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Title: EPOXIDATION OF ALDRIN BY RAINBOW TROUT LIVER MICROSOMES

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Abstract approved: Leon C. Terriere

Assay conditions and analytical methods established for the study of epoxidation of the insecticides aldrin and heptachlor, by rat liver microsomes have been employed to study the same reaction catalyzed by trout liver microsomes. At 37° C and pH 8.0, rainbow trout liver microsomes, incubated with 54.8 millimicromoles of either aldrin or heptachlor in the presence of reduced triphosphopyridine nucleotide (NADPH) and oxygen, produced only 0.36 millimicromole of the corresponding expoxides, dieldrin and heptachlor epoxide. This was only 7.2% of the amount produced by similar assays with male rat liver microsomes.

Increasing the microsomal protein and extending the incubation time failed to increase the epoxide yield of the fish microsomes. The same result was obtained when incubations were carried out at 2, 11, and 22° C, within a pH range of 6.0 to 8.5. Varying the substrate level from 5 to 100 millimicromoles did not affect the activity.

Dieldrin formation remained unchanged when a large excess of an NADPH-generating system or six micromoles of either Fe(II), Mn(II), Co(II), Mg(II) or FMN was added. Incubation of aldrin with freshly prepared trout liver slices instead of microsomes failed to show any improvement in epoxidation. However, no dieldrin was detected when either heat-denatured microsomes, snake venom-treated preparation, steapsin-treated preparation or liver acetone powder was used.

Activities of the components of the microsomal electron transport system, which plays a vital role in epoxidation, were measured. Results indicated their presence in significant levels in trout liver microsomes.

Aldrin and heptachlor conversions by male rat liver microsomes were enhanced two to four times by the addition of either an acetone powder preparation, the supernatant fraction above the 78,000 × g pellet, or microsomes, of trout liver. A reaction is said to be enhanced, when the activity of the combined system is greater than the sum of those of the separate preparations. However, no enhancement was observed when an inactive rat preparation, such as a rat liver acetone powder preparation, was used in combination with a trout fraction. The extent of enhancement in epoxidation depended primarily on the basal activity of the rat liver microsomes. Furthermore, the enhancing ability of the fish fractions

was abolished by treating them with heat, snake venom, or steapsin.

Of the three microsomal electron transport component activities tested, only that involved in neotretazolium (NT) reduction was enhanced by combining rat and trout liver microsomes in the assays. NADPH-cytochrome c and dichlorophenol indophenol (DCPIP) reductase activities were additive in the combined systems. NT reduction is a test reaction for component X_1 in the NADPH-dependent electron transport chain of liver microsomes.

The parallel relationship between enhancement of NT reduction and epoxidation and their relative positions in the established sequence of reactions in the NADPH-dependent chain indicates that the enhancement in epoxidation is brought about by an increase in X_1 relative to the epoxidative enzyme(s) in the combined systems. The evidence collected in this research leads to the conclusion that the low epoxidative activity in trout liver microsomes is a result of a deficiency in the epoxidative enzyme(s).

Epoxidation of Aldrin by Rainbow Trout Liver Microsomes

by

Timothy Ming-Wai Chan

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EPOXIDATION OF ALDRIN BY RAINBOW TROUT LIVER MICROSOMES

I. INTRODUCTION

Aldrin (1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4a, 5, 8, 8a-hexahydro-1, 4-endo-exo-5, 8-dimethanonaphthalene) and heptachlor (1, 4, 5, 6, 7, 8, 8-heptachloro-3a, 4, 7, 7a-tetrahydro-4, 7-methanoindene) are two cyclodiene insecticides widely used in agriculture. In certain organisms, the most important biological fate of these compounds is the conversion to their epoxides, dieldrin and heptoachlor epoxide, which are more toxic than the parent compounds.

That these cyclodienes were converted in vivo to their epoxides was first reported in 1953 (13). These authors found that dogs converted heptachlor to heptachlor epoxide which was stored in the fat tissue of the animal. Following this discovery, conversions of aldrin to dieldrin, and isodrin to endrin by mammals and houseflies were reported (50, 7, 37). These reactions have also been reported in soil microorganisms (26).

Although detoxication has long been known to take place in the liver of mammals, the subcellular location where these reactions occur in an intact biological system was still unexplored until two decades ago. Some of the most significant contributions to the understanding of detoxication came from in vitro studies of these reactions in subcellular fractions of the liver. Of all fractions of the liver

studied, the one composed of "submicroscopic" particles was of particular interest. These particles, isolated from homogenates of liver and other tissues by differential centrifugation, were referred to as "small granules" by Claude (9) and were later given the name "microsomes" (10). The term "microsome" has been used to describe the pellet resulting from the centrifugation at 100,000 to 250,000 × g for 60 to 120 min. of the supernatant fluid after the sedimentation of the mitochondrial fraction (45). With improvements in electron microscopy, the morphological nature of this fraction was revealed. In general this fraction contains membranes called the endoplasmic reticulum. However, the nature of the structures in the microsomal fraction depends on the source of the cells from which the fraction is obtained. With liver, the pellet contains membranes with, and membranes without ribosomes attached to them (45). The former is often called the rough endoplasmic reticulum (RER) and the latter the smooth endoplasmic reticulum (SER). The biological and biochemical significance of the microsomal fraction was recognized when it was found to participate in such vital functions such as protein synthesis, and metabolism of fatty acids, phospholipids, steroids, and nucleotides (44).

In 1954, Axelrod and associates (2) discovered an enzyme system in the microsomal fraction of mammalian liver that deaminated the drug amphetamine. This was the first of a series of discoveries

of NADPH- and oxygen-requiring microsomal systems. Since then, demonstrations of other biological oxidations of foreign materials, especially drugs, in liver microsomes have been numerous. The understanding of these reactions was greatly enhanced by the elucidation of an electron transport chain in microsomes with which these oxidative enzyme systems are intimately associated.

The first of the components of this chain to be discovered was a microsomal NADPH-cytochrome c reductase (21, 38, 39). Since then, three other components, cytochrome b_5, NADPH-cytochrome b_5 reductase and cytochrome P-450, have been confirmed. Furthermore a microsomal ubiquinone has been reported (25). In spite of these advances, the knowledge concerning this electron transfer system is far from complete. Thus, one can either consider it a system containing two separate chains, one involving NADH and another involving NADPH, or one can regard the presently known fragments as one single chain. Most workers treat this system as comprised of two separate chains. The relationships between the components in the two chains and their test reactions are shown in Figure 1. F_{PT} and F_{PD} are two different flavo proteins. Components X_1 and X_2 have not been fully characterized. They have been inserted as a result of a biogenic study of these constitutive enzymes (45). X_2 has been found to be the limiting factor in the reduction of exogenous cytochrome c by cytochrome b_5 in the presence of NADH. Component X_1 is limiting in the detoxication reactions which are assumed to be

catalyzed by hydroxylating and other oxidative enzyme systems in the NADPH chain.

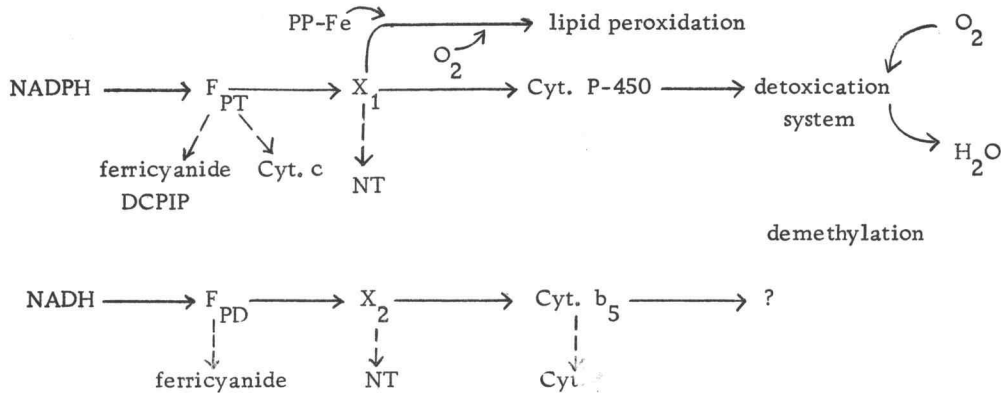

Figure 1. Tentative scheme of sequence of components in the two electron transport chains of rat liver microsomal membranes (11).

Compared to drug metabolism, information on in vitro metabolism of insecticides is scarce. In vitro studies of the metabolism of cyclodienes were reported by Giannotti in 1958 (20), Korte in 1962 (22), Wong and Terriere in 1965 (51) and later by Nakatsugawa et al. (32). However, the previous studies were confined to insects and mammalian liver microsomes. Aquatic species whose environment is often contaminated by insecticides (46) have not been investigated. Moreover, no correlation of epoxidation with the microsomal electron transport system has been attempted. To supplement the understanding of the role of insecticides as pollutants, the research described in this thesis covers the in vitro study of cyclodiene insecticide metabolism in fish. Attempts to correlate the epoxidase system with other microsomal electron transport components have been made.

II. MATERIALS

Two-year old domesticated rainbow trout of both sexes (Salmo gairdnerii) of the Mount Shasta hatchery stock were supplied by the Food Technology and Nutrition Laboratory of Oregon State University. These fish, averaging 8 inches in length, had been on an experimental fish diet (24). Adult white rats used in these experiments were of an inbred Oregon State Wistar strain. The age of these animals ranged from six months to a year old, with an average weight of 380 gm.

NADP (nicotinamide-adenine dinucleotide phosphate-monosodium salt), G-6-P (D-glucose 6-phosphate disodium salt), cytochrome c (from horse heart, 95-100% based on molecular weight 12, 270), DCPIP (2, 6-dichlorophenol-indophenol, sodium salt), snake venom (Trimeresurus flavoviridis), steapsin (crude hog pancreatic lipase), FMN (flavin mononucleotide, sodium salt), FAD (flavin-adenine dinucleotide, disodium salt), and Tris buffer [tris (hydroxymethyl)-aminomethane] were obtained from the Sigma Chemical Company. G-6-P dehydrogenase (specific activity of 166 units/mg) from General Biochemicals, p-neotetrazolium blue (NT) or [2, 2'-di-p-nitrophenyl-(3, 3'-dimethoxy-4, 4'biphenylene)-5, 5' diphenyl ditetrazolium chloride] from Mann Research Laboratory and SKF 525-A (β-diethylaminoethyl diphenylpropylacetate hydrochloride)

from Smith, Kline, and French Laboratories were used.

Aldrin (1, 2, 3, 4, 10, 10-hexachloro- 1, 4, 4a, 5, 8, 8a-hexahydro-1, 4- endo- exo- 5, 8-dimethanonaphthalene), heptachlor (1, 4, 5, 6, 7, 8, 8- heptachloro-3a, 4, 7, 7a-tetrahydro-4, 7-methanoindene), dieldrin (1, 2, 3, 4, 10, 10- hexachloro-6, 7-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-1, 4- endo- exo- 5, 8-dimethanonaphthalene), heptachlor epoxide (1, 4, 5, 6, 7, 8, 8-heptachloro-2, 3- epoxy- 3a, 4, 7, 7a-tetrahydro- 4, 7-methanoindene) were supplied by Shell Chemical Company.

Carbon monoxide, C. P. grade, and methylcellosolve (2-methoxyethanol) were obtained from the Matheson Coleman and Bell Company. Methylcellosolve was distilled twice before use. All other solvents used have also been redistilled and dried.

III. METHODS

Isolation of Microsomes

Rainbow trout of both sexes were transported in plastic tanks from the hatchery to the laboratory where they were killed by a blow on the head and their livers were excised. The livers were rinsed thoroughly with cold 1.15% KCl solution, chilled, and blotted briefly with tissue paper. A 10% (w/v) homogenate in 1.15% KCl solution was prepared with a VirTis "45" homogenizer at full speed for 25 seconds. The homogenate was transferred to polyethylene centrifuge tubes and centrifuged at 9,000 × g for 30 minutes at 4° in a Servall ultracentrifuge. The precipitate containing mostly cell debris and mitochondria was discarded and the post-mitochondrial supernatant was centrifuged at 78,000 × g in type 30 Spinco rotor for 90 minutes in a Spinco model L-2 preparative ultracentrifuge. The supernatant was decanted and the microsomal pellets thus obtained were resuspended in 0.05 M Tris buffer, pH 8.0, to one gm liver equivalent per ml, in a Potter-Elvehjem homogenizer equipped with a teflon plunger.

Male or female rats of the Oregon State Wistar strain were anesthetized with diethyl ether prior to sacrifice. Same procedure as described for trout was followed thereafter.

Further Fractionation of the Post-Mitochondrial Supernatant

In cases where further fractionation was called for, the supernatant obtained after sedimentation of mitochondria and nuclei was divided into two portions for centrifugation, one at 78,000 × g for 90 minutes and the other for 900 minutes at the same speed. The supernatant from the 90 minute spin was again centrifuged at 78,000 × g for 900 minutes. The procedure is summarized in Figure 2.

Preparation of Liver Tissue Slices

After removal from the fish, the liver was thoroughly rinsed with cold isotonic KCl solution. Thin liver slices prepared with a razor blade in a medium of cold KCl. All manipulations were conducted at 4°.

Rat liver slices prepared the same way were found to be active in epoxidation.

Determination of Protein Content

The biuret method modified by Finchem (17) has been employed to determine the protein content in all microsome preparations. The reaction mixture contained 2.4 ml of distilled water, 0.9 ml of 20% (w/v) NaOH, 0.15 ml 25% (w/v) $CuSO_4 \cdot 5H_2O$ and 0.1 ml of the

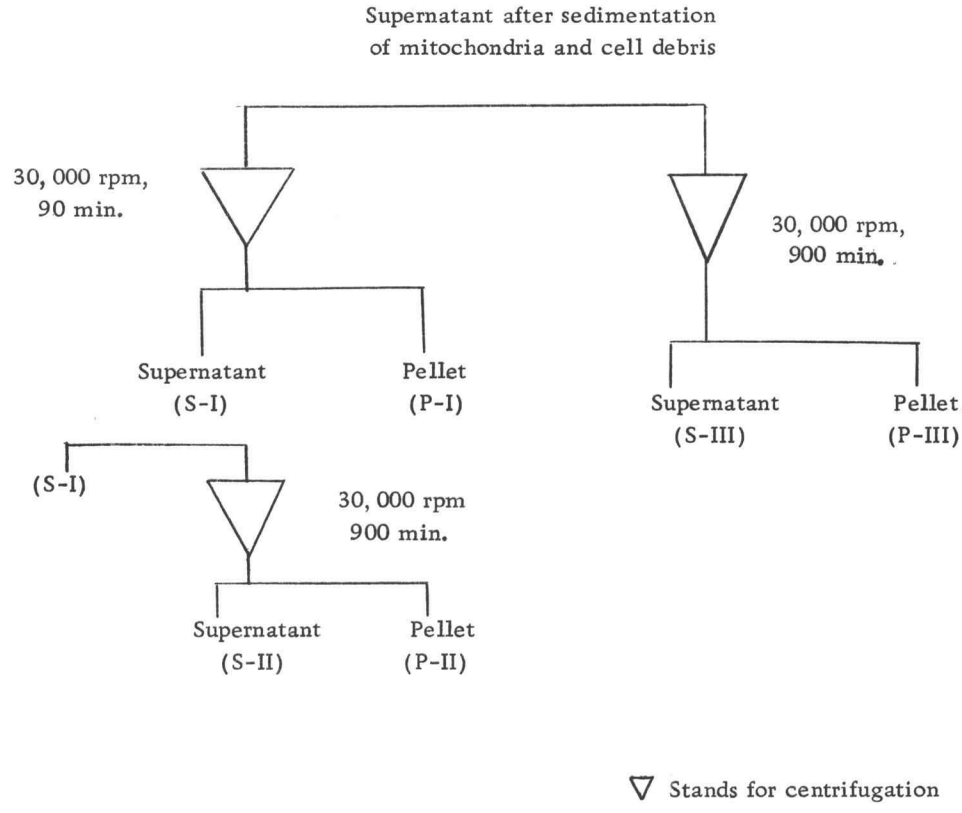

Figure 2. Fractionation of liver post-mitochondrial supernatant by differential centrifugation.

microsomal resuspension. The blue crystals were broken up with a thick glass rod and the excess $Cu(OH)_2$ was sedimented by centrifugation. The absorbency of the clear supernatant was determined at 545 mμ in a Beckman Model B colorimeter. A standard of known concentration was prepared with bovine serum albumin.

Assay of Epoxidative Activity

The epoxidative activity of the microsomal preparations was determined by incubating an appropriate aliquot constituting the amount of protein desired, with aldrin in the presence of an NADPH-generating system and oxygen. The incubation mixture was made up of 54.8 mμ moles of aldrin in 0.1 ml of methylcellosolve, 1.8 μ moles of NADP, 18 μ moles of glucose 6-phosphate, 1.66 units of glucose 6-phosphate dehydrogenase and 2.0 ml of 0.1 M Tris buffer, pH 8.0. Distilled water was added to a final volume of 6.0 milliliters. The reaction was carried out in 50-ml Erlenmeyer flasks fitted with snap-on rubber caps. Incubation was conducted at 37° C for 15 minutes for aldrin and 30 minutes for heptachlor, in a Lab-Line general purpose shaking bath.

The enzymatic reaction was terminated by adding 10 ml of a hexane: 2-propanol (3:2, v/v) solution to the incubation mixture. The residual substrate, aldrin, and the epoxidation product, dieldrin, were extracted into the organic solvent by shaking the flasks

in the shaker at moderate speed for 45 minutes. After separation of the phases, the hexane layer was transferred to a 50-ml volumetric flask with a 10 cc hypodermic syringe. The aqueous phase was again extracted with two ml portions of redistilled hexane. The extracts were combined in the 50-ml volumetric flask and made up to volume with redistilled hexane. Approximately one gm of anhydrous sodium sulfate was added to eliminate moisture in the extracts.

Gas Chromatographic Analysis

Analyses of the extracts for aldrin and dieldrin were performed with a Wilkins Aerograph Hy-FI (model 600) equipped with electron capture detector of the concentric tube design. A pyrex glass column, 1/8 by 60 inches, was packed with 5% Dow Corning 11 for a distance of 20 inches at the exit end and 5% QF 10065 for the rest of the column. Non acid-washed Chromosorb W was used in solid support.

This instrument will detect as little as 5×10^{-12} gm aldrin or dieldrin. The resolution of the peak and the retention time of the compounds depend on column temperature, carrier gas pressure and the condition of the detector cell.

One μl of a 50-ml sample extract was injected into the system and the peak heights of the aldrin and dieldrin peaks were compared to a chromatogram of a standard mixture of aldrin and dieldrin.

Both sample and standard chromatograms were run on the same day. The aldrin chromatogram standard solution was prepared from the same stock solution used in incubation. A typical chromatogram of aldrin and dieldrin is shown in Figure 3.

The amount of aldrin (A) or dieldrin (D) in a 50-ml sample was calculated as follows:

$$\text{m}\mu\text{mole A or D per unit peak height, or } F = \frac{\text{ng A or D standard}}{(\text{molecular weight of A or D}) \times (\text{peak height})}$$

$$\text{m}\mu\text{mole A or D in 50 ml sample of extract} = F \times 10^3\ \mu\text{l/ml} \times 50\ \text{ml} \times \text{peak height}/\mu\text{l sample}$$

The average total recovery, i.e., aldrin plus dieldrin, obtained by this method was 97%.

Similar procedures were followed for heptachlor and heptachlor epoxide.

Assay of NADPH-Cytochrome c Reductase Activity (49)

Since reduced cytochrome c has a characteristic absorption peak at 550 mμ, the ability of the microsomal preparation to reduce exogenous cytochrome c in the presence of added NADPH can be studied spectrophotometrically. This activity was measured by following the change in absorbency at 550 mμ in a Cary model 11 recording spectrophotometer. The sample cell contained microsomal preparation, usually 0.01 to 0.1 ml, 100 mμmoles of NADPH,

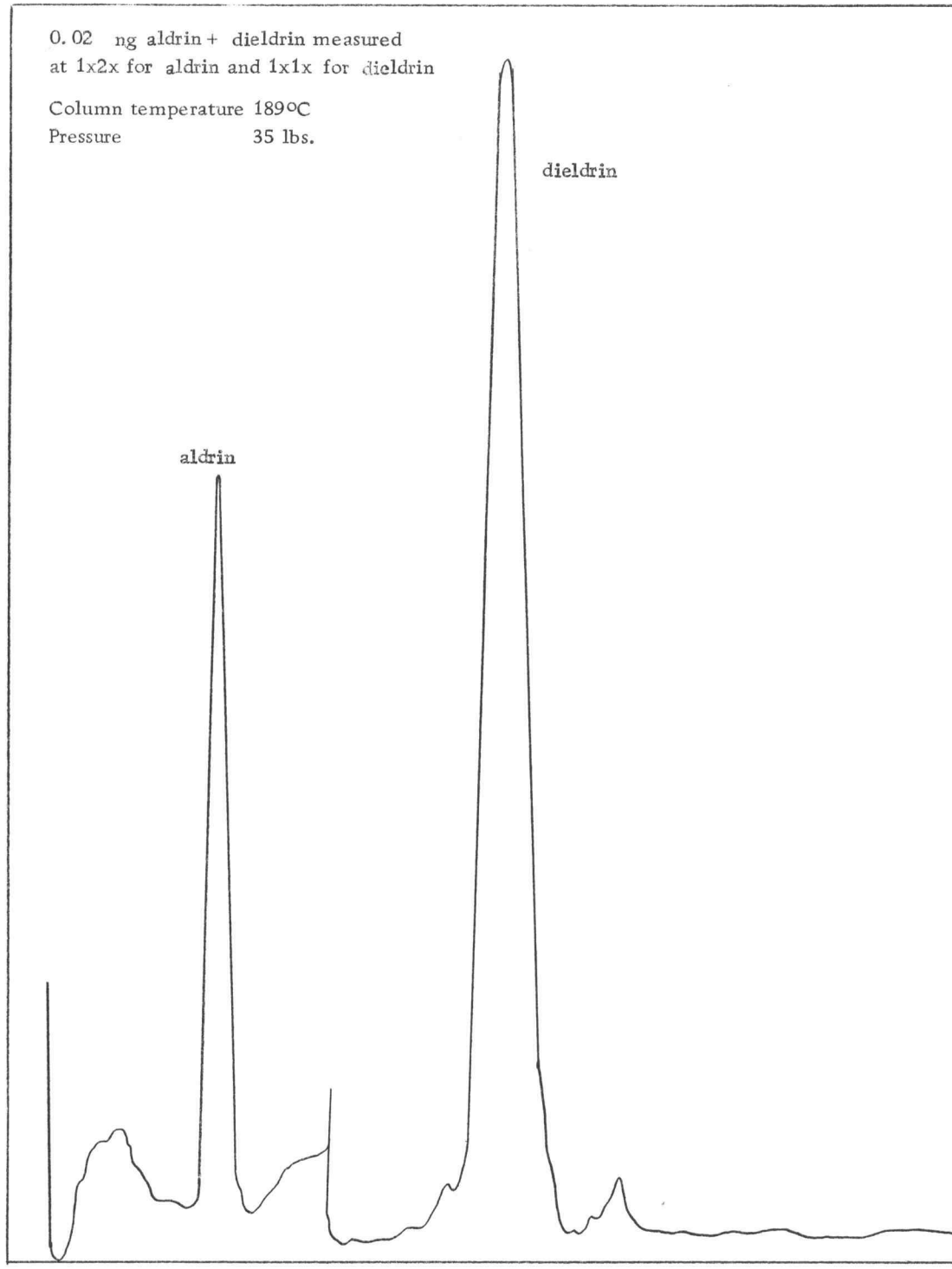

Figure 3. Typical gas chromatographic separation of aldrin and dieldrin.

34 mμmoles of cytochrome c and 0.05 M potassium phosphate buffer, pH 7.7, which was 1×10^{-3} M in potassium cyanide, to make a final volume of 3.01 ml. NADPH was omitted in the reference cell. Reaction in the sample cell was initiated by the addition of NADPH, and was followed for three to four minutes. The comparison of NADPH-cytochrome c reductase activities of different microsomal preparations was based on the change of absorbency per minute per mg microsomal protein or per gm liver equivalent.

Assay of NADPH-2, 6-Dichlorophenol Indophenol Diaphorase (49)

This activity was measured by the decrease in absorbency at 600 mμ as the dye was reduced in the presence of the enzyme in microsomes and added NADPH. Both cuvettes contained 95 mμmoles of DCPIP, 0.01 to 0.1 ml microsomal resuspension, and 0.05 M potassium phosphate buffer, pH 7.7, making a final volume of 3.0 ml. One hundred mμmoles of NADPH were added to the sample cuvette to start the reaction which was followed at 600 mμ in a Cary model 11 recording spectrophotometer. Activity was expressed as change in absorbency per minute per mg of microsomal protein or per gm liver equivalent.

Assay of NADPH-Neotetrazolium Reductase (49)

A reaction mixture containing 0.80 unit of glucose 6-phosphate dehydrogenase, 1.5 μmoles of glucose 6-phosphate, 0.3 μmole of NADP, 15 μmoles of $MgCl_2$ and 0.15 μmole of NT in 0.2 ml of 0.05 M potassium phosphate buffer, pH 7.7, was preincubated in 15 ml glass-stoppered conical centrifuge tube in a Lab-line general purpose shaking bath for five minutes at 37° C. The tubes were returned to ice and 0.01 to 0.1 ml of microsomal suspension was added. Incubation was resumed at 37° for 10 minutes. The reaction was stopped by adding 3.0 ml of redistilled acetone and a few crystals of NaCl. The reduced NT was extracted into the acetone by shaking. The precipitated protein was sedimented by centrifugation. The absorbency of the supernatant was determined in a Beckman model B spectrophotometer at 555 mμ.

Spectral Measurement of Cytochrome P-450 (34)

This cytochrome is peculiar in that its spectral property can be studied only when it is in the reduced state and bound to carbon monoxide. This reduced CO-bound cytochrome has an absorption maximum at 450 mμ. This maximum shifts readily to 420 mμ when P-450 is treated with detergent or sulfhydral reagents (35).

The microsomal resuspension was diluted 10 to 15 times with

0.1M potassium phosphate buffer, pH 7.0, and reduced with a few grains of sodium dithionite. The reduced microsomes were transferred to two cuvettes. Carbon monoxide was then bubbled into the sample cuvette for 30 seconds. Scanning from 600 mμ to 350 mμ was performed in a Cary model 11 recording spectrophotometer on a zero to 0.1 optical density scale. The sample cuvette was also scanned against one containing the non-reduced diluted microsomal resuspension. Cytochrome P-450 content of the preparation was calculated by taking the value of 91 cm^{-1} mM^{-1} for the increment of molar extinction between 450 mμ and 490 mμ in the CO difference spectrum of microsomal bound P-450 (40).

Solubilization of Microsomes

Digestion with snake venom (49): A 1% solution of crude venom of the snake Trimeresurus flavoviridis in 0.05 M Tris buffer, pH 7.4, was heated in a boiling water bath for eight minutes. The resulting precipitate was sedimented by centrifugation. A mixture containing liver microsomes resuspended in 0.1 M Tris buffer, pH 8.5, and 0.1% (in terms of unheated venom) of heated venom was placed in a Thunberg tube which was then evacuated followed by flushing with nitrogen. The mixture was incubated at 4° for 20 hours. A portion of the same microsomal preparation was treated identically but without snake venom for control. The pH of both portions was

adjusted to 8.0 before assaying for enzyme activities.

Steapsin digestion (49): Liver microsomes resuspended in 0.1 M potassium phosphate buffer, pH 7.5, were incubated under the nitrogen atmosphere with 0.07% steapsin in a Thunberg tube at 0° for 16 hours. A control tube, without steapsin, was treated identically. The pH of both tubes was adjusted to 8.0 before use for further studies.

Preparation of Whole Liver Acetone Powder

The following procedure has been followed for both rat and trout livers. Livers removed from the animals were rinsed thoroughly with cold distilled water and were then homogenized in 10 volumes of cold, dry acetone with a Waring blender for three minutes at -15°. The precipitate was collected by vacuum filtration and then resuspended in another 10 volumes of dry acetone. The precipitate then collected was washed with cold peroxide-free ethyl ether and then dried *in vacuo* over sulfuric acid. Mechanical grinding with a mortar and pestle was required to bring it to powder form. The acetone powder was resuspended in buffer at the desired pH and concentration before use. The resuspension was centrifuged at 9,000 × *g* for 30 minutes at 4° in a Servall ultracentrifuge. The precipitate was discarded and the supernatant was reserved for assay.

IV. RESULTS AND DISCUSSION

In their study of the epoxidation of aldrin, Wong and Terriere found that male rat liver microsomes could convert up to 80% of 20 μg (54.8 mμmole) of aldrin to dieldrin in the presence of oxygen and an NADPH-generating system at 37° C, pH 8.0, in 15 minutes (51). Nakatsugawa and associates obtained similar results in aldrin, isodrin and heptachlor epoxidation under slightly different conditions (27). Results in the former investigation were expressed in percent conversion with enzyme concentration based on liver weight. In the latter paper, enzyme concentration was expressed in milligram protein nitrogen.

Since in the anticipated experiments, enzyme concentrations were to be expressed in terms of microsomal protein, the relationship between epoxidative activity and enzyme level needed to be established. Results of these experiments are plotted in Figure 4. The specific activity decreases as enzyme concentration exceed 4 mg of protein. This deviation from linearity could be due to exhaustion of cofactors or oxygen or both. Other possible explanations are product inhibition (32) and the existence of endogenous inhibitors (6).

The effect of pH was also determined since previous works did not indicate whether the values used were those of the buffers or of the reaction media. The pH values in curve A of Figure 5

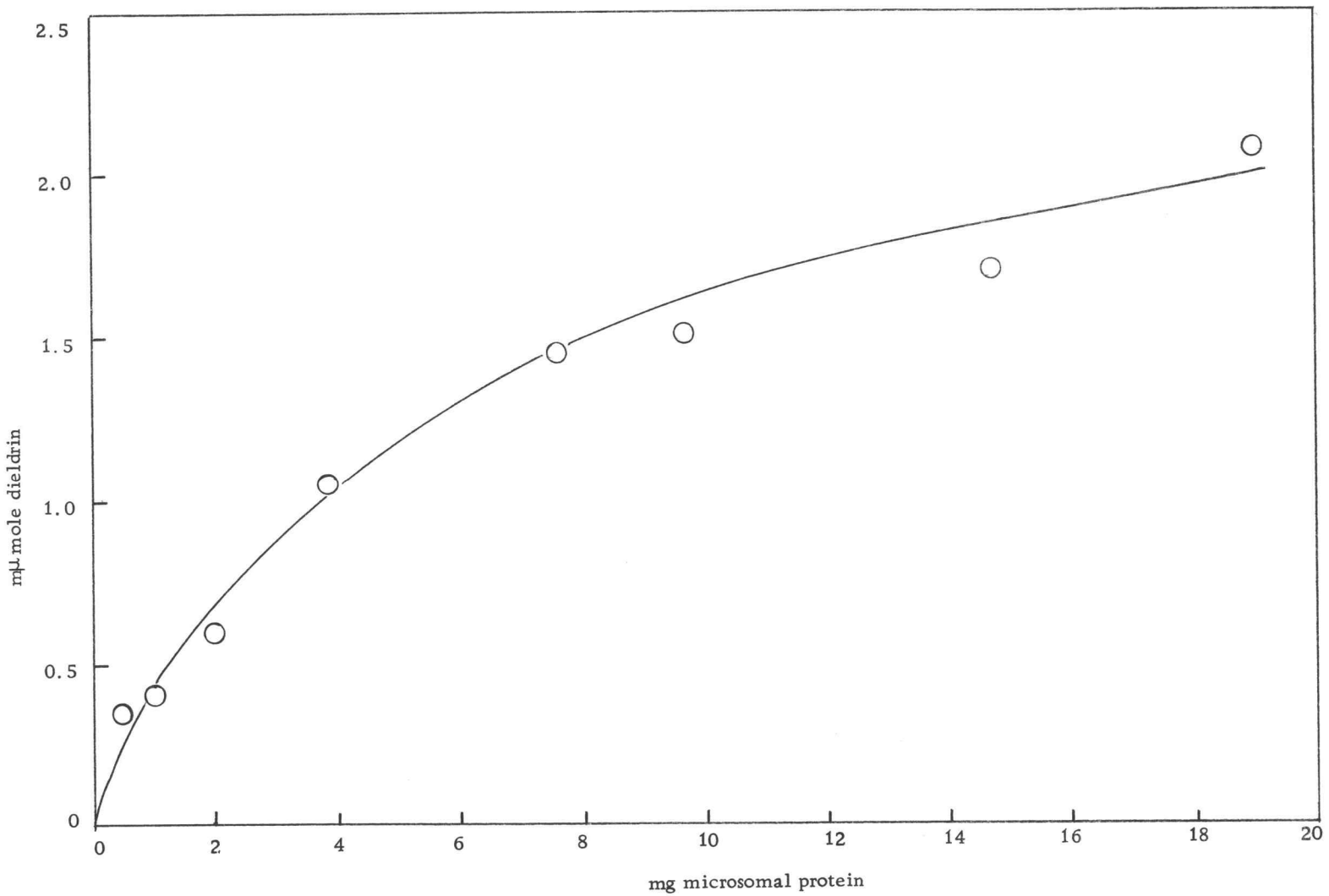

Figure 4. Aldrin epoxidation by male rat liver microsomes. I. Activity vs. protein level.

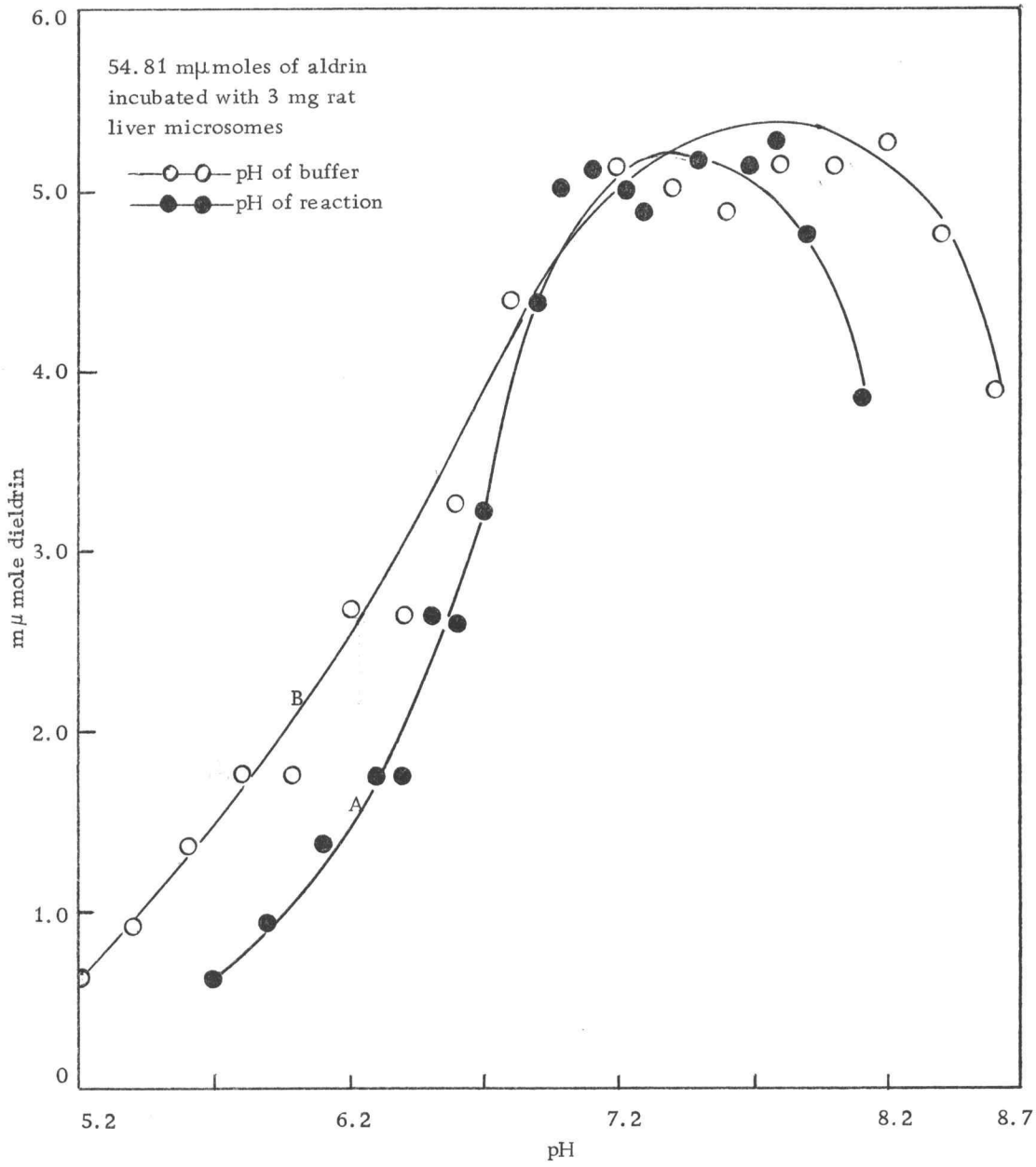

Figure 5. Aldrin epoxidation by male rat liver microsomes.
II. Activity vs. pH.

were obtained by measuring the pH of the reaction mixture at the end of the 15 minute incubation. In curve B, activities have been plotted against pH values of the buffers used. Curve A has a peak covering pH values from 7.0 to 7.7, with an optimum at around 7.3. The broad peak in curve B extends from pH 7.2 to pH 8.2 with an optimum at 7.8. Readjustment of the pH from either extreme on the curve to pH 7.5 after a period of incubation did not help restore activity. This indicates that pH may have affected the stability of the enzyme.

Epoxidation by Trout Liver Microsomes

The optimal conditions for aldrin epoxidation by rat liver microsomes were employed for the study of the same reaction by trout liver microsomes. After 15 minutes' incubation, only a minute amount of dieldrin, along with an average recovery of 98% of the added aldrin was in the hexane extract. An average of 0.36 mμmole of dieldrin (range = 0.12 to 0.47) was formed when 4 mg of trout liver microsomes was incubated with 54.8 mμmoles of aldrin for 15 minutes. Dieldrin formation remained at this low level even when 24 mg of microsomal protein was incubated for two hours. Heptachlor was also included as substrate in these studies; similar results were obtained. Conversion of aldrin by trout liver microsomes is compared with that of other organisms in Table I.

TABLE I. ALDRIN EPOXIDATION IN VITRO BY DIFFERENT SPECIES.

Microsomal source	Specific activity[1]	Reference
Quail liver (male)	108	20
Rat liver (male)	84	20
Housefly (mixed sexes)	41	43
Rat liver (female)	21	20
Quail liver (female)	9	20
Trout liver (mixed sexes)	6	
Blowfly (mixed sexes)	3	43

[1] Specific activity = mμmoles of dieldrin $\times 10^3$ mg^{-1} min^{-1}.

Recently it was reported that aldrin may be metabolized directly, or indirectly through dieldrin, to some other metabolites (12). These metabolites are of a more hydrophilic nature than aldrin or dieldrin and could have escaped detection by the analytical method used. However, since 98% of the added aldrin was recovered, other metabolites, if they exist at all, would not be in significant quantity.

It was then suspected that the small amount of dieldrin could have resulted from a non-enzymatic conversion of aldrin, possibly one similar to the Udenfriend aromatic hydroxylation model system (48). However, Nakatsugawa and co-workers recently reported that such a model system could not epoxidize (32). In the present work,

incubation without microsomes or with heat-denatured microsomes produced no detectable dieldrin.

The low epoxidative activity in trout reported here is in keeping with the finding of other workers concerning oxidative microsomal reactions in fish. La Du et al. reported in 1955 that while compounds such as aminopyrine, methylaniline and hexobarbital underwent extensive oxidative demethylation in mammalian liver microsomes, they were not affected by fish microsomes (23). In their in vivo studies, they found that these compounds were excreted unchanged by fish. Adamson and associates found in a comparative study of several species that fish and amphibians lacked oxidative microsomal drug enzymes (1).

Effect of Isolation Procedures

Hepatic microsomes consist mainly of membranes with and membranes without ribosomes bound to them. The membranes completely enclose a vasicular space (16). A number of enzymes carrying out various oxidative and conjugative reactions have been found either bound to, or included within, the endoplasmic membrane (14), and may be covered by a lipid barrier (19). The disruption of this delicate arrangement during isolation of the microsomes might have inactivated the epoxidative system in trout. To examine this possibility, liver slices of both rat and trout were prepared from fresh

tissues and were incubated with aldrin in the regular reaction medium. Better than 20% of the aldrin was converted to dieldrin in 15 minutes in the case of male rat liver slices, while only 0.4% of the aldrin was converted by the liver slices of trout. Activity in the whole homogenate and supernatant from the low speed spin, as well as that from the high speed centrifugation, was also measured. No dieldrin was obtained with the high speed supernatant but the low level previously obtained was found with the other two fractions.

Effect of Temperature, pH and Substrate Level

Adamson and associates noticed that azo reductase in shark liver homogenate was active at 26° C but was completely abolished at 37° C (1). The difference in the temperature range of the habitats of terrestrial and aquatic animals may explain the difference in enzyme activities. That trout activity is low at 37° C may be due to an unfavorable temperature or an unnatural pH of the reaction medium. Experiments were designed to examine these two factors. The yield of deldrin stayed at around 0.7% (0.38 millimicromole) when incubations were carried out at 2, 11, and 22° C, within a pH range of 6 to 8.5.

Substrate level was also varied. Aldrin concentrations of 5, 13, 27, 40 and 100 mμmoles were all converted to approximately the same low level of dieldrin previously obtained. Thus

inappropriate substrate level was not likely the cause for the poor yield.

Effect of Added Cofactors

The essentiality of NADPH in the in vitro microsomal epoxidation of aldrin has been clearly demonstrated (32). The level of cofactors constituting an NADPH-generating system used in the foregoing investigations were already in excess. Doubling the amounts of these constituents had no effect on either the rat or trout liver microsomal activity.

Metal ions often play the role of activators or essential components in many enzymic systems. Aldrin epoxidation by trout liver microsomes was tested in the presence of 10^{-3} M Fe(II), Mn(II), Co(II), and Mg(II), respectively; no effect on the activity was observed. Similar result was obtained by adding 10^{-3} M FMN to the incubation. The addition of the same level of FAD gave a two-fold increase in activity. This is in agreement with Adamson's report that riboflavin, FMN and FAD at 10^{-3} M accelerated mammalian microsomal nitro and azo reductase (1). Although an FAD-flavoprotein has been found in microsomes (40), the significance of FAD stimulation of epoxidation has yet to be elucidated.

Possible Inhibition by Endogenous Factors

At this point, the possibility that an endogenous inhibitor in trout liver microsomes preventing aldrin epoxidation still remained. Such inhibition exists in the deamination of amphetamine by rat and dog microsomes (6). It was reported that dog and rat liver microsomes converted amphetamine only to p-hydroxyamphetamine, but rabbit liver microsomes deaminated it to yield phenylacetone and ammonia. The deaminase activity of rabbit microsomes was markedly depressed when rat microsomes were added. The authors concluded that the low activity in dog and rat microsomes was due to inhibitory factors rather than to a deficiency of enzymes. Naturally occurring inhibitor(s) of microsomal oxidation has also been found in housefly microsomes (30).

To investigate this possibility, male rat liver microsomes were incubated with trout liver microsomes. Instead of an inhibitory effect, there was an enhancement[1] in epoxidative activity. The enhanced activities obtained by combining various levels of rat liver microsomes and trout liver microsomes were shown in Figures 6 and 7. Results of all combination experiments indicated that the extent of augmentation depended primarily on the original activity

[1] Activity is said to be enhanced when the activity of the combined system exceeds the sum of those of the separate preparations.

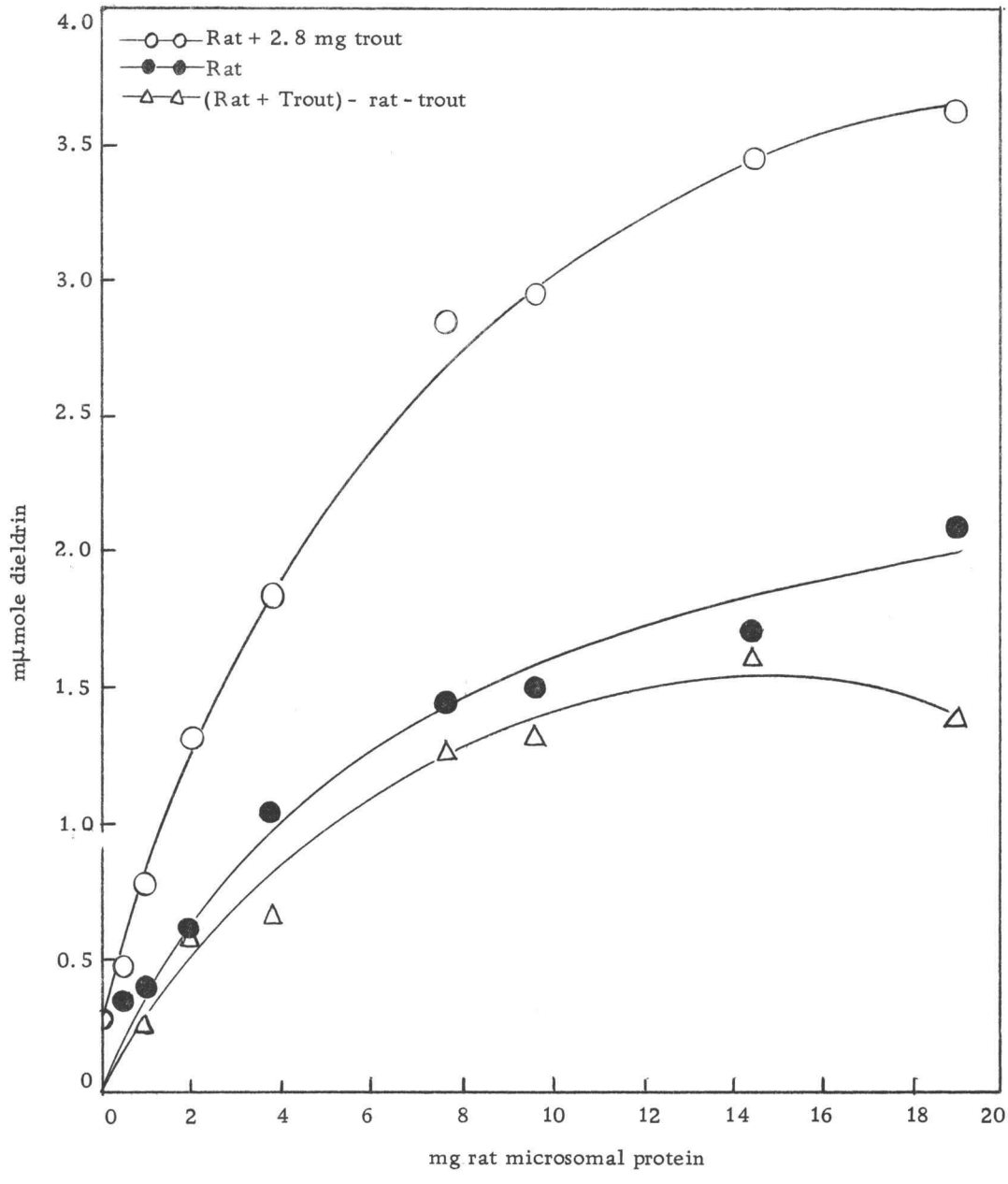

Figure 6. Epoxidation of aldrin by rat liver microsomes added to trout liver microsomes.

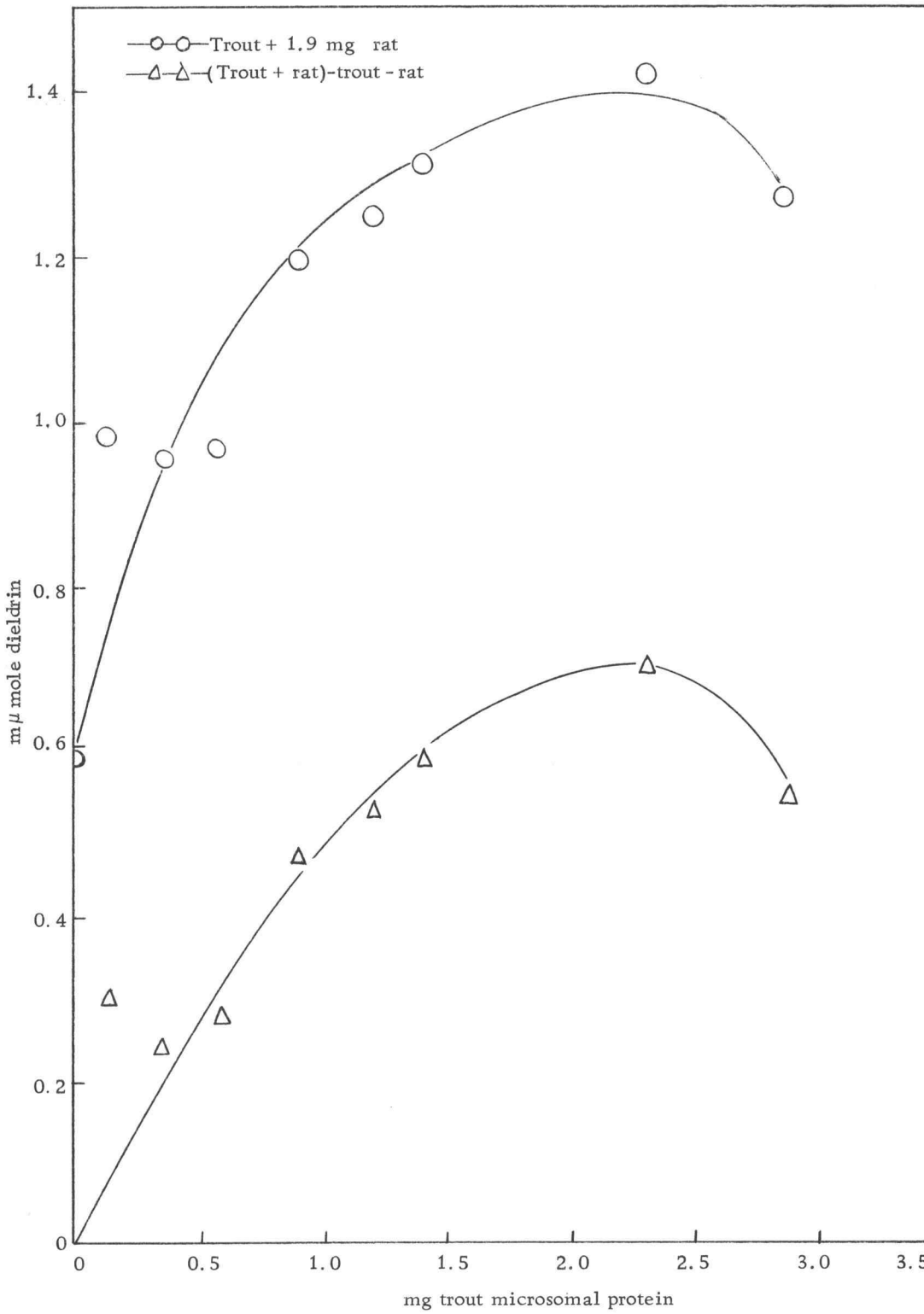

Figure 7. Epoxidation of aldrin by trout liver microsomes added to rat liver microsomes.

of the rat, a point considered later in this thesis. This dependence strongly suggests that it is the fish preparation that contains the enhancing factor(s). According to the interpretation for the deamination studies mentioned above (6), one might infer that the low epoxidative activity in fish was due to a deficiency of certain enzymes. Further work was done with the hope of verifying the above conclusion.

Microsomal Electron Transport and the Epoxidase System

The first example of the *in vivo* demethylation of a synthetic drug was reported by Butler and Bush about 26 years ago (8). Since then, the demonstrations of metabolism of foreign, or xenobiotic (29), compounds, in mammalian systems have been numerous. In 1955 La Du *et al.* (23) and Axelrod (3, 4) reported observations of such oxidative metabolic reactions in an *in vitro* system. These reactions were catalyzed by the microsomal fraction isolated from liver and a few other organs and required oxygen and either NADH or NADPH. Similar *in vitro* oxidations of cyclodiene compounds by liver microsomes have also been discovered (22, 32, 51). But the relationship between these oxidative enzymes and the microsomal electron transport system, as illustrated in Figure 1, was not established until recently (11). In the NADPH-dependent electron

transfer chain, detoxication is preceded by at least three known components. If the epoxidative enzyme(s) occupies that same position as other detoxicative enzymes in the NADPH chain, a deficiency in any of the preceding components in trout liver microsomes would impair aldrin conversion.

However, before these enzyme levels were considered, the relationship between the epoxidative enzyme(s) and the known microsomal electron transport components needed to be established. It would be helpful if one could demonstrate that any agent that prevented the oxidation of NADPH by the flavoprotein or the transfer of electrons from the reduced flavoprotein to X_1 also inhibited aldrin epoxidation (Figure 1). Unfortunately, specific inhibitors of these reactions are not known. However, PCMB and SKF 525-A are known to be general inhibitors of microsomal oxidations (15, 32). When either of these two compounds was present at 10^{-5} M in the incubation mixture, aldrin epoxidation was indeed reduced. Some authors proposed that a sulfhydryl-containing enzyme (27, 38) possibly associated with copper (29) and quinone (33), represented by X_1 in the chain, acts as direct electron acceptor from the reduced flavoprotein. This may account for the PCMB inhibition. However the possibility that the epoxidative enzyme(s) itself may also be PCMB sensitive still remains.

Instead of relying on classical inhibitors, artificial electron

acceptors which compete with the epoxidative enzyme(s) for electrons in the microsomal electron transfer chain were used. Cytochrome c (68 mμmoles), DCPIP (190 mμmoles), and NT (150 mμmoles), when tested separately, completely inhibited aldrin epoxidation. Since cytochrome c and DCPIP received electrons through the reduced flavoprotein and NT from the reduced X_1, thus interrupting the flow of electrons further down the chain, any reaction beyond NT reduction requiring reducing equivalents should be inhibited. These results indicate that aldrin epoxidation, like other microsomal drug oxidations, must take place beyond NT reduction in the NADPH chain.

Microsomal Enzyme Levels in Trout Liver

With the relationship between epoxidation and the microsomal electron transport system established, an investigation of the component enzyme levels was undertaken. Reductions of cytochrome c, DCPIP, and NT by both rat and trout liver microsomes in the presence of NADPH were measured. Cytochrome P-450 contents of the two preparations have also been determined. Results are summarized in Table II. A typical CO-bound reduced P-450 spectrum is shown in Figure 8. The NADPH-dependent enzyme levels of trout liver microsomes are lower than those in rat. However, in comparison with the rat, they should be adequate to bring about

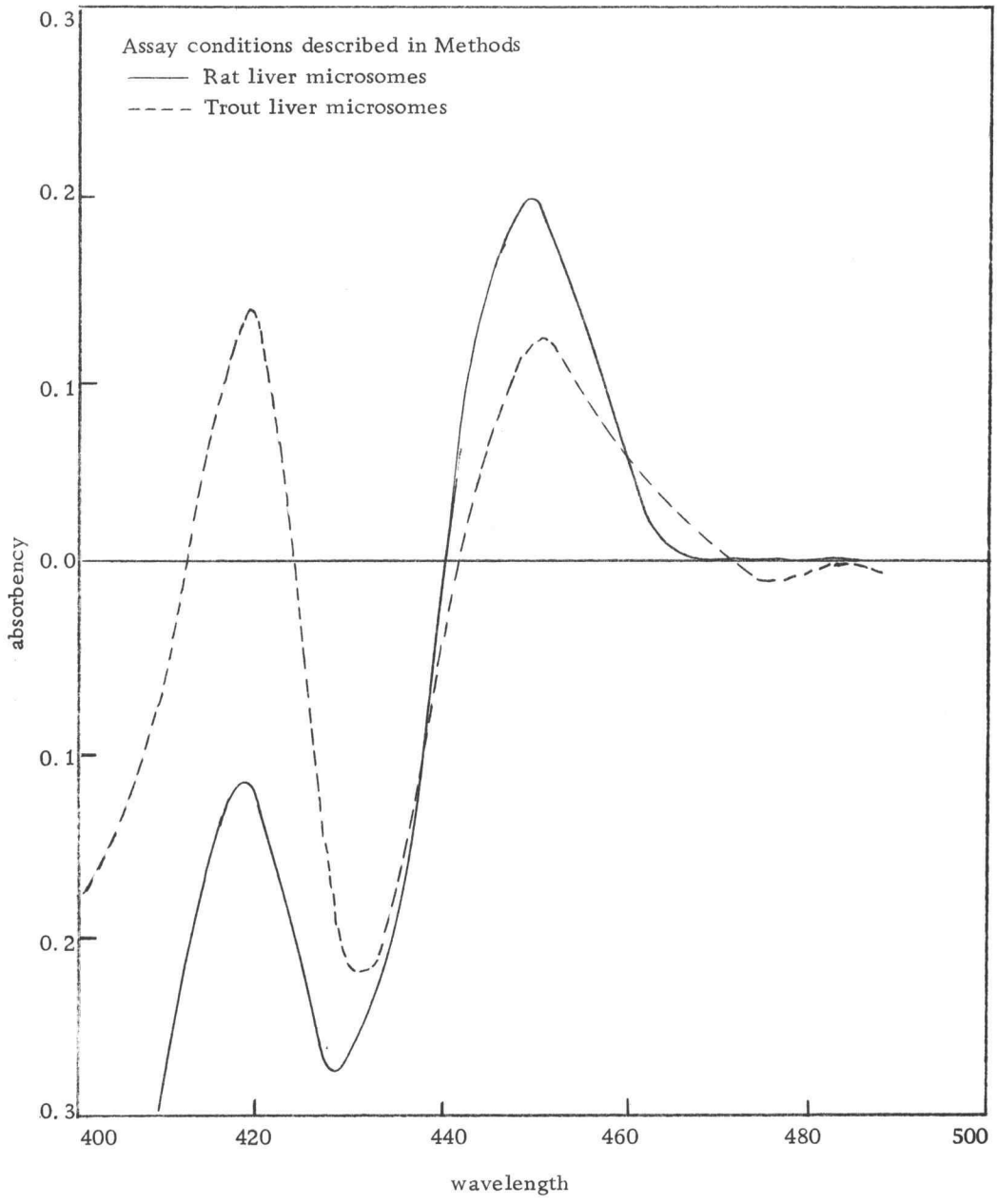

Figure 8. Microsomal cytochrome P-450 difference spectrum.

significant epoxidative activity in trout liver microsomes provided that the epoxidative enzyme(s) was present. Unlike epoxidation, the rate of these NADPH-requiring reactions increases linearly with protein concentration. Therefore it is not likely that aldrin epoxidation in trout liver microsomes is limited by a deficiency of the known components preceding the epoxidative enzyme(s).

TABLE II. HEPATIC MICROSOMAL ENZYME ACTIVITIES OF TROUT AND MALE RAT.

Microsome source	DCPIP reductase[1]	Cytochrome c reductase[1]	NT reductase[2]	Cytochrome P-450[3]
Male rat liver	62.0	88.0	1.25	0.335
Trout liver	9.6	9.5	0.31	0.210

[1] These two reactions are possibly catalyzed by the same enzyme (45).
Activity = change in O.D. $\times 10^3$ mg^{-1} min^{-1}

[2] Activity is expressed as in 1.

[3] P-450 concentration = mμmole mg^{-1} protein. A value of 91 mM^{-1} cm^{-1} has been used as a millimolar extinction coefficient for the difference in absorbence between 450 and 490 mμ in the CO difference spectrum of microsome-bound P-450 (40).

Characterization of the Enhancement Phenomenon

Since the limiting factor for epoxidation in trout liver microsomes is not one of the first three components, it must lie beyond cytochrome P-450 The dependence of the enhancement phenomenon

on the basal epoxidative activity of rat liver microsomes in the rat plus fish system suggested a deficiency of the epoxidative enzyme(s) in fish. This led to a thorough study of the observed enhancement.

Augmentation in certain enzymatic reactions can be caused by the addition of a non-specific protein (47). This was tested by combining male rat liver microsomes with heat-denatured trout liver microsomes, or with female rat liver microsomes. No enhancement was observed. The results shown in Table III indicate that a specific factor was involved.

TABLE III. SPECIFICITY OF ENHANCEMENT IN ALDRIN EXPOXIDATION BY TROUT LIVER MICROSOMES.[1]

Added microsomes	Rat (male)	Rat (female)	Trout	Trout (denatured)
+ none	0.53	0.25	0.1	0
+ rat (male)	--	0.65	1.02	0.30

[1]Activities are expressed in mμmole dieldrin formed in 15 min. 2 mg of protein was used in individual incubations and 2 mg of each was used in the combined incubations.

Rat and trout preparations were combined in assays for NADPH-cytochrome c reductase, NADPH-DCPIP reductase and NADPH-NT reductase activities. Similar enhancement was observed only in NT reduction (Table IV).

Attempts to solubilize both the rat and trout systems were

made. Acetone extraction or treatment with either steapsin or snake venom (49) abolished the epoxidative activity of liver microsomes. Combinations of these treated preparations with fresh trout microsomes showed no activity. Both steapsin- and snake venom-treated trout preparations were slightly inhibitory to aldrin epoxidation when added to native rat liver microsomes. This might have been due to incomplete inactivation of these digestive enzymes in the treated fish preparation. Acetone powder preparations of both trout and rat liver were able to augment aldrin epoxidation and NT reduction in native rat liver microsomes, indicating the non-lipid or possibly proteinaceous nature of the enhancing factor. Results are summarized in Table V.

TABLE IV. NADPH-DIAPHORASE ACTIVITIES OF MICROSOMES IN TROUT AND RAT LIVER.

Preparation	Change in O.D. $\times 10^3$ min^{-1}		
	DCPIP[1]	Cytochrome c[1]	NT[2]
Rat (male)	15.5	22.0	1.60
Trout	8.0	7.9	0.40
Rat (male) + Trout	21.3	30.0	8.75

[1] mg protein: rat = 0.25; trout = 0.83.

[2] mg protein: rat = 1.30; trout = 1.30.

TABLE V. ENZYMIC ACTIVITIES OF SOLUBILIZED MICROSOMES.

Reaction studied	Preparations	Snake venom treatment	Steapsin treatment	Acetone powder
NT reduction	rat	---	---	active
	trout	---	---	active
	rat[1] + trout[2]	---	---	enhanced
Aldrin epoxidation	rat	inactive	inactive	inactive
	trout	inactive	inactive	inactive
	rat[1] + trout[2]	active[3]	active[3]	enhanced

[1] Native (untreated) rat liver microsomes.

[2] Trout liver microsomes treated as indicated.

[3] The combined systems were active but were slightly inhibited.

Functions of Components

The distribution of the component enzymes, namely, NADPH-cytochrome c reductase, NADPH-NT reductase and cytochrome P-450, and the role they play in epoxidation are evident from the results of the fractionation experiments (TABLES VI, VII, and VIII). NADPH-cytochrome c reductase was sedimented in the regular microsomal pellet (P-I), whereas the NT reductase remained mostly in the supernatant fraction which is usually labeled the soluble fraction. Cytochrome P-450 was found in the P-I, P-III and to a slight extent in S-I fractions. This is true for both fish and rat. In the case of aldrin epoxidation, the regular rat liver microsomal pellet (P-I) contained only 70% of the total activity with the rest left in the S-I fraction.

The P-III fraction of rat, which should consist of P-I and P-II fractions, was 3.3 times the sum of P-I and P-II in NT reducing activity and 2.5 times the sum of P-I and P-II in aldrin epoxidation activity. In cytochrome c reduction, P-I and P-II showed the same level of activity. Results were quite different when P-I and P-II fractions were mixed. With this combination the rate of NT reduction was more than twice that of P-III and the rate of aldrin epoxidation was almost identical to that of P-III. This indicates that P-I plus P-II is a more efficient system, perhaps by providing a more

TABLE VI. NADPH-CYTOCHROME C REDUCTION[1] BY VARIOUS COMBINATIONS OF TROUT AND RAT LIVER MICROSOME FRACTIONS.

Source of fractions	Addition	Fractions[2]					
		P-I	P-II	P-III	S-I	S-II	S-III
Rat	None	96	53	96	15.8	5.5	6.0
Rat	Rat P-I	--	--	--	105	--	--
Trout	None	5.4	1.2	58	2.7	0.80	0.98
Trout	Rat P-I	98	--	--	98	--	--

[1] Activity expressed as change in O. D. /20 mg fresh liver equivalent/minute.

[2] Fractions defined in Figure 2.

TABLE VII. NEOTETRAZOLIUM CHLORIDE (NT) REDUCTION[1] BY VARIOUS COMBINATIONS OF TROUT AND RAT LIVER MICROSOME FRACTIONS.

Source of fractions	Addition	Fractions[2]					
		P-I	P-II	P-III	S-I	S-II	S-III
Rat	None	15	7	72	70	40	32
Rat	Rat P-I	--	165	--	520	335	215
Trout	None	tr[3]	tr	14	20	6	12
Trout	Rat P-I	34	430	425	360	380	440

[1] Activity expressed as change in O. D. $\times 10^{-3}$/25 mg fresh liver equivalent/10 min.

[2] Fractions defined in Figure 2.

[3] Activity less than 2×10^{-3} O. D. unit.

TABLE VIII. EPOXIDATION[1] OF ALDRIN BY VARIOUS COMBINATIONS OF TROUT AND RAT LIVER MICROSOME FRACTIONS.

Source of fractions	Addition	Fractions[2]					
		P-I	P-II	P-III	S-I	S-II	S-III
Rat	None	3.05	tr[3]	7.56	1.47	tr	tr
Rat	Rat P-I	--	7.80	--	12.5	12.3	10.2
Trout	None	tr	tr	tr	tr	tr	tr
Trout	Rat P-I	8.28	12.8	12.5	15.1	13.9	14.1

[1]Activities expressed as mμmoles dieldrin/100 mg fresh liver equivalent/15 min.

[2]Fractions defined in Figure 2.

[3]Activity less than 0.1 mμmole dieldrin/15 min.

even distribution of the components. By the addition of any fish liver fraction, or any rat liver fraction, that could reduce NT efficiently, the NT reductase and aldrin epoxidation activities of the P-I fraction of rat are greatly enhanced.

This parallel relationship between the enhancement in NT reduction and aldrin epoxidation indicates that the enhancement factor comes in at the X_1 level. X_1 has been suggested as the most likely limitation in detoxication, or in a broader sense, xenobiotic metabolizing systems (45). That this also holds for epoxidation in rat liver microsomes is evident from the results described above. The enhancing factor is most likely X_1 which is supplied by the fish preparation (Figure 9). The low value shown for NT reduction (component X_1) for trout in Table IV was probably due to a limitation by the preceding component, namely flavoprotein, and not to a deficiency of X_1. The results of fractionation of trout liver homogenate indicate that X_1 is a low molecular weight component. Its proteinaceous nature has been verified by heat-denaturation and acetone precipitation. Similar interpretations have been given to the physical nature of X_1 by other authors (11). It is also loosely bound to the membrane and thus is easily lost to the soluble fraction.

Results of these experiments and the interpretation for the enhancement phenomenon suggest that the low epoxidative activity in trout liver microsomes is due to a deficiency of the epoxidative

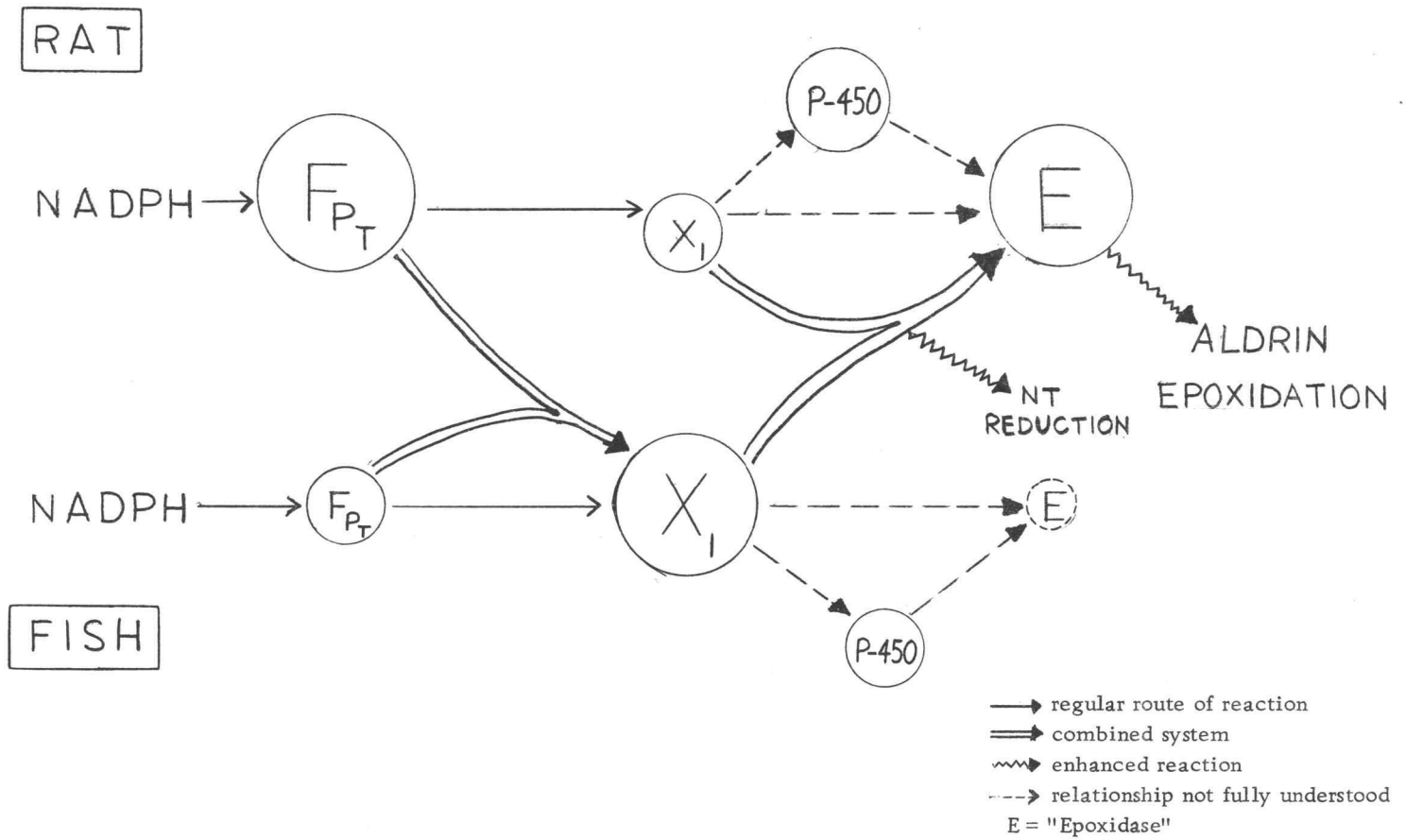

Figure 9. Schematic representation of the interaction between male rat and trout liver microsomal electron transport systems in aldrin epoxidation.

enzyme(s). Support for this conclusion can be summarized as follows:

1. The ability of trout liver microsomes to reduce added cytochrome c, DCPIP and NT, and its cytochrome P-450 content, far exceed that of its activity in epoxidizing aldrin.
2. Trout liver microsomes are able to augment aldrin conversion in male rat liver microsomes only if the latter is active in epoxidation. This is evidenced by the fact that:
   a. no enhancement in aldrin conversion was observed when a rat liver acetone powder preparation active in cytochrome c and NT reductions was combined with native trout liver microsomes (Table V); and
   b. fractions of rat liver homogenate devoid of epoxidative activity showed no aldrin conversion when combined with a fraction of trout liver microsomes which has been proved to be active in augmentation (Table VIII).

In Vivo Production of Dieldrin by Trout

Boyle and co-workers found large quantities of dieldrin in both muscle tissues and viscera of fish exposed to aldrin for 22 days (5). This seems to contradict the conclusion drawn above. However, in another instance when death of fish occurred in five days after a massive dose of aldrin, little dieldrin was found in the viscera. The

slow conversion of aldrin to dieldrin reported in these two instances may be interpreted in at least three different ways. The conversion may have been performed by agents other than the fish; examples are organisms in the medium and microorganisms in the fish. Secondly, the low rate of conversion found in this research, if it is indeed enzymatic, may still be sufficient to produce significant quantities of dieldrin in fish continuously exposed to aldrin. Thirdly, additional epoxidative activity may be induced by the toxicant in such fish, a phenomenon currently under extensive study (31).

Evolution of Detoxicative Enzymes

The evidence collected in this research leads to the conclusion that trout cannot epoxidize because it lacks the specific enzyme system(s). Adamson et al. (1) speculated that in the evolution of life on earth, aquatic species did not require such oxidative systems. This postulate was derived from the results of a series of comparative experiments on the reductive enzymes of fish, amphibians and mammals (1). It was found that azo-reductase exists in the most primitive of fish, elasmobranchii, and in amphibia. Both azo- and nitro-reductase are present in reptile, bird, and mammals. Activity is highest in the latter two. Furthermore, these reductive enzymes are present in the fetal and neonatal rat in which the oxidative enzyme system has not yet developed. An enzyme system

that reduces the nitro group of p-nitro-benzoic acid has been found in E. coli (41, 42). In contrast to reductive enzymes, microsomal oxidative enzyme systems are lacking in fish and amphibians. These findings suggest the evolution of azo- and nitro-reductase which are probably more primitive. These reductive enzymes might have evolved in response to toxic compounds formed during the "primeval atmosphere on earth" in the words of these authors (1). Potentially toxic, lipid-soluble xenobiotics ingested by the aquatic species can diffuse through the membranes of the gills or through the skin into the water unchanged. The xenobiotic oxidizing enzymes were not required until the higher vertebrates migrated from water to land. Since they could no longer rid themselves of these lipid-soluble toxic substances, non-specific enzyme systems had to be developed to convert these materials to less lipid-soluble products which could be more rapidly excreted by the kidney.

The preceding interpretation suggests the evolving of an enzyme system in an organism to survive the changing environment. Indeed, a branch in present research lends support to this phenomenon. Substrates for certain microsomal oxidations induce the synthesis of these enzyme systems (15). Rats fed on a diet fortified with 1, 1, 1-trichloro-2, 2-bis(p-chlorophenyl)ethane (DDT) or received an injection of phenobarbital are able to metabolize DDT,

aldrin, phenobarbital, and numerous other xenobiotics much better than control animals (15, 20, 31). To avoid biological variations and to maintain the availability of animals for experimentation, domesticated fish from the same stock raised under relatively constant environment were used in this research. On the basis of the preceding discussion and cited experimental data, results could have been quite different if fish from highly polluted waters were examined. Furthermore, it would be of interest to see if oxidative enzymes could be induced by subjecting these domesticated fish to xenobiotic treatment.

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