Complexes of Stannous Fluoride and Other Group IVB Dihalides with Mammalian Hemoproteins

Abstract. *Stannous fluoride, the widely used anticaries toothpaste additive, and other tin and germanium dihalides form complexes with hemoproteins such as hepatic cytochrome P-450, hemoglobin, and peroxidase. These complexes are characterized by visible spectra closely similar in shape, molar absorptivity, and absorbance maxima to those obtained with analogous complexes of carbon monoxide. Evidence is presented that the observed complexes are formed with uncharged MX_2 or LMX_2 moieties (where L is a neutral Lewis base, M is a metal, and X is a halogen) acting as π-acceptor ligands.*

This report demonstrates that certain hemoproteins, when treated under proper conditions with tin or germanium dihalides, form complexes characterized by visible spectra closely similar in shape, molar absorptivity, and absorbance maxima to those previously obtained with carbon monoxide (*1*). Lead difluoride, however, does not appear to complex with these enzymes under conditions which result in complexes with germanium or tin dihalides. Evidence is presented which suggests that the observed complexes are formed with uncharged MX_2 or LMX_2 moieties (where L is a neutral Lewis base, M is a metal, and X is a halogen) acting as π-acceptor ligands. Such complexes may be compared to the dialkyl- or diarylgermanium and tin transition metal complexes (*2*) and the well-known trichlorostannite complexes (*3*).

Hepatic microsomes from mice, rabbits, or rats were prepared as previously described (*4*), suspended in 0.5*M* phosphate buffer (*p*H 7.4), and treated with dithionite to reduce all hemoproteins to the ferrous state. Addition of GeF_2 (Pfaltz and Bauer, no purity stated), GeI_2 (Bodman Chemicals, 99+ percent pure), SnF_2 (Ventron, Alfa Products, ultrapure), $SnCl_2$ (Fisher Scientific, certified ACS standard), and $SnBr_2$ (Ventron, Alfa Products, no purity stated) resulted in complexes characterized by spectra such as Fig. 1a for $SnCl_2$. Both GeI_2 and $SnBr_2$ are colored substances which absorb in the region of interest; however, the concentrations used were tested and found to contribute less than 10 percent of the intensity found for their complexes with hemoproteins at the concentrations specified. Absolute or difference spectra, from 350 to 600 nm, were recorded by using an American Instrument Company DW-2 ultraviolet-visible spectrophotometer.

The germanium dihalides gave spectra with intensities attaining 60 percent of that found for the CO complex and with absorbance maxima at 451 nm. For both GeX_2 and SnX_2, spectra were fully developed at $10^{-2}M$. Addition of more dihalide resulted in denaturation of cytochrome P-450 to form cytochrome P-420, which also forms complexes with the dihalides to give spectra similar to those obtained on treatment of cytochrome P-420 with CO.

Unlike CO, the tin and germanium dihalides gave spectra with microsomal hemoproteins without addition of dithionite. The spectrum resulting from the interaction of $SnCl_2$ with microsomal hemoproteins in the unreduced state is shown in Fig. 1b. Although this spectrum is similar in shape to those resulting from some ligand–cytochrome P-450 interactions involving complex formation with the heme iron (*4*), the fact that similar spectra are not obtained with a purified cytochrome P-450 from rabbit liver indicates that this spectrum probably results from partial reduction of microsomal cytochrome b_5 (*5*) by the group IVB dihalide.

Dithionite-reduced cytochrome P-448, purified from rabbit liver and free of other hemoproteins as indicated by spectral analysis (*6*), formed complexes with the tin dihalides to give spectra identical in absorbance maxima to that of the CO complex, but with only 10 percent of the intensity. The spectra were obtained in 0.1*M* phosphate buffer in the absence of glycerol as a stabilizer (*7*) since turbid solutions resulted if glycerol was present. Under these conditions the cytochrome was susceptible to denaturation on addition of tin dihalides at a concentration above that necessary for 10 percent development of the 448-nm peak. Germanium diiodide, however, complexed readily with the purified cytochrome in 0.1*M* potassium phosphate buffer (*p*H 7.4) containing 30 percent glycerol. In this case the absorbance ap-

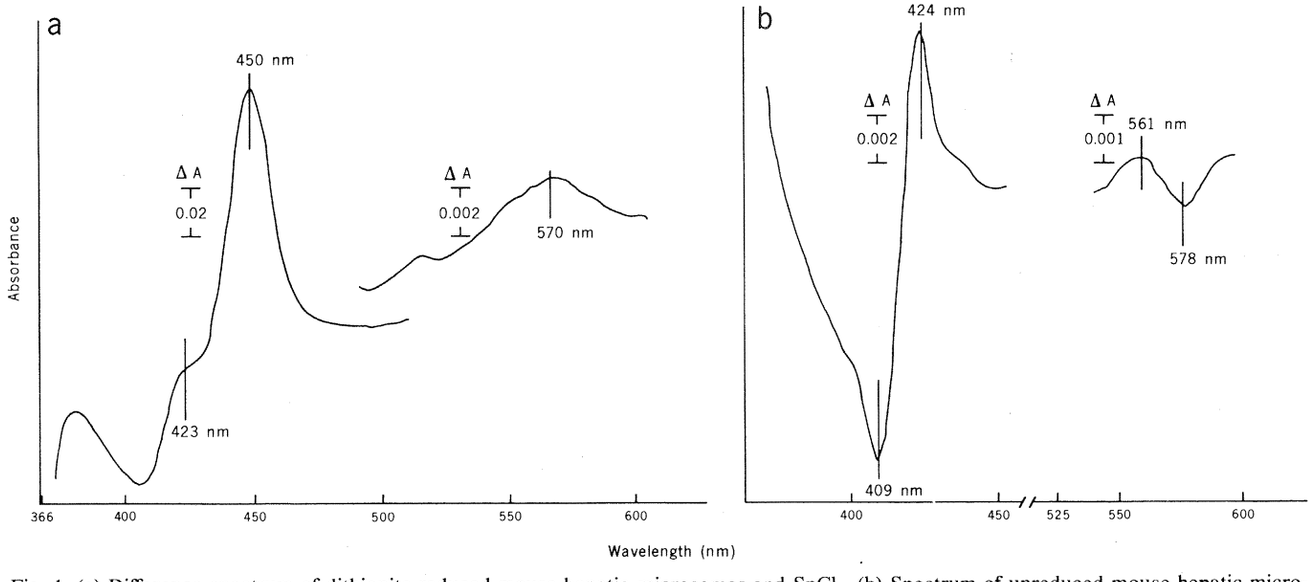

Fig. 1. (a) Difference spectrum of dithionite-reduced mouse hepatic microsomes and $SnCl_2$. (b) Spectrum of unreduced mouse hepatic microsomes and $SnCl_2$. The $SnCl_2$ concentration was about $10^{-2}M$ in each case. Cytochrome P-450 was present at about 1.2 nmole/ml. Difference spectra caused by the addition of SnF_2 or $SnBr_2$ were essentially identical.

proached 80 percent of that expected for the complex with CO. The absorbance maximum was at 448 nm, identical to that for the CO complex.

Solutions of dithionite-reduced hemoglobin (from sheep erythrocytes, Sigma Chemical Company) or peroxidase (from horseradish, Sigma Chemical Company) in phosphate buffer did not show spectral changes on addition of group IVB dihalides. However, addition of tin dibromide to a suspension of lecithin (*8*) (vegetable lecithin, Sigma Chemical Company) and dithionite-reduced hemoglobin in 0.5*M* phosphate buffer (*p*H 7.4) resulted in a spectrum similar to that of carboxyhemoglobin. The other tin dihalides also formed spectra similar to those of the CO complexes with dithionite-reduced peroxidase or hemoglobin if lecithin was added along with sucrose (*8*) or some other oligosaccharide such as trehalose or raffinose.

Figure 2 shows spectra of hemoglobin and $SnCl_2$ in the presence of synthetic dipalmitoyl phosphatidylcholine (*9*). The efficacy of this fully saturated, pure lecithin indicates that evolution of CO from unsaturated fatty acid residues on lecithin (*10*) cannot account for these spectra.

Germanium difluoride or diiodide did not appear to interact with hemoglobin or peroxidase, even in the presence of lecithin and sucrose under conditions effective for the tin dihalides. However, on treatment with GeF_2 or GeI_2, solutions of hemoglobin or peroxidase in suspensions of dithionite-reduced mouse hepatic microsomes gave spectra similar to those of the CO complexes of hemoglobin or peroxidase. The tin dihalides gave spectra identical to those of the CO complexes under these conditions.

The possibility that CO formed chemically under conditions similar to those obtained during the determination of the spectra shown in Fig. 2 seemed remote. However, to check this possibility we prepared a suspension of 25 ml of 0.5*M* phosphate buffer at *p*H 7.5 which contained 1 g of $SnCl_2$, 0.5 g of $Na_2S_2O_4$, 0.5 g of vegetable lecithin, and 1 g of sucrose and was 400 n*M* in hemoglobin. Published extinction coefficients were used for hemoglobin and carboxyhemoglobin (*11*). A stream of air was passed through the suspension, through a condenser packed with glass wool, and into 10 ml of 0.5*M* phosphate buffer that was 4.6 n*M* in dithionite-reduced hemoglobin. After 5 minutes, no carboxyhemoglobin was detected by its spectrum in the 4.5 n*M* hemoglobin detection solution, although the suspension 400 n*M* in hemoglobin gave a spectrum identical to that of a 400 n*M* carboxyhemoglobin solution prepared under the same conditions but with the omission of $SnCl_2$ and the addition of CO. A suspension similar to that described above, when treated with 10 ml of water saturated with CO at 20°C, becomes 295 n*M* in CO [see (*11*, p. 208) for the solubility of CO in water]. When a stream of air was passed through this solution, carboxyhemoglobin was detected spectrally in the detection solution. When $SnCl_2$ was omitted from the suspension and the suspension was made 295 n*M* in CO, the CO could still be detected.

Since CO from carboxyhemoglobin can be detected by this method, this experiment demonstrates that CO, in the unlikely event that it participates at all, cannot be the sole ligand in the production of spectra such as those in Fig. 2. In any case, the rapidity of spectrum formation and the size of the spectra relative to that formed on addition of CO appear to rule out the formation of CO by oxidation of the methylene bridge on the protoheme (*12*). The spectra are observed immediately on mixing the components, which appears to obviate the possibility that oxidation or hydrolysis products of the group IVB dihalides are the complexing agents. Moreover, separate experiments with tin(II) oxide (blue-black allotrope) and tin oxychloride ($SnOCl_2$, produced by the action of oxygen on a solution of $SnCl_2$) produced no spectral changes with dithionite-reduced cytochrome P-450, hemoglobin, or peroxidase under conditions that promoted complex formation with group IVB dihalides.

Further evidence against endogenous CO production by cytochrome P-450 was obtained from observations with human placental microsomes, which are generally contaminated with hemoglobin. Germanium dihalides form complexes with the hemoglobin that yield spectra like that of the CO complex, but do not form complexes with the cytochrome P-450, which can still form a complex with added CO. Tin dihalides, on the other hand, form complexes with both hemoglobin and cytochrome P-450 in human placental microsomes (*13*).

For the soluble hemoproteins hemoglobin and peroxidase, added lipid is necessary for complex formation with the group IVB dihalides. However, for purified cytochrome P-448, a type of cytochrome P-450, no added lipid is required. Since cytochrome P-450 prob-

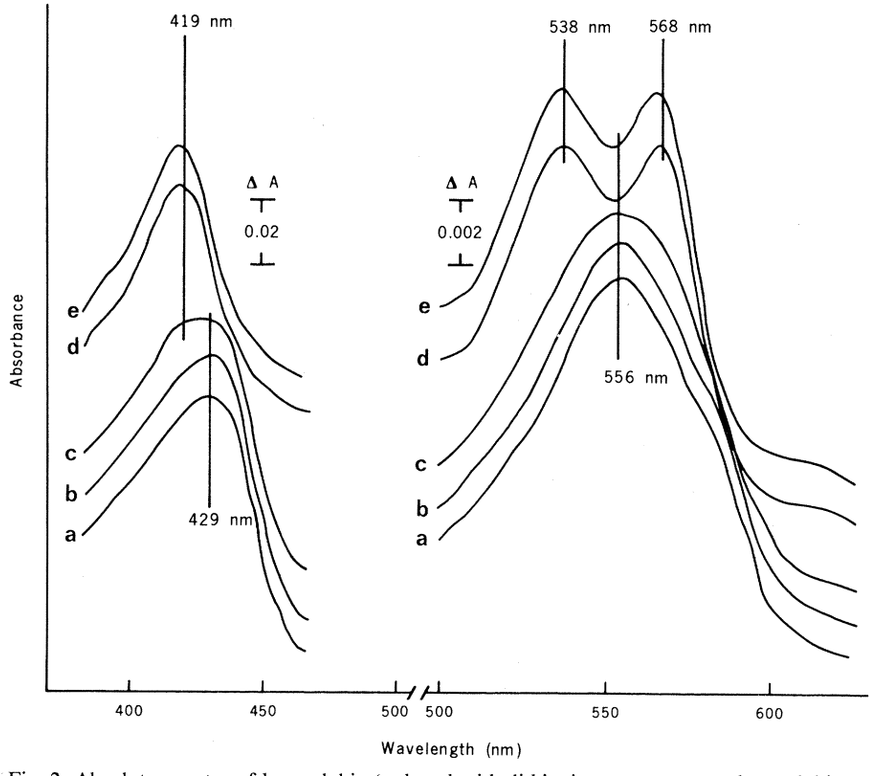

Fig. 2. Absolute spectra of hemoglobin (reduced with dithionite to convert methemoglobin to hemoglobin). (a) Hemoglobin (Hb) and dipalmitoyl phosphatidylcholine (DPC) (1 percent); (b) Hb, DPC (1 percent), and sucrose (10 percent); (c) Hb, DPC (1 percent), sucrose (10 percent) and $SnCl_2$ (20 m*M*); (d) Hb, DPC (1 percent), sucrose (10 percent), and $SnCl_2$ (150 m*M*); (e) Hb, DPC (1 percent), sucrose (10 percent), and CO. Spectra caused by addition of SnF_2 or $SnBr_2$, under the same conditions, are essentially identical. With the exception of hemoglobin, in each case the components in the reference cuvette were identical to those in the sample cuvette.

ably already contains heme iron in a lipophilic environment (*14*), these facts suggest to us that a lipophilic environment is necessary for heme–group IVB complex formation. This suggestion is strengthened by the observation that sucrose is required in some instances for a spectrum to be obtained. Since sucrose is freely water-soluble but not lipophilic, its presence would tend to divert the solvating power of water and to force tin(II) species, probably charged by coordinated phosphate in the aqueous phase, into the lipophilic phase as SnX_2 or $LSnX_2$ species.

The group IVB dihalides, having the ns^2 outer electron configuration and available orbitals, are electronically similar to carbenes, CO, and ethyl isocyanide. Ethyl isocyanide and CO complex with cytochrome P-450 to give spectra having large extinction coefficients and absorption maxima at 455 or 450 nm (*15*). Isocyanide and CO are classified as π-acceptor ligands since each has a free pair of electrons used in dative σ-bond formation and each also has available π-orbitals which are involved in π-bonding by accepting electrons from a filled metal *d*-orbital. We suggest that the similarity in electronic structure between the group IVB dihalides on the one hand and CO and isocyanide on the other leads to similar binding in their heme iron complexes, and consequently similar electronic spectra.

We have investigated the interactions of the group IVB dihalides with artificial systems that behave spectrally like cytochrome P-450 (*16*). We have also examined molecules that appear to produce carbene–cytochrome P-450 complexes after metabolism. These molecules include the methylene dioxyphenyl compounds, which are well-known drug synergists, and their aliphatic analogs the dioxolanes (*17*). No toxicological implications for the use of SnF_2 are immediately apparent from our findings. Further investigations are merited, however, particularly with regard to the possible synergism of toxic materials.

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Molecular Graphics: Application to the Structure Determination of a Snake Venom Neurotoxin

Abstract. *Atomic coordinates have been determined for a snake venom α-neurotoxic protein by fitting a molecular model to a crystallographically derived 2.2-angstrom electron density map. The fitting was carried out entirely on a computer-operated molecular graphics system without going through any mechanical model stage.*

The fitting of a molecular model to a calculated electron density map is an important step in the determination of the structure of a protein. This fitting is commonly done with a device called a Richards box (*1*). Contour lines drawn on a series of transparent sheets represent the map; a molecular model is built in front of the map, but a half-silvered mirror creates the illusion that the model is being built "inside" the map. Since even a small protein has at least 500 atoms, the precise fitting of model to map and subsequent measurement of the coordinates of each atom are tedious and error-prone steps. In this report we describe how the entire process of fitting a model of a protein to its electron density map and then measuring the coordinates was done in a short time by means of a special-purpose interactive computer graphics system, a molecular graphics system, without ever constructing a physical model.

A number of molecular graphics systems have been built, which are each unique in some ways and which together represent a wide variety of design choices (*2*). For the work reported here we used the GRIP (Graphics Interaction with Proteins) molecular graphics system at the University of North Carolina (*3*). GRIP makes available to the user most of the facilities of other systems plus a few unique features. We briefly describe the GRIP system as it appears to the user, stressing features that we found to be effective.

GRIP is operated with explicit commands and with hand controls. The former are given by entering any needed numerical values from a keyboard and then selecting a displayed key word with the light pen or pressing a button on a small console. (The light pen may also be used to select an atom or a contour.) The system's response to these commands is usually not instantaneous. The hand controls are analog devices which specify viewpoint and conformation. The display's response to the use of the hand controls is essentially immediate.

The "seeing" controls of the system include window selection, scaling, detail suppression, suppression of selected contours, viewpoint selection, and creation of a three-dimensional illusion. Execution of several commands specifies the contents of the viewing volume. Once this is done, a hand-controlled lever can be used to change the viewpoint by rotating the contents of the viewed space, which aids the user in comprehending the view and provides a strong depth cue. This is but one of the various monocular and binocular depth cues included in the GRIP system. Of the former, we use intensity depth cueing and continuous changes of viewpoint. (Intensity variation may also be used to distinguish the molecule and the electron density map.)

GRIP uses three analog devices to control smooth changes of the conformation of the displayed molecule. One device, a three-dimensional positional lever, has a knob that may be moved anywhere within a certain vol-