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Michael T. Schwar

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Steven S. Zumdahl, Professor
Instructor in Charge

APPROVED: Steven Zumdahl

M. P. Edmund

HEAD OF DEPARTMENT OF Chemistry

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The Ultraviolet Photolysis of Inositol Hexaphosphate

By

Michael T. Schwar

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THESIS OUTLINE

Michael Schwar

I. INTRODUCTION-SCOPE AND PURPOSE

II. BACKGROUND INFORMATION

- A. Inositol phosphates
- B. Photochemical theory
- C. Other applicable reaction schemes
  1. Diazinon
  2. Ring cleavage

III. EXPERIMENTAL DESIGN

- A. Equipment used
  1. Oxidation
     - a. oxidizer
       - 1) lamp
       - 2) tubing
     - b. pump
  2. TLC
     - a. plates
     - b. UV light
  3. HPLC
     - a. pump
     - b. column
     - c. detector
  4. Chemicals
     - a. IP's
     - b. reagents
- B. Oxidizer runs

C. Thin layer experiments

D. HPLC separations
   1. gradient
   2. ascorbic acid rxn.

IV. RESULTS

A. TLC Separation results
   1. Solvent effects on known samples
   2. Experimental separations

B. HPLC results

V. CONCLUSIONS

A. Proposed mechanism

B. Kinetics

C. Overall conclusions

VI. ACKNOWLEDGEMENTS

TABLE OF CONTENTS

I. INTRODUCTION-SCOPE AND PURPOSE

This study is an investigation of the photooxidation of inositol hexaphosphate. The primary objective is the enhancement of breakdown in an analytical system by understanding the process. Since this chemical is suspected to be present in natural waters, knowledge regarding the photochemical pathway in a laboratory situation may be used as a basis for investigating environmental processes. Thus, the following experiment is a starting point in the investigation of the degradation of a naturally occurring organophosphate compound in a laboratory situaltion, which may also provide insight into the natural breakdown processes.

II. BACKGROUND INFORMATION

A. Inositol phosphates

Although few organophosphorus compounds have been characterized in natural waters, one likely component of such systems is the inositol phos-

phate (IP) group. The IPs have been established as a major source of phosphorus in soils, and research indicates that algal sources can contribute some of the IPs to aquatic systems. In addition, lake water and sediment samples have been shown to contain inositol phosphates (Segars, et al., 1986). Thus, it is probable that IPs exist to some extent in natural water systems.

Inositol is the commnon name for 1,2,3,4,5,6-hexahydroxycyclohexane, and can exist in a number of stereoisomeric forms. The most common isomer in nature, and the form used in this study, is myo-inositol, or cis-1,2, 3,5-trans-4,6-hexahydroxycyclohexane (Figure 1). The IPs are named according to the number of orthophosphate groups substituted onto the hydroxyl locations on the inositol ring: inositol monophosphate for the once substituted, diphosphate for two, tri- for three, tetra- for four, penta- for five, and inositol hexaphosphate (IHP) for the fully substituted phosphate. Isomers can be formed structurally by altering the ring carbons on which the orthophosphate is substituted. In this study, specific structural isomers of each myo-inositol compound are used.

B. Photochemical theory

When light energy is absorbed by a molecule, a number of different types of reaction can occur, depending on both the amount of energy and the molecular characteristics. In the general model of photochemical reactions, the light energy causes the molecule to change into a high-energy intermediate form, which then goes back into the lower energy state by changing the energy into another form. The reaction in this study uses the light energy to oxidize the bonds in the organic inositol hexaphosphate molecule. Although photoreactions can occur by indirect means, that is, the presence of some sensitizing chemical catalyzes the reaction, only the direct means will be dealt with in this paper. Since the reaction is run in pure water, the only possible sensitizers would be the compounds involved and the reaction products, and an investigation is beyond the scope of the current study.

The actual reaction that occurs with inositol hexaphosphate is not predictable from simple calculation using thermodynamic values. Oxidation of the IHP may result in two possible outcomes: ring cleavage, or re-

moval of an orthophosphate group. According to data published by Reutov (1967), both of these bonds have similar energies, around 80-85 kcal/mol. Under different conditions, either could be the more stable bond. As for the illumination involved, the lamp used provides the spectrum shown in Figure 2 (Horspool, 1976). Since the bond energies are 80-85 kcal/mol, light of 335-360 nm would provide the requisite energy.

C. Other applicable reaction schemes

Since the importance of the action of pesticides is widely recognized, some of the most comprehensive studies of photooxidation have dealt with these compounds. Thus, attention was directed to two pesticide degradation reaction types which might be analagous to the IHP photooxidation. These are the photodegradation of diazanon, a phosphorus triester, and a mechanism involving ring cleavage.

Diazanon is the common name for O,O-diethyl-O-(2-isopropyl-4 methyl-6-pyrimidyl) phosphorothioate. This chemical degrades more rapidly when adsorbed onto soil, but degradation also proceeds in soil-free

aquatic environments (Armstrong and Konrad, 1974). Essentially, what occurs is a hydrolysis reaction, with a hydroxyl group replacing the pyrimidyl group on the phosphorus as shown in Figure 3. The probable mechanism (Matsumura, 1982) involves excitation of the molecule followed by attack from a hydroxide ion. Such a mechanism may be applicable to the photolysis of IHP, if the breakdown resulted in removal of phosphates from the ring. Factors which must be taken into account when assessing the applicability of this reaction are the added stabilization of the diazanon phosphorus structure by the ethyl ester groups and the double bonded sulfur instead of oxygen atom in the phosphate structure.

Figure 4 shows an oxidation reaction where a C-C bond in the ring is oxidized, resulting in the removal of orthophosphate. The reaction product is significantly different from the original IHP, and ensuing reactions would not be analagous to the original. Serial phosphorus removal could be accounted for under this model, but the reactions occurring after the first would have different characteristics. In all likelihood, this type of reaction would not result in serial removal.

III. EXPERIMENTAL DESIGN

Since this is an investigation of the ultra-violet oxidation of inositol hexaphosphate, it is important to understand the different methods used, both in the reaction itself and in characterizing it. In this section, the equipment used in the experiments will be specified, and the methods explained for each part of the study.

A. Equipment

Although oxidation is a component of two separate sections of the experimental method, in both cases the equipment and method of oxidation are the same. The oxidizing apparatus consists of two separate units, the oxidizer itself and the pumping system to push the samples through the reactor. The oxidization reactor, in turn is made of four parts: the UV lamp, tubing, platform reactor, and chimney.

The lamp which provides the irradiation of the sample is an Ace-Hanovia photochemical lamp with a power of 450W, supplied by Ace Glass

Inc. The power supply is designed by the manufacturer to power this particular piece of equipment. The lamp is supported within an open metal scaffolding around which is wrapped a length of teflon capillary bore tubing. In the experimental reactor, there are 100 feet of tubing in the oxidizer, while the second reactor, used in chromatographic detection, there are 200 feet of teflon tubing. The tubing is available in .012, .022, and .032 inch diameters, but all of the experiments in this study utilize the .012 inch tubing. In addition, to protect the experimenter from the UV radiation, a metal chimney is used. This chimney, which is placed over and around the platform reactor apparatus (Figure 5 for schematic), has a fan at the top in order to aid air flow, and the bottom has tabs removed for this same purpose. There is a second fan in the base of the platform reactor to force air up and past the lamp. Thus, the two fans and the tabs serve to air-cool the lamp.

The system used to pump the samples through the oxidizer consists of two Scientific Systems units hooked together. A model 200 LC pump is fitted with a micropump head attatchment, and provides the actual pumping force. The second unit is a Model 210 Guardian, which is a pulse sup-

pressor. The fact that the pump has the special micropump head makes it necessary to compensate for the recorded flow rate, as the actual flow rate is approximately one-half the rated value.

The thin-layer technique does not require complicated equipment. However, two different kinds of cellulose chromatographic plates were used. The first cellulose plate was Kodak Chromagram Sheet #13255, and the second was Whatman microcrystalline cellulose plate, 250 micrometer layer, #4820 820. Besides the composition of the medium on the plates, they differ in that the Kodak plates have a flexible plastic backing, while the Whatman plates are glass. The Kodak plates were used primarily in this study, and if the type of plate is not specified as the microcrystalline, then it can assumed to be regular cellulose.

The second piece of equipment used in the TLC step was a hand-held UV lamp which was used in the color development process. The specific model used in this study was a MINERALIGHT short wave lamp produced by Ultra-violet Products, Inc. of San Gabriel CA. The design of the assembly sheilds the light on three sides, and emits the radiation from only one flat

area, but care must be taken to protect the skin, and especially the eyes, from the light.

The high-performance liquid chromatography phase of this experiment is probably the most complex, especially in terms of the number of different pieces of equipment used. Fundamentally, there are three separate sections to the HPLC runs: the separation, the detection, and the data acquisition.

As in many liquid chromatography methods, there is a gradient of solvents involved. In this case, there are two separate solvents, and the rate of flow of each is controlled by a Millipore/Waters pump, model 510. The individual pump flow rate is controlled by a Millipore/Waters model 680 Automated Gradient Controller. The resulting solvent mixture is pumped to the column, where the separation actually takes place. The column is a Biorad Laboratories anion exchange column, AMINEX A-27. It is 250mm long, and has a diameter of 4 mm. The packing material consists of quaternary amino funcionality on polystyrene beads, nominal diameter of 13 micrometers. Essentially, the material is Dowex A1, anion exchange resin.

In order to detect the separation, the eluent is run through two more reactors. The first reactor is an oxidizer identical to the one used in the oxidation runs, except that 200 feet of Teflon capillary bore tubing is used instead of 100 feet. After injection, the flow mixes with ascorbic acid reagent at a t-block and proceeds through another 100 feet of tubing immersed in a Napco Model 210A water bath at 55 C. The ascorbic acid reagent is supplied by an Anspec SSI Model 222 HPLC pump, at a rate of 0.1 ml/min. After leaving the water bath, the sample flow goes into the detector system, which is a Linear UVis 200 ultraviolet/visible detector, which reads the reduced phosphomolybdate reaction product at 668 nm.

The results from the detector are electronically transmitted to a Nelson Analytical Data Acquisition System (DAS). The DAS sends the data to both an Omniscribe recorder, which graphs the results, and an IBM PC which stores the data for later manipulation. The computer gives not only graphing and storing capabilities, but also allows for precise retention time, peak height and peak area to be determined.

All of the chemicals used in this experiment are commercially avail-

able. All of the solvents, reagents, and other chemicals are high-grade materials obtained through various chemical vendors. The inositol phosphates, although more specialized, were also obtained commercially from one of two sources. Sigma Chemical Company is the source of the inositol hexaphosphate and the inositol monophosphate. IHP is listed as the sodium salt of phytic acid (Lot 69B-1500), and 98% pure. The listing of IMP is myo-inositol 2-monophosphate, Di(monocyclohexammonium) salt (Lot 16F-8020), around 95% pure. The other inositol phosphate congeners are Calbiochem Brand of Behring Diagnostics, LaJolla, CA. The pentaphosphate, IPP, is the barium salt of myo-inositol pentaphosphate (Lot 801272). The heptaammonium salt of myo-inositol 1,4,5,6-tetraphosphate (Lot 702640) is the tetra-, and myo-inositol 1,4-diphosphate's tetraammonium salt from lot 702263 is IDP. The tri- is myo-inositol 1,3,4-triphosphate, the K_3 salt (Lot 786116, lyophilized).

B. Oxidation Runs

The basis of this study involves examination of the photooxidation of the compound inositol hexaphosphate. Before the oxidation is actually run,

the pump system must be calibrated, because of the alteration of flow rate caused by the pump head. Calibration, found by measuring flow for different rated values, indicates that actual flow is 0.6 x rated flow.

Initial (unoxidized) samples are 45.5 grams of P/liter concentrations of inositol hexaphosphate. Exposure times in the oxidizer are controlled by flow rate and multiple passes through the unit. Flow rates less than 0.2 ml/min lead to undesirable heating of the solution.

Actual residence time within the oxidizer is computed from the dimensions of the tubing and the flow rate. Table 1 is a listing of the amount of time a sample is exposed to the light as a function of the flow rate, for the .012 inch tubing.

C. TLC separations

Of the several thin-layer separation and detection methods reviewed, that developed by Hong and Yamane (1980), supplemented with Harrap's (1960) detection method, was selected for the TLC experiments. The de-

tailed method begins with preparing the plates by etching five or six lanes in the plate media, starting 2 cm above the bottom of the plate and with a length of 15 cm. At the 15 c point, the media is etched horizontally, but from the bottom of the plate to this point, the media is left continuous, to enable solvent flow. There must be at least 2 cm of space between the edges of the outside lanes and the edge of the plate itself. A mark is made in pencil in the middle of the lower end of each of these channels.

For each sample to be separated, a 25 microliter sample is drawn into a syringe. With care to prevent diffusion on the plate as much as possible, the sample is spotted by placing small drops on the pencil marks and then drying with a hand-held hair dryer. This process is repeated until the entire sample is placed on the plate. After each sample, deionized water is drawn into the syringe twice, in order to rinse it, and another sample is done.

The plate is prepared for separation by being placed upon a glass plate slightly larger than the size of the chromatographic plate itself. The lower edge of the TLC plate must align with an edge of the other. Then a sec-

tion of 3/4 inch diameter flexible Teflon dowling is placed on the top and two sides of the plate, but not in any of the sample channels. A second glass plate is placed on the dowling, and the whole assembly is held together with removable metal clips on the top and two sides. This is then placed, vertically with the bottom down, into a glass separation chamber with approximately 1 cm of the appropriate solvent in the bottom. A glass cover is placed atop the chamber.

After the solvent migrates through the length of the channels, or at least one of them, the plate assembly is removed. If the solvent did not go to the top for all of the samples, the point of solvent migration is marked. Upon disassembly, the TLC plate is removed and dried with the hair dryer, placed in an oven at 90 C for 30 minutes and then developed for compound visualization.

Development, in this case, is a three step process. First, the plate is sprayed with a mixture of 60% perchloric acid, 20% ammonium molybdate, 1N hydrochloric acid, and acetone (1:1:2:16), sprayed from a glass atomizer. The plate is then exposed to light from the hand-held UV source for 15

minutes. The atomizer is rinsed, and is used to spray the plate with a 2.5% solution of alpha-benzoinoxime in methanol. For each spraying, the entire surface of the plate is completely covered with a misting of reagent. As soon as possible after development, the spots that appear are marked permanently on the plate, either by etching or with a pencil.

D. HPLC separations

The HPLC separations begin by simply injecting 250 microliters of the sample into the HPLC system at a point just after the junction of the two solvent pumps. The two solvents used are simply different aqueous concentrations of NaCl. Both solvents contain 0.5 mM of Na_4EDTA, but one has an NaCl concentration of .5M, and the other is .1M NaCl. The solvent flow is constant at 0.5 ml/minute, and the gradient for separation is in Figure 6, and was developed by C. Clarkin et al. (1989).

After the column separates the compunds, the oxidizer breaks them down and produces orthophosphate from the organophosphate chemicals. The ascorbic acid reagent then reacts with the ortho-P in the same reac-

tion used in the soluble reactive phosphorus determination detailed in Strickland and Parsons (1968), to produce the colored product. This reagent is also shown in Table 2.

IV. RESULTS

A. TLC Separation Results

At the onset of this experiment, the TLC work was started because the HPLC separation method had yet to be perfected. The thin-layer technique was to serve as a backup procedure if the HPLC would not work. The TLC method itself was not fully developed when the liquid chromatography method became operative. Because of this, the TLC method was not applied as the final IHP breakdown assay. However the results obtained are reported.

The first notable result is that the order of separation obtained is not the same as reported in the original paper on the method (Hong and Yamane,

1980). When the separations were run with standards of IHP,IMP, and orthophosphate, the IMP traveled farther than the ortho, and IHP barely moved off of the baseline (Figure 7). In Hong and Yamane (1980), it was indicated that the orthophosphate migrated the farthest. The result obtained was confirmed through numerous runs on the regular cellulose plates. Since the original method used microcellulose, these plates were also used to determine if the cellulose type influenced the migration order. Yet in all of the runs the IMP eluted farther than the orthophosphate, confirming the migration order reversal.

A second difference in comparison with the original method was the ability to separate IMP and orthophosphate spots. Although there were obviously two areas representing the two compounds, the resolution was not enough to visualize two distinct spots. In order to increase the resolution, the composition of the solvent was altered by increasing the ammonia and acetic acid concentrations. These changes did tend to differentiate the two substances, but the solvent seemed to be causing breakdown of the compounds, especially the IHP. Therefore, these variations did not seem to represent a viable means improving separations.

Finally, although the separation was incomplete, there was no clear evidence of IMP formed in the oxidation products. The other intermediate IPs were also not evinced, although the action of these was not even tested with standards, but a spot corresponding to the location of orthophosphate was always present and detectable amounts of IHP were present until the second oxidation at 0.2 ml/min (detection limits are in Table 3). While keeping in mind that the procedure is incomplete, these results do not support a serial removal of phosphate groups mechanism of reaction.

B. HPLC Results

Figure 8 shows the the results of running the oxidation products from different experiments through the HPLC separation. It would seem that the products formed do not correspond to the intermediate IPs which comprise the standards. Instead, there is a broad range of peaks which are not located at the elution times of the known substances.

Peak areas can be used to determine relative amounts of a particular substance from run to run, if breakdown rates in the analytical oxidizer

can be assumed to be relatively constant. In this case, the breakdown in the oxidizer will be considered constant. Because of this, analysis using peak areas to correspond to relative concentration of IHP and orthophosphate is done. Table 4 is the peak area data and graphs of these areas, the log and the inverse of the areas, versus retention time in the oxidizer, are in Figures 9, 10 and 11. The results of linear regression, in Table 5, show that the correlation between retention time and both IHP degradation and orthophosphate production.

V. CONCLUSIONS

A. Proposed mechanism

It seems fairly obvious from the data that the oxidation does not proceed as a simple removal of phosphates from the IHP molecule. Rather, some new products are being formed which are different than the ones used in the standards. Although inositol and the IPs have many possible isomeric forms, it is unlikely that different isomers would cause such

distinct shifts in elution time off of the HPLC column. The most probable reaction is then some sort of ring cleavage, such as in the reaction proposed in Figure 4. This would involve excitation of the C-C ring bond, resultant oxidation of a carbon atom, and an obvious change in the character of the compound.

B. Kinetics

Since the highest correlation between the data points and the linear regression lines exists for the inverse of retention time, it is probable that the reaction obeys the second-order rate equation:

$$\text{rate} = k\,[\text{IHP}]^2$$

This would indicate that the rate determing step involves two IHP molecules. One possible reaction is fast excitation by a photon, followed by reaction with another IHP molecule, resulting in the products. The degradation of IHP does not seem to directly coincide with the production of orthophosphate. In fact, when linear regression is run on the PO_4 data, it is apparent that orthophosphate probably does not result directly from IHP degradation. Orthophosphate formation appears to be a zero-order reac-

tion (r^2=0.96). In all cases, the regression fit improves if early data points are omitted (Table 5).

C. Overall conclusions

What this study shows is that the photooxidation of inositol hexaphosphate is more complex than a simple removal of phosphate groups. Further investigation is necessary to grasp the true reaction which occurs, which probably involves cleavage of the inositol ring. The environmental applicablility of this reaction is also questionable, and more study is also needed to determine whether or not the pathway shown actually occurs in nature.

There are many further investigations which would shed insight on the reaction and evaluate the conclusions. If an enzyme were found to remove phosphate groups from IPs in a non-specific manner, a mixture of isomers would result, and the separation of this mixture could be compared against the peaks found in the reaction product. Also, since dilution improves the resolution, samples at a lower concentration can be run to get a more ac-

curate idea of products. Oxidizer run samples could be diluted, to remove the effects that changing the concentration would have on the reaction. A third avenue would involve drawing off the reaction products as they come off of the column and characterizing them. Appropriate analytical tools, such as a mass spectrometer, should give useful information about the products and therefore the reaction itself. Also, some test for the presence of inositol would prove useful in mechanism determination.

Finally, this reaction is run under conditions very different from those encountered in nature, so the applicability to natural processes is questionable. In order to make more accurate predictions concerning these processes, natural conditions can be introduced. For example, "natural waters", with concentrations of possible sensitizing agents may show other reaction mechanisms. Also, the UV lamp can be filtered to remove the light that the atmosphere screens, imparting radiation as it would be encountered in an aquatic system.

Although many specifics concerning this reaction fall beyond the scope of this study, important facts regarding the kinetics and mechanism

of the photooxidation of IHP are now known.

VI. ACKNOWLEDGEMENTS

I would like to thank Dr. Roger Minear and the Institute for Environmental Studies for their assistance on this project, and for the use of their facilities. Also, I'd like to thank Dr. Steven Zumdahl, who advised the project, and Cathy Clarkin, who provided her technical expertise with the HPLC.

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Table 1

Residence Times in Oxidizer

Flow Rate	Oxidation Time
.2 ml/min	11.1 min
.5 ml/min	4.4 min
1.0 ml/min	2.2 min

Table 2

Ascorbic Acid Reagent

Solution	Amount of Reagent	Amount of water	Stability
1. sulfuric acid	140 ml of conc. sulfuric acid	900 ml	indefinite
2. ammonium molybdate	15 g of reagent crystals	500 ml	indefinite
3. antimony potassium tartrate	0.34 g	250 ml	months
4. ascorbic acid	5.4 g of reagent grade	100 ml	one week

The actual reagent is the above reagents, mixed in order, and with a ratio of 5 : 2 : 1 : 1. It was made fresh daily.

Table 3

TLC Detection Limits

Compound	Concentration	Mass
IMP	33.3 mg P/l	.85 microgram P
IHP	9.2 mg P/l	.23 microgram P
ortho	6.7 mg P/l	.17 microgram P

Table 4

time	A	ln A	1/A	regres 1/A	regres ln A	regres A
0	103.1	4.64	0.009699	0.01206	4.4265	84.527
2.2	68.9	4.23	0.014513	0.012467	4.397262	82.37313
4.4	79.7	4.38	0.012547	0.012874	4.368024	80.21926
11.1	66.7	4.20	0.014992	0.014113	4.278981	73.65976
22.2	60.1	4.10	0.016638	0.016167	4.131462	62.79253
33.3	57	4.04	0.017543	0.018220	3.983943	51.92530

ZERO ORDER REGRESSION

Regression Output:

Constant	84.52753
Std Err of Y Est	12.33077
R Squared	0.574324
No. of Observations	6
Degrees of Freedom	4
X Coefficient(s)	-0.97903
Std Err of Coef.	0.421432

FIRST ORDER REGRESSION

Regression Output:

Constant	4.426549
Std Err of Y Est	0.143108
R Squared	0.648874
No. of Observations	6
Degrees of Freedom	4
X Coefficient(s)	-0.01329
Std Err of Coef.	0.004891

SECOND ORDER REGRESSION

Regression Output:

Constant	0.012060
Std Err of Y Est	0.001682
R Squared	0.722192
No. of Observations	6
Degrees of Freedom	4
X Coefficient(s)	0.000185
Std Err of Coef.	0.000057

Table 5

Regression Data Without t=0

time	A	ln A	1/A	regres 1/A	regres ln A	regres A
2.2	68.9	4.23	0.014513	0.013573	4.299259	73.66658	0.232656
4.4	79.7	4.38	0.012547	0.013868	4.279965	72.39566	0.378269
11.1	66.7	4.20	0.014992	0.014766	4.221206	68.52514	0.200204
22.2	60.1	4.10	0.016638	0.016253	4.123859	62.11278	0.096009
33.3	57	4.04	0.017543	0.017741	4.026512	55.70042	0.043051

Regression Output:

Constant	74.93750
Std Err of Y Est	5.327443
R Squared	0.726440
No. of Observations	5
Degrees of Freedom	3
X Coefficient(s)	-0.57769
Std Err of Coef.	0.204675

Regression Output:

Constant	4.270242
Std Err of Y Est	0.020917
R Squared	0.965780
No. of Observations	3
Degrees of Freedom	1
X Coefficient(s)	-0.00707
Std Err of Coef.	0.001332

Regression Output:

Constant	0.013279
Std Err of Y Est	0.000977
R Squared	0.810121
No. of Observations	5
Degrees of Freedom	3
X Coefficient(s)	0.000134
Std Err of Coef.	0.000037

Regression Output:

Constant	4.318553
Std Err of Y Est	0.072087
R Squared	0.770052
No. of Observations	5
Degrees of Freedom	3
X Coefficient(s)	-0.00877
Std Err of Coef.	0.002769

myo-inositol

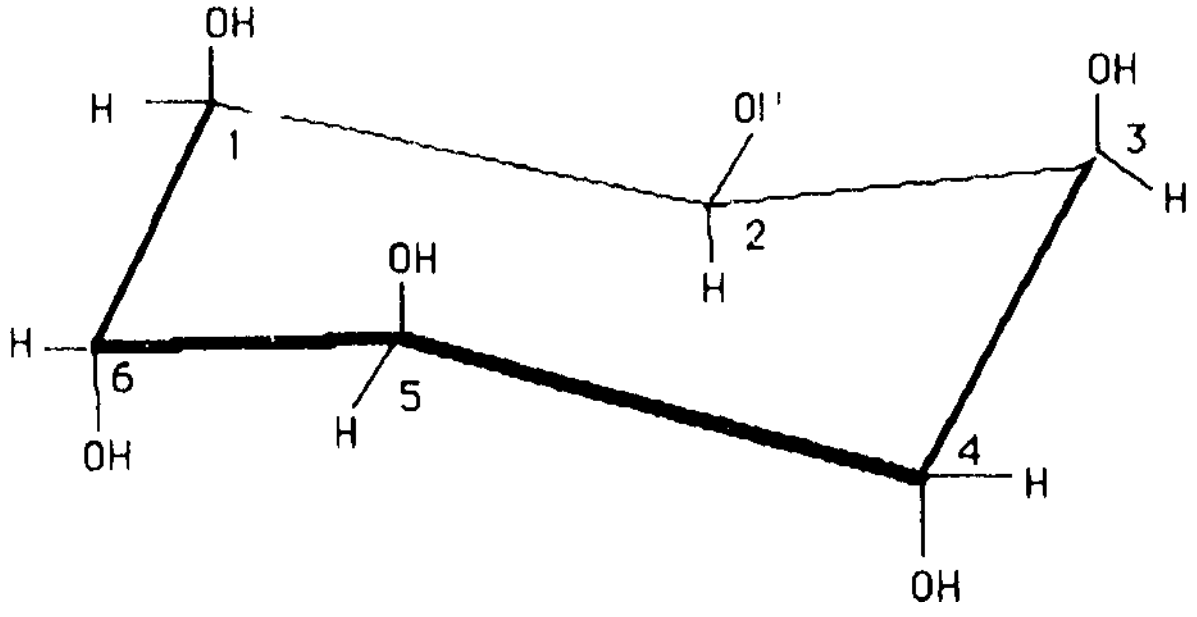


Figure 1

Mercury Lamp Spectrum

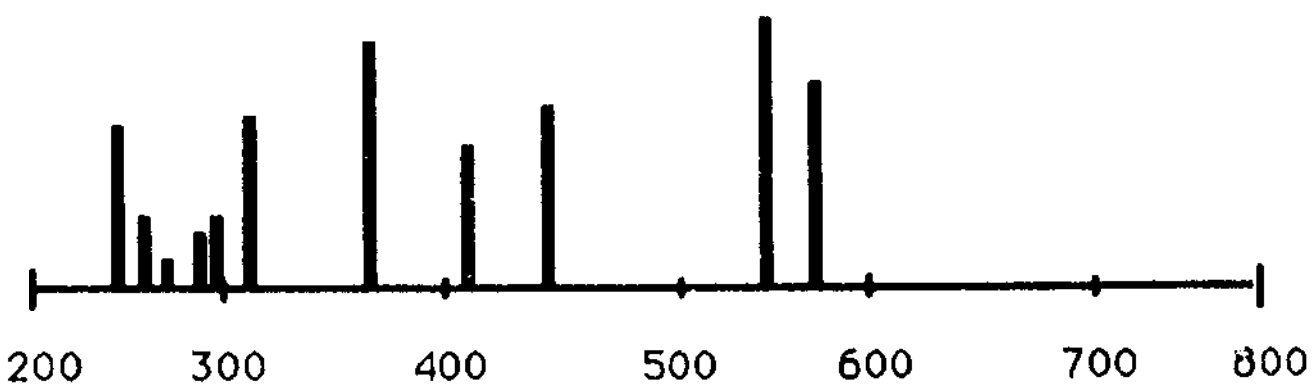
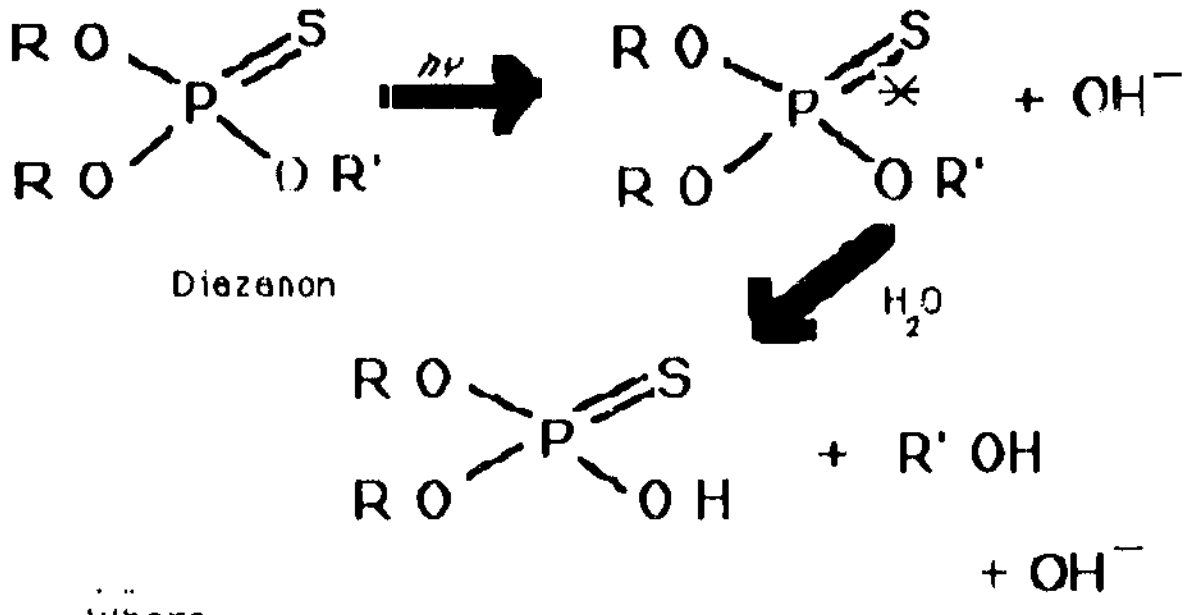


Figure 2



Where:

$$R = C_2H_5$$

and

R' = 2-isopropyl-4-methyl-6-pyrimidine

Figure 3

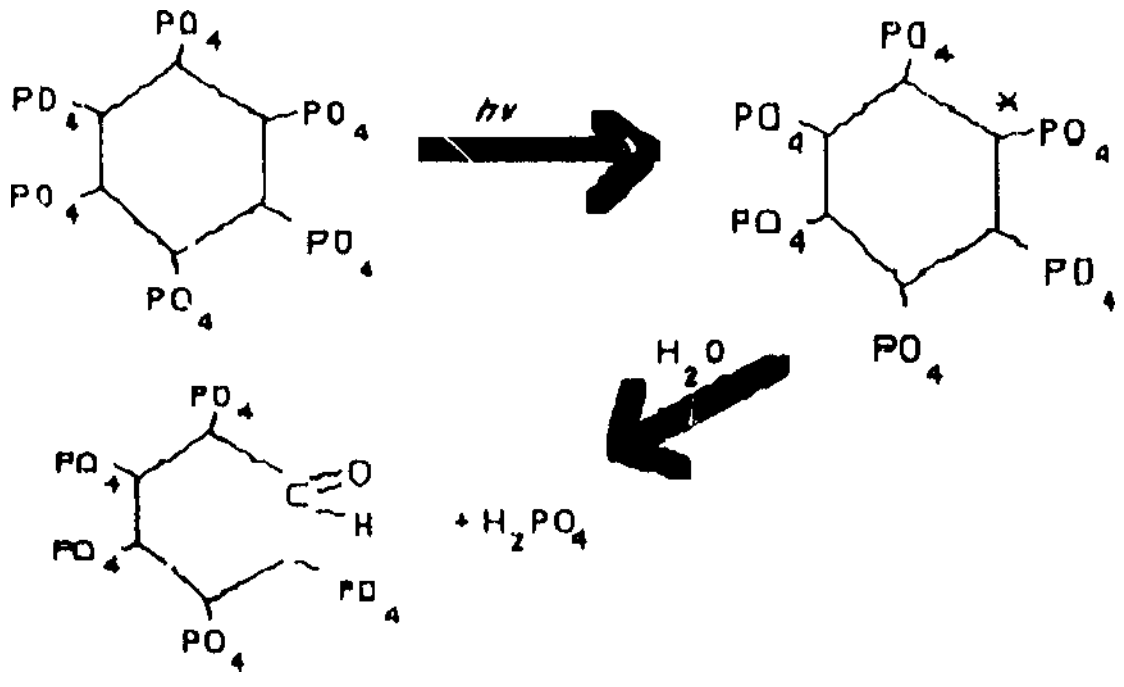


Figure 4

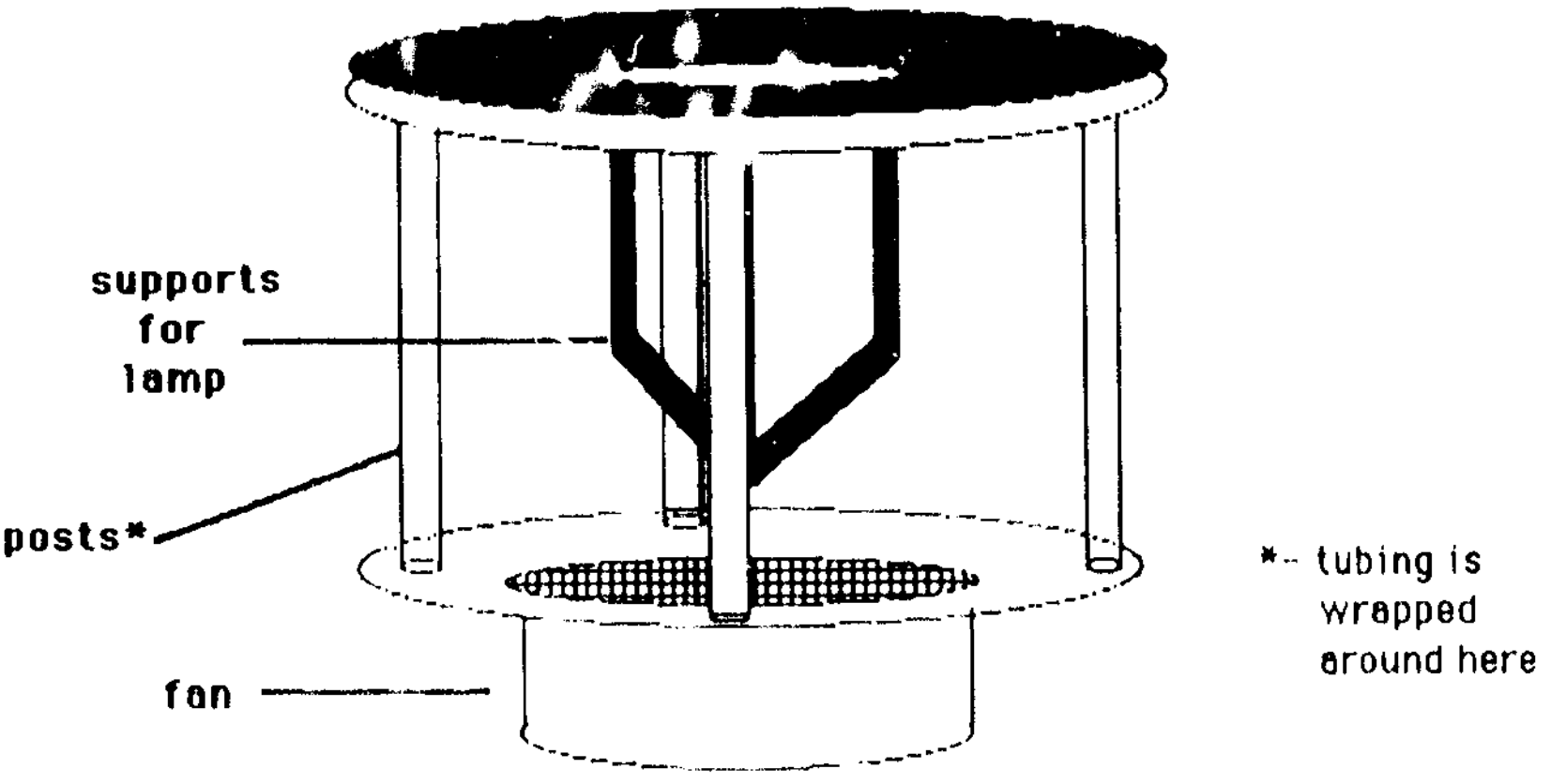


Figure 5

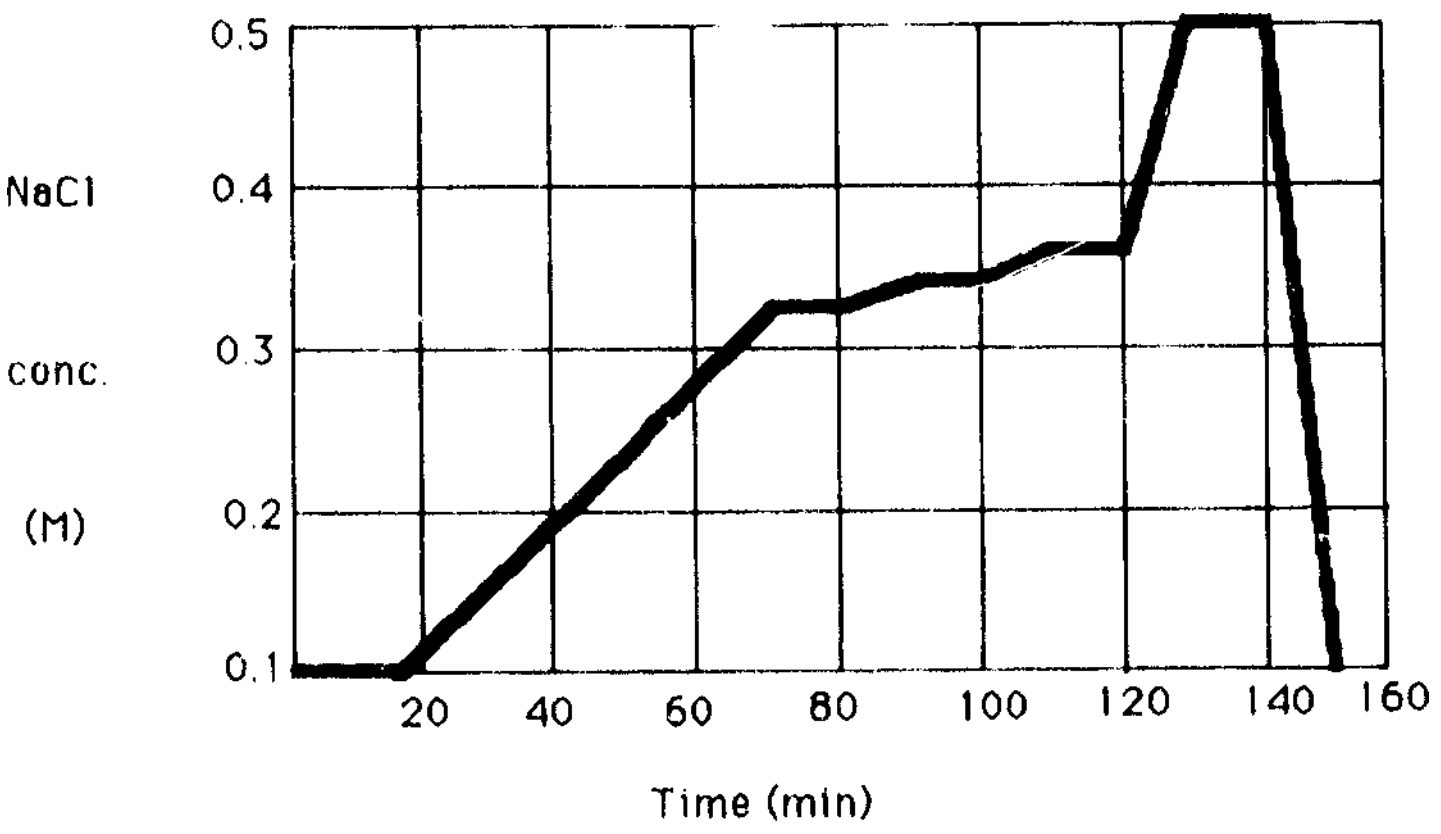


Figure 6

TLC Standards

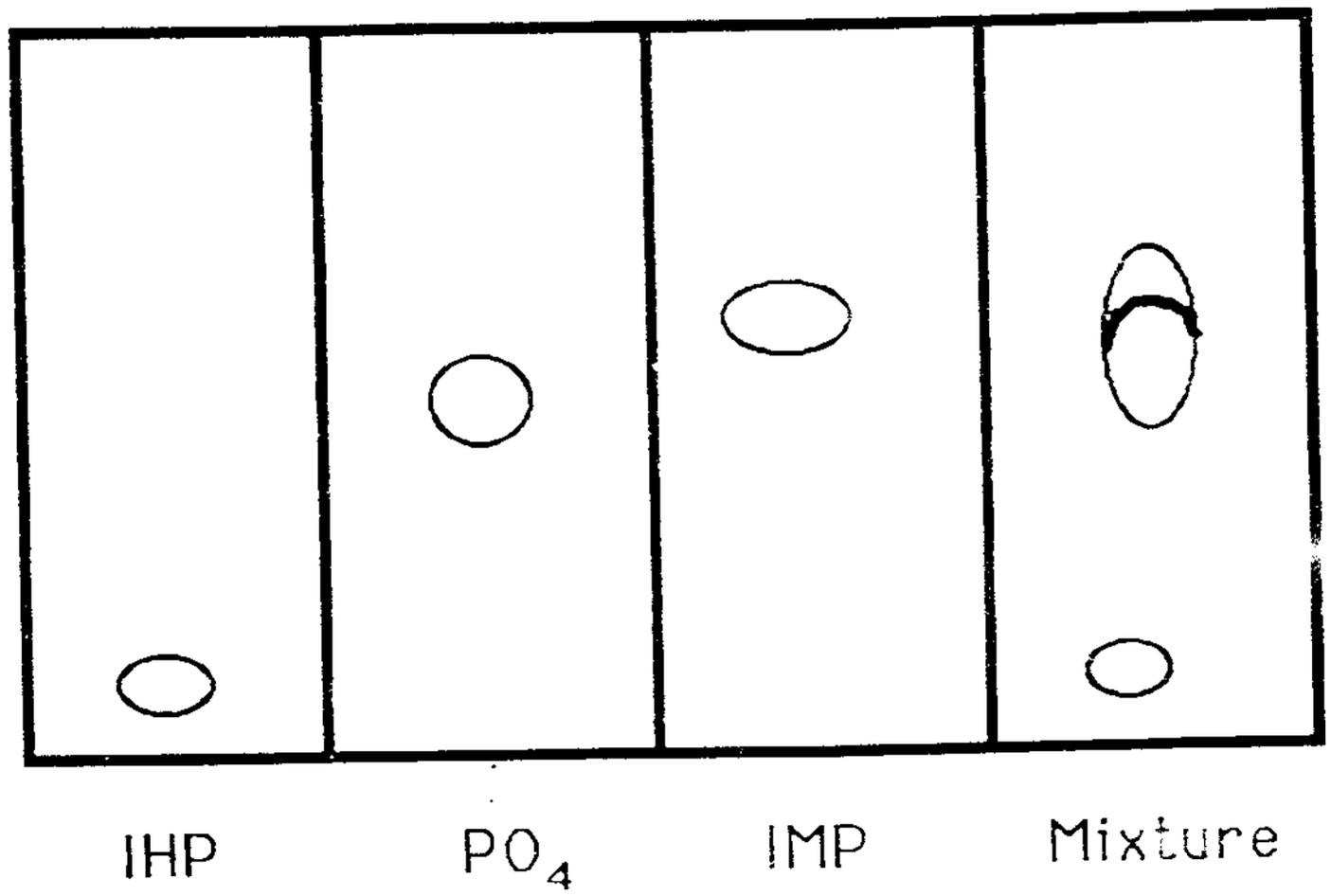


Figure 7

OXIDATION SEPARATIONS

(HPLC)

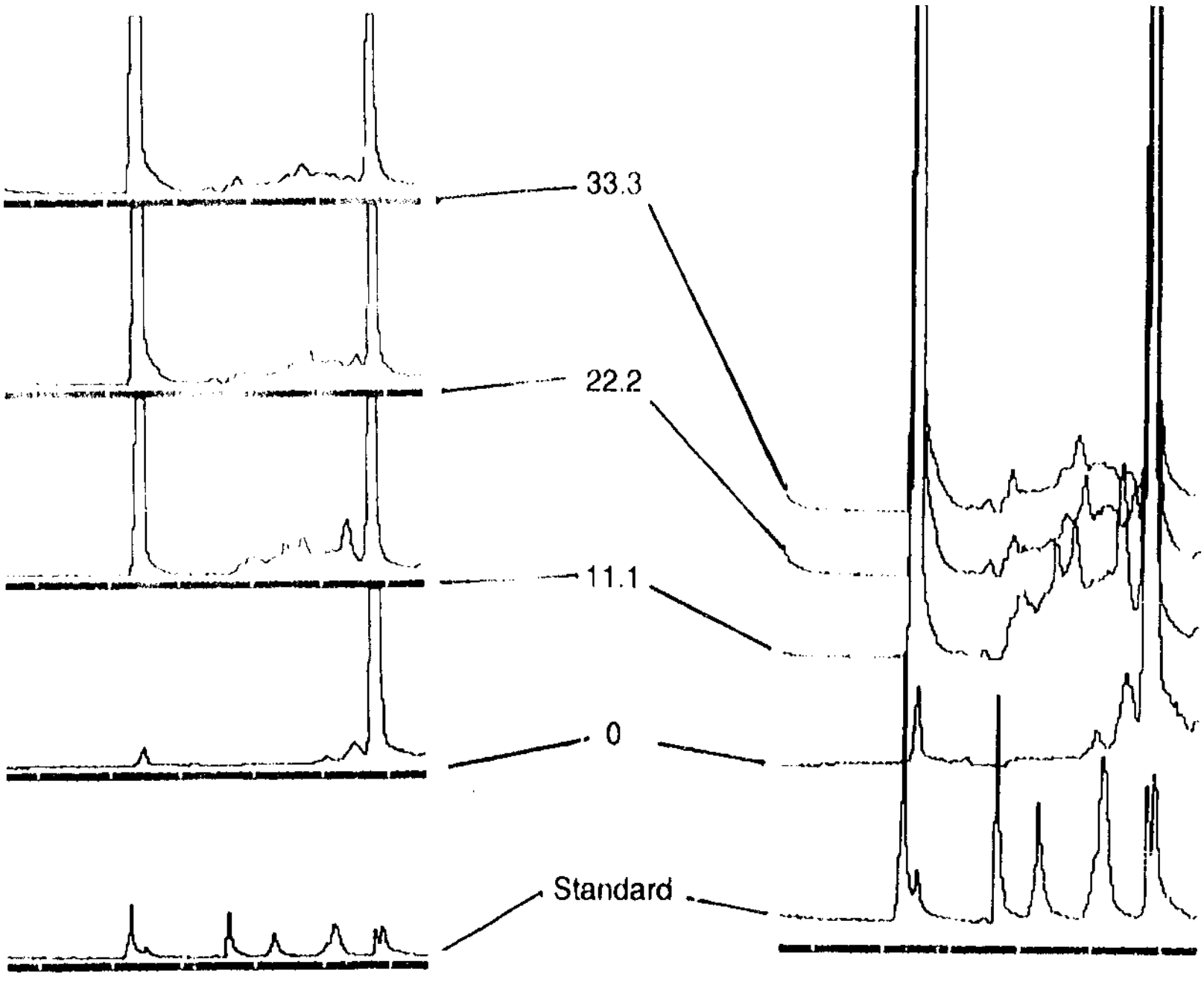


Figure 8

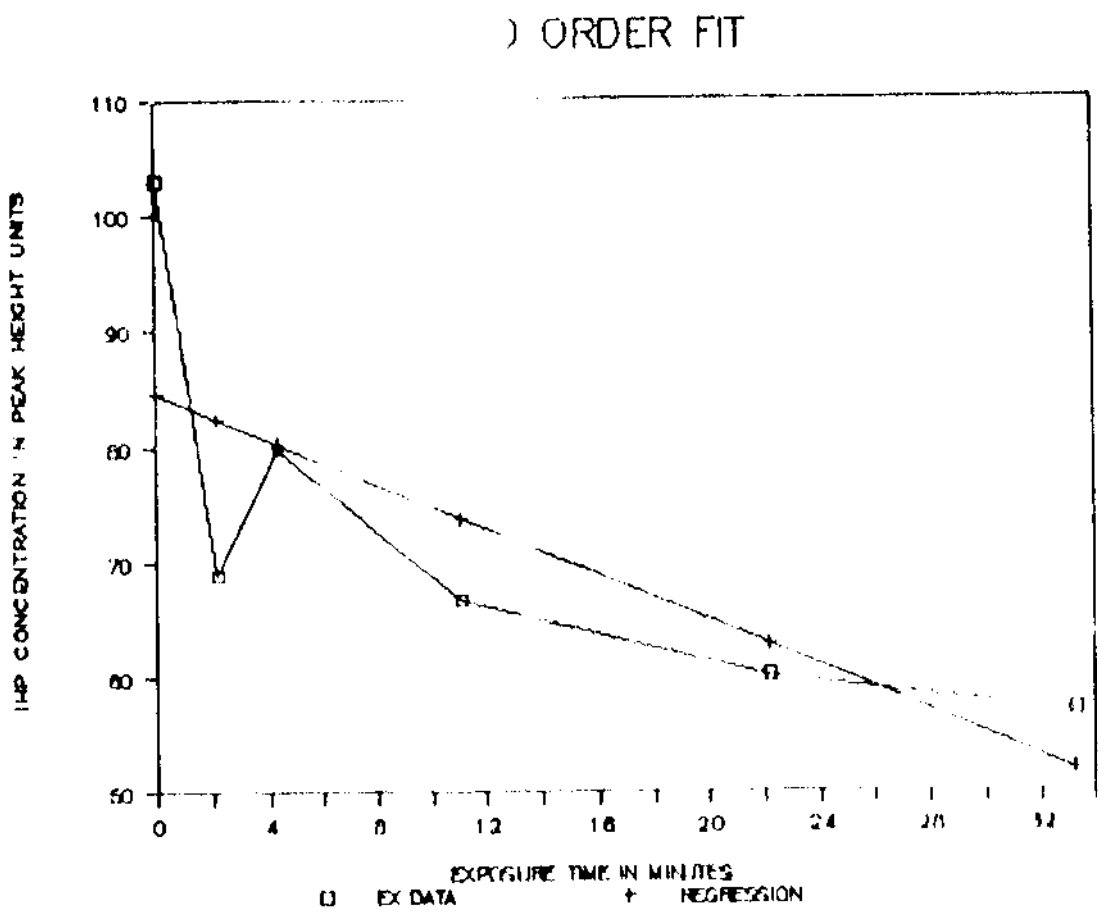


Figure 9

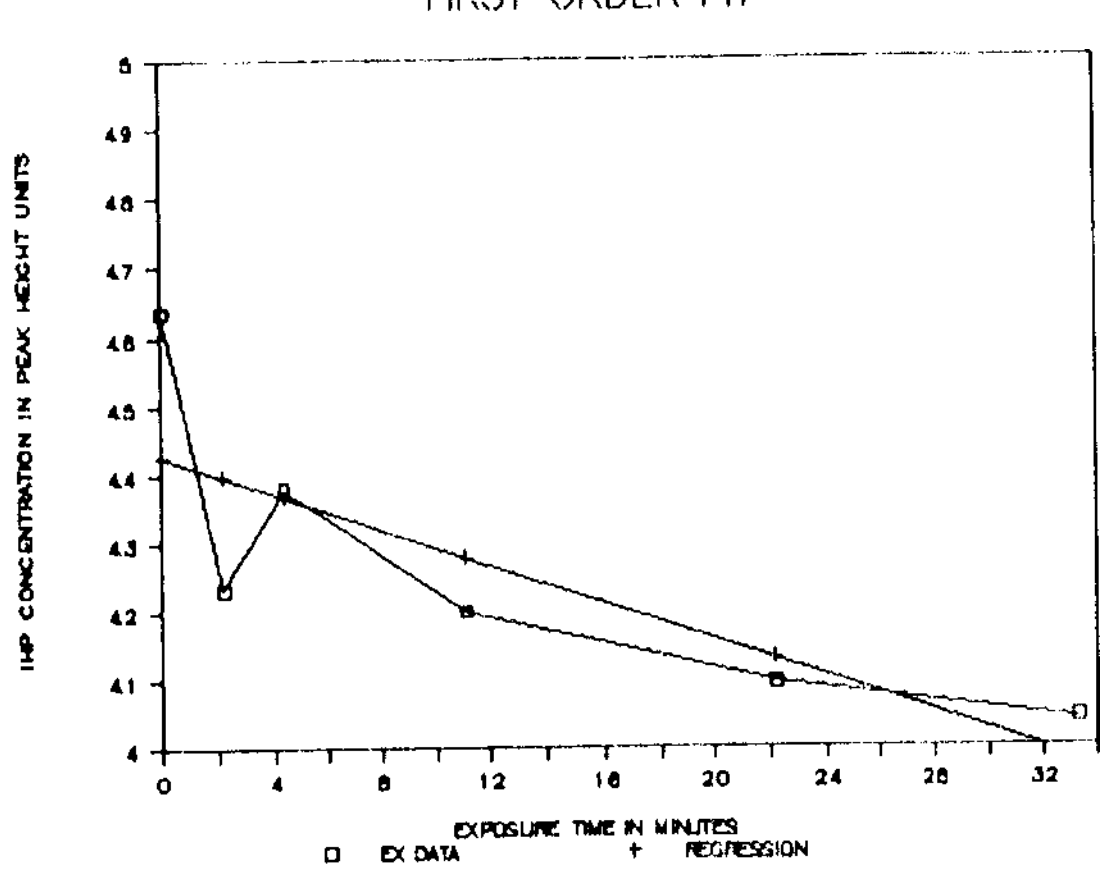


Figure 10

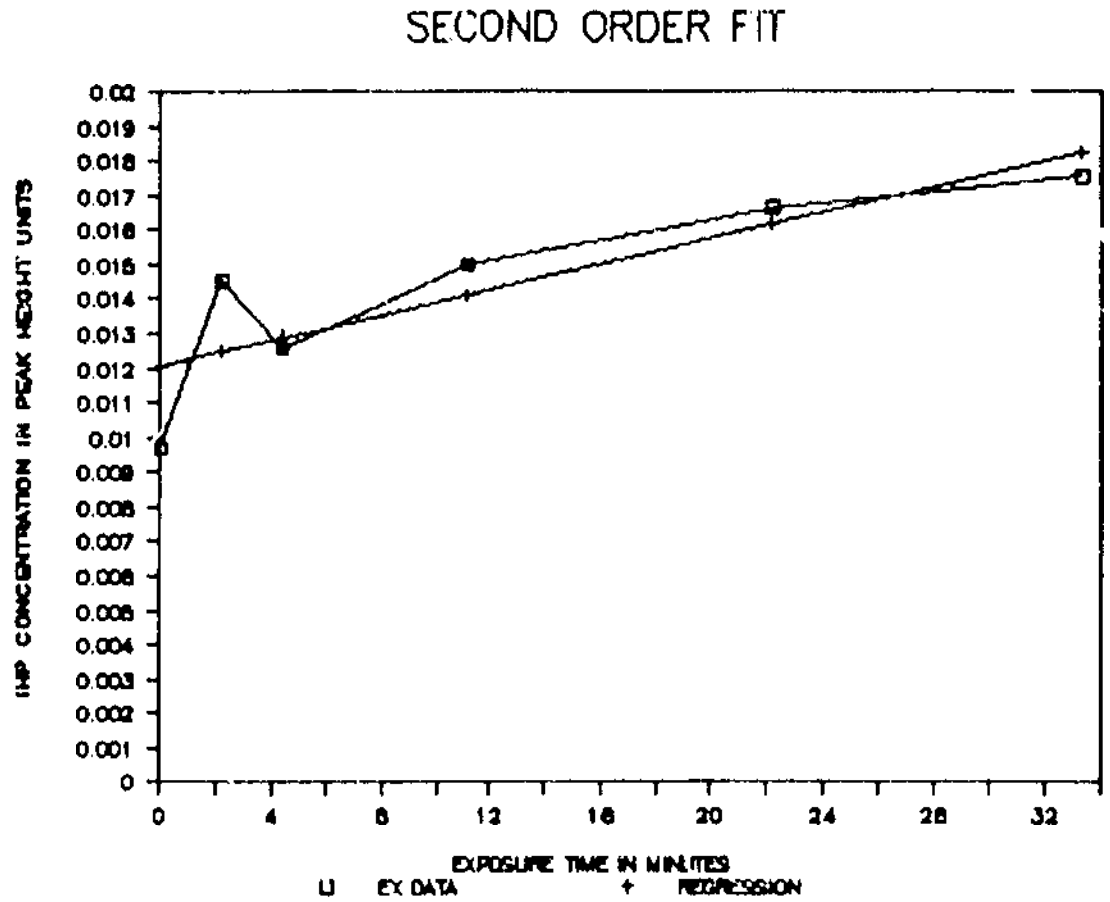

SECOND ORDER FIT
IHP CONCENTRATION IN PEAK HEIGHT UNITS
0.02
0.019
0.018
0.017
0.016
0.015
0.014
0.013
0.012
0.011
0.01
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
0
4
8
12
16
20
24
28
32
EXPOSURE TIME IN MINUTES
EX DATA
REGRESSION

Figure 11