 when it loses its proton. For instance, Table 2 shows that thermolysin, which has no methionine or cysteine, binds DMA, mercurisuccinimide, and $HgCl_2$ through histidines. The mercury-containing compound, mersalyl, has been called a histidine specific reagent as it binds to a histidine in cytochrome *c* and subtilisin BPN.[31] However, neither of these proteins has thiol groups. In calcium-binding protein, mersalyl binds to a thiol group. The specificity, if any, of mersalyl is most likely due to its large size.

The specificity of mercurials seems to be very dependent on their size, shape, and substituent groups. We have seen that in lactate dehydrogenase, the bulky Baker's mercurial binds only one thiol whereas smaller reagents bind two. The binding of mercurials to the immunoglobulin fragment Fab New was also studied by varying the nature of the reagent until ones were found which bound specifically. In alcohol dehydrogenase, which contains 14 thiols, most mercurials denatured the protein, but one, thiomersal, gave a more specific reaction which did not lead to denaturation.[53]

The kind of interaction which may give rise to binding of mercurials to proteins was evident from the early studies of myoglobin and lysozyme. In myoglobin, PCMBS (see Table 2) binds to a histidine. Its negative charge interacts with the positive charge of a lysine on a neighbouring molecule in the lattice. PCMBS does not bind at this site; this has no negative charge. In fact this kind of interaction may easily give rise to binding of PCMBS in a way which does not involve the mercury atom at all. In lysozyme, PCMBS binds only through the sulphonate! In the case of carbonic anhydrase mercury-containing sulphonamides bind as a bivalent ligand with the mercury interacting with the imidazole of His-63 and the sulphonamide group binding to the active site zinc (see Figure 1).[36] This results in strong inhibition.

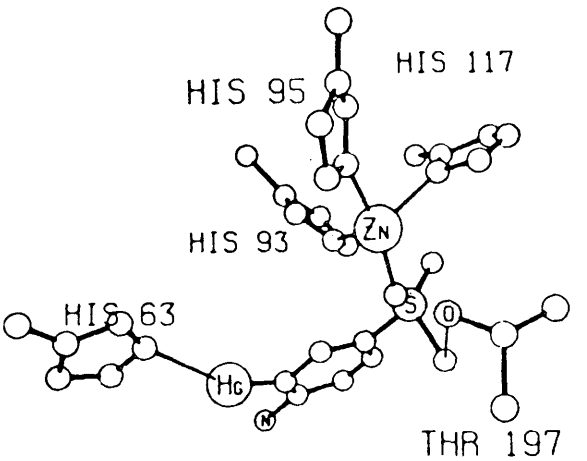

Figure 1 *The interaction of a sulphonamide inhibitor with carbonic anhydrase* (Reproduced by permission from A. Liljas, PhD Thesis, University of Uppsala, 1971)

Anionic mercury complexes such as $HgCl_4^{2-}$, $HgBr_4^{2-}$, or $Hg(SCN)_4^{2-}$ also bind proteins. Despite the fact that the anions have a negative charge the mercury can still become bound by the negatively charged cysteine, as in lamprey haemoglobin. This is probably due to the dissociation of the complex. Thus HgI_4^{2-} dissociates to give rise to HgI_3^-, HgI_2, and I^- in solution, and the reaction may be through an uncharged species. On the other hand the anionic forms can interact electrostatically as in lysozyme or in the minor sites of ferrocytochrome and myoglobin or through hydrophobic interactions as in myoglobin or chironomus haemoglobin. This is discussed in Sections 6 and 7. The hydrophobicity of these and organomercurial reagents may be reflected in a tendency to interact specifically with membrane components. Thus some membrane adenyl cyclases are specifically inhibited by MeHgCl at low concentrations.[11]

B. Mercuration of Disulphides.—Steinberg and Sperling[57] have shown that mercury atoms may be inserted into disulphide bonds of cystines. The mercuration of the cystine may be carried out either by reduction of the disulphide followed by reaction with mercuric ions[58] or in one stage by using the reducing mercurous ions:

$$Hg_2^{2+} + RSSR = 2(RSHg)^+ = RSHgSR + Hg^{2+}$$

The S—Hg—S system formed in this way is linear and is about 3 Å longer than the disulphide bond. Despite this, substitution into one disulphide of ribonuclease[58] or papain[59] appears to have little effect on the conformation or biological activity, and insertion of mercury atoms into immunoglobulin fragments has little effect on antibody binding.[60] However, mercurated ribonuclease crystallized,[58] and the mercurated immunoglobulin fragment, Fab New,[60] crystallized non-isomorphously. Nevertheless, a mercurated Bence–Jones dimer[61] and mercurated α-lactalbumin[62] gave a smaller change in structure of the protein in the crystal. In insulin a selective mercuration of one disulphide, A6—A11, is given.

C. Silver Ions.—Table 3 shows that silver nitrate reacts either with cysteine as in haemoglobin or more often with histidine as in myoglobin, trypsin,[63] and carboxypeptidase. The ions react in a similar way to mercuric ions such as $Hg(NH_3)_2^{2+}$ and probably also form an ammonia complex, $Ag(NH_3)_4^+$, when ammonium sulphate is present. The fact that Ag^+ is less polarizing and not as

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[61] K. R. Ely, R. L. Girling, M. Schiffer, D. E. Cunningham, and A. E. Edmundson, *Biochem. J.*, 1973, **12**, 4233.
[62] R. Aschaffenberg, personal communication, 1976.
[63] J. L. Chambers, G. G. Christoph, M. Krieger, L. Hay, and R. M. Stroud, *Biochem, Biophys. Res. Comm.*, 1974, **59**, 70.

Table 3 *The binding sites of silver in proteins*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*	pH	*Site number*	*Binding site*
$AgNO_3$	Thermolysin (24)	5 mM	5% DMS 0.01M-$CaAc_2$ 0.01M-tris/Ac	5.5	1 2	His-231 His-88
$AgNO_3$	Carboxypeptidase A (25)	5 mM	0.2M-NaAc	8.0	1 2 3 4	His-166, Ser-158 His-120 His-29, Lys-84 His-303
$AgNO_3$	Myoglobin (38)	equimolar with protein	3M-AS	6.5	1	His-B-5 His-GH-1
$AgNO_3$	Haemoglobin (46)		1.9M-AS	7.0	1* 2*	Cys-104α(SH) Cys-93β(SH)
$AgNO_3$	Trypsin (63)	12 mM	$MgSO_4$	7.5	1	His-57, Asp-102

reactive as Hg^{2+} may explain why in acetate buffer Ag^{+} ions bind glugacon in a similar way to the mercuric chloride but with less disordering.[64]

D. Tetrachloroplatinate(II), Tetrachloroaurate(III), and Analogous Reagents.— Many data are available for the reactive tetrachloroplatinate ion, $PtCl_4^{2-}$. The reaction conditions and binding sites of this and related heavy atom reagents are summarized in Table 4.

Platinum, palladium, and gold are fairly soft, forming stable covalent complexes with soft ligands such as chloride, bromide, iodide, ammonia, imidazole, and sulphur ligands. The stereochemistry of their complexes depends critically on the number of '*d*' electrons. Thus d^8 ions of Pd^{II}, Pt^{II}, and Au^{III} are predominantly square-planar. This includes $PtCl_4^{2-}$, $Pt(NH_3)_4^{2+}$, $Pt(CN)_4^{2-}$, and $AuCl_4^{-}$. Occasionally these ions accept one further ligand to give a square pyramid or two ligands to give octahedral co-ordination, but the fifth and sixth ligands are much more weakly bound. On the other hand Pt^{IV} has a d^6 electron configuration and forms stable octahedral complexes such as $PtCl_6^{2-}$ with six covalently bound and equivalent ligands.

In order to understand their protein chemistry we must consider the factors affecting the thermodynamic and kinetic stability of the complexes, not only the potential protein ligands but also the effect of salting-out agent, buffer, and pH on the reaction. Sigler and Blow[74] have drawn attention to the fact that NH_3 from $(NH_4)_2SO_4$ may displace chloride from the square-planar $PtCl_4^{2-}$, and alter the reaction with proteins. They transferred α-chymotrypsin crystals into phosphate and found this led to faster and more reproducible reaction with $PtCl_4^{2-}$. Wyckoff *et al.*[66] found further evidence for the displacement of chloride by ammonia in their study of ribonucleases; only freshly prepared $PtCl_4^{2-}$ reacted.

The platinum complexes found in the presence of ammonium sulphate and phosphate are illustrated in Figure 2.[33] The formation of the phosphate complex may be minimized if a large excess of chloride ions is present. Acetate may also form complexes in time if there is an excess of acetate and no chloride. These complexes vary in the nature of the ligands and in their charge; they will therefore react in very different ways with a protein.[33] The charged groups $PtCl_4^{2-}$, $PtCl_3(PO_4)^{4-}$, $PtNH_3Cl_2(PO_4)^{2-}$, and $Pt(NH_3)_4^{2+}$ do not penetrate into an hydrophobic protein core. The anionic groups do not react with anionic reagents such as RS^{-} but are attacked more readily by neutral nucleophiles such as R—SH, R—imidazole, or $R—NH_2$. The cationic group $Pt(NH_3)_4^{2+}$ is rather inert due to the weak *trans* effect of the ammonia ligands, and it is most likely to form electrostatic complexes with anionic groups such as carboxylate. The neutral $Pt(NH_3)_2Cl_2$ molecule can penetrate into hydrophobic areas. It will require a stronger nucleophile but will be reactive to anionic nucleophiles such as $R—S^{-}$.

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[74] P. B. Sigler and D. M. Blow, *J. Mol. Biol.*, 1965, **12**, 17.

Table 4 *Protein ligands of platinum, palladium, and gold complexes*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*	pH	*Site number*	*Binding site*
K_2PtCl_4	Concanavalin A (41, 65)	0.5 mM	2.1M-phosphate	6.0	1*	Met-129, His-127
					2	Met-129
K_2PtCl_4	Chironomus haemoglobin (43)		3.75M-phosphate	7.0	1*	Met-H17
					2*	His-G2, *C*-terminus
					3	His-G19
$Pt(NO_2)_2(NH_3)_2$	Chironomus haemoglobin (43)		3.75M-phosphate	7.0	1	His-G2
					2*	His-G19
$Pt(en)Cl_2$	Ribonuclease S (66)	2 mM	3.2M-AS	8	1	His-119
$Pt(en)Cl_2$	Ribonuclease S (66)	2 mM	3.2M-AS	5.5	1*	Met-29
$Pt(en)Cl_2$	Lactate dehydrogenase (44)	2.5 mM			1	Cys(SH)
					2*	Cys(SH)
K_2PdCl_4	Lysozyme (20)		0.85M-NaCl	4.7	1	Arg-14, His-15, Asn-93,
K_2PdBr_4						Lys-96, Arg-128
K_2PdI_4	Lysozyme (20)		0.85M-NaCl	4.7	1	Arg-13, Arg-13′ (same as $IrCl_6^{3-}$, HgI_4^{2-}, $AuCl_4^-$)
K_2PtCl_4	Concanavalin (22)	1 mM			1*	His-127, Met-129
					2	Met-42
K_2PtCl_4	Ferricytochrome *c* (horse) (54)		4.6M-phosphate	6.2	1*	Met-65 } Close together
					2*	Met-65 }
					3	His-33
K_2PtCl_4	Tuna ferrocytochrome *c* (67)	0.1 mM	95% AS	6	1	Met-65
K_2PtCl_4	Cytochrome c_{550} (68)	1.3 mM			1*	
K_2PtBr_4 or K_2PtCl_4 or K_2PtI_4	α-Chymotrypsin (69)		3.5M-phosphate 2—4% dioxan	4.2	1, 2*	*N*-terminus and S—S of Cys-1—127
					3, 4*	Met-192

(*continued overleaf*)

Table 4—*continued*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*	pH	*Site number*	*Binding site*
K_2PtCl_4	Subtilisin BPN′ (55)	0.65 mM	2.1M-AS 0.005M-acetate	5.9	1* 2	Met-50 His-64
K_2PtCl_4	Subtilisin novo (27)				1 2 3	Met-50 Trp-241 His-238 Trp-106 Ala-1(*N*-terminus)
K_2PtCl_4	Thermolysin (24)	6 mM	5% DMS 0.01-$CaAc_2$ 0.01M-tris/acetate	5.8	1 2	His-250 His-216
K_2PtCl_4	Carboxypeptidase A (25)		0.2M-LiCl 0.02M-tris	7.5	1 2* 3* 4	Cys-161 (—S—S—) Met-103 *N*-terminus: Ala-1 His-303
K_2PtCl_6	α-Chymotrypsin (69)		3.5M-phosphate	4.2	1, 2*	Terminal amino-group and S—S of Cys-1—127 (same as $PtCl_4^{2-}$)
K_2PtCl_6	Papain (45)	5 mM	Methanol/water	9.3	1 2	*N*-terminus His-159 (same as HgI_4^{2-}, $IrCl_6^{3-}$)
K_2PtCl_6	Concanavalin A (65)	3 mM	2.1M-phosphate	6.0	1* 2 3	Met-129 (same as $PtCl_4^{2-}$) Met-129 Met-42
K_2PtCl_6	Lysozyme (20)		0.85M-NaCl	4.7	1	Arg-14, His15, Asn-93, Lys-96, Arg-128 (same as $HgCl_2$ and $PdCl_4$)

K_2PtI_6	Thermolysin (24)	Sat. soln.	5% DMS 0.01M-$CaAc_2$ 0.01M-tris/acetate	5.6	1 major 5 minor	His-250 Iodination of tyrosine
K_2PtBr_6	High Potential Iron Protein: HiPIP (70)	0.5 mM	3.2M-AS	6.5	1* 2 3	Met-49
$K_2Pt(NO_2)_4$	High Potential Iron Protein: HiPIP (70)	10 mM	3.2M-AS	6.5	1* 2	Met.-49
$K_2Pt(NO_2)_4$	Adenyl kinase (49)	2 mM			1* 2	His-36
$KAuCl_4$	Carbonic anhydrase (36)				1 2 3	Zn, Thr 197, X139 H_2O on Zn, His 128 Arg 25, carbonyl of His 35
$KAuCl_4$	Ferricytochrome c_2 (*Rhodospirillum rubrum*) (15)	10—100 × protein conc.	3.2M-AS	5.8	1* 2	His-42 Asp-3
$KAuCl_4$	Lysozyme chloride (20)		0.85M-NaCl	4.7	1	Arg-12, Arg-13′ (same as HgI_4^{2-})
$NaAuCl_4$	Lactate dehydrogenase (44)	1 mM			1 2	Cys(SH) Cys(SH) Cys(SH)
$HAuCl_4$	Glycera haemoglobin (40)	1.5 mM	2.6M-AS 0.06M-phosphate	6.8	1	Cys-30 (B39) His-72
$KAuCl_4$	Myoglobin (38)	Equimolar with protein	3M-AS	6.5	1	His-B5 (same as $\overset{+}{Ag}$) His-GH1
$KAuI_4$	α-Chymotrypsin (71)				1	Met-192, Cys-191—220 (same as $PtCl_4^{2}$)

(*continued overleaf*)

Table 4—*continued*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*	pH	*Site number*	*Binding site*
$HAuCl_4$	Adenyl kinase (49)				1	His-36
MMTGA + $K_2Pt(CN)_4$	Carbonic anhydrase (36)		2.3M-AS	8.5	1	Zn, Thr-197, X139
$K_2Pt(CN)_4$	Ferrocytochrome *c* (tuna) (72)	6 mM	95% AS	6	1	No near neighbour
					2	Same as HgI_4^{2-} Lys-53, Ala-4, Lys-7, Ser-100, Val-3
					3	Glu-44, Gln-70, Lys-72, Lys-73 Lys-99, Lys-99′, Ser-103,
					4	Ser-103′
					5	Glu-21, Lys-7, Lys-25 Ile-269 (main chain)
$K_2Pt(CN)_4$	Liver alcohol dehydrogenase (73)	1 mM	0.05M-tris/HCl	8.4	1	Asp-223, Lys-228, Arg-47, Arg-369
$K_2Pt(SCN)_4$	Adenyl kinase (49)	2 mM			1*	
					2	Major site near
					3	His-36
					4	
$KAu(CN)_2$	Carbonic anhydrase (36)	20 mM	2.3M-As	8.5	1*	H_2O on Zn, His-128

$KAu(CN)_2$	Lamprey haemoglobin (39)	1 mM	3.6M-AS 20μM-NaCN	6.8	1	Lys-106, Ser-107 Glu-92
					2	Ser-107, Val-8
					3	Cys-141
						Not in presence of phosphate

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[68] R. Timkovich and R. E. Dickerson, *J. Mol. Biol.*, 1973, **79**, 39.
[69] P. B. Sigler, D. M. Blow, B. W. Matthews, and R. Henderson, *J. Mol. Biol.*, 1968, **35**, 143.
[70] C. W. Carter, J. Kraut, S. T. Freer, and R. A. Alden, *J. Biol. Chem.*, 1974, **249**, 6339.
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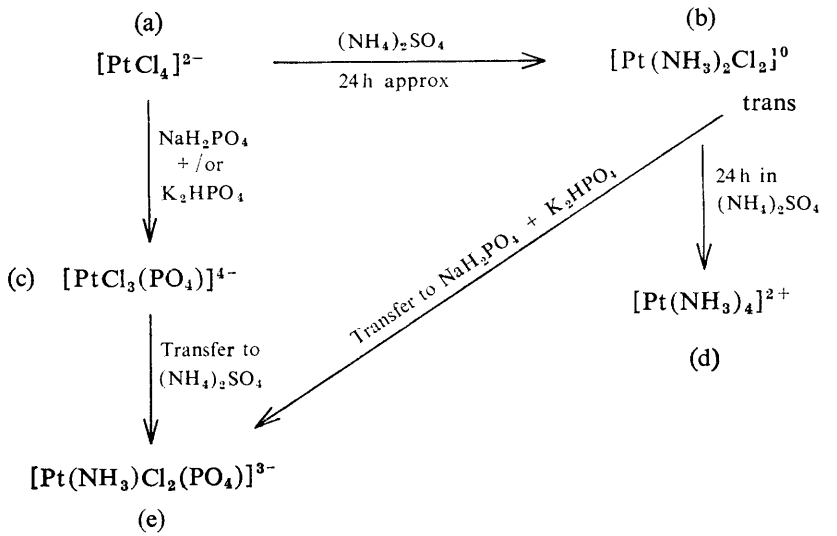

Figure 2 *The complexes of platinum which may exist when* $PtCl_4^{2-}$ *reacts with ammonium sulphate or phosphate*
(Reproduced by permission from G. Petsko, DPhil Thesis, Oxford University, 1973)

These observations provide a rationale for the observed reactions of protein crystals when soaked in $PtCl_4^{2-}$.[33] At acid pH it reacts with methionines, cystine disulphides, *N*-termini, and histidine imidazole, which all form stable complexes. These are good nucleophiles which can displace chloride from platinum complexes. Cysteine —SH groups are less nucleophilic. $PtCl_4^{2-}$ does not react with the cysteines of erythrocruorin or triosephosphate isomerase at about pH 7 in phosphate buffer, or the cysteines of malate dehydrogenase at pH 5.0 in the presence of ammonium sulphate. However, prealbumin and triosephosphate isomerase at about pH 7 in ammonium sulphate react with $PtCl_4^{2-}$ through their cysteine groups, and these reactions occur in one to two days. In these cases the nucleophile is probably $—S^-$ and the reaction may occur with $PtCl_2(NH_3)_2$, which will have formed within 24 h.

The reaction of methionine and ionized cysteine appear to be faster than histidine; and so time of reaction may provide a further variable controlling the specificity.

From the discussion above it can be seen that tetrachloroplatinate is not a very specific reagent but reaction conditions can enhance binding at certain protein ligands relative to others. Thus in ribonucleases $PtCl_4^{2-}$ binds to a methionine at pH 5.5, but a further site at a histidine is partially occupied at higher pH. $PtCl_4^{2-}$ binds different sites in triosephosphate isomerase depending on whether ammonium sulphate or phosphate are present. In most cases a

square-planar platinum complex results. It is possible that a square-pyrimidal complex of platinum(II) is formed but unlikely that these complexes are oxidized to octahedral platinum(IV) complexes as suggested by Dickerson *et al.*[31]

Wyckoff *et al.*[66] have shown that sometimes *cis*-$PtCl_2$(ethylenediamine) prevents substitution by two protein ligands *trans* to each other, a cross-linking reaction which leads to disorder of protein crystals. The rate of the reaction with the protein is slowed down by using the other square-planar anions such as $Pt(NO_3)_4^{2-}$.

$AuCl_4^-$ reacted with sperm whale myoglobin, but only after 6—9 months at pH 6.5 in ammonium sulphate. The reaction which occurred at two histidines may have occurred through an intermediate amine complex such as $AuCl_3(NH_3)$, $AuCl_2(NH_3)_2^+$, *etc.* In aqueous solutions $AuCl_4^-$ is hydrolysed to $Au(OH)_4^-$ in about one hour[34] and $AuCl_4^-$ may also be reduced to free Au^I by the oxidation of methionine.[34] This complicates interpretation of the reactions.

$Pt(CN)_4^{2-}$ does not allow nucleophilic substitution; in ribonuclease S the substitution is quite different from that of $PtCl_4^{2-}$. The binding of stable anions like $Pt(CN)_4^{2-}$ is described in Section 6.

E. Osmium and Iridium Reagents.—The binding sites of osmium and iridium are listed in Table 5. $IrCl_6^{3-}$ can bind proteins through imidazole or amino-groups as in papain where the binding sites are the same as those given by HgI_4^{2-} and $PtCl_6^{2-}$. The anions may also bind basic groups as found in lysozyme and subtilisin novo. In ferrocytochrome c_2, $Ir(NH_3)_6I_3$ appears also to bind to basic groups, predominantly lysines, but the electron density could possibly be due to I^- binding rather than the metal ions.

6 Electrostatic Binding of Heavy Atom Anions to Proteins

Proteins contain a number of positively charged groups, including the terminal α-amino and lysine ε-amino functions and the guanidinium group of arginine, which may form ion pairs with heavy atom anions. Histidine may also bind anions especially at lower pHs where it is positively charged.

We have seen that ions like $PtCl_4^{2-}$ or HgI_4^{2-} tend to be bound covalently by soft ligands such as cysteine, methionine, and histidine by displacement of the halide ligands. However, very often these ions are bound electrostatically. Thus in lysozyme and in the minor site of myoglobin, HgI_4^{2-} dissociates to HgI_3^- and binds ionically to the proteins. The myoglobin site is shown in Figure 3 and involves a lysine, two glutamines, and an asparagine (Table 2). The lysozyme site involves two arginine guanidinium groups. The same site can be taken up by $PtCl_4^{2-}$, $AuCl_4^-$, $IrCl_6^{3-}$, $OsCl_6^{3-}$, and PdI_4^{2-}. The binding as an ionic species gives rise to lack of specificity of these reagents.

If the $Pt(CN)_4^{2-}$ ion is used the ligands are less likely to be displaced by protein ligands, and so an ionic binding becomes the most likely mode of interaction with the protein. Thus Table 4 shows that $Pt(CN)_4^{2-}$ binds at several sites involving lysines in ferricytochrome. In carbonic anhydrase and liver alcohol dehydrogenase $Pt(CN)_4^{2-}$ binds at positively charged sites in the active site.

Table 5 *The ligands of osmium and iridium reagents in proteins*

Reagent	*Protein (Ref.)*	*Conc. of reagent*	*Buffer/salt*	pH	*Site number*	*Binding site*	
$Os(NH_3)_6I_3$	Ferricytochrome c_2 (*Rhodospirillum rubrum*) (15)	10—100 × protein conc.	3.2M-AS	5.8	1*	Glu-37, Lys 112	I^- or
					2*	Lys-56, Met 55	anion
					3	Lys-109	binding
Na_3IrCl_6	Subtilisin novo (27)				1	Lys-136	
					2	Lys-27, Asn-118	
					3	Asn-25	
					4	Trp-241, His Trp-106	
					5	Gln-103, Asn-240	
Na_3IrCl_6	Papain (45)	5 mM	Methanol water	9.3	1*	*N*-terminus	(same as HgI_4^{2-} and $PtCl_6^{2-}$)
					2	His-159	
K_3IrCl_6 K_2OsCl_6	Lysozyme (20)		0.85M-NaCl	4.7	1	Arg-13, Arg-13′ (same as HgI_4^{2-}, PdI_4^{2-} $AuCl_4'$)	

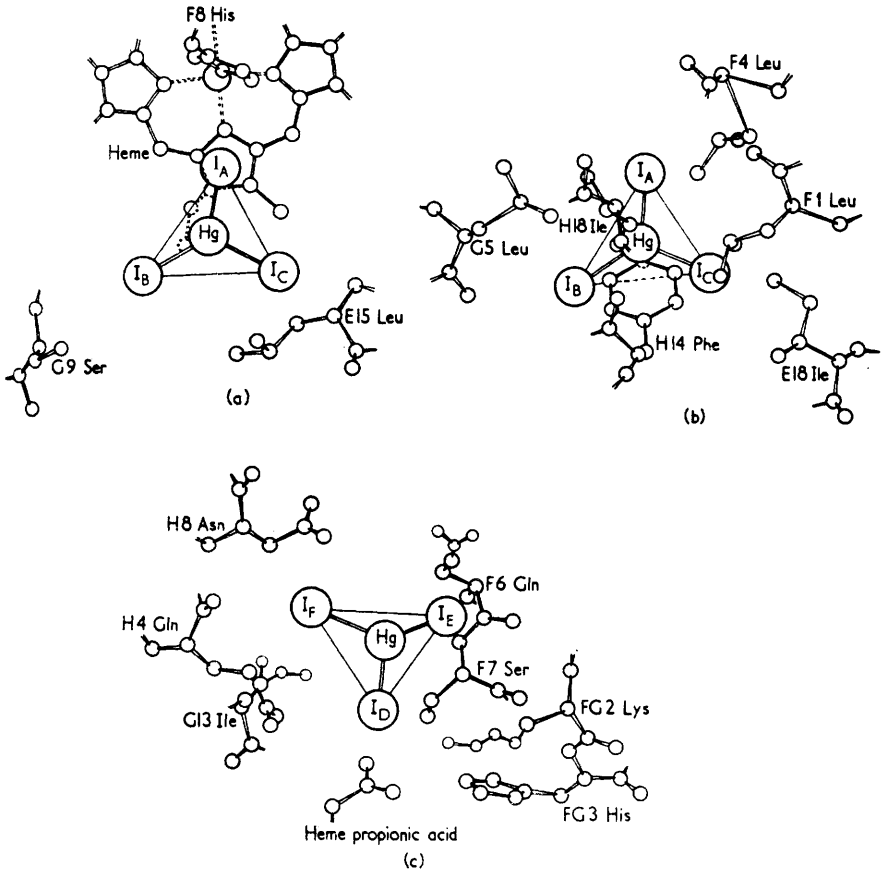

Figure 3 *The binding of* HgI_3^- *to myoglobin*
(Reproduced by permission from *J. Mol. Biol.*, 1968, **31**, 305)

The heavy-atom anions $Pt(CN)_4^{2-}$ and $Au(CN)_2^-$ often act as inhibitors. In carbonic anhydrase they bind close to the zinc atom. In liver alcohol dehydrogenase they bind in strict competition with the coenzyme, NAD. The auricyanide ion binds at two sites. One site is normally occupied by the phosphate groups of the coenzyme and the other by the adenosine part. The tetracyanoplatinate ion binds only to the phosphate site.[53,73] In ribonuclease, $Pt(CN)_4^{2-}$ binds at quite different sites from $PtCl_4^{2-}$. $Au(CN)_2^-$ will also tend to bind at anionic sites, but this is two-co-ordinate and in the presence of soft ligands such as cysteine may give tetrahedral complexes. Similar anion-binding sites may also be occupied by iodide and other halide ions, as occurs in carbonic anhydrase.[75]

[75] J. E. Norne, T. E. Bull, R. Einarsson, B. Lindman, and H. Zeppezauer, *Chemica Scripta*, 1973, **3** 142.

The fact that halide ions bind in similar ways implies that halide in the buffer or salting-out agent could interfere with binding of $Au(CN)_2^-$ or $Pt(CN)_4^{2-}$. Phosphate may also bind in a similar anion pocket. In chironomus haemoglobin[43] $Au(CN)_2^-$ does not bind in the presence of phosphate, but can be bound in other buffers such as acetate.

7 Binding by van der Waals Interactions

Although the early experiments with HgI_4^{2-} on sperm whale myoglobin were designed to bind a mercury to a methionine,[38] it was later found that the ion was bound as HgI_3^- in a hydrophobic pocket close to the haem group[76] as shown in Figure 3. This is not so surprising as the iodine ligands are very soft and would give rather good van der Waals interactions which would stabilize the binding. This description of the binding is consistent with the finding that AuI_4^- and I_3^- also bind at the same site but $HgBr_3^-$ does not. Even a single xenon atom can be bound[77] when myoglobin is equilibrated with xenon at 2.5 atm, and this interaction cannot be through either ionic or covalent links but must be due to London interactions and induced dipole moments which make up van der Waals interactions.

The protein groups are slightly distorted by inclusion of these large groups of atoms, and it is therefore not surprising that the binding depends critically on the nature of the globin. Seal myoglobin binds HgI_3^- in the hydrophobic pocket whereas haemoglobin does not.

8 Metal Ion Replacement in Metalloproteins

A number of metalloproteins have been studied by *X*-ray analysis. Among these are included several zinc proteins (carbonic anhydrase, carboxypeptidase, thermolysin, and insulin) and calcium proteins (staphylococcal nuclease and thermolysin) where the metals are weakly bound and can be replaced by heavy metals.

In some of these, the metal can be replaced by soaking the crystals in a solution of the heavy-metal salt. Thus in nuclease, calcium is directly exchanged for barium by soaking in a solution of barium chloride.[78] Similarly, in subtilisin novo, thallous fluoride replaces a sodium ion although at a slightly different site.[27] In others a more drastic procedure is required as the zinc or calcium is more firmly bound. However, initial attempts at removing zinc cofactors in solution were unsuccessful. For instance, addition of 1,10-phenanthroline to carbonic anhydrase gave a zinc-free enzyme which did not crystallize,[79] and insulin crystallized in a different space group in the absence of zinc.[80] More success was achieved by soaking the crystals themselves in a solution of a suitable chelating agent. Thus dialysis of carbonic anhydrase crystals against 2,3-

[76] R. H. Kretsinger, H. C Watson, and J. C. Kendrew, *J. Mol. Biol*, 1968, **31**, 305.
[77] B. P. Schoenborn, H. C. Watson, and J. C. Kendrew, *Nature*, 1965, **207**, 28.
[78] A. Arnone, C. J. Bier, F. A. Cotton, V. W. Day, E. E. Hazen, D. C. Richardson, J. S. Richardson, and A. Yonath, *J. Biol. Chem.*, 1971, **246**, 2301.
[79] B. Tilander, B. Strandberg, and K. Friborg, *J. Mol. Biol.*, 1965, **12**, 740.
[80] B. W. Low and J. E. Berger, *Acta Cryst.*, 1961, **14**, 82.

dimercaptopropanol in an hydrogen atmosphere produced crystals of the zinc-free enzyme.[79] Use of 5-hydroxyquinoline-8-sulphate and ethylenediaminetetra-acetic acid (edta) with carboxypeptidase[81] and rhombohedral 2-Zn insulin[82], respectively, gave zinc-free crystals. It appears that the crystal packing stabilizes the zinc-free structure and prevents the conformational charge or disaggregation which occurs in solution. Electron-density maps of insulin later showed that the side-chains in the regions vacated by the zinc atoms were rather disordered and were not reordered on addition of heavy atoms with the exception of cadmium.

Imidazole groups of histidines are frequently involved in coordination of replaceable zinc atoms: there are three histidine ligands to zinc in carbonic anhydrase and in insulin, and two histidines and glutamate in carboxypeptidase and thermolysin. Zinc is most easily replaced by transition or B-metal ions such as Fe^{2+}, Co^{2+}, or Cd^{2+}. Dialysis of the crystalline apoenzyme against 0.0003M mercuric acetate for ten days leads to substitution of mercury at the zinc site in carbonic anhydrase.[79] Dialysis against 0.005M mercuric chloride gives a similar mercury substitution for carboxypeptidase.[81]

Cadmium also binds at the zinc site of insulin and the same derivative can be prepared by co-crystallization.[82] Plumbous ions (Pb^{2+}) give low substitution at the zinc sites in insulin, whereas in zinc-free carboxypeptidase two sites 4 Å from the zinc site were given. These results are not unexpected. Although lead is a B-metal, the non-group valency of a plumbous ion means that it is larger than mercuric and less polarizing. It tends to bind electronegative groups such as carboxylate rather than imidazole and will not easily replace zinc. Calcium tends to bind oxygen ligands, such as carboxylate side-chains, and is best replaced by other alkaline earth ions such as Sr^{2+}, Ba^{2+} or tervalent lanthanide ions. Thus in solution the lanthanide, neodymium, will replace calcium in trypsin and trypsinogen[83] and in α-amylase.[84] Colman *et al.*[24] have demonstrated that three of the four calcium ions of thermolysin may be replaced by either lanthanide ions or by strontium or barium. The crystals were first equilibrated with calcium-free tris-acetate buffer at pH 5.5 for one hour and then a solution of metal ions in the same buffer was added. The four calcium sites in thermolysin can be characterised in the following way:

Ca 1, the inner double site, Glu-190, Glu-177, H_2O, Carbonyl 187
Ca 2, the outer double site, Glu-190, Glu-177, Carbonyl 183, H_2O
Ca 3, the single site at Asp-57
Ca 4, the single site at Asp-200.

The lanthanides bind at sites 1, 3, and 4, and calcium is concurrently ejected from site 2 also. This presumably is the result of the higher charge of the lanthanides;

[81] W. N. Lipscomb, J. C. Coppola, J. A. Hartsuck, M. L. Ludwig, H. Muirhead, J. Searl, and T. A. Steitz, *J. Mol. Biol.*, 1966, **19**, 423.
[82] M. J. Adams, E. Collier, G. Dodson, D. C. Hodgkin, and S. Ramaseshan, Abstracts of the Seventh Meeting of the International Union of Crystallography, 1966, A165.
[83] D. W. Darnall and E. R. Birnbaum, *J. Biol. Chem.*, 1970, **245**, 6684.
[84] G. E. Smocka, E. R. Birnbaum, and D. Darnell, *Biochemistry*, 1971, **10**, 4556.

barium and strontium replace all four calciums. The heavy atom usually has a larger radius than the atom it replaces. In thermolysin a more similar substitution is given when the metal ion has a smaller radius than calcium, for example, by the smaller lanthanides such as Lu^{3+}.[85]

Replacement of calcium by barium involves the introduction of a larger metal ion, and it is not surprising to discover that the barium atom in nuclease is 0.75 Å from the calcium ion position.[78] Similarly the mercury positions in carbonic anhydrase[36] and carboxypeptidase[25] differ by 0.7 and 0.25 Å, respectively, from the zinc positions. These small displacements clearly contribute towards the differences of catalytic activity found in the heavy-metal enzymes. Thus esterase activity of Hg-carboxypeptidase is slightly increased in comparison to the zinc enzyme, whereas the lead enzyme has only 50% esterase activity. But neither Hg nor Pb carboxypeptidases have peptidase activity.[5]

9 Biological Implications

The implications of the heavy-atom binding sites found in proteins are rather different for the two classes of metals. The hard cations UO_2^{2+}, Pb^{2+}, Tl^{+}, Ba^{2+}, and the lanthanides can be seen to be bound only by proteins acting as multidentate ligands. These ligands are generally oxygen ligands except for Pb^{2+} when nitrogen ligands are sometimes found. Carboxylates are most prominently found as ligands both in the proteins and in buffers such as citrate which interfere with these reactions. (Thus it is possible that negative staining density is affected by the concentration of carboxylates when Pb^{2+} or UO_2^{2+} are used.) The toxicity may be expected to depend on the nature of the complex. Thus Pb^{2+} is less toxic as the citrate or aspartate complex to *Aspergillus niger*.[86] The diuretic effect of uranyl is reduced by citrate[87] in rats. In the case of lead and thallium this picture is complicated by the possibility that Pb^{IV} and Tl^{III} species will be formed which would tend to bind to softer ligands. However, even in this case it is clear that initially the uptake and distribution of the compounds will depend on their binding to hard ligands such as carboxylates.

The compounds of mercury, cadmium, silver, gold, palladium, and platinum show very different reactivity. However, the range of possible ligands is clearly larger than has been suggested on the basis of reaction with isolated amino-acids. This is again, presumably, due to the protein acting as a multidentate ligand. Thus Hg^{2+} is frequently found to bind to imidazole. The metals show a strong dependence of reactivity on the nature of the other ligands. Thus methylmercury compounds can bind to ligands in hydrophobic pockets not accessible to Hg^{2+}. These compounds can also enter membranes. In protein crystallography iodide complexes of mercury have found use for the same reason. A spectacular instance of the specific requirement for ligands is afforded by the great difference in the toxicity between the *cis* and *trans* isomers of $Pt(NH_3)_2Cl_2$. Although this probably involves reaction with nucleotides rather than proteins it underlines the need to

[86] B W. Matthews and L. H. Weaver, *Biochemistry*, 1974, **13**, 1719.
[86] I. V. Zlochevsbaia and I. L. Rabotnova, *Mikrobiologiya*, 1968, **37**, 691.
[87] V. Nigrovic and E. J. Caftuny, *Nature*, 1974, **247**, 381.

consider biological ligands as multidentate. Protein crystallography also offers evidence of the importance of buffering ions, as many reactions of particularly Pt^{II} and Au^{III} are affected by the presence of NH or amines in the soaking solution. Conversely some complexes such as $Pt(CN)_4^{2-}$ and $Au(CN)_2^-$ are stable and not easily attacked. These bind as anions rather than as electrophiles and may mimic the binding of other anions such as phosphate.

Finally, many cases have now been investigated where a heavy metal has replaced a lighter one in a protein. A generalization from this behaviour is that the exact position and stereochemistry are rarely identical. This may explain the observed differences of specificity and activity.

The detailed description of heavy-metal protein interactions presented here shows that toxicity depends critically not only on the chemistry of the metal ion but also on the nature of the ligands in the reagent and in the medium. The metabolism of the metal ion leading to chemical modification will alter the potential interactions in the organism and change the toxicity of the reagent. Generalizations concerning the 'toxicity' of any heavy atom clearly require the kind of detailed discussion considered proper for other metabolites.

We would like to thank all those who supplied unpublished data concerning heavy metal binding sites in proteins studied by *X*-ray analysis. We are also grateful to Dr P. Sadler and Dr A. Thompson for reading and making helpful comments on the manuscript.