STUDIES IN ENZYME CRYSTALLOGRAPHY

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ABSTRACT

This thesis presents X-ray crystallographic research on chymotrypsin and lysozyme. Seven crystal types of chymotrypsin were identified -- five for the first time. The seven crystal types appear to be different packings of two conformations of the same molecule.

One crystal type of lysozyme was studied. From Patterson projections of crystals diffused with $PtI_6^=$ ions, the x, y, and z coordinates for these ions were determined. After one least squares refinement of these coordinates, the agreement factor between calculated and observed contributions of the $PtI_6^=$ ions was 0.34 for 108 hk0 and h0l reflections. Using these coordinates, an hk0 and an h0l Fourier projection were prepared.

X and y coordinates for the heavy atoms in lysozyme crystals diffused with UO_2^{++} ions, $WO_4^=$ ions, $HgI_4^=$ ions, $HgCl_2$, $ThCl_4$, and p-$ClHg\phi SO_3H$ were determined from Patterson and Fourier projections.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

INTRODUCTION

This report presents research on the structure of two enzymes, chymotrypsin and lysozyme. The object of this research was the preparation of electron density (Fourier) maps for crystals of these enzymes, using the techniques of X-ray diffraction. Two Fourier projections were prepared for crystals of lysozyme; none for crystals of chymotrypsin. No structural information was obtained from either projection.

Chymotrypsin was selected for study because it had been crystallized previously, its enzymatic activity had been investigated extensively, and it might be combined with inhibitors carrying heavy atoms. Inhibited chymotrypsin carrying heavy atoms was of interest for obtaining the heavy atom derivatives required for preparation of Fourier maps.

The research on chymotrypsin consisted of three parts:

1. Crystallization and Preliminary X-ray Studies of DFP*-Inhibited Chymotrypsin.
2. Diffusion of Heavy Atoms into Crystals of DFP-Inhibited Chymotrypsin.
3. Preparation of Inhibitors Carrying Heavy Atoms.

*Di-isopropylphosphorofluoridate

DFP-Inhibited chymotrypsin was used in the first and second parts of the research because it could be crystallized without autolysis at pH's where the free enzyme is active. From the first part of the research, seven crystal types of inhibited chymotrypsin were identified - five for the first time. Two crystal types had been prepared previously (1,2), but not investigated by X-ray diffraction. The present X-ray data from these two crystal types of inhibited chymotrypsin, designated Monoclinic I and Tetragonal, were nearly identical to those from crystals of alpha-chymotrypsin and gamma-chymotrypsin, respectively. Present data from alpha- and gamma-chymotrypsin are in good agreement with published data (3,4). Two crystal forms of inhibited chymotrypsin, the Tetragonal and Trigonal, containing one molecule per asymmetric unit, may be of interest for further study.

In the second part of the research on chymotrypsin, an attempt was made to obtain heavy atom derivatives of the Tetragonal crystals prepared in the first part. Diffusion of these crystals with $HgI_4^=$ ions and $PtI_6^=$ ions failed to produce the heavy atom derivatives required for three-dimensional Fourier maps. Diffusion of Trigonal crystals was not attempted because they were not readily available. Diffusion of Tetragonal crystals of free chymotrypsin (gamma-chymotrypsin) has been undertaken at the National Institutes of Health in Maryland but has not been reported successful. On the

other hand, diffusion of Monoclinic I crystals of free chymotrypsin (alpha-chymotrypsin) has yielded one heavy atom derivative (5).

From the third part of the research on chymotrypsin, one potential inhibitor carrying heavy atoms was obtained but not used to prepare a heavy atom derivative, because it released its heavy atoms under the conditions required for preparation of a heavy atom derivative. Preparation of p-ClHgϕSO$_2$F, an inhibitor carrying a mercury heavy atom, has been achieved recently by Traylor (6). Its use in the preparation of a heavy atom derivative has not been reported. However, the use of an iodo analogue, p-IϕSO$_2$F, in the preparation of a heavy atom derivative has been (7).

The second enzyme studied was lysozyme. Its study was initiated after the above attempts to obtain heavy atom derivatives of chymotrypsin failed. From X-ray structure amplitudes of lysozyme crystals diffused with heavy atoms, Patterson and Fourier projections were prepared. Most of the structure amplitudes were obtained by other workers. From the projections, tentative x, y, and z coordinates for $PtI_6^=$ ions were obtained and tentative x and y coordinates for the heavy atoms in six additional diffused crystals were obtained. The coordinates for the $PtI_6^=$ ions are in close agreement with those obtained at the California Institute for $PtI_6^=$ ions in crystals of lysozyme-$Ta_6Cl_{12}^{++}$. They are symmetrically related to those published for $PdCl_4^=$ ions in crystals of lysozyme (8). The x and y coordinates for two of the six additional heavy atoms are symmetrically related to published coordinates (8).

Subject to confirmation with three-dimensional data, crystals of lysozyme diffused with $PtI_6^{=}$ ions constitute a heavy atom derivative of lysozyme. Data from those crystals were used to prepare an hk0 and h0l Fourier projection of lysozyme. The hk0 projection is in good agreement with that obtained at the Royal Institution in London (6), but contains a number of differences from the hk0 projection of lysozyme-$Ta_6Cl_{12}^{++}$ obtained at the California Institute.

Three-dimensional confirmation of the coordinates of $PtI_6^{=}$ ions and determination of a z coordinate for at least one of the six diffused crystals for which tentative x and y coordinates were obtained will permit preparation of three-dimensional Fourier maps of lysozyme.

I. CHYMOTRYPSIN

Crystallization and Preliminary X-Ray Studies of DFP-Inhibited Chymotrypsin

Introduction

Crystallization of DFP*-inhibited chymotrypsin was first achieved by Gladner and Neurath (1) and Massey and Hartley (2). The object of the present study of the crystallization of DFP-inhibited chymotrypsin was to obtain single crystals suitable for investigation by X-ray diffraction. The inhibited enzyme was selected because it could be crystallized without autolysis at pH's where the free enzyme is active.

Preliminary X-ray diffraction data from crystals obtained by the author and other workers permitted designation of seven crystal types of inhibited chymotrypsin. Five of these crystal types were obtained for the first time. The two remaining crystal types had been prepared previously (1,2), but not investigated by X-ray diffraction. Present X-ray data from these two crystal types, designated as Monoclinic I and Tetragonal, were nearly identical to those from crystals of alpha-chymotrypsin and gamma-chymotrypsin, respectively. Data from the present study were in good agreement with published data (3,4). A third crystal type of inhibited chymotrypsin, Monoclinic III, was also found to be nearly identical

*di-isopropylphosphorofluoridate

to the corresponding crystal type of free chymotrypsin.

The seven crystal types of inhibited chymotrypsin appear to be different packings of two conformations of the same molecule. Each crystal type is discussed in terms of its preparation and potential for future X-ray investigation.

Preparation of Crystals of Inhibited Chymotrypsin

Crystals of DFP-inhibited chymotrypsin were obtained from four batches of DFP-inhibited chymotrypsin, as described below. All chymotrypsin was obtained from Worthington Biochemical Corporation.

Batch 1

The first batch of inhibited chymotrypsin was prepared by mixing a solution of lyophilized alpha-chymotrypsin with a solution of DFP, according to the method of Jansen et al. (9). The solution of chymotrypsin contained 0.856 gm. of enzyme, 17.9 gm. of $NaH_2PO_4 \cdot H_2O$ and sufficient redistilled water and NaOH to form 642 ml. of solution at pH 7.5. Six ml. of an isopropanol solution, 0.01 M in DFP, were mixed with the enzyme solution and the mixture stored for 48 hours at 5°C.

Crystals were obtained from this solution, 0.13% in enzyme, by Miss Battfay. Preparation of the monoclinic prisms that resulted is outlined in Table 1.

Batch 2

As above, a solution of lyophilized enzyme and an isopropanol solution, 0.01 M in DFP, were mixed according to the method of

TABLE 1

PREPARATION OF SEVEN CRYSTAL TYPES OF DFP-INHIBITED CHYMOTRYPSIN

Crystal Type	Crystal Form	Batch	Treatment Before Crystallization	pH of Crystallization
Triclinic	column	3	Monoclinic I crystals dissolved and stored with DFP at pH 7.5 for 6 da.	2.3
Monoclinic I	rhomb	4	precipitation at pH 4.0	4.0
Monoclinic II-1	rhomb	4	precipitation at pH 4.0	4.0, 5.0, 5.2, 5.6
	thick plate	2	precipitation ca. pH 5.0	ca. 4.5
	rhomb	4	precipitation at pH 5.0	5.0
	rhomb	4	precipitation at pH 7.8	4.0
Monoclinic II-2	rhomb	4	precipitation at pH 4.0	4.0
	rhomb	4	precipitation at pH 7.8	4.0
Monoclinic III	prism	1	precipitation at pH 7.5, crystallization as needles at pH 7.0 and recrystallization in 0.1 M acetate	5.6

TABLE 1

(continued...)

Crystal Type	Crystal Form	Batch	Treatment Before Crystallization	pH of Crystallization
Trigonal	trigonal rhomb	2	precipitation at pH 8.5 crystallization at pH 5.9; rhombs appeared after removal of large bipyramids.	5.9
	trigonal rhomb	3	Monoclinic I crystals dissolved and stored with DFP at pH 7.5 for 35 da.	5.0, 5.35, 5.7, 5.8, 5.9
	trigonal rhomb	4	precipitation and crystallization at pH 6.0; rhombs appeared after removal of 1 large rhomb.	6.0
Tetragonal	bipyramid	2	precipitation at pH 8.5	5.9
	bipyramid	4	precipitation and crystallization at pH 5.6, 6.0, 7.0, 8.0	

Jansen et al. (9). But to prepare a larger quantity of inhibited enzyme, an enzyme solution was prepared from 20 gm. of alpha-chymotrypsin, 20.9 gm. of $NaH_2PO_4 \cdot H_2O$ and sufficient redistilled water and NaOH to yield 608 ml. of solution at pH 7.5. The DFP solution (150 ml.) was mixed with the enzyme solution and the mixture, containing 2.6% enzyme, was stored for 48 hours at 5°C.

This mixture was prepared for crystallization by precipitating its inhibited enzyme with 348 gm. of solid $(NH_4)_2SO_4$. Not all of the $(NH_4)_2SO_4$ dissolved, so 180 ml. of redistilled water were added to the solution. Then, after all of the $(NH_4)_2SO_4$ had dissolved, the precipitated enzyme was collected, dissolved, and reprecipitated to free it of isopropanol which had been salted out by the $(NH_4)_2SO_4$. The purified precipitate was dissolved, dialyzed against redistilled water at 5°C and then lyophilized.

Crystallization of the lyophilizate was begun by dissolving it in redistilled water, adjusting the pH to that desired for precipitation and adding sufficient solid $(NH_4)_2SO_4$ to obtain a solution 0.7 saturated with respect to $(NH_4)_2SO_4$. The resulting precipitate was collected by centrifugation and dissolved in redistilled water. Sufficient saturated $(NH_4)_2SO_4$ solution was added to saturate the solution with respect to protein. With less, crystallization did not occur. For crystallization, the protein solution was adjusted to the desired pH, clarified by centrifugation and placed in vials.

From the second batch, tetragonal bipyramids, trigonal rhombs, and thick monoclinic plates resulted. Their crystallization is outlined in Table 1.

Batch 3

To investigate the possibility of inhibiting a solution of crystalline chymotrypsin, batch 3 was prepared. Fourteen ml. of an isopropanol solution, 0.01 M in DFP, were added to 440 ml. of enzyme solution at pH 7.5 and stored for 72 hours at 5°C. The enzyme solution contained 12.5 gm. of $NaH_2PO_4 \cdot H_2O$ and about 600 mg. of chymotrypsin crystals which Miss Battfay had prepared by precipitating and crystallizing alpha-chymotrypsin at pH 4 (10).

Crystals were obtained from the solution of inhibited chymotrypsin by adding 2.9 volumes of saturated $(NH_4)_2SO_4$ to a portion of it, clarifying the resulting solution by centrifugation and adjusting the pH. With less than 2.9 volumes, which saturated the solution with respect to enzyme, no crystals were obtained.

Two hundred crystallizing experiments were prepared from batch 3. Small triclinic columns and trigonal rhombs were obtained. Their preparation is outlined in Table 1.

Batch 4

DFP-inhibited chymotrypsin from Worthington Biochemical Corporation was crystallized from 3% solution by Miss Battfay (10). Monoclinic rhombs, trigonal rhombs and tetragonal bipyramids were obtained. Their preparation is outlined in Table 1.

Preliminary X-Ray Studies

Crystals obtained from the four batches of DFP-inhibited chymotrypsin were studied by X-ray diffraction. Mounted in capillaries, they were exposed to CuKα radiation (λ = 1.542 Å) in precession cameras with crystal-to-film distances of 90 mm. No useful diffraction patterns were obtained from needles grown in each of four batches. Preparation of needles has been reported by earlier workers (1,2). Useful diffraction patterns were obtained from triclinic columns, monoclinic prisms, thick monoclinic plates, trigonal rhombs and tetragonal bipyramids by the author; and from monoclinic rhombs, trigonal rhombs and tetragonal bipyramids by other workers during the present study.

On the basis of these diffraction patterns, seven crystal types of DFP-inhibited chymotrypsin were designated (see Table 2). Five of these crystal types - Triclinic, Monoclinic II-1, Monoclinic II-2, Monoclinic III and Trigonal - were obtained for the first time. The two remaining types, Monoclinic I and Tetragonal, had been prepared previously (1,2), but not investigated by X-ray diffraction. Present X-ray data from these two types are nearly identical to present data from crystals of alpha-chymotrypsin and gamma-chymotrypsin, respectively. Agreement of present data with published data is good, as observed from Table 3. A third crystal type of inhibited chymotrypsin, Monoclinic III, is nearly identical to the corresponding

TABLE 2

PRELIMINARY X-RAY DATA FROM SEVEN CRYSTAL TYPES OF DFP-INHIBITED CHYMOTRYPSIN

Crystal Type	Space Group	a (Å)	b (Å)	c (Å)	β	α	γ	Vol. (Å^3)	Z*	Vol./Mol. (Å^3)
Triclinic	P1	53	94	53	102°	126°	88°	210,000	4	52,000
Monoclinic I	$P2_1$	49.4	67.3	66.1	102°10'	90°	90°	215,000	4	53,700
Monoclinic II-1	$P2_1$	44.7	77.5	65.5	108°50'	90°	90°	215,000	4	53,700
Monoclinic II-2	$P2_1$	44.3	78.4	65.6	106°50'	90°	90°	218,000	4	54,500
Monoclinic III	$P2_1$	43.0	77.4	66.0	109°20'	90°	90°	207,000	4	51,800
Trigonal	$P3_121$	61.2	61.2	107	90°	90°	120°	348,000	6	58,100
Tetragonal	$P4_22_12$	69.8	69.8	97.9	90°	90°	90°	477,000	8	59,600

* Molecules per unit cell

TABLE 3

PRELIMINARY X-RAY DATA FROM CRYSTALS OF FREE AND DFP-INHIBITED CHYMOTRYPSIN

Crystal Type	Space Group	$\underline{a}_o$ (A)	$\underline{b}_o$ (A)	$\underline{c}_o$ (A)	β	Vol. o_3 (A)	Z*
Monoclinic I							
Inhibited	$P2_1$	49.4	67.3	66.1	102°10'	215,000	4
Free Alpha	$P2_1$	49.4	68.1	65.9	102°10'	216,000	4
Free Alpha[3]	$P2_1$	49.6	67.8	66.5	102°	219,000	4
Tetragonal							
Inhibited	$P4_22_12$	69.8	69.8	97.9	90°	477,000	8
Free Gamma	$P4_22_12$	69.6	69.6	97.7	90°	473,000	8
Free Gamma[4]	$P4_22_12$	69.5	69.5	97.5	90°	471,000	8
Monoclinic III							
Inhibited	$P2_1$	43.0	77.4	66.0	109°20'	207,000	4
Free	$P2_1$	42.9	76.9	65.7	109°10'	205,000	4

*Molecules per unit cell

crystal form of free chymotrypsin included in Table 3. The three crystal types of inhibited chymotrypsin presented in Table 3 are nearly identical to the corresponding crystal types of free chymotrypsin with respect to distribution of intensities as well as unit cell data.

Differences in unit cell data and in distribution of intensities were used to establish the seven crystal types. For example, Monoclinic II-1 and Monoclinic II-2 differed in beta angle and in distribution of intensities.

In Tables 2 and 3, the unit cell parameters are derived from at least two different diffraction patterns, except in the cases of c for Monoclinic I and b for Monoclinic II-2, for which only one pattern was available.

Discussion of Seven Crystal Types of Inhibited Chymotrypsin

In Table 1, preparations leading to each of the seven crystal types of inhibited chymotrypsin are collected by crystal type. In Table 2, X-ray data for each of the crystal types are listed. Below are comments on the preparation and crystallographic properties of each type.

Triclinic

Triclinic crystals were obtained only from batch 3 at pH 2.3; no other batch was crystallized at that pH. Because these crystals were small, gave no high-angle reflections and contained 4 molecules per asymmetric unit, further study of them is not recommended.

Monoclinic I

Monoclinic I rhombs were obtained from batch 4 by precipitation and crystallization at pH 4.0. Although high-angle reflections were obtained from these crystals, they are not recommended for further study because all of the crystals thus far have given X-ray patterns indicative of twinning in the ab plane. These crystals are also very fragile and, thus, difficult to mount.

Monoclinic II-1 and Monoclinic II-2

Monoclinic II-1 rhombs were obtained from batches 2 and 4 when either precipitation or crystallization or both were carried out at pH 4-5. Crystallization of batch 4 at pH 4 produced Monoclinic II-2. At pH 4, these two crystal types appeared along with Monoclinic I. Monoclinic II crystals contain 2 molecules per asymmetric unit. The similiarity between these and other monoclinic crystals is discussed later.

Monoclinic III

Monoclinic III crystals were obtained from batch 1 at pH 5.6. The fact that they were not obtained from other batches crystallized at pH 5.6 may be related to the use of 0.1 M acetate in the crystallization of batch 1. This has not been investigated. These crystals gave excellent diffraction patterns. However, further X-ray investigation should await a better understanding of their preparation and relation to other monoclinic crystal types.

Trigonal

Trigonal rhombs were crystallized from batches 2, 3 and 4 using greatly different enzyme concentrations: 0.1%, 0.03%, and 3%, respectively. The treatment of these batches prior to crystallization differed also. Thus, further study of the preparation of trigonal rhombs is suggested. They contain one molecule per asymmetric unit and give good diffraction patterns and, thus, are suitable for further X-ray investigation.

Tetragonal

Tetragonal bipyramids were formed from batches 2 and 4 at or above pH 5.6. No explanation of the failure to obtain them from batches 1 and 3 at the same pH's is possible at present. These crystals contain one molecule per asymmetric unit and give excellent diffraction patterns and, thus, are suitable for further X-ray investigation.

Comparison of crystal types of inhibited chymotrypsin

Monoclinic I and Tetragonal crystals of DFP-inhibited chymotrypsin contain different conformations of the same molecule (10). These crystals differ in volume per molecule - 54,000 and 60,000 Å^3, respectively - probably because their two conformations differ in volume. Crystals with volumes per molecule of about 54,000 Å^3 would be expected to contain the conformation present in Monoclinic I crystals; and those of about 60,000 Å^3, the conformation present in

Tetragonal crystals. Thus, the Triclinic and Monoclinic crystals, with volumes per molecule of between 52,000 and 54,000 $\AA^3$, appear to contain the conformation found in Monoclinic I crystals. Similarly, the Trigonal crystals, with a volume per molecule of 58,000 $\AA^3$, appear to contain the conformation found in Tetragonal crystals whose volume per molecule is 60,000 $\AA^3$. Differences in unit cell parameters among crystals within the two groups probably arise from differences in packing of the conformation in each group. Considerations like the above suggest that Monoclinic I and Monoclinic III crystals of free chymotrypsin are different packings of the same conformation.

All of the Monoclinic crystals contain 2 molecules per asymmetric unit and were grown from solution at pH 5.6 or below. Both trigonal and tetragonal crystals contain 1 molecule per asymmetric unit and were grown from solution at or above pH 5.0. Apparently, a low pH conformation of inhibited chymotrypsin was crystallized in the Monoclinic crystals and a high pH form of greater volume was crystallized in Trigonal and Tetragonal crystals.

The existence in solution of two conformations of chymotrypsin which differ in size is supported by Egan's analysis of the electrophoretic mobilities of alpha- and gamma-chymotrypsin (11). Crystals of alpha- and gamma-chymotrypsin are nearly identical to Monoclinic I and Tetragonal crystals of inhibited chymotrypsin. Thus, properties

of free and inhibited chymotrypsin in crystals and in solutions appear related. Further investigation of crystallographic and electrophoretic properties of free and inhibited chymotrypsin may lead to a better understanding of the structural relationship between the enzyme in crystals and in solution.

Details about the conformation of the enzyme in each crystal type may be obtainable from low resolution Patterson maps. If these maps support the existence of two conformations of chymotrypsin which can be crystallized in a number of forms, then only one form of each conformation need be the subject of further X-ray investigation.

Diffusion of Heavy Atoms into Crystals of DFP-Inhibited Chymotrypsin

Diffusion of heavy atoms into Tetragonal crystals of DFP-inhibited chymotrypsin was attempted by other workers to obtain heavy atom derivatives of inhibited chymotrypsin. From X-ray intensities obtained by other workers, the author prepared hk0 and h0l Patterson projections from which the location of heavy atoms was not possible. Thus, no heavy atom derivaties were obtained.

Tetragonal crystals of inhibited chymotrypsin were selected because they contain one enzyme molecule per asymmetric unit and give excellent diffraction patterns. These crystals were diffused with $HgI_4^=$ ions and $PtI_6^=$ ions because location of ions with their large

number of electrons was more likely than location of ions with fewer electrons. X-ray patterns from crystals diffused with $HgI_4^=$ ions contained minor intensity changes from patterns of undiffused crystals. But corresponding Patterson projections from diffused and undiffused crystals were essentially identical and an hk0 Difference Patterson failed to reveal specific heavy atom sites. X-ray patterns from crystals diffused with $PtI_6^=$ ions were nearly identical to those from undiffused crystals. Therefore, no Patterson projections were prepared for crystals diffused with $PtI_6^=$ ions.

Diffusion of $HgI_4^=$ ions at concentrations higher than those used above has yielded diffraction patterns little different from those obtained above. Thus, further diffusion of Tetragonal crystals with $HgI_4^=$ ions is not recommended. Further diffusion with $PtI_6^=$ ions or ions with fewer electrons is not recommended either. However, diffusion of other crystal types of inhibited chymotrypsin is recommended because diffusion of Monoclinic I crystals of free chymotrypsin (alpha-chymotrypsin) has yielded one heavy atom derivative (5).

Preparation of Inhibitors Carrying Heavy Atoms

Introduction

Heavy atom derivatives are required for the preparation of electron density maps of chymotrypsin. One way to obtain heavy

atom derivatives is to prepare inhibitors carrying heavy atoms, inhibit chymotrypsin with them and crystallize the resulting inhibited enzyme. This approach also provides a method of tagging the active site of chymotrypsin in the crystalline state.

The following section describes attempts to obtain inhibitors carrying heavy atoms from the reactions of:

p-HOØHgCl and $POCl_3$, p-HOØHgCl and $SOCl_2$, p-HOØHgCl and SO_2Cl_2;

$SOCl_2$ and PCMBS*, $SOCl_2$ and C_4I_4NH**;

CHI_3 and $(NaO)_2P(O)F$, CHI_3 and $(AgO)_2P(O)F$; and

$AgNO_3$ and $(NaO)_2P(O)F$.

These eight reactions were attempted for the purpose of obtaining phosphoryl halide, sulfonyl halide or thionyl halide inhibitors carrying mercury, silver or iodine heavy atoms. From only one of these reactions, the one between $AgNO_3$ and $(NaO)_2P(O)F$, was such a compound obtained. It was not used to prepare a heavy atom derivative because it appeared to release heavy atoms under the conditions required for inhibition of chymotrypsin.

Each of the eight reactions is described in detail below.

The Reaction between p-HOØHgCl and $POCl_3$

The reaction between p-HOØHgCl and $POCl_3$ was undertaken for the purpose of obtaining $ClP(O)(OØHgCl)_2$, a potential phosphonyl inhibitor

*p-chloromercuribenzenesulfonic acid
**tetraiodopyrrole

carrying mercury atoms:

$$ClP(O)Cl_2 + 2HO\phi HgCl + 2Me_2(\phi)N = ClP(O)(O\phi HgCl)_2 + 2Me_2(\phi)N\cdot HCl.$$

The potential inhibitor was not obtained.

However, a crystalline substance was obtained. It was extracted by acetone from the solid present after stirring and refluxing the mixture of 10 gm. of practical grade p-$HO\phi HgCl$, 400 ml. of dry ether, 5 ml. of $Me_2(\phi)N$ and 1.4 ml. of $POCl_3$. The stirring and refluxing were continued for 17 hours. Acetone was one of seven organic solvents used in an attempt to obtain possible products from the solid phase, which had increased 0.7 gm. during the 17 hours. Acetone was selected because it yielded the largest amount of extract.

The extract was crystallized from ethanol as microscopic plates which were laboriously separated from the amorphous material deposited with them. The plates contained mercury and melted between 136.7 and 137.2°C. They were analyzed by Elek Micro Analytical Laboratories:

phosphorous-nil, chlorine-24.56%
carbon-20.18%, hydrogen-2.50%

No analysis for mercury was possible because the sample was limited.

Without phosphorous, the plates could not contain the desired phosphonyl inhibitor. Because the inhibitor had not been extracted from the solid phase by acetone, and was not expected to be soluble in the liquid phase, no further work on the reaction was undertaken. No attempt was made to purify or analyze extracts of the solid phase obtained with solvents other than acetone, because these extracts

were obtained in even smaller amounts than the extract from acetone.

Discussion

The reaction between p-HOØHgCl and $POCl_3$,

$$ClP(O)Cl_2 + 2HO\emptyset HgCl + 2Me_2(\emptyset)N \Rightarrow ClP(O)(O\emptyset HgCl)_2 + 2Me_2(\emptyset)N\cdot HCl,$$

was a modification of a successful reaction (12):

$$FP(O)Cl_2 + 2HO\emptyset + 2Me_2(\emptyset)N = FP(O)(O\emptyset)_2 + 2Me_2(\emptyset)N\cdot HCl.$$

Mercury phenol was substituted for phenol, and $POCl_3$ was substituted for $POCl_2F$ in an attempt to obtain a potential phosphonyl inhibitor carrying mercury atoms. The inhibitor was not obtained although the solid phase increased 0.7 gm. during the reaction. If the inhibitor had been formed and deposited in the solid, it was not soluble in acetone. Perhaps this lack of success in obtaining an inhibitor is related to the substitution of an essentially insoluble mercury phenol for the soluble phenol.

The role of solubility was not investigated because the primary object of this reaction was to obtain a potential inhibitor, rather than to study the reaction in detail.

The Reaction between p-HOØHgCl and $SOCl_2$

The reaction between p-HOØHgCl and $SOCl_2$ was attempted to obtain p-ClHgØOS(O)Cl, a potential thionyl chloride inhibitor carrying a mercury atom. The potential inhibitor was not obtained. However, a

crystalline substance, probably $HgCl_2$, was. These conclusions are based upon three experiments with p-HOØHgCl and $SOCl_2$, as described below.

First experiment

Mercury-containing crystals were obtained from the first experiment with p-HOØHgCl and $SOCl_2$. When a few hundred mg. of p-HOØHgCl were dissolved in boiling $SOCl_2$, a yellow solution formed. The solution deposited a white solid which yielded clear crystals from ether. Because there were too few of these crystals for quantitative elemental analysis, a second experiment was undertaken to obtain sufficient crystals for analysis.

Second experiment

Sufficient crystals were obtained from the second experiment with p-HOØHgCl and $SOCl_2$ for quantitative elemental analysis. The analysis showed that the crystals were not the desired inhibitor, p-ClHgØOS(O)Cl, but probably were $HgCl_2$.

Two batches of crystals were obtained: one from the solid phase and the other from the liquid phase present after a boiling mixture of 33 ml. of $SOCl_2$, 200 ml. of chloroform, and 2.5 gm. of p-HOØHgCl was allowed to cool. The solid was crystallized from ether as rhombs. The liquid was evaporated to a pool of white liquid which, upon further evaporation, deposited rhombs and needles.

Crystals from the solid and the liquid were combined and recrystallized from acetone in order to obtain the largest possible

amount of pure crystals. During an attempt to find their melting point, the crystals gave off white fumes which deposited needles upon a cool surface. The needles were resublimed for further purification.

The twice-sublimed crystals were analyzed by Elek Micro Analytical Laboratories:

sulfur-nil, chlorine-25.57%
carbon-nil, hydrogen-nil

The limited amount of needles did not permit a quantitative analysis for mercury.

Because the sublimate contained no sulfur, carbon, or hydrogen, it could not be the desired inhibitor. However, the sublimate appeared to be $HgCl_2$. It contained 25.57% chlorine, whereas $HgCl_2$ contains 26.2%. It, like $HgCl_2$, contained mercury, appeared as colorless rhombs or white powder, sublimed as white vapor and dissolved in ether and warm water, but was only sparingly soluble in cold water.

Because the analysis had been performed on sublimed cyrstals, the possibility existed that the sublimation had done more than simply purify the crystalline product of the reaction between p-HOØHgCl and $SOCl_2$. Therefore, a third experiment was undertaken to settle this point.

Third experiment

The solid present after adding powdered p-HOØHgCl to $SOCl_2$ with

stirring was collected and repeatedly crystallized from ether. The crystals were analyzed by Elek Micro Analytical Laboratories:

sulfur-nil, chlorine-23.03%
carbon-0.50%, hydrogen-0.30%

Their composition was similar to that of sublimed crystals from the second experiment:

sulfur-nil, chlorine-25.57%
carbon-nil, hydrogen-nil.

Apparently, sublimation had only purified the crystalline product of the reaction between p-HOØHgCl and $SOCl_2$. Thus, the crystalline product from both the second and third experiments was probably $HgCl_2$. The crystalline product definitely was not the desired inhibitor, p-ClHgØOS(O)Cl, because it contained no sulfur.

The probable product of both the second and third experiments was the same. But in the second, chloroform was present and the $SOCl_2$ boiled, whereas in the third, chloroform was not present and the $SOCl_2$ was not boiled. Therefore, formation of the product does not depend upon the presence of chloroform or the boiling of $SOCl_2$. In both experiments, a molar excess of $SOCl_2$ was used. The third experiment was the final experiment with p-HOØHgCl and $SOCl_2$.

Discussion

The above attempt to obtain p-ClHgØOS(O)Cl, a potential thionyl chloride inhibitor carrying a mercury atom, was unsuccessful. However, another attempt, using KOØHgCl and $SOCl_2$ has been reported successful (13). Investigation of this attempt is recommended.

An undesired result of the reaction of p-HOØHgCl and $SOCl_2$ was the removal of mercury from p-HOØHgCl, probably as $HgCl_2$, by an excess of $SOCl_2$. Perhaps this reaction would be reduced by use of a stoichiometric quantity of $SOCl_2$. Another way to reduce removal of mercury is to substitute mercury aniline for mercury phenol in the reaction with $SOCl_2$(14). This substitution should lead to p-ClHgØN(H)S(O)Cl, a potential inhibitor.

Although the removal of mercury from mercury phenol with boiling aqueous KI has been reported previously (14), removal by excess $SOCl_2$ apparently has not.

The Reaction between p-HOØHgCl and SO_2Cl_2

For comparison with the reaction of p-HOØHgCl and a stoichiometric excess of $SOCl_2$, a reaction between p-HOØHgCl and a stoichiometric excess of SO_2Cl_2 was conducted. The solid that was formed in the reaction with SO_2Cl_2 dissolved completely in ether, forming colorless crystals. The same observations had been made for the reaction with $SOCl_2$ from which crystalline $HgCl_2$ was the probably product. Therefore, the probable product from the reaction with SO_2Cl_2 was $HgCl_2$.

A stoichiometric excess of SO_2Cl_2 apparently removed mercury from mercury phenol.

Summary of Reactions with p-HOØHgCl

Three reactions were attempted with p-HOØHgCl: one with $POCl_3$,

one with $SOCl_2$, and one with SO_2Cl_2. From none of these reactions was the desired ester of p-HOϕHgCl obtained. From the reaction with a stoichiometric quantity of $POCl_3$, a non-phosphorous crystalline product was obtained; from the reactions with excesses of $SOCl_2$ and SO_2Cl_2, $HgCl_2$ was probably the product.

The Reaction between $SOCl_2$ and PCMBS

The reaction between $SOCl_2$ and PCMBS was attempted for the purpose of producing ClHgϕSO_2Cl, a potential inhibitor carrying a mercury atom (15). No evaluation of this attempt was possible, because the product from it was obtained in a quantity insufficient for identification. The product was deposited as crystalline chunks when a boiling mixture of 7 ml. of $SOCl_2$, 0.07 gm. of PCMBS, and 10 ml. of chloroform was allowed to cool and evaporate.

Discussion

Although the above attempt to prepare ClHgϕSO_2Cl was not proven successful, another attempt was. Traylor (6) prepared ClHgϕSO_2Cl from $POCl_3$ and PCMBS, and showed that its sulfonyl fluoride, ClHgϕSO_2F, inhibited chymotrypsin stoichiometrically.

The use of the inhibited chymotrypsin in the preparation of a heavy atom derivative has not been reported. Preparation of a heavy atom derivative from chymotrypsin inhibited with ClHgϕSO_2F may be attempted by the author in the near future.

However, no further work on the reaction between $SOCl_2$ and PCMBS is planned, because its desired product, $ClHg\emptyset SO_2Cl$, can now be obtained by Traylor's method.

The Reaction between $SOCl_2$ and C_4I_4NH

Reaction between $SOCl_2$ and C_4I_4NH was attempted to obtain $C_4I_4NS(O)Cl$, a potential thionyl chloride inhibitor carrying iodine atoms. The attempt was unsuccessful. However, there was one indication that a reaction might have occurred. Bubbles of vapor were generated by stirring a mixture of 9 ml. of $SOCl_2$, 3.7 gm. of C_4I_4NH, and 66 ml. of chloroform. Because there was no other indication of a reaction, there was no attempt to isolate possible products from the mixture.

In a second attempt to obtain $C_4I_4NS(O)Cl$, $SOCl_2$ and C_4I_4NH were mixed in ether, instead of the original chloroform. This attempt was also unsuccessful. Aside from formation of a residue unfit for identification, the only other indication of reaction was the generation of bubbles, as before. Therefore, no isolation of possible products was attempted.

No other attempts to obtain $C_4I_4NS(O)Cl$ are planned because they seem unlikely to succeed.

The Reaction between CHI_3 and $(NaO)_2P(O)F$

For 3 months, a benzene solution of CHI_3 stood in contact with

solid $(NaO)_2P(O)F$ in an attempt to obtain $(I_2HCO)_2P(O)F$, a potential inhibitor carrying iodine atoms. The attempt was unsuccessful.

However, in the search for products, deep-yellow needles were obtained when crystals from an ethanol extract of the solid present after three months were recrystallized from ethanol. But these needles did not contain phosphorous and, therefore, were not $(I_2HCO)_2P(O)F$. Because $(I_2HCO)_2P(O)F$ was not obtained, no further work on the reaction between CHI_3 and $(NaO(_2P(O)F$ was undertaken.

The Reaction of CHI_3 and $(AgO)_2P(O)F$

The reaction between CHI_3 and $(AgO)_2P(O)F$ was attempted to obtain $(I_2HCO)_2P(O)F$, a potential inhibitor carrying iodine atoms. The attempt was unsuccessful.

However, there were two indications of reaction between CHI_3 and $(AgO)_2P(O)F$. The first, formation of yellow solid, was observed shortly after a mixture, of 7.7 gm. of $(AgO)_2P(O)F$ in 150 ml. of benzene with a filtered solution of 53 gm. of CHI_3 in 500 ml. of benzene, was set to stirring and refluxing in a three-neck flask equipped with high-speed stirrer and condenser. The yellow solid was essentially insoluble in water, $(NH_4)OH$, and warm conc. NaOH, and was therefore assumed to be AgI.

The second indication of reaction, a 2 gm. increase in solid phase, was observed after 3 hours of stirring and refluxing.

These two indications suggested the possibility of isolating

product(s) from the solid phase present after 3 hours of stirring and refluxing. Therefore, the solid phase was extracted with acetone and with ethyl acetate. From these extracts only a brown viscous liquid of insufficient quantity for distillation was obtained. No products were isolated.

Neither were products isolated from the liquid phase present after 3 hours of stirring and refluxing. Therefore, no further work with the reaction between CHI_3 and $(AgO)_2P(O)F$ was undertaken.

Summary of the Reactions with CHI_3

When CHI_3 was reacted with $(NaO)_2P(O)F$, needles which did not contain phosphorous were obtained. When CHI_3 was reacted with $(AgO)_2P(O)F$, an increase in solid resulted, part of which probably resulted from the formation of AgI. But from neither of these reactions was $(I_2HCO)_2P(O)F$, the desired potential inhibitor carrying heavy atoms, obtained. Therefore, neither reaction was investigated in detail. No explanation of the above results was attempted.

The Reaction between $AgNO_3$ and $(NaO)_2P(O)F$

A reaction between $AgNO_3$ and $(NaO)_2P(O)F$ was conducted according to the method of Saunders and Stacy (16) for preparing $(AgO)_2P(O)F$, a potential phosphoryl fluoride inhibitor carrying silver atoms. Eight and one-half grams of $(AgO)_2P(O)F$ were precipitated by adding

saturated aqueous $AgNO_3$ to a solution of 10.0 gm. of $(NaO)_2P(O)F$ in 260 ml. of water.

Thus, the potential inhibitor $(AgO)_2P(O)F$ had been made available for inhibition of chymotrypsin. But no inhibition was attempted because $(AgO)_2P(O)F$ appeared to release its heavy atoms when allowed to stand at the pH required for inhibition. Within the time required for inhibition, an insoluble sample of $(AgO)_2P(O)F$ placed in water at pH 7.4 had dissolved and a second insoluble substance had formed. Apparently, the second insoluble substance was AgO formed from the silver ions released by $FP(O)(OAg)_2$ as it dissolved as $FP(O)O_2^{=}$. In summary, $(AgO)_2P(O)F$ was not likely to be of use as a carrier of heavy atoms after it inhibited chymotrypsin. Therefore, $(AgO)_2P(O)F$ was not studied further.

Conclusion

None of the reactions described in this section appears to merit further consideration for the preparation of inhibitors carrying heavy atoms. However, two related reactions may be of value. One, related to the reaction between $SOCl_2$ and PCMBS, is Traylor's reaction of $POCl_3$ and PCMBS for the preparation of p-$ClHg\phi SO_2F$, a proven inhibitor of potential value in preparing heavy atom derivatives.

A second reaction, related to the reaction between p-$HO\phi HgCl$ and $POCl_3$, may be of value in the preparation of an inhibitor carrying heavy atoms:

$$ClP(O)Cl_2 + 2HO\phi I + 2Me_2(\phi)N \rightarrow ClP(O)(O\phi I)_2 + 2Me_2(\phi)N\cdot HCl$$

This reaction differs from the one between p-HOØHgCl and $POCl_3$ only in the use of p-HOØI in place of p-HOØHgCl. This change should allow isolation of a product, $ClP(O)(ØI)_2$, which is more soluble than $ClP(O)(OØHgCl)_2$, the desired product from the reaction with p-HOØHgCl. However, after reaction of $ClP(O)(ØI)_2$ with chymotrypsin and crystallization of the inhibited enzyme, location of iodine atoms in the crystal will be required before the crystal can be used as a heavy atom derivative. And locating iodine atoms is extremely difficult. Nevertheless, Sigler et al. (17) have reported locating iodine atoms bound to an inhibitor in crystals of inhibited chymotrypsin.

One additional type of reaction, unrelated to any of the attempted reactions, may deserve investigation: the preparation of inhibitors with groups capable of complexing with heavy atoms. These inhibitors can be reacted with chymotrypsin, complexed with heavy atoms, and the resulting complex crystallized to obtain heavy atom derivatives. With this approach, the inhibitors should be soluble enough to be isolated and the heavy atoms heavy enough to be located.

II. LYSOZYME

Introduction

The following X-ray investigation of crystals of lysozyme hydrochloride diffused with heavy atoms was undertaken to locate heavy atoms for the preparation of electron density maps of the lysozyme molecule. The crystals are tetragonal: a = 79.1, c = 37.9 Å, space group 4_12_12 or 4_32_13 with eight molecules per unit cell (8). This investigation was also undertaken to permit comparison of its findings with two current investigations: one, of the same crystal, at the Royal Institution (8); and the other, of tetragonal crystals of lysozyme-$Ta_6Cl_{12}^{++}$, at the California Institute (17).

From analysis of hk0 and h0l structure amplitudes, most of which were collected by other workers, tentative x, y and z coordinates were obtained for the heavy atoms in crystals diffused with $PtI_6^=$ ions and tentative x and y coordinates were obtained for the heavy atoms in crystals diffused with UO_2^{++} ions, $WO_4^=$ ions, PCMBS*, $HgCl_2$, $HgI_4^=$ ions, and $ThCl_4$. The coordinates for $HgI_4^=$ ions are symmetrically equivalent to published ones (8). Those for $PtI_6^=$ ions and PCMBS are equivalent to published coordinates for $PdCl_4^=$ ions and MHTS**, respectively (8). The coordinates for $PtI_6^=$ ions are also in close agreement with those obtained at the California Institute for the same ions diffused into crystals of lysozyme-$Ta_6Cl_{12}^{++}$.

*p-chloromercuribenzenesulfonic acid
**o-mercurihydroxytoluene-p-sulfonic acid

One hk0 and one h0l electron density projection were prepared. The hk0 projection in Figure 1 agreed with one obtained at the Royal Institution but contained a number of differences, along the unit cell diagonal, from the hk0 projection of lysozyme-$Ta_6Cl_{12}^{++}$ obtained at the California Institute. For the preparation of three-dimensional electron density maps, two heavy atom derivatives are required. Subject to confirmation with three-dimensional data, the crystals diffused with $PtI_6^{=}$ ions constitute one. The other may be obtained by determining the z coordinate for the heavy atom in one additional diffused crystal. Crystals diffused with UO_2^{++} ions, $WO_4^{=}$ ions, or PCMBS appear most promising.

The tentative x and y coordinates reported above were derived from centrosymmetric hk0 Fourier and Patterson projections; and the z coordinates from centrosymmetric h0l projections. Agreement of coordinates from Fourier and Patterson projections was required for acceptance of tentative coordinates. Some of the tentative coordinates were further tested for acceptability by scaling heavy atom scattering factors based upon them to observed scattering factors.

The following work on diffused lysozyme crystals is grouped according to the heavy atom diffused into the crystal.

Crystals Diffused with $PtI_6^{=}$ Ions

Introduction

Structure amplitudes from crystals diffused with $PtI_6^{=}$ ions were

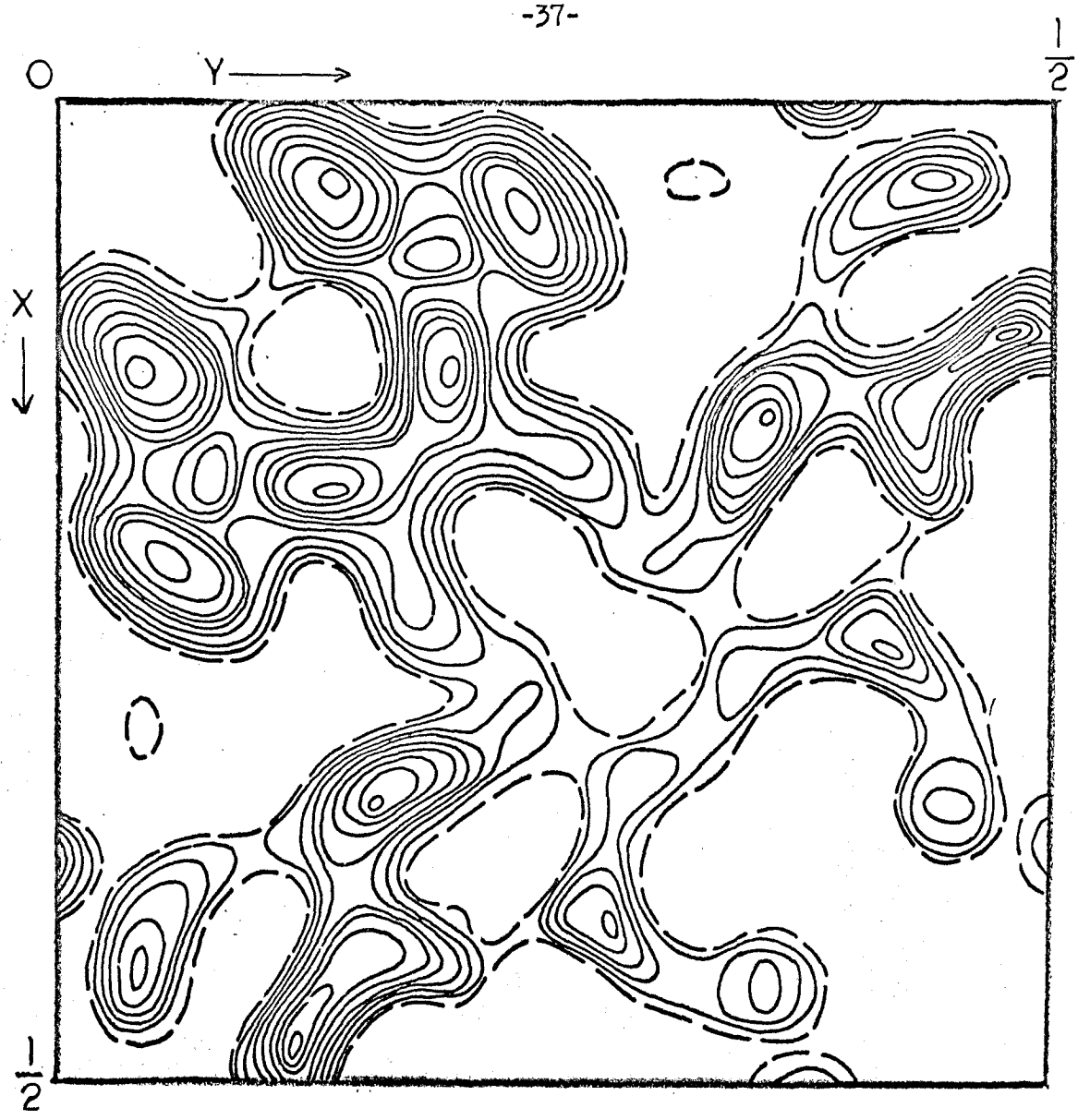

Fig. 1 - An hk0 Fourier for crystal of lysozyme hydrochloride,

dmin = 6Å.

Phase angles from Phillips at Royal Institution.

Data collected by other workers.

obtained by other workers and used by the author to prepare hk0 and h0l Patterson and Fourier projections from which tentative x, y, and z coordinates for the $PtI_6^=$ ions were obtained. These coordinates were refined and used to calculate the scattering contribution of the $PtI_6^=$ ions. Good agreement of these calculated contributions, f_{PtI_6}, and observed contributions, F_{PtI_6}, was indicated by an R factor of 34% for 108 hk0 and h0l reflections. The R factor was calculated as follows:

$$\frac{\Sigma \left| |f_{PtI_6}| - |F_{PtI_6}| \right|}{\Sigma |f_{PtI_6}|}$$

On the basis of this good agreement for projection data, collection of three-dimensional data is recommended. If the tentative coordinates are confirmed with this data, the crystals diffused with $PtI_6^=$ ions would constitute one of the two heavy atom derivatives required for the preparation of three-dimensional electron density maps.

Determination of Coordinates for $PtI_6^=$ Ions

From five hk0 Patterson projections and two hk0 Fourier projections, tentative coordinates for $PtI_6^=$ ions of x = y = 0.35 were obtained. The hk0 projections will be discussed following the section on refinement of coordinates. At that time coordinates obtained from each of the projections will be compared with the refined coordinates.

From the h0l Patterson presented in Figure 2, a z coordinate of 0.21 was obtained; the x and y coordinates were equal to those obtained from hk0 projections. The type of amplitude for this h0l Patterson, $(k'|F_{lys} + PtI_6| - |F_{lys}|)^2$, was selected in preference to $(k|F_{lys} + PtI_6|^2 - |F_{lys}|^2)$ and $|F_{lys} + PtI_6|^2$ because the latter yield Pattersons with interactions other than those between heavy atoms. The calculation and acceptability of the value of k' used in Figure 2 will be considered later.

A second Patterson was prepared with amplitudes of the type used in Figure 2, but with k' equal to 0.7 times that used in Figure 2. In it, two of the peaks present in Figure 2 were missing. The importance of the scale factor k' is evident.

Because the coordinates obtained from Figure 2 were confirmed by subsequent refinement, no h0l Fourier projection was prepared to confirm the coordinates.

Data for the two h0l Pattersons which were prepared were obtained by other workers from crystals of lysozyme which had been diffused with $PtI_6^=$ ions at a $PtI_6^=$:lysozyme molar ratio of 0.25:1. This molar ratio was selected for the reasons enumerated below.

Selection of Molar Ratio of $PtI_6^=$:Lysozyme

Diffraction patterns from crystals diffused with $PtI_6^=$ ions at $PtI_6^=$:lysozyme molar ratios of 0.25:1, 0.75:1, and 1:1 were compared

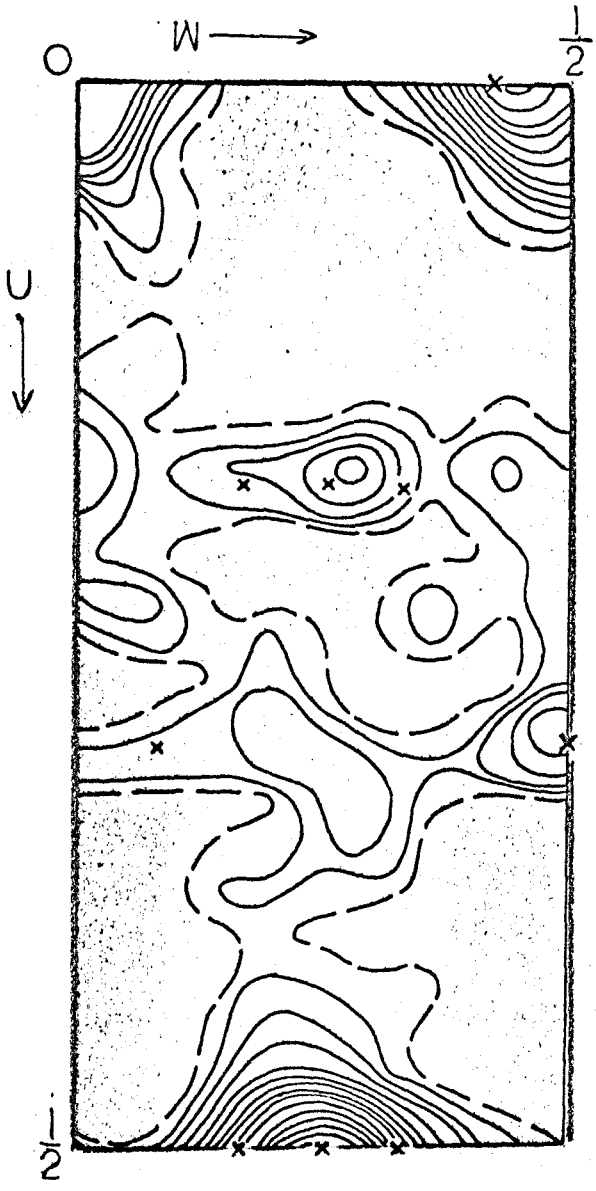

Fig. 2 - An h0l Patterson for lysozyme crystal diffused with $PtI_6^=$ ions for 28 da. at a molar ratio of 0.25:1, dmin = 5Å.

Amplitudes: $(k' \left|F_{lys} + PtI_6\right| - \left|F_{lys}\right|)^2$, $k' = 1.2$.

Crosses mark projection of interactions between heavy atoms at x = y = 0.35, z = 0.21 and symmetrically-related locations.

Data collected by other workers.

to select the minimum ratio above which no apparent change in high-angle reflections occurred. This selection was made to obtain maximum occupancy of heavy atom sites and a minimum of additional heavy atoms in the crystal.

The high-angle intensities for hk0 patterns from crystals diffused at 0.75:1 and 1:1 were apparently identical. Patterns at both ratios differed at high angles from those at 0.25:1. Thus, the desired ratio was between 0.25:1 and 0.75:1. This conclusion from hk0 patterns was supported by h0l patterns also.

Data from crystals diffused at 0.25:1, rather than at 0.75:1, were used for preparation of h0l Pattersons and for refinement of coordinates, because workers at the California Institute had shown that unit cells of crystals diffused at 0.25:1 contained a number of $PtI_6^=$ ions closer to the eight required by full occupancy of symmetrically-related sites than did the unit cell of crystals diffused at 0.75:1. The good agreement between calculated and observed contributions of $PtI_6^=$ ions, discussed below, supports the acceptability of the selection of the 0.25:1 ratio.

Refinement of Coordinates

Refinement of coordinates obtained from Fourier and Patterson projections was undertaken with hk0 and h0l data from lysozyme crystals diffused with $PtI_6^=$ ions at a molar ratio of 0.25:1. The refinement

was undertaken to evaluate these coordinates. They were shown to be essentially correct because their refinement led to an R factor of 34% for the agreement of observed and calculated contributions of $PtI_6^=$ ions for 108 hk0 and h0l reflections.

The observed contributions of $PtI_6^=$ ions were obtained by scaling $|F_{lys} + PtI_6|$ and $|F_{lys}|$, by the method of least squares, to the calculated contributions for $PtI_6^=$ ions centered at x = 0.355, y = 0.345, and z = 0.200. These coordinates are essentially equal to the x = 0.34, y = 0.35, and z = 0.21 which were obtained from Fourier and Patterson projections. The calculated contributions were obtained by Dr. Stanford. He adjusted them for the scattering matter replaced by the $PtI_6^=$ ions by multiplying the Pt and I scattering factors by 0.6717. The observed contributions yielded an R factor of 38% with the calculated contributions.

The refinement of coordinates for $PtI_6^=$ ions was conducted with the observed contributions obtained by the above scaling and with calculated contributions prepared by a Structure Factor - Least Squares program written by Dr. Stanford and modified by the author. After one cycle of coordinate refinement, using observed and calculated contributions for 108 hk0 and h0l reflections, the R factor for 67 hk0 reflections was 35% and for 41 h0l reflections was 31% - an improvement over the initial R factors of 38%. The R factor for all 108 hk0 and h0l reflections was 34%. The refined coordinates were: x = 0.346, y = 0.348, z = 0.212, in very close agreement with

the coordinates obtained from hk0 and h0l projections.

A second set of refined coordinates was obtained by refinement of 41 h0l reflections, but no hk0 reflections. These coordinates yielded hk0 and h0l calculated contributions for $PtI_6^=$ ions which were compared with hk0 and h0l observed contributions. The R factor from the comparison was greater than that obtained with the first set of refined coordinates. Thus, the first set of refined coordinates was accepted.

The calculated contributions for $PtI_6^=$ ions at the first set of refined coordinates, x = 0.346, y = 0.348, and z = 0.212, and the observed contributions of $PtI_6^=$ ions are presented for hk0 reflections in Table 4 and for h0l reflections in Table 5.

TABLE 4

SCALED hk0 $PtI_6^=$ DATA, dmin = 5Å

Index	$F_{lys+PtI_6}$	F_{lys}	F_{PtI_6}	f_{PtI_6}	$\lvert F_{PtI_6} - f_{PtI_6} \rvert$
4 0 0	-2305	-1352	-953	-1291	338
8	-717	-1069	+352	+204	148
10	-1757	-1148	-609	-896	287
12	-167	-759	+592	+342	250
3 1 0	-1557	-819	-738	-995	257
4	-704	+292	-996	-874	122
5	-799	-974	+175	+74	101
6	-1312	-936	-376	-576	200
7	-379	+281	+660	+662	2
8	+184	-730	+914	+922	8
12	-1067	-911	-156	-488	332
14	+794	+308	+486	+410	176
15	-664	-385	-279	-59	220
2 2 0	-578	-999	+421	+214	207
3	-220	-759	+539	+424	115
5	-1968	-713	-1255	-1400	145

Index	$F_{lys+PtI_6}$	F_{lys}	F_{PtI_6}	f_{PtI_6}	$\lvert F_{PtI_6} - f_{PtI_6} \rvert$
6	-270	+306	-576	-403	173
7	+802	+421	+381	+490	109
8	+995	+1354	-359	-68	291
10	+635	+504	+131	+298	167
11	-862	-251	-611	-660	49
12	-869	-810	-59	-113	54
13	+366	+451	-85	-50	35
14	+1006	+1148	-142	-108	34
15	-432	-889	+457	+369	88
3 3 0	+815	-783	+1598	+1538	60
4	+629	+1123	-494	-258	236
7	-498	+768	-1266	-1052	214
8	+172	-270	+442	+277	164
9	+886	+211	+675	+659	16
10	+161	+394	-233	-38	195
14	(-)480	(-)497	+17	+94	77
4 4 0	-498	-1745	+1247	+858	389
5	+486	-428	+914	+868	45
6	-1334	-481	-853	-831	22
7	+164	+662	-498	-307	191
8	+159	+188	-29	-140	111
10	+1085	+459	+626	+622	4
13	-266	-608	+342	+32	310
14	+213	+355	-142	-224	82
5 5 0	(-)664	(-)668	+4	+8	4
6	+612	+197	+415	+589	174
7	-984	-1146	+162	+83	79
8	-1438	-566	-872	-957	85
10	-235	-676	+441	+130	311
12	+877	+283	+614	+512	102
13	-698	-887	+189	+42	147
14	+224	+418	-194	-324	130
15	+626	+1042	-406	-6	400
7 6 0	+198	+494	-296	-212	84
11	+897	+611	+286	+290	4
14	+550	+305	+245	+223	22
7 7 0	-164	-979	+815	+759	56
8	+838	+611	+227	+345	118
10	+773	+988	-215	-46	169
13	-149	-564	+415	+393	22
14	-368	-449	+81	+116	35
8 8 0	+393	+379	+14	+22	8
9	+889	+209	+620	+480	140

Index	$F_{lys+PtI_6}$	F_{lys}	F_{PtI_6}	f_{PtI_6}	$\lvert F_{PtI_6} - f_{PtI_6} \rvert$
10	+681	+943	-262	-106	156
11	+144	+690	-546	-476	70
10 9 0	-791	-617	-174	-66	108
13	-713	-432	-281	-247	34
10 10 0	+1182	+586	+596	+474	122
11	-342	-477	+135	+64	71
12	+512	+1275	-763	-179	584
11 11 0	+1026	+738	+288	+67	221

TABLE 5

SCALED h01 $PtI_6^=$ DATA, dmin = 5Å

Index	$F_{lys+PtI_6}$	F_{lys}	F_{PtI_6}	f_{PtI_6}	$\lvert F_{PtI_6} - f_{PtI_6} \rvert$
8 0 0	-395	-616	+221	+204	17
10	-1159	-616	-543	-896	353
3 0 1	156	138	18	57	39
4	147	92	239	154	85
5	395	478	83	245	162
7	257	156	101	115	14
8	653	763	110	178	68
9	404	423	19	95	76
10	653	515	138	6	132
11	82	230	148	130	18
13	174	322	148	18	130
1 0 2	312	542	854	840	14
2	147	248	101	7	94
4	653	662	9	8	1
5	460	174	216	101	185
7	607	257	864	916	48
8	496	579	103	19	84
9	818	340	478	576	98
10	561	469	92	3	89
12	404	294	110	15	95
1 0 3	82	404	486	519	33
2	386	128	514	582	68
3	147	266	119	166	47
4	653	432	221	376	155
6	230	469	239	234	5
9	266	506	240	279	39
0 0 4	257	285	542	647	105
1	257	220	37	3	34
5	349	423	74	20	54

Index	$F_{lys+PtI_6}$	F_{lys}	F_{PtI_6}	f_{PtI_6}	$\lvert F_{PtI_6} - f_{PtI_6} \rvert$
6	165	524	359	443	84
8	506	386	120	76	44
10	524	294	230	339	109
1 0 5	322	128	450	466	16
2	358	82	440	525	85
3	202	294	92	137	45
5	184	515	331	487	156
6	211	220	431	234	197
1 0 6	276	230	46	52	6
2	184	156	28	6	22
3	349	303	46	83	37
4	239	294	55	7	48

For these tables dmin = 5Å. In both Tables, the scaled values of $\lvert F_{lys + PtI_6} \rvert$ and $\lvert F_{lys} \rvert$ are presented. Their signs were selected to obtain the best agreement between the observed contribution F_{PtI_6}, which equals $(F_{lys + PtI_6} - F_{lys})$, and the calculated contribution f_{PtI_6}. The Tables also include F_{PtI_6}, f_{PtI_6} and $\lvert F_{PtI_6} - f_{PtI_6} \rvert$. The R factors mentioned above were calculated from $R = \frac{\Sigma \lvert F_{PtI_6} - f_{PtI_6} \rvert}{\Sigma \lvert f_{PtI_6} \rvert}$.

The calculated contributions of $PtI_6^=$ ions, f_{PtI_6}, presented in both Tables were calculated for $PtI_6^=$ ions in random orientation (18). Random orientation was selected because the orientation of the $PtI_6^=$ ions was not known. As before, the calculated contributions were adjusted for the scattering matter displaced by the ions, by multiplying the Pt and I scattering factors by 0.6717.

In Table 4, reflections with both $\lvert F_{lys + PtI_6} \rvert$ and $\lvert F_{lys} \rvert$ greater than 150 are presented. Reflections with scaled amplitudes below 150 were not included because they were subject to a relatively large percent of error and thus were not used in the refinement. In Table 5,

the minumum included value of $|F_{lys} + PtI_6|$ and $|F_{lys}|$ is 80.

In Table 4, the signs represent phase angles: plus for 0° and minus for 180°. The phase angles for F_{lys} were compared with those obtained from Phillips at the Royal Institution. Of the 47 compared, 39 were the same, indicating a good agreement.

Discussion of hk0 Patterson and Fourier Projections

After a consideration of the refinement of $PtI_6^=$ data, we are prepared to discuss the coordinates obtained from hk0 Patterson and Fourier projections in terms of the refined coordinates.

The refined x and y coordinates, 0.346 and 0.348, respectively, were closely matched by the x = y = 0.344, the x = y = 0.346 and the x = y = 0.350 which were derived from a Fourier with amplitudes of $|F_{lys} + PtI_6|$, a Patterson with amplitudes of $(k'|F_{lys} + PtI_6| - |F_{lys}|)^2$ and a Patterson with amplitudes of $|F_{lys} + PtI_6|^2$, respectively. The $|F_{lys} + PtI_6|$ values for these projections came from crystals diffused at a molar ratio of 0.25:1. The first two projections are presented in Figures 3 and 4.

The quality of the 0.25:1 data is supported by the close agreement of coordinates obtained from three different treatments of the data: Patterson, Fourier and Least Squares Refinement. Another indication of the quality of the 0.25:1 data is that the peaks at u = 1/2, v = 0.15, and u = v = 0.35 in the two Pattersons using this

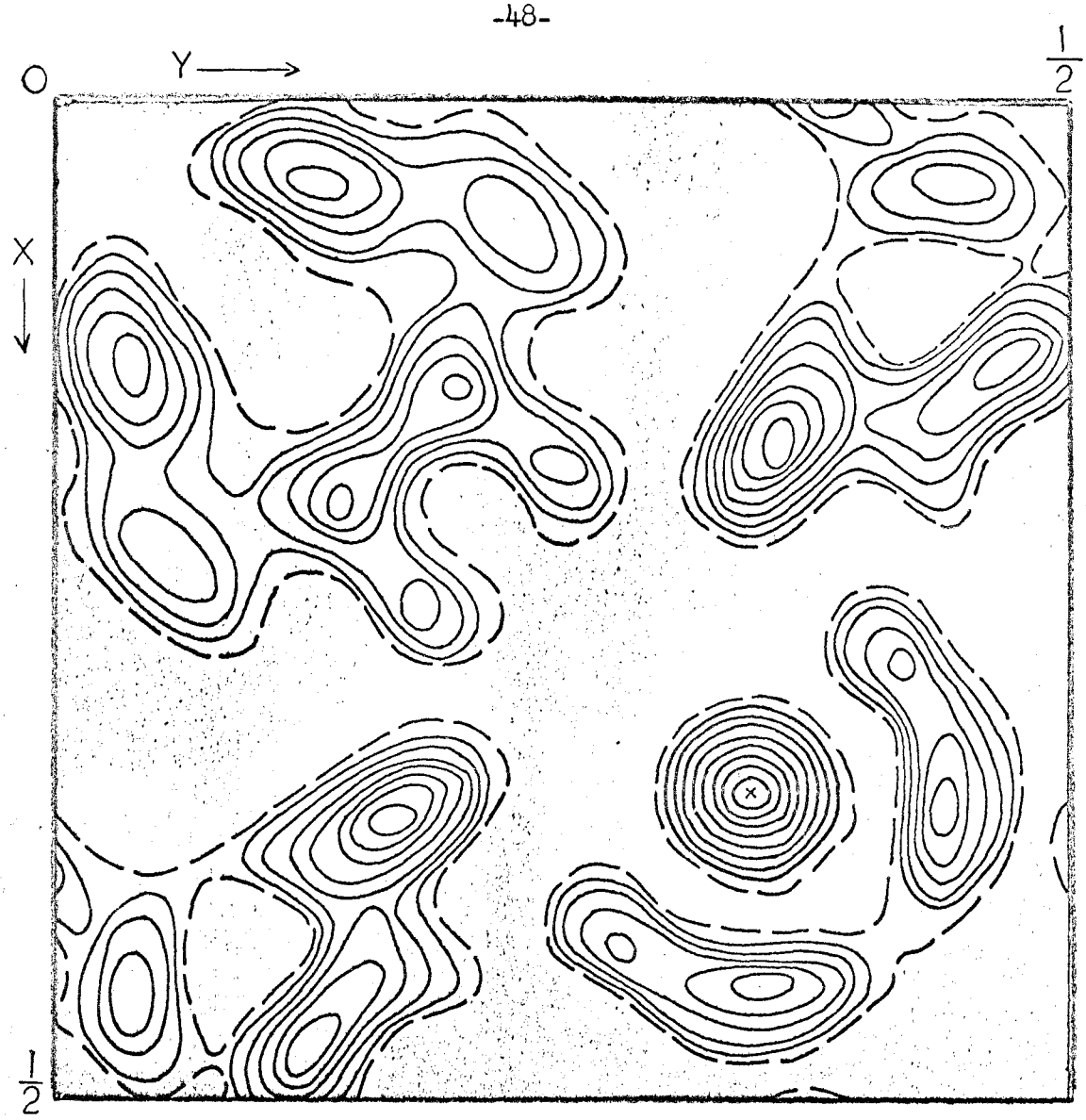

Fig. 3 - An hk0 Fourier for lysozyme crystal diffused 28 da. with $PtI_6^{=}$ ions at a $PtI_6^{=}$: lysozyme molar ratio of 0.25:1, dmin = 6Å.

Phase angles from Phillips at Royal Institution.

The cross marks heavy atom at x = y = 0.344.

Data collected by other workers.

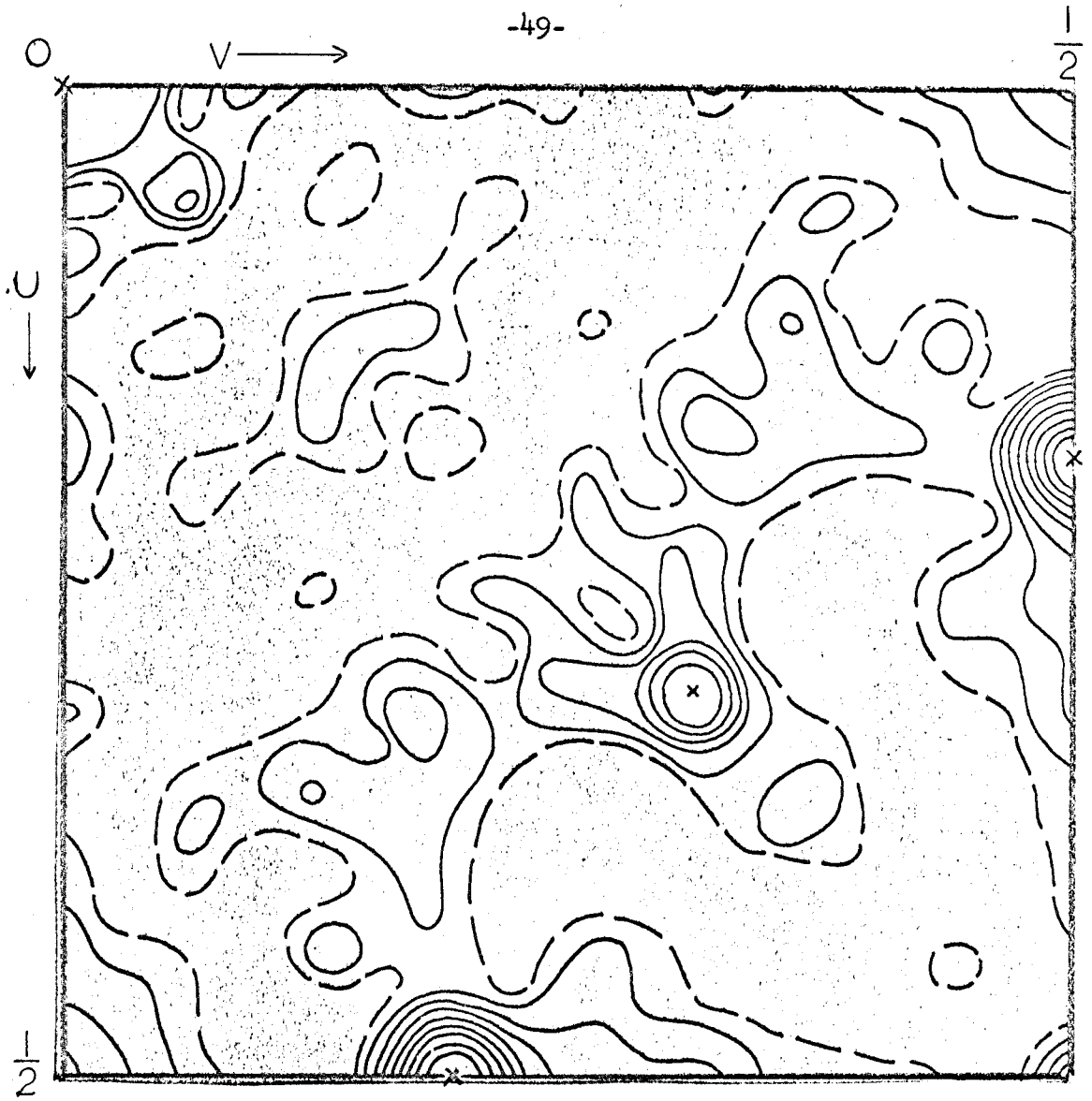

Fig. 4 - An hk0 Patterson for lysozyme crystal diffused 28 da. with $PtI_6^=$ ions at a $PtI_6^=$: lysozyme molar ratio of 0.25:1, dmin = 5A.

Amplitudes: $(k' \left|F_{lys + PtI_6}\right| - \left|F_{lys}\right|)^2$, $k' = 0.55$.

Crosses mark projection of interactions between heavy atoms at x = y = 0.346 and symmetrically-related locations.

Data collected by other workers.

data have the 2:1 relative height required by crystal symmetry.

In the Fourier mentioned above, the peak associated with $PtI_6^=$ is 3 times as high as that of surrounding peaks, whereas the peaks associated with $PtI_6^=$ in the two Pattersons are 1.5 times as high as surrounding peaks. Thus, the Fourier is more powerful for location of $PtI_6^=$ ions. However, the correctness of the Fourier depends strongly upon the essential correctness of the phase angles used in its preparation. The essential correctness of the phase angles from Phillips at the Royal Institution, which were used to prepare the Fouriers for diffused and undiffused crystals (Figures 1 and 3), is supported qualitatively by the Figures and quantitatively by the phase angles presented in Table 1. The lysozyme portions of the Fourier projections in Figures 1 and 3 are nearly identical for diffused and undiffused crystals. The phase angles from Phillips are in about 80% agreement with those for diffused and undiffused crystals. This strong evidence of correctness of Phillip's phase angles allows confidence in their use with subsequent diffused crystals.

The scale, k', used in the preparation of the $(k'|F_{lys} + PtI_6| - |F_{lys}|)^2$ Patterson from 0.25:1 data was derived from: $k' = (\Sigma|F_{lys}|^2 / \Sigma|F_{lys} + PtI_6|^2)^{1/2}$. This and other values of k' obtained from the same data are presented below:

k'	Source
0.55	$(\Sigma\|F_{lys}\|^2/\Sigma\|F_{lys} + PtI_6\|^2)^{1/2}$
0.60	$(\Sigma\|F_{lys}\|/\Sigma\|F_{lys} + PtI_6\|)$
0.79	Scaling by method of least squares.

Apparently, the second formula provides a better estimate of the least squares values than does the first. Summation was carried out with hk0 data, dmin = 5Å.

Data from crystals diffused at molar ratios of 3.4:1 and 6.8:1 appeared to be of poorer quality than those from crystals diffused at 0.25:1, because Pattersons from crystals diffused at the higher ratios yielded coordinates for $PtI_6^=$ ions which differed more from the refined ones than did those from crystals diffused at 0.25:1. In a Patterson from 6.8:1 data, the $PtI_6^=$ -$PtI_6^=$ interactions were less prominent than those in a Patterson from 3.4:1 data.

Discussion of h0l Scaling

The scale, k', used to prepare the h0l Patterson which yielded the z coordinate of 0.21 was obtained as follows:

$$\left(\frac{1/2\Sigma_{h00,00l}\left|F_{lys}\right|^2 + \Sigma_{h0l \neq h00,00l}\left|F_{lys}\right|^2}{1/2\Sigma_{h00,00l}\left|F_{lys} + PtI_6\right|^2 + \Sigma_{h0l \neq h00,00l}\left|F_{lys} + PtI_6\right|^2} \right)^{1/2}$$

This formula was selected to equate the height of origin peaks in Pattersons from diffused and undiffused crystals. Other values of scale, prepared from the same data, are presented below:

Scale	Source
1.1	$(\Sigma\left\|F_{lys}\right\|^2/\Sigma\left\|F_{lys} + PtI_6\right\|^2)^{1/2}$
1.2	k', as above

Scale	Source
1.3	$(\Sigma \lvert F_{lys} \rvert / \Sigma \lvert F_{lys + PtI_6} \rvert)$
1.4	Scaling by method of least squares

as with hk0 data, the $(\Sigma \lvert F_{lys} \rvert / \Sigma \lvert F_{lys + PtI_6} \rvert)$ provides the best estimate of the values obtained by the method of least squares. Summation was carried out with h0l data, dmin = 5Å.

Discussion of Crystals Diffused with $PtI_6^{=}$ Ions

The isomorphism of undiffused lysozyme crystals with lysozyme crystals diffused with $PtI_6^{=}$ ions is supported by a 34% R factor for the agreement of calculated and observed contributions of the $PtI_6^{=}$ ions. The prospect for three-dimensional conformation of the tentative coordinates for $PtI_6^{=}$ ions obtained from hk0 and h0l data is excellent. Thus, collection of three-dimensional data is recommended.

The refined tentative coordinates for $PtI_6^{=}$ ions in crystals of lysozyme (0.346, 0.348, 0.212) are nearly equal to those obtained at the California Institute for the same ions in crystals of lysozyme-$Ta_6Cl_{12}^{++}$ (0.345, 0.355, 0.200). The refined coordinates are symmetrically equivalent to published coordinates for $PdCl_4^{=}$ ions in crystals of lysozyme (0.35, 0.34, 0.21) (8).

Crystals Diffused with UO_2^{++} Ions

Introduction

Structure amplitudes from crystals diffused with UO_2^{++} ions were obtained by other workers and used by the author to prepare hk0

Fourier and Patterson projections from which tentative x and y coordinates for UO_2^{++} ions were obtained. Additional structure amplitudes were obtained by the author and used to prepare h0l Fourier and Patterson projections from which no satisfactory z coordinate was obtained. Determination of a z coordinate from three-dimensional data is recommended.

hk0 Fourier and Patterson Projections

Both the hk0 Fourier Projection in Figure 5 and the hk0 Patterson projection in Figure 6 support the coordinates x = 0.41 and y = 0.38 for the UO_2^{++} ions. The Fourier was prepared with amplitudes of $|F_{lys} + UO_2|$ and phase angles from Phillips at the Royal Institution. The Patterson was prepared with amplitudes of $(k' |F_{lys} + UO_2| - |F_{lys}|)^2$.

A second Patterson was prepared with the value of k' used for the first but with amplitudes of $(k' |F_{lys} + UO_2|)^2 - |F_{lys}|^2$. Because the second Patterson did not support the above coordinates, it was assumed a less reliable type of Patterson for location of UO_2^{++} ions.

Comparison of the Fourier from crystals diffused with UO_2^{++} ions (Figure 5) with the Fourier from undiffused crystals (Figure 1) suggested an additional heavy atom location at x = 0.30 and y = 0.17. This location was rejected because it was not consistent with the Patterson in Figure 6.

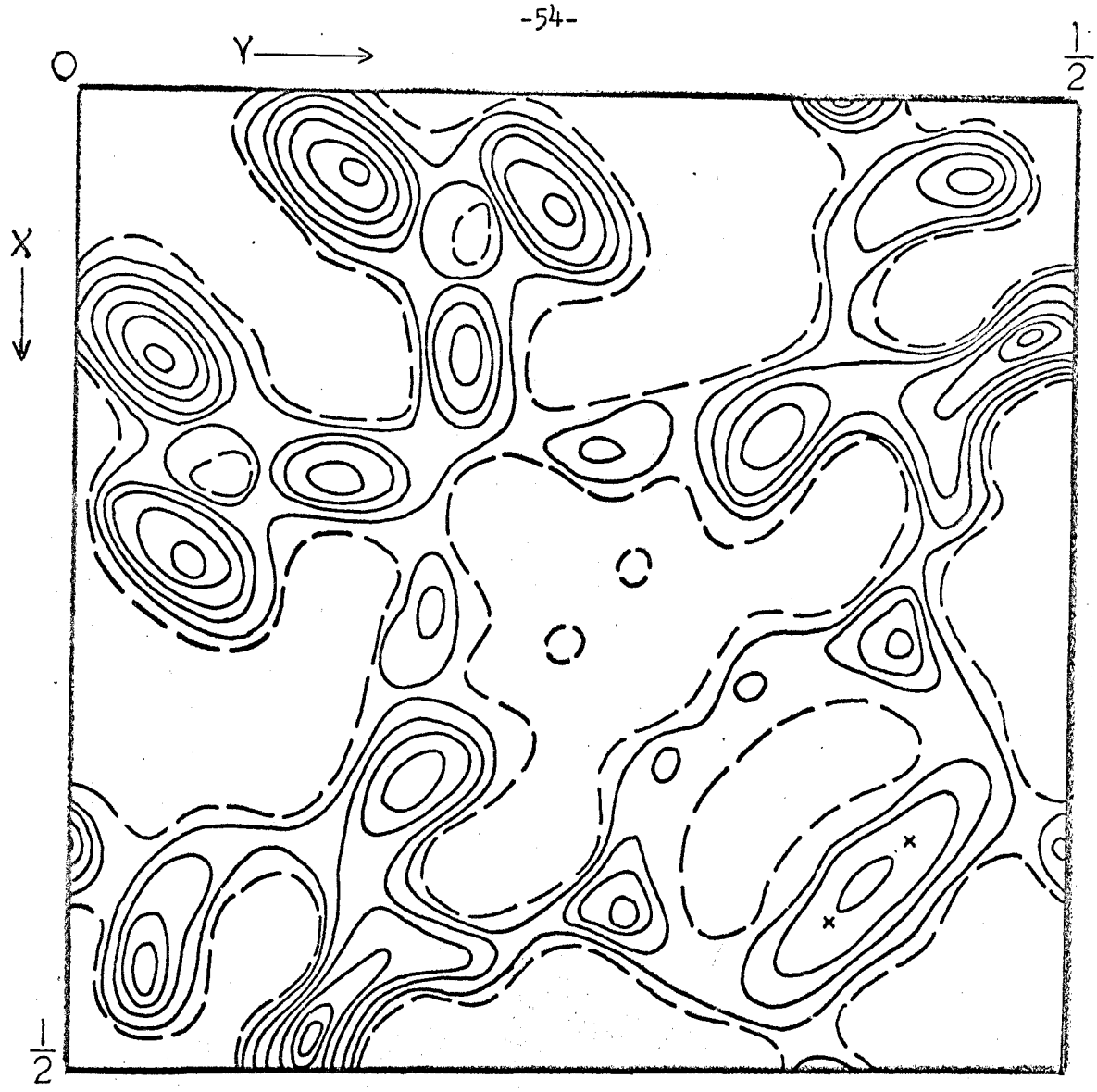

Fig. 5 - An hk0 Fourier for lysozyme crystal diffused 2-1/2 mo. with UO_2^{++} ions at a UO_2^{++}:lysozyme molar ratio of 2:1, dmin = 6Å.

Phase angles from Phillips at Royal Institution.

Crosses mark heavy atom at x = 0.41, y = 0.38 and symmetrically-related location.

Data collected by other workers.

Fig. 6 - An hkO Patterson for lysozyme crystal diffused 2-1/2 mo. with UO_2^{++} ions at a UO_2^{++}:lysozyme molar ratio of 2:1, dmin = 5Å.

Amplitudes: $(k' \left| F_{lys + UO_2} \right| - \left| F_{lys} \right|)^2$, $k' = 1.4$.

Crosses mark projection of interactions between heavy atoms at x = 0.41, y = 0.38 and symmetrically-related locations.

Data collected by other workers.

UO_2^{++}:Lysozyme Molar Ratio Studies

After determination of tentative x and y coordinates from hk0 data, h0l data were collected for determination of a tentative z coordinate. The h0l data were from lysozyme crystals diffused with UO_2^{++} ions at a molar ratio of 1.5:1, because the following study indicated that location of the UO_2^{++} ions would be favored at that ratio.

Both hk0 and h0l diffraction patterns were obtained from crystals diffused at molar ratios of 1:1, 1.5:1, 2:1 and 3:1. The 2:1 hk0 pattern was obtained by other workers. The 1:1 hk0 and h0l patterns appeared identical to those from undiffused crystals. Patterns from crystals diffused at higher ratios appeared identical to each other but different from the 1:1 patterns. Thus, the 1.5:1 ratio was selected for collection of h0l data because it was the minimum ratio producing patterns different from the undiffused patterns.

The lack of difference at low angles between patterns from 1.5:1 and from higher ratios is striking, but inexplicable at present. Similarly, the apparent low-angle and high-angle identity of patterns from undiffused crystals and crystals diffused at 1:1, is striking but inexplicable.

h0l Projections

Two h0l Fouriers and one h0l Patterson were prepared from data collected from crystals diffused at a molar ratio of 1.5:1. Two

Difference Fouriers were prepared: one, using phase angles for lysozyme derived from $PtI_6^=$ data, and the other using phase angles from scattering factors for uranium atoms with coordinates from the first. The two Fouriers were qualitatively similar. In the second, heavy atom peaks appeared in the positions derived from the first, apparently unshifted. But the correctness of the coordinates from the first projection is questionable because the phase angles derived from them, and used in the second projection, differed from those used in the first, for 23 out of 57 reflections compared. The phase angles used in the first projection were those for lysozyme derived from $PtI_6^=$ data and should show much greater agreement with the phase angles from a genuine heavy atom derivative than is the case above.

Further, the third h0l projection, a Difference Patterson, did not support the coordinates obtained from the Difference Fouriers. In summary, no satisfactory z coordinate was obtained.

Difference projections were prepared because they were expected to offer the best opportunity for location of heavy atoms. The value of k' used for the three difference projections was an average of six independently-derived values. Because the range of these six values was relatively small, no additional values of k' were used in the preparation of h0l projections.

Scaling of h0l Data

To further evaluate the coordinates derived from the first h0l

Difference Fourier, scaling of h0l data to scattering factors for uranium atoms at those coordinates was attempted. The method of least squares failed to scale the data, giving additional evidence of an unsatisfactory z coordinate.

Discussion

Satisfactory x and y coordinates were obtained from hk0 Patterson and Fourier projections, but a satisfactory z coordinate was not obtained from h0l Patterson and Fourier projections. The inability to obtain a satisfactory z coordinate may result from the relatively smaller contribution of heavy atoms to the scattering matter projected through 80Å for the h0l projection than to the scattering matter projected through 40Å for the hk0 projection. It may also result from a random distribution of heavy atoms in the direction of the c axis. If the former is the case, then determination of a satisfactory z coordinate may be possible with three-dimensional data. If the latter is the case, then no z coordinate can be obtained. Collection of three-dimensional data is recommended for settling the question.

Crystals Diffused with $WO_4^=$ Ions

Introduction

Structure amplitudes were obtained by the author and other workers from crystals diffused with $WO_4^=$ ions and used by the author

to prepare hk0 and h0l Patterson and Fourier projections. From the hk0 projections, tentative x and y coordinates were obtained, but no satisfactory z coordinate was obtained from the h0l projections. Determination of a satisfactory z coordinate from three-dimensional data is recommended.

hk0 Fourier and Patterson Projections

Four hk0 projections were prepared for crystals diffused with $WO_4^{=}$ ions. For two of them, a Fourier and a Difference Patterson, data were obtained by other workers from crystals diffused at a molar ratio of 4:1. The Fourier supported x = 0.264 and y = 0.25, but the Difference Patterson did not. However, at a molar ratio of 2:1, both the Fourier, presented in Figure 7, and the Difference Patterson, presented in Figure 8, supported x = 0.27 and y = 0.25. Consequently, tentative coordinates of x = 0.27 and y = 0.25 were adopted. The 2:1 data were collected by the author.

Discussion of hk0 Projections

The consistency of coordinates from Fouriers and Difference Pattersons is strongly influenced by molar ratio. Coordinates from the Fourier at the higher ratio are consistent with those from both the Fourier and Difference Patterson at the lower ratio, but those from the Difference Patterson at the higher ratio are not. The Fourier is less influenced by molar ratio than the Patterson. No

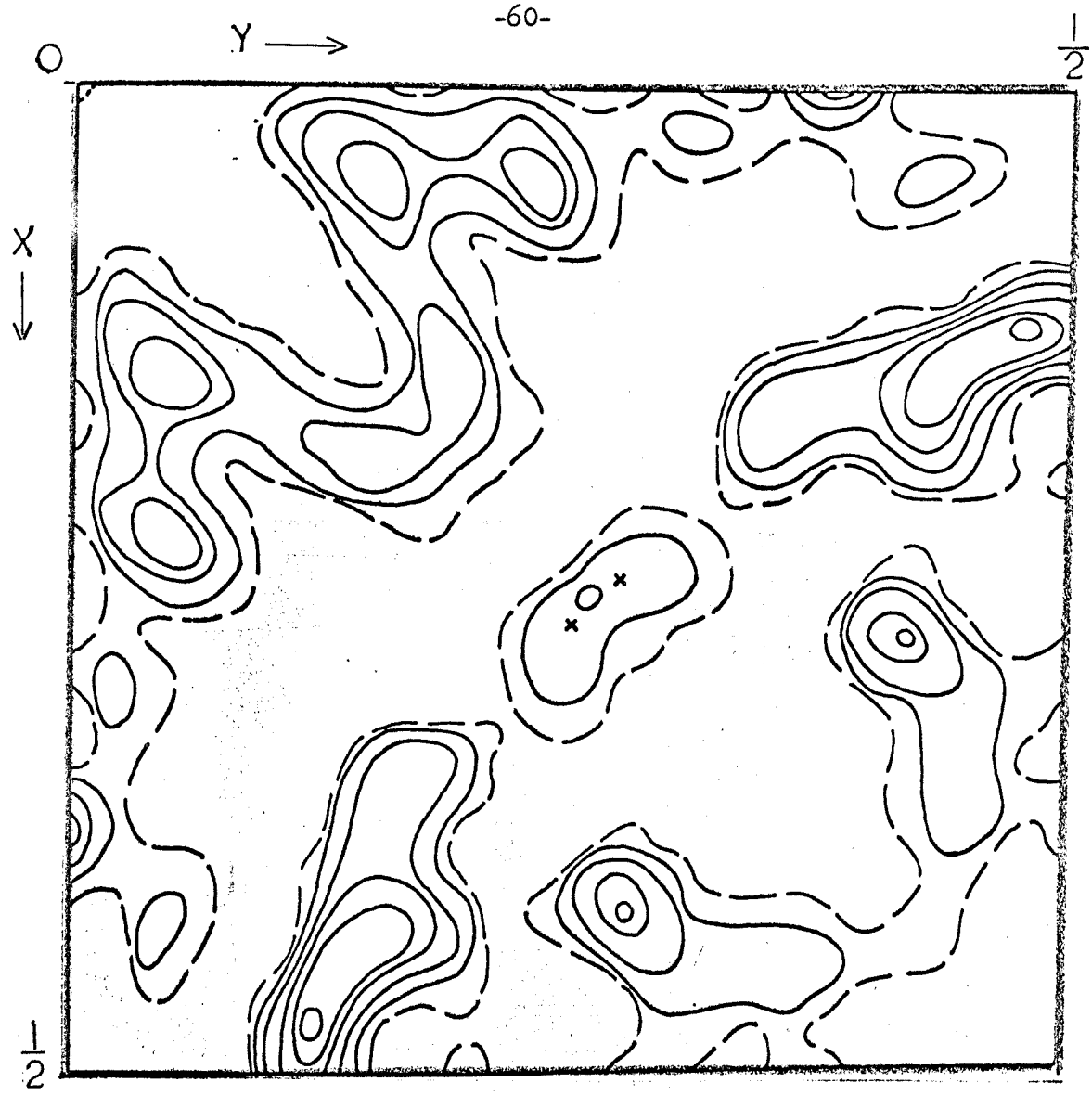

Fig. 7 - An hkO Fourier for lysozyme crystal diffused 55 da. with $WO_4^=$ ions at a $WO_4^=$:lysozyme molar ratio of 2:1, dmin = 6Å.

Phase angles from Phillips at Royal Institution.

Crosses mark heavy atom at x = 0.27, y = 0.25 and symmetrically-related location.

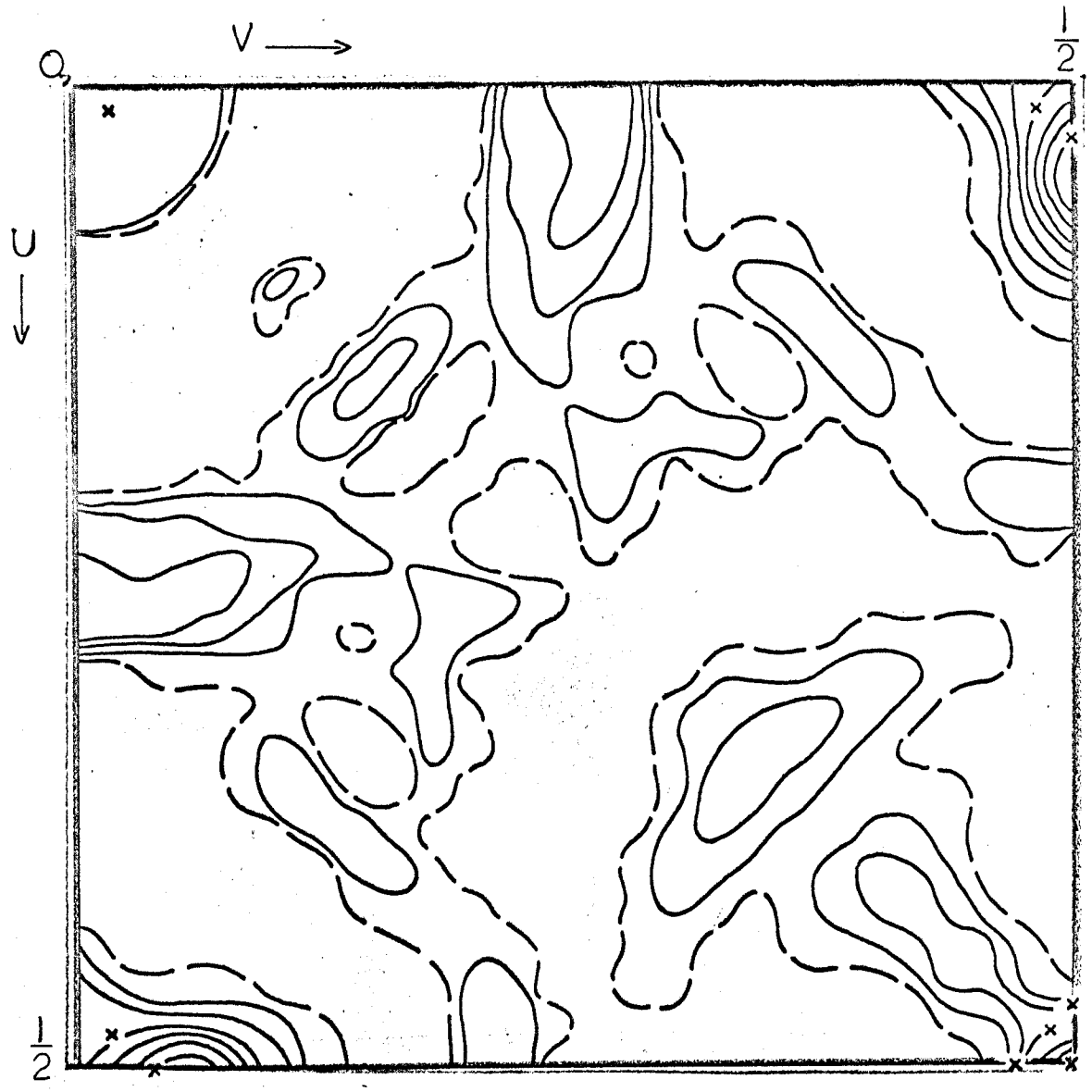

Fig. 8 - An hk0 Patterson for lysozyme crystal diffused 55 da. with $WO_4^=$ ions at a $WO_4^=$:lysozyme molar ratio of 2:1, dmin = 5Å.

Amplitudes: $(k' \left| F_{lys + WO_4} \right| - \left| F_{lys} \right|)^2$, $k' = 1.06$.

Crosses mark projection of interactions between heavy atoms at x = 0.265, y = 0.25 and symmetrically-related locations.

$\left| F_{lys} \right|$ collected by other workers.

doubt, this difference arises from the Fouriers' dependence upon the same, essentially correct, phase angles and upon the Pattersons' dependence upon a scale factor derived from data which are affected by the number of $WO_4^=$ ions randomly distributed in the crystal. At the higher ratio, the number of $WO_4^=$ ions in the crystal would be greater than at lower ratio, and, thus, the scale factor would be expected to deviate more from the correct value.

The Fourier in Figure 7 contains the 220 reflection. The apparently spurious peaks at (0,0) and (1/2, 1/2) were smaller than those in a second Fourier prepared without this reflection.

h0l Data

Data of the h0l projection were obtained by the author from crystals diffused with $WO_4^=$ ions at a $WO_4^=$:lysozyme molar ratio of 3:1. This ratio was selected after comparison of hk0 and h0l diffraction patterns from crystals diffused at a number of molar ratios. The 3:1 ratio was the lowest above which there was little further change in the high-angle diffraction pattern from that of undiffused crystals. Consequently, the 3:1 ratio was expected to yield the highest possible occupancy of heavy atom sites with the lowest possible number of additional heavy atoms in the crystal.

h0l Fourier and Patterson Projections

The contribution of $WO_4^=$ ions to six h0l Fourier and Patterson

projections was at or below the noise level of the projections. Two Fouriers, one for the diffused and the other for the undiffused crystal, were qualitatively similar, except for a small peak in the first at x = 1/4 and z = 5/8. This peak probably represented a small fluctuation in electron density amplified by the center of symmetry at that point.

To investigate quantitatively the possible contribution of $WO_4^=$ ions to Fouriers, two Difference Fouriers were prepared. The scale for the second was set at 1.4 times that for the first in an effort to improve the chance of locating $WO_4^=$ ions. The same phase angles were used for the preparation of both Fouriers. The first Difference Fourier did not permit unique determination of a z coordinate, although the second did. However, in both Fouriers a number of large peaks were unexplained by the assigned values of z.

To test the doubtful coordinates obtained from the Difference Fouriers, the amplitudes from the two Difference Fouriers were squared and used to prepare two Difference Pattersons. From the Pattersons no coordinates consistent with these obtained from the Difference Fouriers were obtained. Thus, the coordinates obtained from the Fouriers were not confirmed. The contribution of $WO_4^=$ ions must be at or below the noise level of the h0l projections investigated.

h0l Scaling

The coordinates obtained from the second Difference Fourier

were tested further by preparing scattering factors for $WO_4^=$ ions at these coordinates and scaling the scattering factors by the method of least squares to structure amplitudes from the diffused and undiffused crystals. Although an R factor of 48% was obtained for the scaled data, only 36% of the phase angles for undiffused crystals derived from the scaled data agreed with the phase angles for undiffused crystals derived from $PtI_6^=$ data. Therefore, the coordinates from the second Difference Fourier were rejected.

Discussion

No satisfactory z coordinate for $WO_4^=$ ions was obtained from h0l data. This may result from their relatively small contribution to the h0l projection or from their random distribution in the direction of the c axis. Study of three-dimensional data is recommended to settle the question.

Crystals Diffused with PCMBS*

Introduction

Structure amplitudes from crystals diffused with PCMBS were obtained by other workers and used by the author to prepare hk0 Fourier and Patterson projections from which tentative x and y

*p-chloromercuribenzenesulfonic acid

coordinates for the heavy atoms in the crystal were obtained. These coordinates were symmetrically related to published coordinates for MHTS** (8).

Determination of a z coordinate from three-dimensional data is recommended.

hk0 Fourier and Patterson Projections

Four hk0 projections were prepared from data from the same diffused crystal. The Fourier in Figure 9 and the Difference Patterson in Figure 10 are consistent with heavy atoms at x = 0.305 and y = 0.08, whereas two other Difference Pattersons prepared are not. The scale for the first Patterson was about 1.2 times that for the second and third. The larger scale was selected when the smaller failed to yield Pattersons which supported the coordinates derived from the Fourier.

Tentative coordinates of x = 0.305 and y = 0.08 were adopted for the heavy atoms in crystals diffused with PCMBS.

Discussion

The tentative coordinates of x = 0.305 and y = 0.08 for the heavy atoms in crystals diffused with PCMBS are nearly equal to the coordinates of x = 0.28 and y = 0.10, which are symmetrically related

**o-mercurihydroxytoluene-p-sulfonic acid

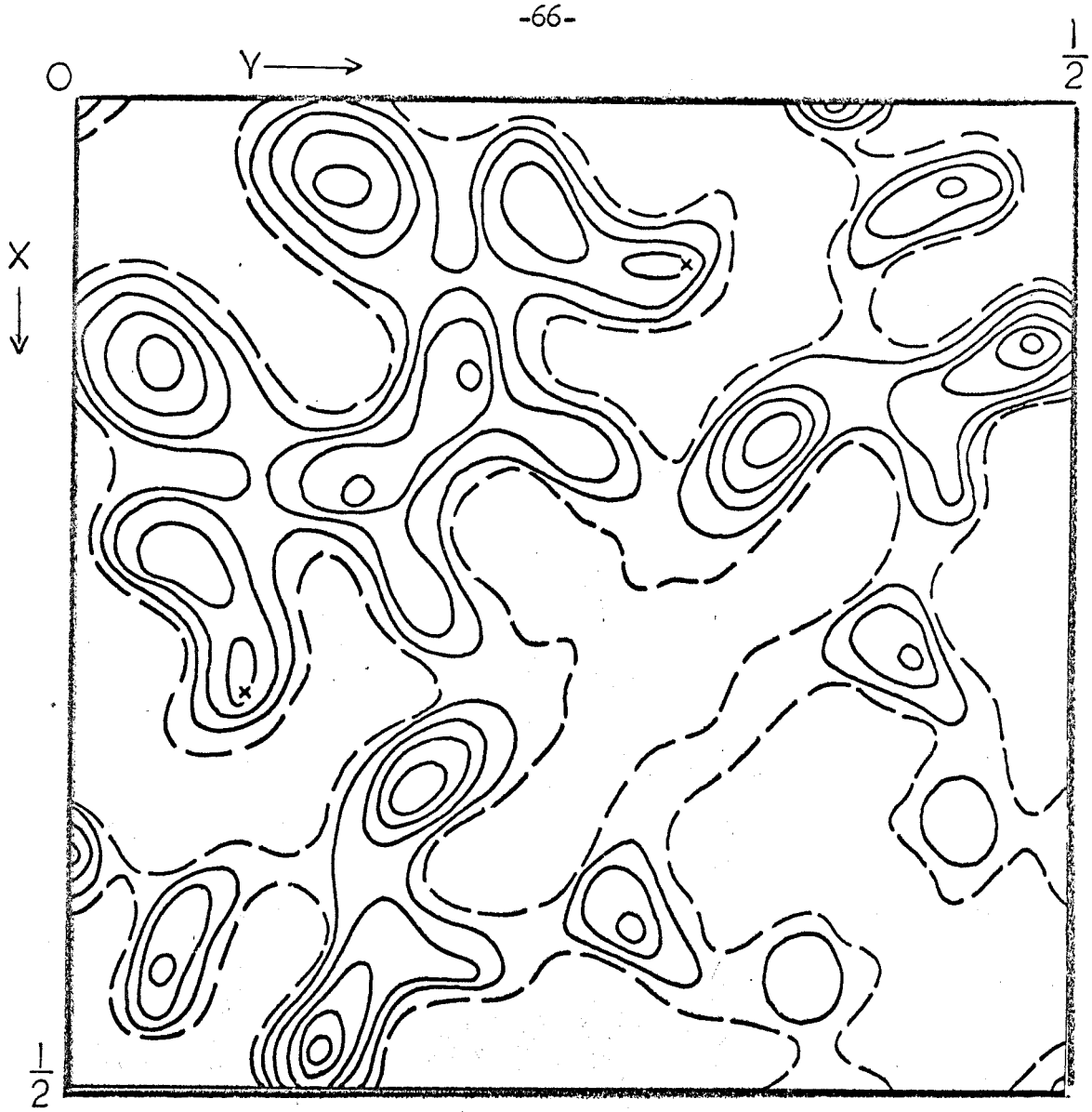

Fig. 9 - An hk0 Fourier for lysozyme crystal diffused 3 mo. with PCMBS at a PCMBS:lysozyme molar ratio of 2-1/2:1, dmin = 6Å.

Phase angles from Phillips at Royal Institution.

Crosses mark heavy atoms at x = 0.306, y = 0.08 and symmetrically-related position.

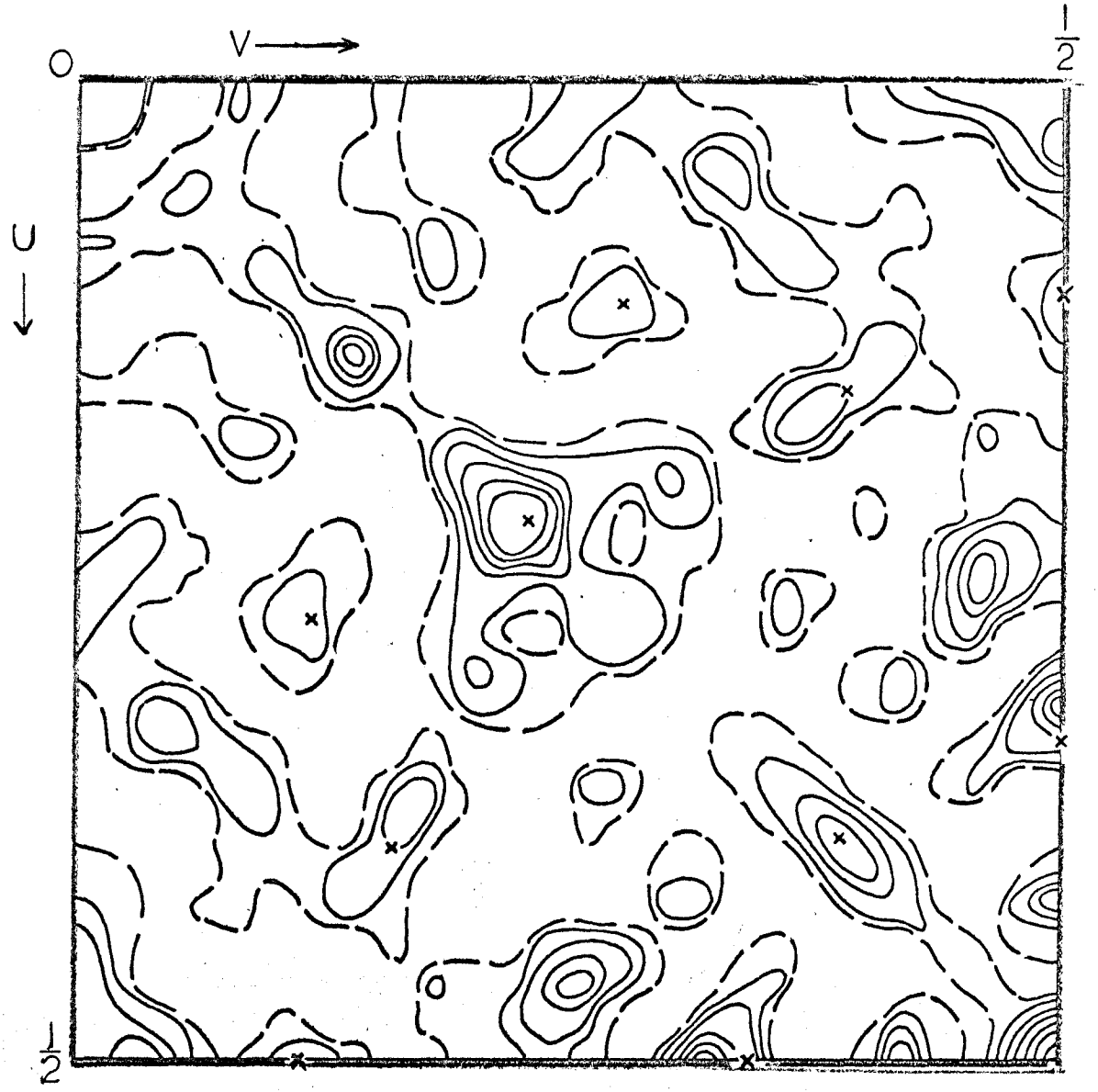

Fig. 10 - An hk0 Patterson for lysozyme crystal diffused 3 mo. with PCMBS at a PCMBS:lysozyme molar ratio of 2-1/2: 1, dmin = 5Å.

Amplitudes: $(k' \left| F_{lys + PCMBS} \right| - \left| F_{lys} \right|)^2$, $k' = 1.2$.

Crosses mark projection of interactions between heavy atoms at x = 0.305, y = 0.08 and symmetrically-related locations.

Data collected by other worker.

to published coordinates for heavy atoms in crystals diffused with MHTS (8).

Determination of a z coordinate from three-dimensional data is recommended. The indication from crystals diffused with $WO_4^{=}$ ions or UO_2^{++} ions is that no satisfactory z coordinate can be determined from h0l data for heavy atoms which yield hk0 Fourier peaks of the height found for PCMBS.

Preliminary comparison of diffraction patterns from crystals diffused with PCMBS at a number of molar ratios suggests that three-dimensional data should be collected from crystals diffused at a molar ratio of approximately 4.5:1.

Crystals Diffused with $HgCl_2$

Introduction

Structure amplitudes from crystals diffused with $HgCl_2$ were obtained by other workers and used by the author to prepare hk0 and h0l Patterson and Fourier projections from which tentative x, y and z coordinates were obtained for the heavy atoms in the crystal. Scaling of h0l structure amplitudes to calculated heavy atom scattering factors was achieved. However, no further study of these crystals is recommended because structure amplitudes from these crystals appear to contain considerable random error and their heavy atoms may have the same coordinates as $PtI_6^{=}$ ions. With the same coordinates, they would not constitute as independent heavy atom derivative.

All structure amplitudes from crystals diffused with $HgCl_2$ were obtained from crystals diffused at a 20:1 molar ratio of $HgCl_2$: lysozyme because that ratio was required to produce diffraction patterns different at high angles from those of undiffused crystals.

hk0 Fourier and Patterson Projections

From an hk0 Fourier and an hk0 Difference Patterson, tentative coordinates of $x = y = 0.35$ were obtained. In the Fourier, the heavy atom peak with these coordinates was oval-shaped. The structure of the heavy atom was not known. In the Difference Patterson, a number of large peaks were not accounted for by the tentative coordinates. Because these peaks were not consistent with any coordinates supported by the Fourier, they were assumed indicative of random errors in the data. Additional indication of random error in the data came from the departure of the ratio of the height of the Patterson peak at $u = 1/2$, $v = 0.15$ to the peak at $u = v = 0.35$ from the ratio required by crystal symmetry.

Determination of a z Coordinate for Heavy Atoms

From an h0l Difference Patterson, a tentative z coordinate of 0.19 was obtained. There was some doubt about the correctness of this coordinate because several of the heavy atom-heavy atom interactions required by it were of low height. Evaluation of this coordinate is considered below.

No h0l Fourier was prepared to evaluate the z coordinate because no further work on crystals diffused with $HgCl_2$ was undertaken, for reasons stated in the Discussion.

Evaluation of the z Coordinate for Heavy Atoms

To evaluate the z coordinate for heavy atoms in crystals diffused with $HgCl_2$, h0l structure amplitudes were scaled by the method of least squares to the scattering factors for $PtI_6^=$ ions with coordinates of x = 0.345, y = 0.355 and z = 0.200. Scattering factors for $PtI_6^=$ ions were used because the structure of heavy atoms in crystals diffused with $HgCl_2$ was unknown. The above coordinates are essentially those obtained from hk0 and h0l Fourier and Patterson projections for the heavy atoms in crystals diffused with $HgCl_2$.

The scaling of data from 60 h0l relections led to an R factor of 43% for the agreement between calculated heavy atom scattering factors, f_{calc}, and observed heavy atom scattering factors, f_{obs}:

$$R = \frac{\Sigma \left| |f_{obs}| - |f_{calc}| \right|}{\Sigma |f_{calc}|}$$

Although this agreement was encouraging, the agreement between the resulting phase angles for lysozyme and those for lysozyme derived from $PtI_6^=$ data was not. Of 37 phase angles compared, only 24 agreed. The disagreements in phase angles may have resulted from an incorrect z coordinate for the heavy atoms in crystals diffused with $HgCl_2$, or from random error in the data.

Discussion

No further study of crystals diffused with $HgCl_2$ is recommended. Structure amplitudes from these crystals appear to contain considerable random error and their heavy atoms may occupy the same locations as $PtI_6^=$ ions. With the same heavy atom coordinates, the crystals diffused with $HgCl_2$ would not constitute an independent heavy atom derivative.

Crystals Diffused with $HgI_4^=$ Ions

Structure amplitudes from crystals diffused with $HgI_4^=$ ions were obtained by other workers and used by the author to prepare an hk0 Fourier which revealed two heavy atom sites per asymmetric unit. The coordinates of these sites, $x = y = 0.32$ and $x' = y' = 0.38$, are in close agreement with $x = y = 0.32$ and $x' = y' = 0.37$, which are symmetrically related to published coordinates for $HgI_4^=$ ions in crystals of lysozyme (8).

Further work on lysozyme crystals with two heavy atom sites per asymmetric unit should be postponed until all crystals with one site per asymmetric unit have been investigated fully, because phase angles from crystals with two sites will be adversely affected by uncertainties in the relative occupancy of sites, whereas crystals with one site will not.

Crystals Diffused with $ThCl_4$

Structure amplitudes from crystals diffused with $ThCl_4$ were obtained by other workers and used by the author to prepare an hk0 Fourier and two hk0 Pattersons. These three hk0 projections were consistent with two heavy atom sites per asymmetric unit. As mentioned in the previous section, no further work on crystals with two sites per asymmetric unit is recommended until all crystals with one site per asymmetric unit have been investigated fully.

Comparison of hk0 Difference Pattersons

Two types of hk0 Difference Pattersons were used in the preceding studies: one with amplitudes of $(k' \left| F_{lys + \text{heavy atom}} \right| - \left| F_{lys} \right|)^2$ and the other with amplitudes of $(k \left| F_{lys + \text{heavy atom}} \right|^2 - \left| F_{lys} \right|^2)$. Pattersons with the first amplitudes were prepared for crystals diffused with 6 of the 7 heavy atoms studied. From each of the 6 were derived x and y coordinates consistent with coordinates obtained from hk0 Fouriers whose amplitudes included no scale as a source of error and whose phase angles had been found essentially correct during the study of $PtI_6^=$ crystals. Thus Difference Pattersons with the first amplitudes were extremely reliable for location of heavy atoms in the hk0 projection.

Four Difference Pattersons with the second type of amplitude were prepared in the preceding studies. For 3 of these, the scale, k, was the square of the k' which had been used in a Difference Patterson of

the first type to yield coordinates consistent with a Fourier. In only two of these three cases did the second type of Patterson yield coordinates consistent with the Patterson of the first type and with the Fourier. Therefore, Difference Pattersons of the second type were not as reliable for location of heavy atoms as were Difference Pattersons of the first type.

Discussion of Diffused Lysozyme Crystals

Determination of a z coordinate for heavy atoms was attempted with h0l data from crystals diffused with $PtI_6^{=}$ ions, UO_2^{++} ions, $WO_4^{=}$ ions and $HgCl_2$. Only for crystals diffused with $PtI_6^{=}$ ions was a z coordinate determined with reasonable certainty. The ability to determine z coordinates for the above heavy atoms is apparently related to their relative contribution to hk0 Fourier projections. In Figure 1, the hk0 Fourier for lysozyme crystals diffused with $PtI_6^{=}$ ions, the contribution of $PtI_6^{=}$ ions is greater than that of surrounding lysozyme, whereas the contribution of the heavy atoms in crystals diffused with UO_2^{++} ions, $WO_4^{=}$ ions or $HgCl_2$ is about equal to that of surrounding lysozyme (see Figures 5 and 7). An inability to determine a z coordinate was associated with a heavy atom contribution about equal to that of surrounding lysozyme.

The contribution of heavy atoms to Figure 9, the hk0 Fourier for crystals diffused with PCMBS, is about equal to that of surrounding lysozyme. Therefore, an inability to determine a z coordinate for

heavy atoms for hOl data for these crystals is predicted. Collection of three-dimensional data is recommended. Collection of three-dimensional data is also recommended for crystals diffused with UO_2^{++} ions or $WO_4^{=}$ ions. It is not recommended for crystals diffused with $HgCl_2$, because data from these crystals will probably contain considerable error.

Of the crystals diffused with UO_2^{++} ions, $WO_4^{=}$ ions or PCMBS, those diffused with $WO_4^{=}$ ions appear most promising for determination of a z coordinate from three-dimensional data, because hkO and hOl diffraction patterns from these crystals show the greatest difference at high angles from the corresponding diffraction patterns of undiffused crystals. Further, the crystals diffused with PCMBS appear more promising than those diffused with UO_2^{++} ions because diffraction patterns from the former differ more at high angles from the patterns for undiffused crystals.

Only after three-dimensional data from crystals diffused with $WO_4^{=}$ ions, PCMBS, or UO_2^{++} ions have been investigated fully is collection of three-dimensional data from crystals diffused with $HgI_4^{=}$ ions or $ThCl_4$ recommended. Crystals diffused with $HgI_4^{=}$ ions or $ThCl_4$ have two heavy atoms sites per asymmetric unit and probably will yield phase angles less certain than those from crystals diffused with $WO_4^{=}$ ions, PCMBS or UO_2^{++} ions which have one site per asymmetric unit. Thus, immediate study of crystals diffused with $HgI_4^{=}$ ions or $ThCl_4$ is not recommended.

When the tentative x, y and z coordinates for $PtI_6^=$ ions have been confirmed with three-dimensional data, and at least one additional heavy atom derivative has been obtained, then three-dimensional electron density maps for lysozyme crystals may be prepared.

III. CONCLUSION

No heavy atom derivatives were obtained for chymotrypsin, but, subject to confirmation with three-dimensional data, one was obtained for lysozyme. And, except for the possible preparation of inhibitors of chymotrypsin carrying heavy atoms, no immediate source of heavy atom derivatives for chymotrypsin is available. However, several diffused crystals of lysozyme may be useful as heavy atom derivatives of lysozyme if z coordinates for their heavy atoms can be determined from three-dimensional data. Consequently, obtaining the minimum of two independent heavy atom derivatives required for preparation of three-dimensional electron density maps is much more likely, in the near future, for lysozyme than chymotrypsin.

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