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by

Ronald Lee Hybertson

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major Subject: Zoology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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Biochemical and cytological aspects of in vitro metabolism of parathion by rat liver cell fractions

Ronald Lee Hybertson

Under the supervision of P. A. Dahm
From the Department of Zoology and Entomology
Iowa State University

This study presents a combined biochemical and cytological study of the in vitro metabolism of the organophosphate insecticide parathion by hepatic cell fractions of the laboratory rat. The cytological work includes an electron microscopical examination of liver homogenate and centrifugally prepared debris, mitochondrial, crude microsomal, rough microsomal, and smooth microsomal fractions. The biochemical work includes an analysis of the in vitro metabolism of parathion by gas-liquid chromatography, thin-layer chromatography, and liquid scintillation spectrometry. Protein determinations were made on each cell fraction, and RNA content of the microsomal fractions was determined and used in conjunction with electron microscopy to assess the purity of the rough and smooth microsomes. The effects of varying several incubation parameters on parathion metabolism by crude microsomes were examined. Regression analyses and analysis of variance tests were performed on these results.

A comparison of the enzymatic activity by the six cell fractions showed the highest specific activity in the smooth microsomal fraction. The mean smooth microsome:rough microsome response ratio for both paraoxon production and aqueous metabolite production was 1.4:1. After adjusting
the nitrogen level in the specific activity calculations to exclude the contribution by the ribosomal component, the ratio was 1.2:1.

Specific activity differences as a function of the resuspension of crude microsomal pellets in several different aqueous solutions were not significant. The cesium chloride employed in the subfractionation of microsomes did not affect activity. An incubation mixture pH of 7.5 was optimal for parathion metabolism and maximal enzymatic activity occurred around the physiological temperatures of the rat (34.5-40.0 C). The specific activity of crude microsomes decreased with increasing microsomal concentration over a range of 0.3-3.1 mg protein/ml incubation mixture.

The metabolism of parathion was not linear with time and showed a decreasing rate for both metabolic pathways. Crude microsomes retained their enzymatic activity without significant loss while stored at -20 C for six weeks. Microsomal activity towards parathion was destroyed by several methods of solubilization.
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INTRODUCTION

In a world with increasing numbers of humans, food and fiber become of paramount importance. For many years, insects have competed with man for these basic needs. In the past half century man has marshalled a number of chemicals in an effort to control agriculturally and medically important insect-pest species. Unfortunately, his arsenal contains naturally occurring poisons and synthetic poisons, most of which are detrimental to living systems in general and not species-selective as one might hope. The continued use of insecticides of many different chemical forms poses problems for the biologist who is concerned with their impact and influence on the physiology of nontarget as well as target organisms. Toxicological research today must include among its considerations cellular structures and functions as they relate to the phenomena of intoxicative and degradative metabolism of insecticidal compounds.

An enzyme system found in microsomes prepared from the livers of adult rats (Nakatsugawa and Dahm 1967, Neal 1967a), mice, and guinea pigs (Neal 1967a) metabolized parathion in vitro to paraoxon. Parathion, O,0-diethyl O-p-nitrophenyl phosphorothionate, is an organophosphate insecticide; paraoxon, O,0-diethyl O-p-nitrophenyl phosphate, is a powerful anticholinesterase metabolite of parathion. Concurrent with the formation of paraoxon, a portion of the parathion was converted to diethyl phosphorothioic acid and p-nitrophenol by a reaction that also required a reduced nucleotide cofactor and molecular oxygen. Kojima and O'Brien (1968) have reported that paraoxon is degraded to diethyl phosphoric acid and p-nitrophenol by microsomal enzymes of rat liver in a reaction that is
unaffected by several different cofactors.

The objective of this investigation has been to continue these studies by examining certain subcellular characteristics and physiological parameters associated with the in vitro metabolism of parathion by rat liver homogenates and centrifugally prepared cell fractions. Abbreviations of selected compounds referred to in the text are summarized on page 3 together with the principal pathways of the in vitro metabolism of parathion by microsomal enzymes of the rat (Nakatsugawa and Dahm 1967, Neal 1967a, 1967b, Kojima and O'Brien 1968).

An outline of the fractions used, of the metabolic pathways considered, and of the analytical procedures employed is presented in Figure 1.
Abbreviations

DEPA (O,O-diethyl hydrogen phosphate = diethyl phosphoric acid)

DEPTA (O,O-diethyl hydrogen phosphorothionate = diethyl phosphorothioic acid)

DFP (diisopropylphosphorofluoridate)

GSH (reduced glutathione)

NAD = DPN (nicotinamide adenine dinucleotide)

NADH₂ = DPNH₂ (reduced nicotinamide adenine dinucleotide)

NADPH₂ = TPNH₂ (reduced nicotinamide adenine dinucleotide phosphate)

Parathion Metabolism
**Rat Liver Preparations**

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<tr>
<td>Mitochondria</td>
<td>RNA</td>
<td>TLC</td>
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<tr>
<td>9,340 g avg Supernatant</td>
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<td>Total Microsomes</td>
<td>EM</td>
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<td>Rough Microsomes</td>
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<tr>
<td>Smooth Microsomes</td>
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<tr>
<td>105,200 g avg Supernatant</td>
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<tr>
<td>269,000 g avg Supernatant</td>
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**Reactions**

Parathion → Paraoxon

- → p-nitrophenol

DEPTA → DEPA

→ Dealkylation

Products

Figure 1. Research scheme outlining enzyme preparations, analytical methods used and potential metabolic pathways. Broken arrows indicate metabolites not specifically identified.
REVIEW OF LITERATURE

To introduce this study of the metabolism of parathion, I shall review briefly the development, mode of action, and metabolism of insecticidal organophosphorus esters of which parathion is an example.

Organophosphates and their Metabolism

The last two decades have been marked by the marketing of a new class of insecticidal compounds known as organophosphates. The term organophosphates is used loosely to apply to all compounds that contain carbon and are derivative of phosphoric acid. Organophosphates have become the insecticides of choice for reasons of low mammalian toxicity and growing problems of insect resistance and environmental persistence of chlorinated hydrocarbons. Consequently, chemical companies have turned to "safe" organophosphates such as Diazinon, malathion, and Sumithion.

The first esterification of phosphoric acids and alcohol is attributed to Lassaigne in 1820 (Kosolapoff 1950). In the early 20th century Michaelis was a leader in the synthesis of compounds containing phosphorus and nitrogen, particularly amidates of phosphoric, phosphorothionic and phosphinic acids. Arbusow (1906) reported the synthesis of a number of phosphonates and phosphates which were forerunners of the compounds used as anticholinesterases today. Lange and von Krueger (1932) prepared several dialkyl-phosphorofluoridates. However, no mention was made of the poisonous nature of these compounds. It became apparent sometime in the 1930's that these compounds were potential chemical warfare agents, and work began on the synthesis of more compounds both in England and Germany in
the early days of World War II. The English workers first studied diisopropylphosphorofluoridate (DFP) which they made in 1941 and were particularly attracted to the miotic effects and high toxicity of this compound. As a result, DFP and similar compounds became known as "nerve gases" and were applied as mists with concentrations of the order of 1 part per million. Gerhardt Schrader of I. G. Farbenindustrie headed the work in Germany. These workers realized the insecticidal importance of these compounds and by the end of the war had made many of the insecticidal phosphates in use today, including parathion which was synthesized in 1944 (O'Brien 1960).

The anticholinesterase property of organophosphates was first found in 1941 by a British group at Cambridge who published their findings after World War II was over (Adrian et al. 1947). Their work suggested that organophosphates were similar in action to eserine which had long been known as a potent anticholinesterase. Another Cambridge group, Mackworth and Webb (1948), studied the anticholinesterase activity of DFP and found that DFP inhibition was progressive and irreversible, unlike that of eserine.

The mode of action of organophosphates is only partially understood. Most mammalian physiologists accept the fact that in acute poisoning the action of organophosphates is almost exclusively attributed to an inhibition of the cholinesterase of the nervous system. However, debate is vigorous on the mode of action in insects. A fairly good knowledge of the mechanisms by which the compounds are metabolized in plants and animals is available. The biological significance of cholinesterase, particularly in
mammalian systems, is well understood. This important enzyme catalyzes the hydrolysis of acetylcholine released by stimulation of cholinergic portions of the nervous system. Therefore neuromuscular junctions, parasympathetic effectors, sympathetic synapses, and the sympathetic innervation of the adrenal and sweat glands are affected by organophosphate poisons. The main signs of poisoning resulting from the accumulation of acetylcholine are lacrimation, salivation, bronchoconstriction, bradycardia, muscular twitchings and paralyses, convulsions, hyperexcitability, vasoconstriction, and central paralysis of the respiratory system with the ultimate cause of death usually being asphyxia (O'Brien 1960, Heath 1961).

Phosphorothionates and phosphoramidates are typically weak anticholinesterase inhibitors in vitro (Aldridge and Barnes 1952). However, their conversion to potent inhibitors, known as "activation", was first suggested for schradan in 1950 by Gardiner and Kilby and demonstrated by them to be caused by metabolism in the liver (Gardiner and Kilby 1950). Diggle and Gage (1951) showed a similar activation of parathion by liver slices, and Gage (1953) demonstrated that the anticholinesterase produced in vivo was paraoxon. The degradation of organophosphates in animals was first studied by Mazur (1946). He found a DFP-hydrolyzing enzyme which occurred principally in liver tissue.

Much information exists in regard to the metabolic pathways and terminal residues of organophosphorus compounds in mammals, insects, and plants (Lykken and Casida 1969). Recent concentration of research effort in the insecticide field has been focused on metabolism of this class of compounds, resulting in extensive literature on this subject (Fukuto and
Metcalf 1969). The following conversions of dialkyl aryl phosphorothio- nates were described by Plapp and Casida (1958a): (1) activation;
(2) degradation;

\[
\begin{align*}
(RO)(HO)P(S)OAr & \rightarrow (RO)_{2}P(S)OAr \rightarrow (RO)_{2}P(S)OH \\
(RO)(HO)P(O)OAr & \rightarrow (RO)_{2}P(O)OAr \rightarrow (RO)_{2}P(O)OH
\end{align*}
\]

O'Brien (1962) reviewed the desulfuration of organic compounds involving the enzymatic substitution of oxygen for sulfur. Phosphorothionates such as parathion are converted to phosphates which are generally more toxic products. The relative toxicity of a particular compound in a given species will depend on the rate of desulfuration of the parent compound, on the rate of reaction of the desulfurated product with cholinesterase, and on the rates of inactivation of the parent compound and its desulfurated product. Davison (1955) observed that livers of male rats activated (desulfurated) parathion 1/10 as rapidly as did livers of female rats, and correspondingly the males were 1/2 as sensitive as females to the compound. SKF-525A (2-diethylaminoethyl 2:2-diphenyl-valerate hydrochloride) inhibits the desulfuration of phosphorothionates (Davison 1955) as do several insecticide synergists such as MGK 264, piperonyl butoxide, and certain steroids such as testosterone propionate and androstanoioline (Dahm et al. 1962). Since the balance between activating and inactivating processes seems to determine the toxicity of phosphorothionates, their toxicity should be decreased by SKF-525. O'Brien observed (1961, 1962) that parathion was not antagonized in vivo; rather the level of paraoxon in female mice was increased. By injecting
radioactive paraoxon into mice and cockroaches with and without pre-treatment with SKF-525, it was determined that the hydrolysis of paraoxon was decreased in the treated animals, but a potentiation of toxicity was not observed. It appears that in vivo SKF-525 blocks both activation and hydrolysis with the blocking of hydrolysis as the major effect. The lack of potentiation can be interpreted to mean that whole body levels of toxicant may not be closely correlated with toxicity (O'Brien 1961, 1962).

Other conversions of organophosphates include the oxidation of the thioether sulfur, oxidation of the phosphoramidate moiety, deamidation, C-S bond cleavage and possible N-dealkylation (Fukuto and Metcalf 1969). The aryl portion in the preceding scheme can be replaced by X to indicate a more inclusive scheme of metabolism. More extensive discussions of organophosphate insecticides, their history, chemistry, biological effects, and metabolism have been written by O'Brien (1960, 1967) and Heath (1961).

Metabolism of Parathion

Parathion (0,0-diethyl 0-P-nitrophenyl phosphorothionate, C_{10}H_{14}O_{5}NPS, m. w. 291.3) is a biologically active compound with an acute intraperitoneal LD_{50} of 3.6 mg/kg for adult male rats and 1.5 mg/kg for weanling male rats (Brodeur and DuBois 1963), and an acute oral toxicity of 13 mg/kg and an acute dermal LD_{50} of 21 mg/kg for adult male rats (Gaines 1960). Parathion, also known as E 605, Thiophos® and by several other trade names, has become important throughout the world as a broad spectrum insecticide. Numerous efforts have been made to replace it by
better products, yet, it became firmly established as a standard product over two decades and it continues to gain importance from year to year in world agriculture (Lorenz and Sasse 1968). It has also served as a model compound in numerous studies concerning the bioactivation of phosphorothionate insecticides (Dahm and Nakatsugawa 1968).

Diggle and Gage (1951) observed that pure parathion had no appreciable in vitro inhibition of cholinesterase when incubated with a 1% rat brain homogenate. They found that when pure parathion was administered intraperitoneally to male rats there was a lag of a few minutes before cholinesterase inhibition developed. They suggested that parathion was converted by the liver to an active inhibitor. Likewise, Aldridge and Barnes (1952) found that parathion was converted to a more active inhibitor of cholinesterase when administered either intravenously or intraperitoneally to rabbits.

A substance with in vitro anticholinesterase activity was extracted from the livers of rats dosed with parathion (Gage 1953). The inhibitory compound was identified as paraoxon, based on evidence from paper-chromatography, absorption spectra, and rates of hydrolysis in alkaline solution. Concurrently, Metcalf and March (1953) studied the in vitro conversion of parathion and methyl parathion by mouse liver slices and various organs of the American cockroach. These highly purified thionophosphates were converted enzymatically to their oxygen analogues under aerobic conditions; conversion was prevented by heating, by homogenizing the gut tissue, and by various enzyme inhibitors. Davison (1955) demonstrated that rat-liver suspensions can convert parathion into a compound
inhibitory to cholinesterase by preparing the liver in solutions containing nicotinamide with addition of diphosphopyridine nucleotide or a boiled liver extract. He also found that the major part of the enzyme activity remained in the supernatant after centrifugal removal of intact cells, nuclei, and mitochondria. When this fraction was further separated into microsomes and a clear supernatant, the microsomal fraction was more active than the supernatant fraction but neither was as efficient alone as the two fractions together.

The pyrimidine nucleotide requirement for the activation of phosphorothoniates by liver microsomes was studied by O'Brien (1959). Reduced triphosphopyridine nucleotide \( \text{TPNH}_2 = \text{NADPH}_2 \) was found to be at least as good as reduced diphosphopyridine nucleotide \( \text{DPNH}_2 = \text{NADH}_2 \) while NAD was considerably less effective.

Dahm et al. (1962) compared the in vitro microsomal activation of several organophosphorus insecticides by manometrically assaying their incubation products with fly head and rat brain cholinesterase. The greatest increase in cholinesterase inhibition occurred with methyl parathion, Diazinon®, Co-Ral®, ronnel, Dowco 199, and Guthion® with lesser activation occurring with malathion and Trithion®. Nakatsugawa et al. (1968) studied analogs of parathion with substituted and unsubstituted 4-nitrophenyl ester structures for their metabolism in vitro by microsomes of rat and rabbit livers and housefly abdomens. All phosphorothoniates were metabolized via two oxidative pathways, activation to phosphate analogs and cleavage at the aryl phosphate bond. An oxidative degradation of \( \text{n-} \)propyl paraoxon was observed which required \( \text{NADPH}_2 \).
Plapp and Casida (1958a) found that in vitro and in vivo hydrolysis of Dow ET-57®, dicapthon, Chlorthion®️, methyl parathion, and parathion occurred at both the alkyl-phosphate and the aryl-phosphate bonds. The hydrolytic degradation of parathion, methyl parathion, and fenitrothion in mammals and plants leads to the formation of all possible hydrolytic products of dialkyl aryl phosphorothionates (Möllhoff 1968).

Plapp and Casida (1958a) studied the products formed by in vitro and in vivo hydrolysis of parathion and other dialkyl aryl phosphorothionate insecticides. The hydrolysis products from rats after treatment with $^{32}$P-parathion were 16% diethyl phosphoric acid (DEPA), 78% diethyl phosphorothioic acid (DEPTA), 4% desethyl parathion and only 2% desethyl paraoxon. Hydrolysis of all compounds occurred at both the alkyl-phosphate and the aryl-phosphate bonds. The percentages indicate that the proportion of alkyl-phosphate hydrolysis was very small for parathion but proportionally greater for methyl parathion in rats and under alkaline in vitro conditions. No consistent differences were noted between male and female rats.

Shishido and Fukami (1963) studied the metabolism of parathion by rat liver subcellular fractions. Production of desethyl parathion in the mitochondrial, microsomal, and supernatant fractions was very small. Contrarily, when methyl parathion and methyl paraoxon were incubated with rat liver supernatant the principal metabolites were demethyl parathion and demethyl paraoxon (Shishido and Fukami 1963, Fukami and Shishido 1963, Fukunaga et al. 1969).

Nakatsugawa and Dahm (1965) studied the activation of parathion in
vitro by enzymes in the fat body microsomes of the American cockroach, *Periplaneta americana* (L.). Activation was estimated manometrically and the anticholinesterase product was identified as paraoxon. Oxygen and NADPH₂ were required for the microsomal oxidation. These investigators later reported (Nakatsugawa and Dahm 1967) the activation of ³⁵S-parathion by rat and rabbit liver microsomes with a ³⁵S-metabolite being bound onto the microsomes. They found that rabbit liver microsomes degraded parathion by splitting at the aryl phosphate bond into an acidic metabolite that co-chromatographed with potassium 0,0-diethyl phosphorothionate. An independent but corroborative study by Neal (1967a) reported that the major metabolites of parathion by liver microsomes of rats, mice, and guinea pigs were paraoxon, diethyl phosphate (DEPA), diethyl phosphorothionate (DEPTA), and p-nitrophenol.

Recently the metabolism of parathion by two species of *Rhizobium*, nitrogen-fixing bacteria, was described as being primarily nitro-reduction with about 10% of the parathion converted to DEPTA (Mick and Dahm 1970). Their discovery that reduced glutathione (GSH) enhanced the production of DEPTA, whereas NADPH₂ did not, suggests that the aryl-phosphate cleavage of parathion may be accomplished for these bacterial species similarly to the O-demethylation of methyl parathion by the supernatant fraction of rat liver and horn beetle larva midgut homogenates (Fukami and Shishido 1966).

With the metabolism of parathion to paraoxon confirmed, information concerning the degradation of paraoxon is necessary for a more complete understanding of the metabolism of parathion.

The O-demethylation products of methyl paraoxon in mouse liver
supernatants were identified as demethyl methyl paraoxon and S-methylglutathione (Hollingworth 1969). The highest concentration of glutathione tested (3.3mM) resulted in increased monodemethylation of methyl paraoxon. The incubation of (ethyl) paraoxon with mouse liver supernatant showed only a small enhancement of degradation on adding glutathione.

The metabolism of methyl paraoxon by mitochondrial and microsomal preparations from mouse liver was also studied by Hollingworth (1969). Ion exchange separation of the water-soluble metabolites showed only one metabolite, dimethyl phosphate. With the addition of NADPH₂, very small amounts of demethyl methyl paraoxon were formed. Unlike the supernatant, neither mitochondrial nor microsomal fractions showed increased degradation when glutathione was included in the incubation mixture. In striking contrast is the NADPH₂-dependent and O₂-requiring dealkylation of chlorfenvinphos [2-chloro-1-(2',4'-dichlorophenyl)vinyl diethyl phosphate] by rabbit liver microsomes reported by Donninger et al. (1967). This disparity in the O-dealkylating capacity of mouse and rabbit liver microsomes is not clear.

Metabolism of paraoxon by enzymes in subfractions of rat liver homogenates was reported by Kojima and O'Brien (1968). They found at least three enzymes that hydrolyze paraoxon to DEPA and p-nitrophenol. An enzyme in the mitochondrial fraction had a pH optimum of 7.0, was stimulated by Ca²⁺, and inhibited by the tetrasyodium salt of ethylene diamine-tetraacetate (EDTA) and p-chloromercuribenzoate (p-CMB). The microsomal enzyme had a pH optimum of 7.7, was stimulated by Ca²⁺, inhibited by EDTA, but was not sensitive to p-CMB. The enzyme in the final supernatant,
called crude soluble, had a pH optimum of 8.8, was stimulated by Ca\(^{++}\), was unaffected by EDTA, but was inhibited by p-CMB. After subjecting the crude soluble fraction to ammonium sulfate fractionation, an enzyme was found in two precipitates (P 40 and P 80) and one soluble fraction (S 40) that degraded paraoxon to desethyl paraoxon and ethyl alcohol. Its properties were not described. Kojima and O'Brien (1968) did not report a cytological examination of their fractions.

Lauwerys and Murphy (1969) examined the in vitro metabolism of paraoxon by spectrophotometric, manometric, and anticholinesterase techniques. They studied the kinetics of the reactions, enzyme activity in various tissues, differences between sexes and among species, the effects of calcium, magnesium, and EDTA, and the effects of pretreatment in vivo with parathion and tri-o-tolyl phosphate. The results of their study suggested that the three systems do not measure the same mechanism of paraoxon metabolism. Spectrophotometric and manometric techniques measure enzymatic hydrolysis while tissue binding is likely to be the principal mechanism of paraoxon inactivation operating in the anticholinesterase system.

Paraoxon is highly toxic to rats; its approximate intraperitoneal acute LD\(_{50}\) = 1.2 mg/kg (Lauwerys and Murphy 1969) and its intravenous acute LD\(_{50}\) is < 0.5 mg/kg (O'Brien 1960). Lauwerys and Murphy (1969) suggest that the concentration of paraoxon in vivo, when an LD\(_{50}\) dose is administered, is closer to the substrate concentration of 10\(^{-7}\)M employed in their anticholinesterase assay system than the concentration of 4 x 10\(^{-4}\)M in their spectrophotometric technique. They hypothesize that tissue binding may play an important role in the inactivation of paraoxon.
In vivo, the major urinary metabolites of mice dosed orally with methyl paraoxon (4 mg/kg) are dimethyl phosphate (54%) and demethyl methyl paraoxon (33%) (Hollingworth 1969). In rats, $^{35}$S- and $^{32}$P-para-thion were metabolized into six urinary metabolites as determined by ion-exchange chromatography (Nakatsugawa et al. 1969). With a dose of 0.5 mg/kg, 39% of the radioactivity was identified as $^{32}$P-DEPTA and 30% as $^{32}$P-DEPA. At 1 mg/kg, 43% DEPTA and 22% DEPA were found while at 5 mg/kg, 45% DETA, 21% desethyl paraoxon, and 19% DEPA were observed. The $^{35}$S was identified as inorganic sulfate. When $^{35}$S- or $^{32}$P-DEPTA was injected intraperitoneally, > 98% was recovered 24 hr after injection as unchanged DETA. When $^{35}$S-desethyl parathion was injected, desulfuration was low, i.e., radioactivity of the sulfate peak was less than 5% of the total radioactivity.

The distribution of parathion-degrading enzymes among tissues of rats was also studied by Nakatsugawa et al. (1969). Tissues from nine organs were homogenized and the degradation of $^{35}$S-parathion assayed for each in the presence of $10^{-3}$ M NADPH$_2$ or GSH, or in the absence of such cofactors. Degradation by extrahepatic tissues never reached 10% that of liver.

An in vitro study of parathion degradation was done in conjunction with an in vivo study in order to determine the distribution of parathion-degrading enzymes in liver homogenates and to compare metabolites collected in both studies (Nakatsugawa et al. 1969). The principal findings were (1) of the subcellular fractions, the post-microsomal supernatant (F-4) showed the highest activity when no cofactor was added; (2) NADPH$_2$
greatly enhanced microsomal (F-3) activity but only slightly increased that of the F-4 fraction; (3) the F-4 fraction showed a linear time course of reaction while the microsomal fraction slowed down with time; (4) the only metabolite of the particulate fractions was DEPTA while F-4 produced a minor metabolite that appeared to be desethyl parathion; and (5) whereas anaerobic conditions enhanced the activity of the F-4 fraction in the presence of GSH, the F-3 fraction was suppressed to 33% of the control.

The in vivo findings of Nakatsugawa et al. (1969) confirm the earlier report of Plapp and Casida (1958a) with some qualitative and quantitative differences. Nakatsugawa et al. (1969) did not find desethyl parathion; they did find that desethyl paraoxon was an important metabolite at 1 and 5 mg/kg dosages. They also identified DEPA, ethyl phosphoric acid, and phosphoric acid in significant quantities. Their results regarding desulfuration of DEPTA and desethyl parathion argue that DEPA, ethyl phosphoric acid, and phosphoric acid were produced from paraoxon rather than from corresponding sulfur analogs. These investigators concluded that metabolism of parathion in the rat is primarily initiated by liver microsomal oxidases and that soluble enzymes play a minor role.

Centrifugal Fractionation of Cell Components

Cytologists have appreciated for many years that plant and animal cells, far from being homogeneous masses of protoplasm, contain discrete structures that can be seen and identified. Perfection of microscopic lenses by Amici permitted morphological details in plant and animal tissues to be seen. Similarly, critical determination of the chemical
composition and function, in terms of enzymatic activity, of the structural components of cells followed the development of techniques for mechanical rupture of cells and the isolation of cell components by differential centrifugation. Claude (1948), Schneider and Hogeboom (1951), Hogeboom et al. (1953), and Duve and Berthet (1954) have reviewed the early history of differential centrifugation as applied to the isolation of nuclei, mitochondria, chromosomes, submicroscopic forms of glycogen, submicroscopic particles including microsomes, and soluble material. The use of differential centrifugation in the study of tissue enzymes and a partial tabulation of enzyme studies on subcellular fractions obtained by this method were reviewed by Duve and Berthet (1954) and Roodyn (1965).

Thomson and Mikuta (1954) and Kuff and Schneider (1954) employed density-gradient centrifugation to achieve a more selective separation of cytoplasmic particulates from liver homogenates of rats and mice.

**Fractionation of endoplasmic reticular membranes**

For a while the centrifuge surpassed the light microscope in the exploration of cellular elements, morphologically speaking, because of lack of microscopic resolving power. With the development of the electron microscope, resolving power was no longer a problem. However, improvements in techniques such as fixation, thin-sectioning, and staining were necessary before the capabilities of the new instrument could be realized in the study of cells in situ and isolated cell elements (Claude 1948).

The importance of electron microscopy in an integrated morphological and biochemical analysis of cytoplasmic particulates in liver homogenates is evident in the reports of Palade and Siekevitz (1956) and Kuff et al.
(1956). The latter investigators used a small sucrose gradient and obtained cytoplasmic particles of three major size groups which were composed of more or less heterogenous particles based on enzymatic activity and RNA analysis. The vesicular structures derived from the endoplasmic reticulum (ergastoplasm) of the intact cell were found principally in the groups of intermediate- and small-sized particles. Earlier studies, as review by Claude (1948), had referred to these vesicular structures as microsomes, a noncommittal term meaning small bodies. Palade and Siekevitz (1956) demonstrated rather conclusively that microsomes are a centrifugal product derived mainly from the endoplasmic reticulum. Perhaps their most significant finding was that microsomes contain two structurally different components: one membranous and the other particulate in nature. Small dense particles with high RNA content were observed, in the case of liver, to be in close association with the membranous component. Microsomes appeared as isolated, membrane-bound vesicles, tubules and cisternae; they often contained a seemingly homogenous material of noticeable density, and attached to their outer surface were small, dense particles 100 to 150 Å in diameter with high RNA content. Those vesicles free of attached particles were thought to be derived from smooth-surfaced parts of the endoplasmic reticulum (Palade and Siekevitz 1956).

An extensive review of microsomes - their isolation, their chemical composition, their role in cellular metabolism, their transport and transmission function, and their enzymatic components - was prepared by Siekevitz (1963). In an earlier publication (1962) he described the usual
method of preparing mammalian microsomes and pointed out the difficulties encountered in translating fractionation methods from one tissue to another or from one organism to another. Such a difficulty was reported by Brindley and Dahm (1970) for the preparation of a microsomal fraction from American cockroach fat body homogenates. They found that their insect tissue required much less vigorous centrifugation than rat liver, for example. Szarkowski et al. (1960) also reported differences in the morphology of sediments when onion roots were studied. Consequently, the term microsomes has only a methodological meaning, describing a pellet obtained after high-speed centrifugation of a mitochondrial supernatant. One cannot assume that a preparation from another tissue, made in exactly the manner routinely used for liver or pancreas, will be of the same biochemical and morphological nature as the methodologically corresponding fraction from liver or pancreas (Siekevitz 1962).

From a morphological point of view, microsomes arise through a fragmentation or pinching-off process of the endoplasmic reticulum into "rough" vesicles with ribosomes attached to the outer surface and "smooth" vesicles lacking ribosomes (Palade and Siekevitz 1956). Several centrifugal procedures have been described to subfractionate these two forms of microsomes (Moulé et al. 1960, Chauveau et al. 1962, Rothschild 1963, Hallinan and Munro 1965, Dällner et al. 1966a, Bergstrand and Dallner 1969, and Lee et al. 1969). These procedures basically involve a stepwise removal of the unbroken cells, nuclei and mitochondria followed by a layering of the mitochondrial supernatant over a discontinuous or continuous sucrose gradient with or without cations and centrifuging at varying
gravitational (g) forces for a selected period of time. Lee et al. (1969) employed zonal centrifugation with a continuous sucrose gradient. All investigators reported successful subfractionation of "rough microsomes" from "smooth microsomes". Moulé et al. (1960), Hallinan and Munro (1965), and Lee et al. (1969) reported contamination by RNA in the smooth-surfaced microsomes. In the last study, the RNA level of the smooth microsomes surpassed the level in the rough microsomes. Electron microscopic evidence suggested that this was the result of contamination with free ribosomes. Hallinan and Munro (1965) found that they could satisfactorily separate smooth-surfaced membranes from ribosomes by a procedure in which a mixture of agranular membrane and free ribosomes obtained by the method of Chauveau et al. (1962) was treated with iso-octane. On centrifugation, the iso-octane treatment caused physical separation of the ribosome and membrane fractions. Bergstrand and Dallner (1969) were able to subfractionate further the smooth microsomes into what they termed smooth I and smooth II microsomes. Both of the latter preparations had low RNA-protein ratios.

The successful separation of rough-surfaced membranes from smooth-surfaced membranes has facilitated the discovery of their biochemical differences (Dallner et al. 1966a, Glaumann and Dallner 1968, Glaumann et al. 1968, Bergstrand and Dallner 1969) including differences in enzyme distribution (Decken 1967, Holtzman et al. 1968, Bergstrand and Dallner 1969, Gram et al. 1967), differences in pattern of enzymic differentiation (Dallner et al. 1966b), and antigenic differences (Decken 1967). A study of the distribution of microsomal enzymes which metabolize parathion has
not been reported.

Studies on the metabolism of drugs by subfractions of hepatic microsomes have shown that significant enzyme activity of rat and rabbit liver is in the smooth-surfaced fraction (Gram et al. 1967, Gram and Fouts 1967, Holtzman et al. 1968). In the rabbit, Holtzman et al. (1968) found that the smooth membrane/rough membrane activity ratios were significantly greater than one whether the activities were expressed per g of liver, per mg of protein, per g of phospholipid phosphorus, per unit of cytochrome P-450, or per unit of NADPH-cytochrome c reductase. However, when the ethylmorphine \textit{N}-demethylase activity per unit of NADPH-cytochrome P-450 reductase activity was measured, there was no significant difference between the rough and smooth microsomes. They observed for the rabbit that the rate at which cytochrome P-450 is reduced is lower in the rough membranes and that the fraction of the total cytochrome P-450 that is reducible by NADPH is lower in the rough than in the smooth membranes. The ethylmorphine \textit{N}-demethylase activity for rough and smooth microsomes from rat livers could be explained on the basis of different concentrations of cytochrome P-450 in each of the fractions (Holtzman et al. 1968). The latter workers employed a No. 40 Spinco rotor in their subfractionation procedure. The No. 40 rotor has a tube-to-axis angle of only 26°. Bergstrand and Dallner (1969) state that an angle rotor with a tube-to-axis angle of < 34° increases contamination of the rough fraction to a nonacceptable level because of side wall impaction. Neither RNA levels nor electron microscopic evidence accompanied the report of Holtzman et al. (1968).
Electron Microscopy

The importance of electron microscopy in corroborating biochemical data in studies employing centrifugally prepared cell fractions cannot be overstated. For a thorough discussion of the theory of electron microscopy, preparation of samples, microtomy, staining and high resolution work, the reader is referred to Wischnitzer (1962), Pease (1964) and Sjöstrand (1967). Sabatini et al. (1963) have been instrumental in popularizing a fixation procedure employing a buffered aldehyde in primary fixation with osmium tetroxide (OsO₄) postfixation. Of the aldehydes studied, glutaraldehyde (GA) gave the best general preservation of cellular detail, especially the cellular membranes, when the tissues were postfixied in OsO₄. Furthermore, with this fixation, the size of the tissue block is not so critical since relatively large blocks can be fixed and stored in cold buffered GA for extended periods; later, smaller blocks can be trimmed for postfixation in OsO₄ without loss of quality. This flexibility in fixation schedule facilitates combined biochemical and cytological investigations.

Analysis of Metabolites

A brief commentary on three analytical methods used in this study follows.

Gas-liquid chromatography

Gas chromatography has developed rapidly during the last two decades into a major tool of the analytical chemist and has become increasingly
important to the biologist (Karmen 1963). Gas chromatography is a general term covering both gas-liquid and gas-solid chromatography. Gas-liquid chromatography (GLC), one of the more recently developed forms of chromatography, accomplishes a separation by partitioning a sample between a moving vapor phase and a stationary liquid phase held on a solid support. When a solid absorbent is employed as the stationary phase, the technique is called gas-solid chromatography. A description and review of improvements in the use of ionization detection systems in the gas chromatography of insecticides have been reported by Giuffrida et al. (1966). Beroza and Bowman (1968) have reviewed the gas chromatography of three major types of insecticides. Guidelines for the reporting of gas chromatographic methods have been prepared by Beroza and Hornstein (1970). The quantitative detection of insecticidal compounds, particularly chlorinated hydrocarbons and organophosphates, has become accepted practice and is extensively reported in the literature. In summary, the determinations by GLC are accomplished rapidly and easily with sensitivities in the pico- to nanogram range (Beroza and Bowman 1968).

Thin-layer chromatography

Thin-layer chromatography (TLC) is an efficient, rapid, and inexpensive technique for the identification and semiquantitation of insecticidal compounds and their metabolites. It serves as a useful adjunct to other methods of analysis. Walker and Beroza (1963) reported the $R_f$ values of 62 pesticides in 19 different solvent systems and chromogenic reagents for making the compounds visible. More recently Getz and Wheeler (1968) investigated the separation by TLC of 42 organophosphorus insecticides,
including several oxidative metabolites. They employed 5 different ternary solvent systems and compared four different adsorbents. They observed that with more polar insecticides, the polarity of the developing solvents must be greater to bring about migration from the origin; silica gels were the preferred adsorbent. Stenersen (1968) determined the $R_f$ values for 25 degradation products of dialkyl aryl phosphorothionates on silica gel, type G, thin-layer chromatography plates in a single solvent system consisting of technical acetonitrile and water in a ratio of 88:12 v/v. For most analyses with chromogenic sprays, the limit of detection was 100 nanograms or more of the insecticide (Walker and Beroza 1963, Getz and Wheeler 1968, Stenersen 1968); with serum cholinesterase inhibition treatment, five nanograms and more of organophosphorus insecticides with $\text{P}=\text{O}$ configuration were detected.

**Liquid scintillation spectrometry**

Liquid scintillation counting has become widely used for estimating radioactive compounds and their metabolites. A limited number of references were available prior to 1963. A review of literature highlights in the field of liquid scintillation counting from 1957–1963 was prepared by Rapkin (1964). A thorough discussion of scintillation counting from a theoretical view was made by Birks (1964), while a general and practical account of counting with organic scintillators was given by Schram (1963). Recently Parmentier and Ten Haaf (1969) have reviewed advances in the field over the past 5 to 6 years with emphasis on developments in instrumentation, quench corrections, and sample preparations as they apply to use in chemical, biochemical, and medical research. They
concluded that advances are due mainly to improved instrumentation.

**Solubilization of Microsomes**

The purification of enzymes from natural sources is sometimes a formidable task. If the enzyme to be studied is bound to a particulate portion of the cell, isolation of that particulate fraction is advisable. A purification of the enzyme from the cellular component necessitates a release of the enzyme. Since enzymes are proteins and are fragile molecules, careful control of temperature, pH, and protein concentration during the fractionation process is essential (Mahler and Cordes 1966). Conventional methods employed in the release of membrane-associated enzymes such as ultrasonication, freeze-thaw, glycerol extraction, enzymatic hydrolysis, or treatment by surfactants generally inactivate many microsomal enzymes (Shuster 1964). Consequently, very few microsomal enzymes have been isolated in a form sufficiently pure to allow proper characterization. Shuster (1964) has reviewed the limited number of microsomal drug-metabolizing enzymes that have been solubilized. Song and Bodansky (1967) reported the localization of 5'-nucleotidase of rat liver in the membranous component of the microsomal fraction with a major fraction residing in the plasma membrane fragments that constituted a part of the microsomal fraction. This enzyme was present in the lipoprotein residue that was not extractable with 0.9% NaCl solution; 5'-nucleotidase was readily solubilized from the membrane with 0.5% sodium deoxycholate. These investigators did not report the effect of solubilization on enzyme activity. More recently Ljunggren and Åkeson (1968) reported the
solubilization and identification of a peroxidase from the microsomal fraction of beef thyroid. Their most promising results were obtained by treating an acetone powder derived from microsomes with trypsin in combination with deoxycholate. The recovery of activity after trypsin treatment was 76% of the microsomal fraction.

It is not known whether decrease or total loss of activity for many microsomal enzymes following solubilization is a function of (1) the denaturation of the enzyme, (2) a requirement for association with lipoidal material, or (3) a disruption of associated subunits requisite for function. Song et al. (1967) were able to solubilize 5'-nucleotidase from human liver with 3% deoxycholate in sodium diethylbarbiturate buffer with 71% of the activity remaining. When 5'-nucleotidase was precipitated from the deoxycholate supernatant fluid by cold ethanol, approximately 100% of the activity was recovered. When the precipitate was resuspended and dialyzed against three changes of distilled water and pelleted by centrifugation, an electron microscopic examination of the pellet revealed a collection of membrane vesicles resembling the smooth membranes present in the original material. They concluded that a reconstitution of various solubilized components into membranes had taken place.
MATERIALS AND METHODS

Animals

Adult, male, albino rats (250 to 300 g) were purchased from Dan Rolfsmeyer Co., Madison, Wisconsin. At the time of sacrifice the rats ranged from approximately 275 g to over 500 g with an average of approximately 350 g. The animals were fed ad libitum on a diet of Purina Lab Chow for Rats, Ralston Purina Co., St. Louis, Mo., and water and were kept in an air-conditioned room at 82 ± 1°C and 50 ± 2% relative humidity under artificial lighting (12 hr light, 12 hr darkness).

The animals were fasted, but with water available, for 18 to 22 hr prior to sacrifice. They were killed by decapitation with a guillotine, allowed to bleed freely, and the livers quickly removed and placed on aluminum foil over ice.

Homogenization and Centrifugation

The livers were immediately minced with a razor blade or scissors, rinsed in cold 0.25M (isotonic) sucrose, blotted, weighed, and placed in cold isotonic sucrose in a total volume equivalent to 2.5X the original wet weight of the liver. After three successive homogenizations, each for six seconds, at maximum rpm in a Sorvall Omnimixer®, the homogenate was further diluted with sucrose solution to a volume equal to 5.0X the original wet weight. The homogenate was transferred to a Potter-Elvehjem (P-E) type glass tube and six passes were made with a Teflon® pestle powered by a Black & Decker, U-100, 1/8" utility drill with a maximum rpm of 2500. All
containers and solutions were kept in an ice bath. Preparation of the liver cell fractions employed in this study is summarized in Figure 2. The gravitational forces ($g$-values) refer to forces at the top, middle, and bottom of the tubes, respectively. Each small sample pelleted for electron microscopical examination was located at a position in the centrifuge tube that was equivalent to the maximum gravitational force applied ($g_{\text{max}}$ in Figure 2). In the Spinco #30 rotor, 30 ml, Oak Ridge type, Autoclear® polycarbonate tubes with Teflon® screw caps were used. Identical tubes of 10 ml capacity were used in the Spinco #40 rotor. Beckman® polyallomer tubes of approximately 5 ml capacity were used in the Spinco SW39L rotor. The centrifugation times include acceleration and deceleration of the rotors. If liver fractions were not to be used immediately, they were quickly frozen and stored at -20°C, with the pellets being frozen wet after decanting supernatants. All fractionations were performed in Beckman® models L and L2-65 preparative ultracentrifuges (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.).

Electron Microscopy

Rat liver homogenate and centrifugal fractions were prepared by two procedures summarized in Table 1. Procedure A incorporates the methods of Sabatini et al. (1963) and Omura et al. (1967) but was modified to include sucrose in the buffered glutaraldehyde (GA) fixing solution. The buffer used was a phosphate ($\text{PO}_4^-$) buffer prepared as a 0.1M buffer ($0.08M \text{Na}_2\text{HPO}_4; 0.02M \text{KH}_2\text{PO}_4$) with a pH of 7.2. Procedure B follows the method of Sabatini et al. (1963) more closely with slight differences in
Figure 2. Preparative scheme for rat liver cell fractions employed in the study of in vitro metabolism of parathion
Decapitate rat. Exsanguinate. Remove liver. Chill. Dice. Wash twice in cold 0.25M sucrose. Blot. Weigh. Homogenize (Omnimixer, 6 sec, 40% w/v in cold 0.25M sucrose). Homogenize (P-E with Teflon pestle, 6 passes, 20% w/v in cold 0.25M sucrose). All rotors and models L and L2-65 preparative ultracentrifuges were from Spinco Division, Beckman Instruments, Inc., Palo Alto, California.

### Homogenate (1)

- **Centrifuge**
  - 10 min
  - 4 C
  - #30 rotor
  - $g_{\text{min}}$: 982
  - $g_{\text{avg}}$: 1,532
  - $g_{\text{max}}$: 2,062

  Supernatant brought to volume with cold 0.25M sucrose

### Debris (2)

- **Centrifuge**
  - 15 min
  - 4 C
  - #30 rotor
  - $g_{\text{min}}$: 5,990
  - $g_{\text{avg}}$: 9,340
  - $g_{\text{max}}$: 12,570

  Supernatant brought to volume with cold 0.25M sucrose

### Mitochondria (3)

- **Centrifuge**
  - 90 min
  - 4 C
  - #40 rotor
  - $g_{\text{min}}$: 67,900
  - $g_{\text{avg}}$: 105,200
  - $g_{\text{max}}$: 144,800

  CsCl added to supernatant to final concentration of 15mM

  Layered 3.3 ml of 15mM CsCl:0.25M sucrose containing supernatant over 2 ml of 15mM CsCl:1.3M sucrose

### Crude Microsomes (4)

- **Centrifuge**
  - 210 min
  - 4 C
  - SW39L rotor
  - $g_{\text{min}}$: 79,800
  - $g_{\text{avg}}$: 124,000
  - $g_{\text{max}}$: 166,400

  Discarded upper 2 ml of clear supernatant

### Resuspended pellet in 0.25M sucrose

- **Centrifuge**
  - 60 min
  - 4 C
  - #40 rotor
  - $g_{\text{min}}$: 67,900
  - $g_{\text{avg}}$: 105,200
  - $g_{\text{max}}$: 144,800

### Rough Microsomes (5)

- **Centrifuge**
  - 100 min
  - 4 C
  - #40 rotor
  - $g_{\text{min}}$: 67,900
  - $g_{\text{avg}}$: 105,200
  - $g_{\text{max}}$: 144,800

### Smooth Microsomes (6)

- Collected fluffy layer and added cold isotonic 0.25M sucrose
Table 1. Electron microscopy preparative scheme

<table>
<thead>
<tr>
<th>Procedure A</th>
<th>Procedure B</th>
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**Fixation**

1. 2.5% GA:0.1M PO₄:0.25M sucrose (775 milliosmols) pH 7.2, 19 hr at 4 C, 105,200 gav 60 min

2. Washed pellets in 0.1M PO₄: 0.25M sucrose (495 milliosmols) pH 7.2, 3 changes, 20 min each

3. 1% OsO₄:0.1M PO₄:0.25M sucrose (535 milliosmols) pH 7.2, 16 hr at 4 C

4a. Washed pellets with 0.1M PO₄ (220 milliosmols) pH 7.2, 3 changes, 3 min each

4b. 0.5% uranyl acetate (aqueous) 7 hr at 4 C

4c. Washed with 0.1M PO₄ pH 7.2, 3 changes of 3 min

**Dehydration**

5. 5 min in 25, 50, 70, 95% EtOH. 3 changes of 5 min in 100% EtOH. 3 changes of 5 min in propylene oxide (PO)

5. Variable time in 25% EtOH (<60 min) 5 min in 50, 70, 95% EtOH. 3 changes of 5 min in 100% EtOH. 3 changes of 5 min in propylene oxide (PO)
Table 1. (Continued)

<table>
<thead>
<tr>
<th>Infiltration</th>
<th>Procedure A</th>
<th>Procedure B</th>
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<tbody>
<tr>
<td>6. PO-resin mixture</td>
<td>(3:1), 15 min</td>
<td>6. PO-resin mixture (shaking until Schlieren lines disappeared)</td>
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<tr>
<td>6. PO-resin mixture</td>
<td>(1:1), 30 min</td>
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<td>6. PO-resin mixture</td>
<td>(1:3), 60 min</td>
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<tr>
<td>6. Resin mixture</td>
<td>(Pure), 22 hr</td>
<td>Resin mixture (Pure), 1 to 2 hr</td>
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<tr>
<th>Polymerization</th>
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<tr>
<td>7. 36 C, 30 hr</td>
<td>45 C, 20 hr</td>
<td>60 C for 56 hr</td>
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<tr>
<td>7. 60 C, 60 hr</td>
<td>7. Room temperature for 24 hr</td>
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<th>Staining</th>
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<tr>
<td>8. 2% uranyl acetate</td>
<td>15 min</td>
<td>8. 2% uranyl acetate in absolute methanol, 15 min</td>
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<tr>
<td>9. 2.66% lead citrate</td>
<td>11 min</td>
<td>9. 2.66% lead citrate, 11 min</td>
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Resin mixture:

- Araldite 502: 10 ml
- Epon 812: 13 ml
- DDSA (dodecanyl succinic anhydride): 30 ml
- D. E. R. 732 (a polyglycol diepoxide): --
- DMP-30 [2,4,6-tri(dimethylaminomethyl)phenol]: 1.2 ml

<table>
<thead>
<tr>
<th>Procedure A</th>
<th>Procedure B</th>
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<tr>
<td>10 ml</td>
<td>33.0 g</td>
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<tr>
<td>13 ml</td>
<td>45.9 g</td>
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<tr>
<td>30 ml</td>
<td>60.0 g</td>
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<tr>
<td>--</td>
<td>15.4 g</td>
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<tr>
<td>1.2 ml</td>
<td>6.0 ml</td>
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concentrations of ingredients. Graphs relating molarity with pH and osmolality (Maser et al. 1967) were employed in procedure B to prepare solutions, containing Sorensen's phosphate buffer C (0.288M Na$_2$HPO$_4$: 0.112M NaH$_2$PO$_4$) appropriately diluted, with a final tonicity slightly below 300 milliosmols.

Procedures A and B differed also in the manner in which the cell fractions were introduced into the first fixative. In procedure A the pellets were resuspended in 10 ml of 4 C buffered 2.5% GA solution with a homogenizer, stored at 4 C for approximately 3 hr and centrifuged at 105,200 g$_{avg}$ for 60 minutes. The buffered GA solution was left over the pellet overnight at 4 C. After decanting, the pellets were rinsed and postfixied in buffered OsO$_4$ (Step 3, Table 1) and carried through the remaining steps to embedding. In procedure B the cell fractions were frozen and stored prior to processing. In preparation for incubation with parathion, the pellets were thawed and suspended in 0.125M sucrose: 0.01M PO$_4$:0.15M KCl. Either 0.5 or 1.0 ml aliquots of these suspensions were brought to 10 ml with buffered GA and fixed at 4 C for 3.5 hr including centrifugation time. The flexibility in treatment times for fixatives and washes was based on the findings of Sabatini et al. (1963, 1964) (Table 1). The variability in procedures resulted, in part, from my adaptation to procedures and materials used in separate electron microscopy laboratories. Dehydration was carried out by an ethanol-propylene oxide series. The embedding epoxy resin mixtures used were modifications of the mixture reported by Anderson and Ellis (1965).

All sections were cut with glass knives on an LKB Ultratome III.
Thin sections exhibiting gray to silver interference colors were flattened with chloroform vapors and picked up on uncoated 400 mesh copper grids. The sections were stained for 15 min in 2% uranyl acetate in absolute methanol, washed for 5 sec or less in absolute methanol followed by two washes in 1:1 methanol:water (5-10 sec each) and two washes in water (5-10 sec each). The individual grids were allowed to dry prior to an 11 min post-staining with lead citrate (Reynolds 1963).

RCA electron microscopes (models EMU-3F and EMU-4A) were used in this study. All micrographs were taken with the EMU-4A. The fractions described in Figure 2 were examined at least once, with the crude microsomes being studied from three separate preparations and rough and smooth endoplasmic reticulum samples from two fractionations.

Micrographs were mounted on poster board and photographed with Polaroid® P/N Type 55 film for thesis reproduction on Kodak polycontrast light weight paper.

In Vitro Analytical Procedures

**Incubation parameters**

Chemicals used in this study were of reagent grade. For gas-liquid chromatography (GLC) Nanograde n-hexane (Mallinckrodt Chemical Works, St. Louis, Mo.) was employed. Unless otherwise stated the following components were used to make an incubation volume of 4 ml (final concentration in the incubation mixture):
2.8 ml buffer (8 x 10^{-3}M Na_2HPO_4:2 x 10^{-3}M KH_2PO_4:0.15M KCl) pH 7.4
0.1 ml cofactors in buffer (10^{-3}M NADPH_2:10^{-2}M nicotinamide)
0.1 ml parathion (2.5 x 10^{-5}M ethyl-1-{^14}C-parathion)
1.0 ml cell fraction
4.0 ml Total

The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH_2) was purchased from P-L Biochemicals, Inc., Milwaukee, Wisc., nicotinamide from Eastman Organic Chemicals, Rochester, N.Y., and {^{14}}C-parathion from Amersham/Searle, Amersham, England. The latter compound was purchased December 1967 with an initial specific activity of 8.84 mc/mmole. It was purified in our laboratory by Dr. T. Nakatsugawa on a silica gel column with chloroform as the eluent after which it yielded a single GLC peak and chromatographed as a single spot in a 2:1 hexane:ethyl acetate solvent system. After evaporation of the solvent, an acetone solution of approximately 10^{-3}M was stored in darkness at -15 C. To prepare a stock solution from this purified preparation, the acetone was evaporated, parathion was weighed by difference and a 1 x 10^{-3}M solution was prepared in absolute ethanol containing 1% (v/v) Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), a nonionic surfactant.

Nonradioactive parathion (American Cyanamid Co., Princeton, N.J.) was column purified as previously described and a 1 x 10^{-3}M stock solution was gravimetrically prepared. Identical dilutions of these stock solutions produced identical peak heights and retention times when assayed by GLC. A purity check of radiolabeled stock solutions by thin-layer chromatography (TLC) indicated > 98% purity for {^{14}}C-parathion by
radiometric determination. The cold parathion chromatographed as a single spot in a 2:1 hexane:ethyl acetate solvent system.

Paraoxon, kindly supplied by American Cyanamid Co., Princeton, N.J., was gravimetrically prepared as a $10^{-3}$M stock solution in absolute ethanol. It produced a single spot by TLC and was detected as a single peak by GLC by methods described later. Ethyl-$l^{14}C$-paraoxon, prepared by Dr. T. Nakatsugawa by bromine-water oxidation of $^{14}C$-parathion and purified on a silica gel column with hexane:isopropanol (10:1) as the eluting solvent, was subsequently re-chromatographed by Dr. T. Y. Ku of our laboratory and made up as a hexane solution. When co-chromatographed with the unlabeled paraoxon, identical $R_f$ values were observed in several solvent systems tried.

The incubations were performed, unless otherwise stated, at room temperature ($22 \pm 1$ C) in 35 ml glass, screw-cap centrifuge tubes with caps removed. An initial swirling of the ingredients was the only shaking applied. The incubations were 1 hr in length at which time 1 ml of 10% trichloroacetic acid (TCA) in 3N hydrochloric acid (HCl) was added to stop the reaction.

The pH was measured at the beginning of the incubation and just prior to the addition of the acid with either a Corning® model 12 or a Beckman® model GS pH meter equipped with a Corning® combination electrode, #476050.

Control studies

Duplicate samples of control incubations were run for all of the metabolism studies unless otherwise stated. Control incubations were
established by substituting a volume of buffer equivalent to that of the liver cell fraction added to the experimental tubes. Control incubations containing enzyme fraction but lacking cofactors were also tried in conjunction with those lacking enzyme in order to ascertain any influence on recovery or metabolism by the mere presence of enzyme without cofactor.

Metabolite extraction

After stopping the reaction the nonpolar compounds were extracted by adding 10 ml of n-hexane and shaking for 30 min on a Fisher-Kahn shaker at approximately 280 oscillations per min. Separation of the phases was facilitated by spinning in an International centrifuge (model K) at approximately 2000 rpm for 10 min.

Approximately 9 ml of the hexane layer were pipetted into 10 ml screw-cap, glass centrifuge tubes (plastic caps with Teflon liners) for subsequent analyses by GLC, TLC, and liquid scintillation spectrometry. No additional extractions were made before analysis of the aqueous phase by TLC and liquid scintillation spectrometry.

Analyses

Protein nitrogen determination The nitrogen content of the rat liver cell fractions was estimated by a micro-Kjeldahl method (Yuen and Pollard 1953; Bremner 1960). A 5.5 cm Buchner funnel was lined with a 9.0 cm Whatman no. 44 filter paper and 10 ml of 10% TCA in 3N HCl was added. Then 0.1 to 0.2 ml of liver homogenate or 0.5 to 1.0 ml of the resuspended centrifugal fractions was pipetted into the TCA-HCl. The protein precipitate was filtered with the aid of a water aspirator and washed
with another 10 ml of 10% TCA in 3N HCl. The filtrate was discarded and the filter paper bearing the precipitate was digested in a 30 ml micro-
Kjeldahl flask containing 3 ml of concentrated sulfuric acid and 0.1 g of catalyst. The catalyst consisted of a mixture of \( \text{K}_2\text{SO}_4 \), \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \), and selenium metal in a ratio of 8:1:1. At least two blank samples containing TCA-wetted filter paper were assayed for each experiment. At least two standard solutions of urea or \( (\text{NH}_4)_2\text{SO}_4 \) (1 mg/ml) or one of each were also analyzed and served as controls. The flasks were heated until the contents reached a pale green color. After digestion the contents of each flask were transferred with a minimum of three rinses to a distillation flask to which were added 20 ml of 10N NaOH and glass distilled water (g.d. \( \text{H}_2\text{O} \)) to a final volume of 80 ml. The ammonia was steam distilled into an Erlenmeyer flask containing 5 ml of a 1% boric acid-mixed indicator solution. A final volume of 35 ml was adequate for complete distillation of all ammonia. The boric acid-indicator solution was prepared as follows: 19.8 mg of bromocresol green and 13.2 mg methyl red were dissolved in 20 ml absolute ethanol (100% EtOH) and added to 980 ml of g.d. \( \text{H}_2\text{O} \) containing 10 g of boric acid. The distillate was titrated against a standard HCl solution prepared from a stock solution, the latter having been standardized by titration against anhydrous sodium carbonate.

A minimum of duplicate estimates of the nitrogen in each liver cell fraction was made and an average value computed after correcting for nitrogen in the blank. The nitrogen (N) levels were calculated as follows:

\[
\varepsilon_N = \left( \frac{\text{ml} \times \text{normality} \times \text{meq weight}}{\text{titrant}} \right) - \varepsilon_N \quad (\text{N}) - \varepsilon_N \quad (\text{blank})
\]
The estimated values were then converted to mg N/ml of each liver cell fraction assayed.

**RNA analysis**

Ribonucleic acid (RNA) was measured according to the method of Schneider (1945). One-ml aliquots of centrifugally prepared rat liver cell fractions (3) through (6) (Figure 2) were assayed in duplicate from two separate experiments. Each aliquot was sequentially extracted twice with 5 ml of cold 10% TCA, two 5 ml extractions with 95% ethanol, and extracted with 10 ml hot 5% TCA (90°C) for 20 min. The tissue residue was removed from the final extraction by the filtration procedure described for protein nitrogen determination and was rinsed with a total of 15 ml 5% TCA bringing the filtrate to a final volume of 25 ml. Two ml of filtrate and 2 ml of orcinol reagent were heated 20 min in a boiling water bath. The orcinol (3,5-dihydroxytoluene) was grayish-white in color as supplied by the manufacturer. It was purified by dissolving in boiling benzene, decolorized with activated charcoal by repeated filtration, and finally crystallized from hexane by evaporation of solvent. A very white crystalline product was obtained. One gram of purified orcinol was dissolved in 100 ml of concentrated HCl containing 0.5 g of FeCl₃. The reagent was prepared fresh for each experiment as it rapidly darkened after several hours.

To prepare an RNA standard curve, yeast RNA (Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in 5% TCA to make a final concentration of 100 μg RNA/ml and held at 90°C for 20 min. This stock preparation was diluted with final concentrations ranging from 2 to 70 μg RNA/ml and treated with the orcinol reagent. RNA-containing samples were measured at
660 nm with a Beckman model DB® spectrophotometer in a 1 cm square cuvette. Sample values were interpolated from a standard curve relating absorbance and concentration of the yeast RNA preparation (Figure 3). The instrument was set at zero absorbance with orcinol reagent in both cells. Because transmission of this solvent exceeded 50%, the band-width selector was adjusted to the pre-set narrow position.

**Gas chromatographic analyses**  A Packard dual-column gas chromatograph, model no. 7821, equipped with electron capture detectors was used. Coiled glass columns (120 cm x 2 mm (i.d.)) were packed with 68-80 mesh silylated Chromosorb® G (Johns-Manville Products Corp., New York, N.Y.) coated with Apiezon® N (AEI, Ltd., ordered from Analabs, Inc., Hamden, Conn.). The Chromosorb G was either washed and silylated according to the procedure described by Nakatsugawa et al. (1968) or was purchased from Analabs, Inc., as an acid washed and silylated preparation. A quantity of silylated solid support was weighed and Apiezon N equivalent to approximately 10% (w/w) was dissolved in Nanograde™ n-hexane. The liquid phase, Apiezon N, was applied to the solid support by a filtration process as follows: The solid support and Apiezon N solution were placed in a side-arm filter flask, swirled, and the pressure was reduced with a water aspirator for 5 min after which shaking dislodged bubbles of air. Atmospheric pressure was returned to the flask and suction repeated, followed by transfer of the slurry into a Büchner funnel with filter paper; the slurry was allowed to drain freely. Suction by water aspirator was applied to the flask until the cake appeared damp, but not wet, at which time the cake was removed and spread for drying at room temperature followed by
Figure 3. Standard curve relating concentration of RNA reacted with orcinol reagent (Schneider 1945) and absorbance at 660 nm.
drying overnight at approximately 80 C. The dried support flowed freely and had a powdery appearance.

The glass columns were cleaned with dichromate-sulfuric acid cleaning solution, silylated with hexamethyldisilazane (HMDS, Analabs Solution B), and packed with coated support. Packing was facilitated by vibration (model V-73, Burgess Vibrocrafters, Inc., Grayslake, Ill.) under reduced pressure. The columns were conditioned at approximately 200 C with a nitrogen gas flow rate of 20 to 30 ml/min for varying periods of time ranging up to 9 days.

Standard operating conditions were: carrier gas, 50 ml N₂/min; inlet temperature, 215 C; column temperature, 195 C; detector temperature, 210 C; outlet temperature, 222 C; and range setting of 1 x 10⁻⁸ amperes. Optimum voltages (20 to 40 volts) for each detector were determined by applying a tangent to the step-curve response obtained by varying the voltage to each detector.

Samples in n-hexane were dried over anhydrous Na₂SO₄ and at least two injections of either 3 µl or 6 µl aliquots were made for each sample. Standard peak height ratio curves (Figure 4) were prepared from stock solutions of known concentrations of appropriate organophosphates. The concentration of 10⁻⁶ M served as the standard against which other concentrations of standard solutions and experimental samples were compared. These curves were prepared each time changes in columns or detectors were made and concentrations of compounds were read directly from them. The values obtained from the duplicate injections (single injection in each column) were averaged.
Figure 4. Typical standard curves used in gas chromatographic analyses of parathion and paraoxon samples relating the ratios of sample and standard peak heights to parathion and paraoxon concentrations. Standard concentrations were $10^{-6}$ M.
Thin-layer chromatography. Precoated, 250 μ-thick, silica gel F-254 glass plates, 5 x 20 cm and 20 x 20 cm (Brinkmann Instruments, Inc., Westburg, N.Y.), were employed for quantitation and identification of metabolites and for determination of purity of the parathion and paraoxon stock solutions. The solvent systems employed by Neal (1967a) gave optimum separation of parathion and its metabolites. Several other solvent systems were tried and gave poorer separations. Using the nomenclature of Neal, solvent system A consisted of methanol:chloroform:10%(v/v) NH₄OH in a 24:75:3.5 ratio by volume and effectively separated aqueous metabolites. Solvent system B was composed of n-hexane:chloroform:methanol in a 7:2:1 ratio by volume and effectively separated parathion and paraoxon.

Microcaps (Drummond Scientific Company, Broomall, Pa.) were employed to spot 10- or 20-μl samples of reference compounds and polar and nonpolar incubation samples. Before removing aqueous samples for TLC analyses, the small amount of hexane remaining was evaporated with nitrogen and the protein precipitate was removed by filtration.

The coating on the plates was scored into as many as ten, 2- x 10-cm rectangles; therefore, as many as 10 samples could be spotted on the same plate. Usually duplicate spots of aqueous samples and single spots of reference samples were spotted on the same plate. Cool air blown over the plate aided the application of samples.

The plates were subjected to ascending chromatography in tanks lined with filter paper (Whatman no. 3). The plates were removed from the tanks when the solvent front had migrated 10 cm past the origin. A brief
observation under an ultraviolet lamp permitted a recording of fluorescent spots indicating the location of sample components. Either 0.5- or 1-cm wide bands were scraped crosswise into scintillation counting vials for counting. Spots with $R_f$ values identical to authentic parathion and paraoxon samples were eluted with n-hexane and the extracts of these spots were analyzed by GLC for corroborative identification. The recovery of radioactive samples was compared for identical aliquots which had been pipetted directly into scintillation counting vials or which were spotted and immediately scraped into scintillation counting vials.

The parathion and paraoxon standards were previously described in the gas chromatography section.

$^{35}$S-labeled DEPTA used as a reference standard had been prepared previously (Nakatsugawa et al. 1968) and stored as an aqueous solution at -15 C. $^{14}$C-DEPA was graciously provided by Dr. T. Y. Ku of our laboratory. The latter compound was prepared by ion-exchange chromatography of metabolites found in urine collected from $^{14}$C-paraoxon-treated rats as described by Nakatsugawa et al. (1969) based on the procedure of Plapp and Casida (1958b).

**Liquid scintillation spectrometry** Radioactivity was measured by a Packard model 3003 Tri-Carb Liquid Scintillation Counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The optimal settings for our system were a gain of 25% for the red channel from which sample counts were taken and 6% gain for the blue channel from which was recorded the automatic external standardization values. Background subtraction was set at zero; however, 10-min counts were taken periodically to monitor background
levels and total counts were adjusted for samples of low activity, e.g.,
several hundred to two thousand counts per minute (cpm). For most 0.5 ml
nonpolar and aqueous samples, the background radiation accounted for <1%
of the total counts and was disregarded.

The scintillation counting mixture consisted of 50 g of scintillation
grade PPO (2,5-diphenyloxazole), a primary scintillator, and 2 g of scin­
tillation grade dimethyl POPOP {1,4-bis-2-(4-methyl-5-phenyloxazolyl)-
benzene}, a secondary scintillator, dissolved in 5 liters of reagent grade
toluene. To this mixture was added 500 ml of methanol:aminooethanol (40:6)
and brought to a total volume of 10 liters with methanol. This counting
fluid was thoroughly mixed with a magnetic stirrer and stored in a dark
cabinet in brown glass bottles.

All counting vials were of the low-potassium-1 type; their screw caps
were lined with cork and tin foil (Packard Instrument Co., Inc.). Radio­
activity in sample solutions containing either aqueous metabolites or
hexane-extractable compounds was measured by adding 0.5 ml of a sample
to 20 ml of scintillation fluid. Duplicate counting samples were prepared
from each sample. The hexane-extracts were taken from the same sample
that was used for GLC analyses; careful removal of 1 ml from the extracted
aqueous layer was accomplished by gently expelling a few air bubbles as a
pipette was lowered into the aqueous layer; the tip of the pipette was
wiped with Kleenex® tissue before transferring the fluid to the counting
vials. All vials were tightly capped, shaken, and cooled for at least 30
min in the sample chamber to about 2 C. All fluid samples were counted
for one min as the activity generally ranged from about 2000 to 140,000
cpm. Dark adaption and cooling stabilized the background counts of blanks containing scintillation fluid to about 20 to 30 cpm.

The TLC bands or spots to be assayed were scraped with the aid of a funnel into vials containing scintillation solution; the vials were shaken well, cooled, and counted. The relatively low activity of the TLC samples necessitated 10 min counts; the background counts of vials of scintillation fluid containing silica gel scrapings from plates without samples averaged 48 cpm.

Enzyme Activity Studies

Liver cell fractions vs parathion metabolism

The rat liver cell fractions previously described were incubated with parathion. The fine structure of these fractions was examined by electron microscopy in order to correlate biochemical findings with cytological observations.

Incubation conditions affecting enzyme activity

Resuspending media Rat liver tissue was homogenized and centrifugally fractionated in 0.25M sucrose. Since CsCl was employed in the fractionation of the post-mitochondrial supernatant into rough-surfaced membranes and smooth-surfaced membranes (Figure 2) and since a review of the literature showed a lack of uniformity of media used in the preparation of microsomes, various media were tested for their influence on the parathion-metabolizing activity of crude microsomes. The following resuspending solutions were tried: (a) 0.008M Na₂HPO₄:0.002M KH₂PO₄:0.15M KCl, (b) 0.08M Na₂HPO₄:0.02M KH₂PO₄:0.15M KCl, (c) 0.8M Na₂HPO₄:0.2M
Enzyme activity vs pH  To assess the influence of pH on microsomal metabolism of parathion in vitro, a series of 0.01M phosphate:0.15M KCl: 0.125M sucrose buffers of pH's ranging from 3.0 to 10.4 were prepared, the pH being adjusted by the relative proportions of $H_3PO_4$, $KH_2PO_4$, $Na_2HPO_4$, and $Na_3PO_4$. The final pH values of the incubation mixture containing freshly prepared microsomes ranged from 4.3 to 8.6. Within this range the pH values were maintained within ± 0.1 pH unit of the initial reading during the 1 hr incubation. Replicate experiments were run on separate days except that incubation mixtures with pH values of 4.3 and 8.6 were tested in only one experiment. Duplicate incubations were run in all experiments. Duplicate controls were run at each pH tested with the exceptions of pH 4.3 and pH 8.6 where a single control was run for each. Other incubation parameters and analyses of the products were as described.

Temperature  The metabolism of parathion by rat liver microsomes
was undertaken at 4, 17, 22, 34, 37, 40, 45, and 58 C. All temperatures were maintained ± 1.0 C. Microsomes that had been stored frozen (-20 C) either 2 weeks or 4 weeks were thawed and resuspended to a 20% concentration (equivalent to 20% w/v of liver tissue) in 0.008M Na₂HPO₄:0.002M KH₂PO₄:0.15M KCl:0.125M sucrose. The standard incubation buffer, 0.008M Na₂HPO₄:0.002M KH₂PO₄:0.15M KCl, was replaced with the resuspension medium containing sucrose; the cofactors were dissolved in the latter buffer plus sucrose mixture. The incubation mixtures minus the microsomes were equilibrated at appropriate temperatures and the microsomes were added, starting at the lowest temperature and warmed in a water bath to within 1 C of the next higher temperature before addition. Replicate experiments were performed on separate days with duplicate incubations on each day. Duplicate controls were established for 4, 22, 37, and 58 C incubations only. Other incubation parameters and procedures of analysis were as previously described.

Enzyme concentration The effect of various levels of TCA-precipitable microsomal protein, expressed as mg of nitrogen (mg N), on the in vitro conversion of parathion was assessed. Rat liver microsomes that had been frozen for one week were thawed and suspended in 0.008M Na₂HPO₄:0.002M KH₂PO₄:0.15M KCl:0.125M sucrose solution. One ml of this 20% (w/v) microsomal suspension was found to contain 0.964 mg N (average value) by micro-Kjeldahl analysis. Aliquots of microsomal suspensions, ranging from 0.1 ml to 2.0 ml, were added and the total incubation volume of 4 ml was maintained by adjusting the volume with 0.008M Na₂HPO₄:0.002M KH₂PO₄:0.125M sucrose solution. All other conditions and analyses were as
Parathion metabolism vs time  The incubation interval of one hr was identical to that used by Nakatsugawa and Dahm (1967), Nakatsugawa et al. (1969), and Neal (1967a, 1967b). A single experiment with duplicate incubations was conducted to assess the progress of parathion conversion over shorter and longer periods of incubation within my experimental system. Crude microsomes that had been freshly prepared and stored briefly in an ice bath after pelleting were resuspended in the standard resuspension medium as a 10% suspension. In one pair of incubations the 10% TCA in 3 N HCl solution used to stop enzymatic activity was added prior to the addition of 1.0 ml of microsomes. In all other incubations the experiments began with the addition of microsomes and stopped with addition of the acid solution at intervals ranging from 10 sec to 120 min after initiation.

Microsomal storage  Microsomes prepared from the pooled livers of six rats, that averaged 280 g with an average liver weight of 7 g, were assessed for their capacity to metabolize parathion as freshly prepared microsomes and as microsomes that had been stored as frozen pellets at -20 C for periods ranging from 1 to 6 weeks. All pellets were resuspended in the standard medium of 0.008M Na$_2$HPO$_4$:0.002M KH$_2$PO$_4$:0.15M KCl:0.125M sucrose and were incubated for 1 hr. The incubation products were analyzed as previously described.

Paraoxon hydrolysis

The conversion of paraoxon to DEPA and p-nitrophenol by several liver
cell fractions was studied in replicate experiments with duplicate incubations in each. The metabolism of paraoxon by the mitochondrial fraction, crude microsomes, rough microsomes, and smooth microsomes was determined by GLC. The hydrolysis of paraoxon was assessed by comparing the quantity of paraoxon extractable by 10 ml of hexane from incubations containing 1.0 ml of the respective cell fractions with the quantity of paraoxon in control incubations in which enzyme had been deleted. The incubation conditions were the same as those used for parathion studies, with the final concentration of paraoxon being $2.5 \times 10^{-5}$ M.

**Solubilization of microsomal enzymes**

Several liver cell fractions were treated as follows in an effort to dissociate the enzymes responsible for the metabolism of parathion from their membranous site: (1) Crude, rough, and smooth microsomal pellets (Figure 2) were resuspended in 5 ml of 0.5% (w/v) sodium deoxycholate (Nutritional Biochemicals, Corp., Cleveland, Ohio) or 5 ml of 0.5% (v/v) Tween 80® (Sargent-Welch Sci. Co., Skokie, Ill.). They were treated at 4 C with a 30 min shaking on a wrist-action shaker (Burrell Corp., Pittsburgh, Pa.). (2) Crude microsomes were treated for 1 hr with 0.01% (w/v) lysozyme (9950 U/mg, Worthington Biochemical Corp., Freehold, N.J.) in glass distilled water. (3) A portion of the microsomes treated in (2) was subjected to sonication for approximately 1 min (Sonifier®, 6 amp D.C., Branson Instruments, Inc., Stamford, Conn.). (4) Crude microsomes were treated for varying periods by sonication at 2.5 amp. (5) Crude microsomes were suspended in 0.008M Na$_2$HPO$_4$:0.002M KH$_2$PO$_4$:0.15M KCl buffer containing 0.1% (v/v) Triton X-100. (6) A portion of (5) was sonicated
for either 1 or 5 min. (7) Crude microsomes suspended in 0.008M Na₂HPO₄: 0.002M KH₂PO₄: 0.15M KCl: 0.25M sucrose were incubated at 36 to 37 C for 6 hr followed by sonication for 10 min at 6 amp. (8) A sample of the 9,340 g avg supernatant (Figure 2) was incubated 12 hr at 37 C, made 0.5% by volume with Triton X-100 and sonicated at 2.5 amp for 1 hr. The temperature of the samples was depressed with an ice bath during sonication.

After treatments 1 through 8 were completed, the preparations were centrifuged at 105,200 g avg for 60 to 90 min. The supernatants were collected and 1 ml aliquots were incubated with the standard incubation mixture; parathion metabolism was compared to incubation mixtures with untreated microsomes from the same sources incubated concurrently with the treated samples. The supernatant of (8) was concentrated approximately 30% by a Diaflo® apparatus equipped with a UM-2 filter (Amicon Corp., Lexington, Mass.) prior to incubation. The enzyme activities of the undissociated pelleted fractions of treatments (7) and (8) were assessed.

Data Calculations

A total of 100 nanomoles of ¹⁴C-parathion was added to each incubation mixture, i.e., 0.1 ml of a 1 x 10⁻³ M stock solution was added. To estimate radiometrically the amount of parathion and its metabolites after the incubation of parathion with cell fractions of rat liver, standard hexane and standard aqueous solutions were prepared by adding 100 nanomoles of ¹⁴C-parathion to 10 ml of hexane and 5 ml of water, respectively. Two 0.5 ml samples of each of these standards were counted and the mean
cpm values were determined. The average cpm values that were determined from duplicate 0.5 ml samples from each experimental incubation were divided by the mean cpm values of the related, standard solutions. The quotients obtained from the hexane samples were multiplied by 100; these products were compared to the amounts of parathion and paraoxon estimated by GLC. The quotients obtained from the aqueous samples were also multiplied by 100; the products became the estimates of the amount of parathion converted to aqueous metabolites.

The specific activity of the enzyme systems found in the liver cell fractions employed in this study was expressed as moles of substrate reacting per unit of enzyme per unit of time or moles of product per unit of enzyme per unit of time (Dixon and Webb 1958). Inasmuch as the enzyme sources employed were heterogenous systems, the best representation of enzyme unit was weight of nitrogen or weight of protein. I have chosen to express results in terms of nanomoles of substrate or product/mg N/hr; nitrogen values were consistently obtained by micro-Kjeldahl analyses.

It was previously described how parathion and paraoxon were quantified by GLC through the interpolation of standard peak-height-ratio curves.

Pentose analysis by the method of Schneider (1945) was used to estimate the RNA content of the following liver cell fractions: mitochondria, crude microsomes, rough microsomes, and smooth microsomes (Figure 2, fractions 3, 4, 5, and 6, respectively). Assuming rat liver ribosomes were the principal source of RNA in these experiments and that these ribosomes were approximately 50% RNA and 50% protein by weight (Siekevitz
1967), the total precipitable N measured by micro-Kjeldahl can be ad­justed by subtracting the N contributed by ribosomal RNA and protein. Protein has been reported to be about 16% by weight (Mahler and Cordes 1966) while RNA was reported to be 16.1% nitrogen by weight (Schneider 1945). After measuring the RNA content for each of the four fractions and making the above assumptions, the nitrogen values were adjusted and these values were used in the calculations of specific enzyme activity.

In TLC studies of radioactive substrate and metabolite standards and samples, an aliquot equal to that spotted at the origin was placed some distance above the solvent front after the plate had been removed from the solvent system and air dried. An area of silica gel exceeding but including the spot was scraped into a vial containing scintillation solution. The cpm values of all chromatographed spots, usually scraped as 1 x 2 cm areas, were divided by the cpm value of the nonchromatographed sample. The quotient was multiplied by 100 to estimate the percent of radioactivity attributable to each compound. Sometimes the standard and sample spots had Rf values that overlapped two bands. In this case the cpm values were the sum of the two adjacent bands.

To estimate the quantities, in nanomoles, of each of the compounds identified after TLC separation, two methods of calculation were used, i.e., both methods were used where applicable for hexane extracted com­pounds and aqueous soluble compounds. One method, abbreviated as RA, expressed the total radioactivity in the extraction phase contributed by a particular component. The formula used was:
(cpm) x (ml extract) x (10^3 μl/ml)
(μl spotted on TLC plate) = Total radioactivity of component in extract

For example, the incubation mixture was extracted with 10 ml of n-hexane; 20 μl of this extract was chromatographed; a spot of Rf 0.3 was scraped into a vial containing scintillation fluid and counted. The estimated total cpm value for the particular component with Rf 0.3 that had produced a net cpm value of 200, after adjustment for background radiation, would be:

\[
\frac{(200 \text{ cpm}) \times (10 \text{ ml}) \times (10^3 \mu l/ml)}{20 \mu l} = 10^5 \text{ cpm}
\]

The mean value for the 10^{-3} M ^{14}C-parathion stock solution used was 16,934 cpm/μl. Since 100 μl of ^{14}C-parathion was added to the incubation mixture, the recovery (percent) of radioactivity at Rf 0.3 on TLC plate was:

\[
\frac{10^5 \text{ cpm}}{(1.6934 \times 10^4 \text{ cpm/μl}) \times (10^2 \mu l)} = \frac{10^5}{1.6934 \times 10^6} \times 100 = 5.9%
\]

It was fortuitous that 100 nanomoles of parathion were added to the incubation mixture; consequently, percent recovery values were numerically equivalent to nanomole quantities.

The second method, abbreviated SA, estimated the amount of metabolism of parathion to metabolites, based on the specific activity of the radioactive parathion as reported by the supplier. The specific activity of this lot of parathion was 8.84 mc/m mole. The decrease in specific activity of the parathion over the period of these experiments was negligible, owing to the long half-life of ^{14}C (5,770 years). The disintegration
constant for $^{14}\text{C}$ is $2.22 \times 10^9$ dpm/mc (Hendricks 1968). The quantity of radioactivity in the TLC sample described above, expressed as nanomoles, was estimated by method SA as follows:

\[
\frac{(\text{cpm}) \times (\text{ml extract}) \times (10^3 \mu l/ml)}{(\mu l \text{ spotted} \times (^{14}\text{C-parathion}) \times (\text{disintegration} \times (\text{efficiency}) \text{ on TLC plate}) \times (\text{specific activity}) \times (\text{constant})} = \text{quantity of sample}
\]

i.e.,

\[
\frac{(200 \text{ cpm}) \times (10 \text{ ml}) \times (10^3 \mu l/ml)}{(20 \mu l) \times (8.84 \text{ mc/mmole}) \times (2.22 \times 10^9 \text{ dpm/mc}) \times (86\%)} = 5.9 \text{ nanomoles}
\]

When this formula was used to estimate the number of nanomoles of parathion in 0.1 ml of the $10^{-3}$ M parathion solution, the mean cpm value was found to be about 86% of the theoretical value. Three separate gravimetric preparations of $10^{-3}$ M parathion solutions produced identical peak heights by GLC analysis. Consequently, a correction factor of 1.16 was used for adjusting estimates to compensate for counting efficiency. The estimate of the component described above became 5.9 nanomoles ($5.1 \times 1.16$). Thus, the results of two methods are in agreement.
RESULTS AND DISCUSSION

Electron Microscopy

The morphologies of cytoplasmic particulates in rat liver homogenates and centrifugal fractions have been ably described (Kuff et al. 1956, Palade and Siekevitz 1956, Moulé et al. 1960, Rothschild 1963, Gram et al. 1967, Bergstrand and Dallner 1969). The morphologies of the cell fractions (Figure 2) used in the study of parathion metabolism are presented in Figures 5 through 18 and are in general agreement with these earlier reports in regard to the identity and characterization of the principal components of the rat liver fractions.

Homogenate (1)

The homogenate (Figure 5) shows mitochondria in varying states of disintegration and rough and smooth endoplasmic reticulum fragments in the form of vesicles.

Debris (2)

Figures 6 and 7 are magnifications of 19,600X and 42,300X, respectively, of the debris pellet. Mitochondria are the predominant component with some rough and smooth endoplasmic reticulum vesicles sandwiched between the mitochondria. Most of the mitochondria do not exhibit the characteristic infolding of the inner membrane, and some appear to have the outer membrane detached.

Mitochondria (3)

Contrasting aspects of the mitochondrial pellet are seen in Figures 8
Figure 5. Homogenate (1), procedure B, containing rough endoplasmic reticulum fragments (RER), smooth endoplasmic reticulum fragments (SER), and mitochondria (M). 42,300X
Figure 6. Debris pellet (2), procedure B; predominantly mitochondria (M) with some rough endoplasmic reticulum (RER) fragments. 19,600X

Figure 7. Same as figure 6. 42,300X
and 9. The mitochondria and lysosomes sediment toward the bottom of the pellet while the rough endoplasmic reticulum vesicles collect near the centripetal pole of the pellet. Most ribosomes are bound as opposed to free.

The mitochondria observed in fractions 1, 2, and 3 (Figure 2) were atypical by classical description. The main changes appear to be an expansion of the inner membrane compartment, an alteration in the folding of the inner membrane, and a change in the appearance of the matrix material. Packer (1970) has examined the conformational changes that occur in rat liver mitochondria in relation to energy coupling. He observed conformational changes, as a function of ion accumulation, by light scattering changes and by electron microscopy.

The structure of the mitochondria observed in this study, and represented in Figures 5 through 8, is very similar to the morphology of expanded mitochondria described by Packer (1970) that occurs at the peak of the first oscillation after ion accumulation.

**Crude microsomes (4)**

A portion of the crude microsomal pellet exhibited numerous ribosomes together with a relatively small number of smooth endoplasmic reticulum vesicles (Figure 10). Many of the ribosomes were aggregated in groups numbering from 3 to 7. These aggregates appear to be held together in a beaded, strand-like fashion and morphologically resemble polysomes. The precise location of these structures within the pellet was not determined. A more characteristic appearance of crude microsomes obtained from a different section of the pellet is seen in Figures 11 and 12 with rough
Figure 8. Mitochondrial pellet (3), procedure B. Mitochondria (M) in various states of disintegration. Lysosome (L). 42,300X

Figure 9. Mitochondrial pellet (3), procedure B. Note predominance of rough endoplasmic reticulum vesicles (RER). 42,300X

Figure 10. Crude microsomal pellet (4), procedure B. Ribosomes predominate with polysomes indicated by arrows or circles. 42,300X
Figure 11. Crude microsomes (4), procedure B. Pellet frozen for 6 weeks prior to processing. Both rough and smooth endoplasmic reticulum vesicles present (RER and SER). 42,300X

Figure 12. Crude microsomes (4), procedure B. Pellet frozen overnight. 42,300X
and smooth endoplasmic reticulum vesicles and free ribosomes randomly assorted.

**Rough microsomes** (5)

The subfractionation of the postmitochondrial supernatant with a discontinuous sucrose gradient containing CsCl yields two fractions. The rough endoplasmic reticulum vesicles are selectively sedimented to the bottom of the tube and are the predominant component in Figures 13 and 15. Figures 13 and 15 represent the rough microsomes derived from two different liver sources.

When crude microsomes are resuspended in isotonic sucrose and 15mM CsCl, the subfractionation of fraction 4 yields a pellet composed primarily of rough endoplasmic reticulum vesicles also (Figure 17).

**Smooth microsomes** (6)

The components of the postmitochondrial supernatant that collect at the interface between the sucrose densities can be recentrifuged into a smooth microsomal pellet. This pellet is presented in Figures 14 and 16 showing smooth endoplasmic reticulum vesicles or fragments as the predominant component. Most ribosomes appear to be free. The average diameter of the smooth vesicles is considerably smaller than the diameter of the rough vesicles.

The smooth microsomal pellet obtained by subfractionation of crude microsomes resuspended in isotonic sucrose and 15mM CsCl is represented in Figure 18.

The contamination by smooth membranes in the rough microsomal
Figure 13. Rough microsomes (5), procedure A. Predominantly rough endoplasmic reticulum fragments (RER). 42,300X

Figure 14. Smooth microsomes (6), procedure A. Tightly packed smooth endoplasmic reticulum fragments (SER) with slight ribosomal contamination. 42,300X
Figure 15. Rough microsomes (5), procedure B. Rough endoplasmic reticulum vesicles (RER) predominate. 42,300X

Figure 16. Smooth microsomes (6), procedure B. Smooth endoplasmic reticulum vesicles (SER) predominate. Slight contamination with free ribosomes (R). 42,300X
Figure 17. Rough microsomes (5), procedure B. Prepared by subfractionation of crude microsomes (4). Rough endoplasmic reticulum vesicles (RER). 42,300X

Figure 18. Smooth microsomes (6), procedure B. Prepared by subfractionation of crude microsomes (4). Smooth endoplasmic reticulum vesicles (SER). 42,300X
fraction was small and a similar relationship held for the contamination by rough membranes in the smooth microsomal fraction. Generally, the smooth microsomal fraction was quite free of ribosomes whether bound or unbound. This differs from the reports of Moulé et al. (1960), Chauveau et al. (1962), and Lee et al. (1969) where the concentration of RNA in smooth microsomes was approximately the same or greater than that found in rough microsomal fractions.

Rough and smooth microsomes prepared by Procedure A (Table 1) have profiles that differ from the same fractions prepared under Procedure B. The two procedures were not used strictly for comparative purposes. Procedure B was an attempt to maintain near-isotonic solutions throughout fixation and differed in several other ways from Procedure A. The microsomal profiles prepared under Procedure A (Figures 13 and 15) were more elongated and slender than those prepared under Procedure B (Figures 14 and 16). After "isotonic" fixation (Procedure B), these profiles were oval or circular. Similar observations were reported for crude microsomes by Palade and Siekevitz (1956). The vesicular elements of both rough and smooth microsomes have varying densities internally, i.e., some appear to be practically empty while others contain relatively dense material. It is not known from this study whether these variations reflect different metabolic functions or heterogeneous origins within the rough and smooth endoplasmic reticulum or possible other membranous organelles. This study did not include an enzymatic assessment of the origins of the membrane fragments.
In Vitro Analytical Procedures

Control studies

In 58 separate control incubations in which liver cell fractions were replaced with equivalent volumes of buffer, no paraoxon was detected and an average of 0.44% of the initial substrate concentration was not extracted by \( n \)-hexane from the aqueous layer. The amount of parathion and breakdown products in the aqueous fraction was measured by scintillation counting.

In 3 separate control incubations, crude microsomes were added to the standard incubation mixture but NADPH\(_2\) and nicotinamide were omitted. No paraoxon was detected after 1 hr of incubation, and radioactivity equivalent to 0.40% of the initial \(^{14}\)C-parathion concentration was detected in the \( n \)-hexane extracted aqueous fraction.

The data from the control studies indicated that (1) efficient extraction of parathion from the incubation mixture was accomplished by a single, 10-ml volume of \( n \)-hexane, (2) nonenzymatic breakdown of parathion to water-soluble products in the incomplete incubation mixture was negligible, and (3) omission of the reduced nucleotide cofactor was as effective in preventing the metabolism of parathion as omission of the enzyme itself.

Analyses

Protein nitrogen The nitrogen content of all 6 cell fractions described in Figure 2 was determined on 3 separate occasions and recorded in Table 2. In addition the supernatant above the crude microsomal pellet
Table 2. TCA-precipitable nitrogen values determined by micro-Kjeldahl analyses of rat liver cell fractions prepared from 20% homogenates for in vitro metabolism of parathion. Mean values for duplicate determinations expressed as mg N/ml of 20% (w/v) suspension.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Homogenate (1)</th>
<th>Debris (2)</th>
<th>Mitochondria (3)</th>
<th>Crude Microsomes (4)</th>
<th>Rough Microsomes (5)</th>
<th>Smooth Microsomes (6)</th>
<th>Su₁a (7)</th>
<th>Su₂b (8)</th>
<th>Summation of columns 2, 3, 4, 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.509</td>
<td>0.163</td>
<td>0.297</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2d</td>
<td>5.645</td>
<td>2.408</td>
<td>1.288</td>
<td>0.503</td>
<td>0.271</td>
<td>0.302</td>
<td>1.419</td>
<td>1.360</td>
<td>5.618</td>
</tr>
<tr>
<td>3d</td>
<td>5.601</td>
<td>1.938</td>
<td>1.202</td>
<td>0.995</td>
<td>0.416</td>
<td>0.357</td>
<td>1.639</td>
<td>1.575</td>
<td>5.774</td>
</tr>
<tr>
<td>4c</td>
<td>6.053</td>
<td>3.385</td>
<td>0.342</td>
<td>0.466e</td>
<td>0.164</td>
<td>0.162</td>
<td>1.271</td>
<td>1.115</td>
<td>5.464</td>
</tr>
<tr>
<td>5d</td>
<td>-</td>
<td>-</td>
<td>0.492e</td>
<td>0.926e</td>
<td>0.420</td>
<td>0.290</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6d</td>
<td>-</td>
<td>-</td>
<td>0.836e</td>
<td>0.840e</td>
<td>0.425</td>
<td>0.302</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mean Value</td>
<td>5.766</td>
<td>2.577</td>
<td>0.832</td>
<td>0.706</td>
<td>0.310</td>
<td>0.285</td>
<td>1.443</td>
<td>1.350</td>
<td>5.558</td>
</tr>
</tbody>
</table>

aFraction (7) abbreviated Su₁ = 105,200 gavg supernatant.

bFraction (8) abbreviated Su₂ = 269,000 gavg supernatant prepared by centrifuging Su₁ at 269,000 gavg for 60 min.

cThree livers pooled.

dSingle liver.

e10% (w/v) suspensions used for incubations.
(Su₁ = 105,200 gavg supernatant, fraction 7) was analyzed for nitrogen content before and after centrifuging at 269,000 gavg for 1 hr in a Spinco #65 rotor. The latter supernatant (Su₂) is referred to as fraction 8. A very small amount of particulate material was sedimented; this is reflected in the small decrease in nitrogen content. The classical fractions derived from rat liver homogenate are "debris", "mitochondrial", "microsomal", and "soluble" fractions (Palade and Siekevitz 1956). A summation of these 4 fractions is recorded in the right-hand column of the table. Recovery of the TCA-precipitable nitrogen content of the original homogenate averages >96%. However, there is variation among preparations within a given fraction.

**RNA analysis**  
RNA levels measured for fractions (3), (4), (5), and (6) in 2 experiments are summarized in Table 3. The mitochondrial fraction and crude microsomal fraction were 10% suspensions while the rough and smooth microsomes were 20% suspensions. The crude microsomes exhibit the highest level of RNA per g of tissue. Since the rough and smooth microsomal fractions are derived from the postmitochondrial supernatant also, their summation should approximate the crude microsomal value; the recovery is approximately 92%. The relatively high mitochondrial RNA values seen in Table 3 are attributed to the contamination by rough endoplasmic reticulum fragments and free ribosomes in the mitochondrial fraction. Attention has been called to this contamination in the mitochondrial fraction under Electron Microscopy.

The protein and RNA concentrations and RNA:protein ratios observed in this study (Tables 2 and 3) were compared to values obtained by
Table 3.  RNA determinations for rat liver cell fractions

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Exp. No.</th>
<th>mg RNA/ml&lt;sup&gt;a&lt;/sup&gt;</th>
<th>mg RNA/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>5</td>
<td>0.10</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.12</td>
<td>1.20</td>
</tr>
<tr>
<td>Crude Microsomes</td>
<td>5</td>
<td>0.25</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.22</td>
<td>2.20</td>
</tr>
<tr>
<td>Rough Microsomes</td>
<td>5</td>
<td>0.38</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.33</td>
<td>1.65</td>
</tr>
<tr>
<td>Smooth Microsomes</td>
<td>5</td>
<td>0.09</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.07</td>
<td>0.35</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean value of duplicate measurements.
several other investigators (Table 4). It can be seen that the average microsomal concentration of 22 mg protein/g wet liver tissue is similar to that determined by others. The RNA concentration found in this study was slightly lower for crude and rough microsomal fractions but comparable to others for smooth fractions. The low RNA:protein ratio observed in this study indicates adequate separation of rough from smooth membranes with approximately 4.5X as much RNA in the rough fraction as in the smooth.

The mean value for mg N/g of tissue in the liver homogenate was 28.8; converted to mg protein/g of tissue, this is 180 mg protein/g of wet tissue. Palade and Siekevitz (1956) reported 24.4 mg N/g of tissue and 152 mg protein/g of wet weight liver pulp.

Based on the similarities between my findings and those reported in the literature, I conclude that my measurements are valid and can be used as a basis for comparisons of activity between fractions.

**Metabolite identification and quantitation** The reaction mixtures resulting from the incubation of $^{14}$C-parathion with male rat liver cell fractions were examined for metabolic breakdown products retaining the $^{14}$C-label in the ethoxy groups. After extracting the hexane and the removal of this layer, the reaction mixture was deprotei nized by filtration and 10 µl samples of the aqueous layer and reference compounds were spotted and co-chromatographed in solvent system A. Aliquots of 20 µl from the hexane layer were spotted and developed with solvent system B. The average $R_f$ values for samples and standards are summarized in Table 5. Paraoxon and parathion were not separated in solvent system A but were
Table 4. A comparison of protein and RNA levels in microsomal fractions derived from rat liver as reported in independent studies

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Protein (mg/g tissue)</th>
<th>RNA (mg/g tissue)</th>
<th>RNA/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude Microsomes</td>
<td>18.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>21.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>19.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>22.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.35&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rough Microsomes</td>
<td>10.9</td>
<td>3.31</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>10.4</td>
<td>2.48</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1.78</td>
<td>0.18</td>
</tr>
<tr>
<td>Smooth Microsomes</td>
<td>6.7</td>
<td>0.54</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>7.1</td>
<td>0.20</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.40</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<sup>a</sup>Glaumann and Dallner, 1968.
<sup>b</sup>Bergstrand and Dallner, 1969.
<sup>c</sup>Palade and Siekevitz, 1956.
<sup>d</sup>Data from this research.
Table 5. *R*$_f$ values of parathion and metabolites in two solvent systems$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent system A$^b$</th>
<th>Solvent system B$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean$^d$</td>
</tr>
<tr>
<td>$^{14}$C-Parathion</td>
<td>0.70-0.90</td>
<td>0.82</td>
</tr>
<tr>
<td>$^{14}$C-Paraoxon</td>
<td>0.70-0.90</td>
<td>0.82</td>
</tr>
<tr>
<td>$^{35}$S-DEPTA</td>
<td>0.18-0.32</td>
<td>0.26</td>
</tr>
<tr>
<td>$^{14}$C-DEPA</td>
<td>0.12-0.17</td>
<td>0.15</td>
</tr>
</tbody>
</table>

$^a$Solvent front traveled 10 cm from origin.

$^b$Solvent system of methanol:chloroform:10% (v/v) NH$_4$OH (24:75:3.5).

$^c$Solvent system of hexane:chloroform:methanol (7:2:1).

$^d$Means based on a minimum of 10 determinations.

$^e$Not determined.
separated well in solvent system B. DEPTA and DEPA were adequately separated in system A.

The extraction of parathion from the incubation mixture by a single 10-ml volume of n-hexane was essentially complete (99.6%). When compounds in the aqueous extract with a mean \( R_f \) value of 0.82 in solvent system A or 0.28 in solvent system B were partitioned into n-hexane and analyzed by GLC, paraoxon only was detected. Experimental data based on GLC analyses indicated that approximately 87% of the paraoxon was extracted by a single 10-ml volume of n-hexane. Adjustments for this error in the estimation of specific enzyme activity values were not made.

The quantities of DEPTA, DEPA, paraoxon, and parathion from experiments in which more than one method of detection and quantitation had been employed were tabulated, according to the methods used, for comparison (Table 6). The values for paraoxon in the hexane extract as determined by GLC and radiometric-TLC methods were similar. Likewise, parathion values determined by GLC and radiometric-TLC methods were in agreement. Unextracted paraoxon and parathion in the aqueous phase were not separated by the radiometric-TLC method employed in this analysis and are included under paraoxon (aqueous) in Table 6. Thus, the radiometric-TLC estimation of the compounds remaining in the aqueous phase after extraction with an average \( R_f \) of 0.82 (solvent system A) provided an indication of the efficacy of a single, 10 ml extraction of the incubation mixture.

The DEPTA:DEPA ratios from 60 min incubations in separate experiments ranged from 1.2 to 2.2 with an average ratio of 1.8 (Table 6). The ratios obtained from incubations of parathion with crude, rough, and smooth
Table 6. Estimated quantities in nmoles, of each of the compounds identified after incubation of 100 nmoles of $^{14}$C-parathion with rat liver cell fractions. Other incubation conditions are described in the text. Mean GLC values are from 2 incubations in each experiment each with duplicate samples; radiometric-TLC (RA-TLC) estimates represent mean values from at least two determinations from 1 of the 2 incubations in each experiment. Solvent systems are described in the text.

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>No. of Exps.</th>
<th>Length of Incubation (min)</th>
<th>DEPTA RA-TLC Aqueous</th>
<th>DEPA RA-TLC Aqueous</th>
<th>Paraoxon GLC Hexane</th>
<th>Paraoxon RA-TLC Aqueous Hexane</th>
<th>Parathion GLC Hexane</th>
<th>Parathion RA-TLC Hexane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mito-</td>
<td>1</td>
<td>60</td>
<td>1.2</td>
<td>1.4</td>
<td>8.6</td>
<td>0.8</td>
<td>N.D.</td>
<td>73.2</td>
</tr>
<tr>
<td>ochondria</td>
<td>1</td>
<td>10</td>
<td>2.1</td>
<td>0.6</td>
<td>7.1</td>
<td>1.6</td>
<td>6.2</td>
<td>69.2</td>
</tr>
<tr>
<td>Crude</td>
<td>3</td>
<td>60</td>
<td>7.0</td>
<td>3.9</td>
<td>15.8</td>
<td>5.0</td>
<td>14.6</td>
<td>43.1</td>
</tr>
<tr>
<td>Microsomes</td>
<td>1</td>
<td>120</td>
<td>8.0</td>
<td>5.8</td>
<td>17.1</td>
<td>6.1</td>
<td>16.5</td>
<td>37.6</td>
</tr>
<tr>
<td>Rough</td>
<td>2</td>
<td>60</td>
<td>5.0</td>
<td>3.2</td>
<td>15.3</td>
<td>4.0</td>
<td>12.3</td>
<td>50.6</td>
</tr>
<tr>
<td>Microsomes</td>
<td>2</td>
<td>60</td>
<td>6.9</td>
<td>4.8</td>
<td>15.8</td>
<td>5.6</td>
<td>12.8</td>
<td>49.3</td>
</tr>
</tbody>
</table>

*Not determined.
microsomes were very similar in the same experiment but varied from one experiment to another. The quantity of DEPTA and DEPA produced by the smooth microsomal fraction exceeded the quantity produced by the rough microsomal fraction. A comparison of the hydrolytic degradation of para-thion to DEPTA and p-nitrophenol and of paraoxon to DEPA and p-nitrophenol by crude, rough, and smooth microsomal fractions has not been reported previously. Neal (1967a) found the ratio of DEPTA:DEPA to be approximately 1.2 after 1 hr of incubation of para-thion with crude microsomes prepared from rat liver.

The mean DEPTA:DEPA ratio of 1.8 after 1 hr of incubation observed in this study is less than that reported by Plapp and Casida (1958a) for metabolism of para-thion in vivo. At a dosage of 1.5 mg para-thion/kg of rat body weight, the hydrolysis products collected from the urine were 78% DEPTA, 16% DEPA, 2% desethyl paraoxon, and 4% desethyl para-thion, i.e., they found the ratio of DEPTA:DEPA was 4.9. Nakatsugawa et al. (1969) found the ratio of DEPTA:DEPA varied depending on the dosage administered. A dosage of 0.2 mg para-thion/kg of rat body weight gave a ratio of 1.3; a dosage of 1 mg/kg produced a ratio of 2.0; and a dosage of 5 mg/kg produced a ratio of 2.4 for these two degradation products. They also detected phosphoric acid, ethyl phosphoric acid, and desethyl paraoxon at 1 and 5 mg/kg levels of para-thion administration. Thus, independent in vitro studies of the metabolism of para-thion by crude microsomes prepared from livers of male rats have produced similar results; furthermore, the DEPTA:DEPA ratios obtained from in vitro studies are practically identical to the ratios obtained from in vivo studies in which the
parathion dosage was 1 mg parathion/kg of rat body weight or less.

The DEPTA:DEPA ratio in incubations of parathion with crude microsomes varied with the length of incubation (Table 6). With 10 min incubation time, the ratio was 3.5, at 60 min it was 1.8, and at 120 min it was 1.4. The decrease of the DEPTA:DEPA ratio with time during the incubation suggests that a hydrolytic degradation of paraoxon is occurring.

Nakatsugawa and Dahm (1967) reported that paraoxon was degraded by paraoxon-treated rabbit microsomes whether a reduced pyridine nucleotide cofactor was present or absent. An enzymatic degradation of paraoxon is favored by findings in this study which will be described later.

- Enzyme Activity Studies

**Liver cell fractions vs parathion metabolism**

The results of the study of the metabolism of parathion by cell fractions (Figure 2) is reported in Table 7. The metabolism of parathion is expressed as specific enzyme activity for paraoxon production ($Y_1$) and for parathion conversion to aqueous metabolites ($Y_2$). The highest specific enzyme activity was found in the smooth microsomal fraction. By placing the specific enzyme activity value for paraoxon produced by smooth microsomes equal to 1, the ratio of values for mitochondria, crude microsomes, rough microsomes, and smooth microsomes is 0.43:0.68:0.73:1.00. Similarly, the ratio of specific enzyme activities for the metabolism of parathion to aqueous metabolites for these same fractions is 0.37:0.73:0.70:1.00.

Since the number of experimental samples were not equal for cell
Table 7. A comparison of parathion metabolism by cell fractions. Nitrogen values were determined by micro-Kjeldahl analyses without adjustment for ribosomal RNA and ribosomal protein. Incubation conditions are described in the text.

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>No. of Expts</th>
<th>mg N/ml (^a)</th>
<th>Paraoxon produced ((Y^1)) (^b) nmoles/mg N/hr</th>
<th>Aqueous metabolites produced ((Y^2)) (^c) nmoles parathion converted/mg N/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate (1)</td>
<td>3</td>
<td>5.766</td>
<td>1.7</td>
<td>3.5</td>
</tr>
<tr>
<td>Debris (2)</td>
<td>3</td>
<td>2.577</td>
<td>4.2</td>
<td>5.3</td>
</tr>
<tr>
<td>Mitochondria (3)</td>
<td>5</td>
<td>0.699</td>
<td>19.7</td>
<td>20.6</td>
</tr>
<tr>
<td>Crude Microsomes (4)</td>
<td>5</td>
<td>0.523</td>
<td>30.9</td>
<td>40.7</td>
</tr>
<tr>
<td>Rough Microsomes (5)</td>
<td>5</td>
<td>0.339</td>
<td>33.2</td>
<td>38.9</td>
</tr>
<tr>
<td>Smooth Microsomes (6)</td>
<td>5</td>
<td>0.283</td>
<td>45.6</td>
<td>55.7</td>
</tr>
<tr>
<td>105,200 g avg Supernatant</td>
<td>3</td>
<td>1.443</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>269,000 g avg Supernatant</td>
<td>3</td>
<td>1.350</td>
<td>0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

\(a\)Average mg N/ml of cell fraction suspension.

\(b\)Determined by GLC.

\(c\)Determined by scintillation counting.
fractions (1) through (6), here referred to as treatments, the fractions were split into two groups for analysis based on the number of replicate experiments. A statistical analysis (Kennedy 1970) was performed with the assistance of the Iowa State University (ISU) Computation Center on the data for the homogenate (1) and debris (2) fractions. The mean values are given in Table 7. The results of this analysis are summarized in Table 12 (Appendix). When only the treatment variates were considered, the $Y_1$-values for the homogenate and debris fractions were significantly different but the $Y_2$-values were not.

An identical analysis was performed on the raw data for the mitochondrial (3), crude microsomal (4), rough microsomal (5), and smooth microsomal (6) fractions. The results of the statistical analysis are summarized in Table 13 (Appendix). All of the F-values obtained for fractions 3 to 6 were highly significant.

Because the Treatment-Experiment (TE) interaction appeared to contribute a significant portion of the variance, it was decided to subject the data from a single experiment to an analysis of variance test. An experiment with triplicate incubations per treatment was selected because it included rough and smooth microsomes prepared according to the standard procedures in Figure 2 and also rough and smooth microsomes prepared by fractionating resuspended crude microsomes instead of the 9,340 $g_{avg}$ supernatant. Furthermore, the morphology of each of these fractions had been examined by electron microscopy. Only the results obtained from incubations of parathion with crude, rough, and smooth microsomal fractions (Table 8) were assessed statistically (Table 14, Appendix). An
Table 8. A comparison of parathion metabolism by crude microsomes and rough and smooth microsomes prepared according to procedures described in the text. Nitrogen values were determined by micro-Kjeldahl analyses without adjustment for ribosomal RNA and ribosomal protein. Incubation conditions are described in the text. Footnotes in Table 7 also apply to this table.

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Source</th>
<th>mg N/ml$^a$</th>
<th>Paraoxon produced (Y$^b$) nmoles/mg N/hr</th>
<th>Aqueous metabolites produced (Y$^c$) nmoles parathion converted/mg N/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>9,340 g$_{avg}$</td>
<td>0.233</td>
<td>45.8</td>
<td>52.1</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rough</td>
<td>9,340 g$_{avg}$</td>
<td>0.164</td>
<td>51.8</td>
<td>61.6</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rough</td>
<td>Crude</td>
<td>0.234</td>
<td>53.1</td>
<td>68.1</td>
</tr>
<tr>
<td>Microsomes</td>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smooth</td>
<td>9,340 g$_{avg}$</td>
<td>0.162</td>
<td>73.8</td>
<td>86.8</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smooth</td>
<td>Crude</td>
<td>0.052</td>
<td>80.8</td>
<td>83.3</td>
</tr>
<tr>
<td>Microsomes</td>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
analysis of variance test on the data in Table 10 indicated that the variability in enzyme specific activity values was significant. Consequently, a Scheffe Test (Walker and Lev 1969) based on an F-value of 3.480 was performed on the data in Table 8 to test for significant differences between specific enzyme activity values. For both \( Y_1 \) (nmoles paraoxon produced/mg N/hr) and \( Y_2 \) (nmoles parathion converted to aqueous metabolites/mg N/hr) values, the smooth microsomes prepared from both sources showed significantly higher values than rough microsomes or crude microsomes. Rough microsomes prepared from either source produced significantly higher \( Y_2 \)-values than crude microsomes.

In order to assess the influence of ribosomal nitrogen on the specific enzyme activity values, measurements were made according to the method of Schneider (1945) of the RNA content of the mitochondrial and crude, rough, and smooth microsomal fractions (Table 3). The RNA measurements were then used as described in the MATERIALS AND METHODS section to adjust the TCA-precipitable nitrogen values for each fraction to exclude the nitrogen contribution by the ribosomes. The specific enzyme activity values for these 4 liver cell fractions used in 2 separate experiments were adjusted for their nitrogen values and then compared (Table 9). The data that produced Table 9 were subjected to an analysis of variance test, the results of which are summarized in Table 15 (Appendix).

The values in Table 15 (Appendix) suggest that the variance contributed by differences in specific enzyme activity values of liver cell fractions (3) through (6), i.e. "treatments", is highly significant. When a least significant difference test, \( \text{lsd} = t_{.95} \frac{\sqrt{2 \times S^2}}{n} \), (Snedecor and
Table 9. A comparison of parathion metabolism by cell fractions. Nitrogen values of all fractions were adjusted for ribosomal RNA and ribosomal protein\(^a\). Specific enzyme activity values are mean values obtained from duplicate incubations in each experiment. Incubation conditions are described in the text.

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Expt. No.</th>
<th>Adjusted (\text{mg N/ml})</th>
<th>Paraoxon produced ((Y_1))(^c)</th>
<th>Aqueous metabolites produced ((Y_2))(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\text{nmoles/mg N/hr})</td>
<td>(\text{nmoles parathion converted/mg N/hr})</td>
</tr>
<tr>
<td>Mitochondria (3)</td>
<td>5</td>
<td>0.214</td>
<td>40.4</td>
<td>29.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.380</td>
<td>22.4</td>
<td>20.1</td>
</tr>
<tr>
<td>Crude Microsomes (4)</td>
<td>5</td>
<td>0.383</td>
<td>44.1</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.348</td>
<td>42.1</td>
<td>55.8</td>
</tr>
<tr>
<td>Rough Microsomes (5)</td>
<td>5</td>
<td>0.299</td>
<td>57.2</td>
<td>73.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.319</td>
<td>45.1</td>
<td>53.6</td>
</tr>
<tr>
<td>Smooth Microsomes (6)</td>
<td>5</td>
<td>0.240</td>
<td>64.4</td>
<td>82.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.282</td>
<td>54.4</td>
<td>70.4</td>
</tr>
</tbody>
</table>

\(^a\)Nitrogen adjustment described in Data Calculations of MATERIALS AND METHODS.

\(^b\)Adjusted \(\text{mg N/ml}\) of cell fraction suspension.

\(^c\)Determined by GLC.

\(^d\)Determined by scintillation counting.
Cochran (1967) was employed to test differences between "treatment" mean values, all specific enzyme activity values were significantly different, except \( Y_2 \)-values for crude and rough microsomes. Smooth microsomes exhibited the highest specific enzyme activity values and mitochondria exhibited the lowest. Thus, both the Scheffe test on equivalent data from a single experiment and a test of least significant difference between means drawn from replicate experiments supported the observation that the specific enzyme activity values of smooth microsomes were significantly higher than those of crude and rough microsomes in this study. The Scheffe test provided the additional information that the specific enzyme activity values of rough and smooth microsomes fractionated from 9,340 \( g_{\text{avg}} \) supernatant and of rough and smooth microsomes fractionated from crude microsomes were not significantly different. The cytological evidence suggested that the additional centrifugation and resuspension necessary to fractionate rough and smooth microsomes from pelleted crude microsomes resulted in greater disintegration and fragmentation of the microsomal vesicles than occurred in their direct preparation from the 9,340 \( g_{\text{avg}} \) supernatant (Figures 13 through 18). This decrease in structural integrity was not correlated with a significant loss in enzymatic activity. The additional preparative work and the lack of any significant increase in activity argue against a more lengthy and complicated preparative method.

No activation to paraoxon and a very small amount of degradation to aqueous metabolites occurred in incubations of parathion with microsomal supernatants (Table 7). Shishido and Fukami (1963) reported that
degradation of parathion and the production of desethyl parathion in the supernatant of the rat liver homogenate was very slight. It seems certain that both pathways of parathion metabolism (Figure 1) involve membrane-associated enzymes while "soluble" enzymes play a very minor role, if any, in the in vitro conversions of parathion (Nakatsugawa et al. 1969).

Earlier studies reported that the hepatic enzymes that catalyze the metabolism of parathion are localized in the microsomal fraction (O'Brien 1959, Nakatsugawa and Dahm 1967, Neal 1967a, 1967b, and Nakatsugawa et al. 1969). In none of these earlier studies were the biochemical assessments of the enzymatic activity of liver cell fractions towards parathion correlated with cytological observations of the fractions as made in this study.

Another unique feature of this study has been the fractionation of the mitochondrial supernatant into rough and smooth microsomal fractions in order to localize within the membranes of the endoplasmic reticulum the distribution of enzymes involved in the metabolism of parathion. Studies on the metabolism of certain drugs by fractions of rat hepatic microsomes have demonstrated greater enzymatic activity in the smooth microsomal fraction (Gram et al. 1967, Gram and Fouts 1967, and Holtzman et al. 1968). The ratios of smooth:rough specific enzyme activity values in drug studies ranged from 1.5 to 3.0; species differences, cofactor variations, and fractionation methods affect the smooth:rough activity ratios (Gram and Fouts 1968). Employing the Dallner method of fractionation (Dallner et al. 1966a), I observed that the smooth:rough specific enzyme activity ratio for parathion metabolism ranged from 0.9 to 1.5 with a mean of 1.4
for paraoxon production ($Y_1$) and from 0.9 to 1.7 with a mean of 1.4 for aqueous metabolite production ($Y_2$). Cytological examination of the rough and smooth microsomal fractions indicated that cross contamination was small (Figures 13 through 18). The low level of RNA detected in the smooth microsomal fraction (Table 3) supported the cytological observations. After adjusting the nitrogen values of rough and smooth microsomal fractions to exclude the contribution by ribosomes the smooth:rough specific enzyme activity ratios were 1.2 for both $Y_1$- and $Y_2$-values.

I conclude that the hepatic enzymes of the rat that metabolize parathion in vitro to paraoxon or to DEPTA and \( p \)-nitrophenol are distributed in both the rough and smooth membranes making up the endoplasmic reticulum of the hepatic cell. The analyses of the data support the concept that the smooth microsomal fraction exhibits greater enzymatic activity in the metabolism of foreign compounds. The disparity in specific enzyme activity values between rough microsomes and smooth microsomes for the metabolism of parathion does not appear to be as great as has been observed for the metabolism of certain drugs (Gram et al. 1967, Gram and Fouts 1967, and Holtzman et al. 1968).

**Incubation conditions affecting enzyme activity**

**Resuspending media** Rat liver is commonly homogenized in 0.25M sucrose. After precipitation of the microsomes, the pellet is resuspended before incubation. Several media have been employed for this purpose. They include 0.25M sucrose (Nakatsugawa et al. 1969, Dallner et al. 1966a); 0.1M phosphate buffer, pH 7.4, and 1.15% KCl, pH 7.4 (Gram et al. 1967); 0.15M KCl:0.02M Tris (Holtzman et al. 1968); distilled water (Neal 1967a);
and 0.1M Hepes (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) buffer (Peters and Fouts 1970). A lack of standardization at this stage of preparation was apparent and two questions arose: (1) In this study would the resuspending medium chosen or the concentrations of its ingredients influence the enzyme activity of crude microsomes for parathion metabolism?, and (2) What effect if any does CsCl, employed in the fractionation of rough and smooth microsomes, have on parathion metabolism by crude microsomal enzymes?

Thirteen different resuspending media were tested. The average specific enzyme activity values for parathion metabolism by crude microsomes are presented in Table 10. The first 6 media, treatments (A) through (F), were studied in two replicate experiments, with duplicate incubations in each experiment, performed on separate days with separate microsomal preparations. The remaining 7 media, treatments (G) through (M), were tested with four incubations for each treatment in the second replicate experiment only. An AARDVARK (Mexas 1968) analysis of variance test was applied separately to the two groups of data (treatments A through F and G through M). The results of this analysis are summarized in Tables 16 and 17 (Appendix).

The analysis of the data from the first 6 treatments indicated that the effects of different resuspending media did not contribute significantly to the observed variance (Table 16, Appendix). When specific enzyme activity values were compared by the lsdw test previously described, incubations with crude microsomes resuspended in 0.25M sucrose showed significantly lower values for paraoxon production only at the _t_ .95 level.
Table 10. A comparison of the metabolism of parathion by crude microsomal enzymes as influenced by the media used to resuspend the crude microsomal pellets. Incubation conditions are described in the text. Footnotes in Table 7 also apply to this table.

<table>
<thead>
<tr>
<th>Resuspending Medium (Treatment)</th>
<th>mg N/ml&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Paraoxon produced (Y&lt;sub&gt;i&lt;/sub&gt;)&lt;sup&gt;b&lt;/sup&gt; nmoles/mg N/hr</th>
<th>Aqueous metabolites produced (Y&lt;sub&gt;j&lt;/sub&gt;)&lt;sup&gt;c&lt;/sup&gt; nmoles parathion converted/mg N/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass distilled H&lt;sub&gt;2&lt;/sub&gt;O (A)</td>
<td>0.856</td>
<td>23.0</td>
<td>32.8</td>
</tr>
<tr>
<td>0.25M sucrose (B)</td>
<td>0.816</td>
<td>21.7</td>
<td>33.1</td>
</tr>
<tr>
<td>0.25M sucrose:15mM CsCl (C)</td>
<td>0.877</td>
<td>22.1</td>
<td>31.9</td>
</tr>
<tr>
<td>0.25M sucrose:0.01M PO&lt;sub&gt;4&lt;/sub&gt;:0.15M KCl (D)</td>
<td>0.856</td>
<td>22.8</td>
<td>34.7</td>
</tr>
<tr>
<td>0.125M sucrose:0.01M PO&lt;sub&gt;4&lt;/sub&gt;:0.15M KCl (E)</td>
<td>0.900</td>
<td>23.6</td>
<td>33.2</td>
</tr>
<tr>
<td>0.01M PO&lt;sub&gt;4&lt;/sub&gt;:0.15M KCl (F)</td>
<td>0.876</td>
<td>22.5</td>
<td>33.2</td>
</tr>
<tr>
<td>0.01M PO&lt;sub&gt;4&lt;/sub&gt;:0.15M KCl 1% ethanol (G)</td>
<td>0.992</td>
<td>27.0</td>
<td>38.3</td>
</tr>
<tr>
<td>0.01M PO&lt;sub&gt;4&lt;/sub&gt;:0.15M KCl 1% isobutanol (H)</td>
<td>0.967</td>
<td>22.9</td>
<td>23.8</td>
</tr>
<tr>
<td>0.10M PO&lt;sub&gt;4&lt;/sub&gt;:0.15M KCl (I)</td>
<td>0.972</td>
<td>24.8</td>
<td>37.1</td>
</tr>
<tr>
<td>1.0M PO&lt;sub&gt;4&lt;/sub&gt;:0.15M KCl (J)</td>
<td>1.016</td>
<td>12.4</td>
<td>18.4</td>
</tr>
<tr>
<td>0.01M Tris (K)</td>
<td>1.049</td>
<td>21.7</td>
<td>32.2</td>
</tr>
<tr>
<td>0.10M Tris (L)</td>
<td>1.120</td>
<td>19.4</td>
<td>34.6</td>
</tr>
<tr>
<td>1.0M Tris (M)</td>
<td>1.985</td>
<td>3.4</td>
<td>6.4</td>
</tr>
</tbody>
</table>
At a $t_{0.975}$ level, all means were equal. CsCl used in the fractionation procedure did not depress or enhance significantly the metabolism of parathion by crude microsomes. Analysis of the data from the remaining 7 treatments indicated that the effect of the resuspending medium was highly significant. The LSD test used to investigate differences between mean specific activity values for paraoxon production and aqueous metabolite production, respectively, showed the following relationships:

(1) For paraoxon production, the significantly different treatment means, placed in decreasing order of size, were 0.01M $P_4:0.15M KCl:1\%$ ethanol (G) > 0.1M $P_4:0.15M KCl$ (I) > 0.01M $P_4:0.15M KCl:1\%$ isobutanol (H) = 0.01M Tris (K) > 0.1M Tris (L) > 1.0M $P_4:0.15M KCl$ (J) > 1.0M Tris (M);

and (2) For aqueous metabolite production, the significantly different treatment means, placed in decreasing order of size were 0.01M $P_4:0.15M KCl:1\%$ ethanol (G) = 0.10M $P_4:0.15M KCl$ (I) > 0.1M Tris (L) > 0.01M Tris (K) > 0.01M $P_4:0.15M KCl:1\%$ isobutanol (H) > 1.0M $P_4:0.15M KCl$ (J) > 1.0M Tris (M).

If the mean square error estimate used in the LSD test comparisons was based on the mean square value for the treatment-experiment (TE) interaction in Table 16, i.e., a mean square of 5.515 was used instead of 1.372, the relationships between treatments (G) through (M) were changed to the following: (1) For paraoxon production, the specific enzyme activity value of 0.01M $P_4:0.15M KCl:1\%$ ethanol (G) = 0.10M $P_4:0.15M KCl$ (I); 0.10M $P_4:0.15M KCl$ (I) = 0.01M $P_4:0.15M KCl:1\%$ isobutanol (H) = 0.01M Tris (K); 0.01M $P_4:0.15M KCl:1\%$ ethanol (G) > 0.01M $P_4:0.15M KCl:1\%$ isobutanol (H) and 0.01M Tris (K); 0.01M $P_4:0.15M KCl:1\%$ isobutanol (H) =
0.01M Tris (K); 0.01M PO₄:0.15M KCl:1% isobutanol (H) > 0.1M Tris (L);
0.01M Tris (K) = 0.1M Tris (L) > 1.0M PO₄:0.15M KCl (J) > 1.0M Tris (M);
and (2) For aqueous metabolite production, the specific enzyme activity
value of 0.01M PO₄:0.15M KCl:1% ethanol (G) = 0.10M PO₄:0.15M KCl (I);
0.01M PO₄:0.15M KCl:1% ethanol (G) > 0.10M Tris (L), 0.01M Tris (K),
0.01M PO₄:0.15M KCl:1% isobutanol (H), 1.0M PO₄:0.15M KCl (J), and 1.0M
Tris (M); 0.1M PO₄:0.15M KCl (I) = 0.1M Tris (L); 0.1M Tris (L) = 0.01M
Tris (K) > 0.01M PO₄:0.15M KCl:1% isobutanol (H) > 1.0M PO₄:0.15M KCl (J)
> 1.0M Tris (M). Stated another way, the specific enzyme activity values
Y₁ and Y₂ for the crude microsomes resuspended in 0.01M PO₄:0.15M KCl:
1% ethanol (G) buffer were the highest but were not significantly greater
than the values obtained from crude microsomes resuspended in 0.10M PO₄:
0.15M KCl (H) buffer. The lower concentrations of phosphate-containing
buffers resulted in equivalent data. The 0.10M PO₄:0.15M KCl (I) buffer
did not differ significantly from the 0.01M Tris (K) buffer for paraoxon
production. Similarly, the 0.10M PO₄:0.15M KCl (I) buffer did not differ
significantly from the 0.1M Tris buffer for aqueous metabolite production.
Buffers of highest ionic strength depressed the enzyme activity the most.

The results in Table 16 show that a highly significant portion of the
variance was contributed by differences between replicate experiments,
supporting the observation frequently made during the study that micro­
somal preparations varied in their enzyme activity, other factors being
held the same.

In studying the distribution of aniline hydroxylase activity in
rabbit liver microsomal fractions, Gram et al. (1967) observed identical
responses for smooth microsomes and similar responses for rough microsomes in 0.1M phosphate and 0.1M Tris buffers of pH 7.4. Fouts (1970) found that Tris buffers (0.1 to 0.2M, pH 7.4) allowed better rates of hexobarbital, aminopyrine, and aniline metabolism by crude microsomes than phosphate buffers (0.1 to 0.2M, pH 7.4).

The highest specific enzyme activity values for parathion metabolism by crude microsomes occurred with 0.01M PO₄:0.15M KCl:1% ethanol as the resuspending medium. Every other incubation mixture contained a final concentration of approximately 2.5% absolute ethanol, since the stock parathion was dissolved in absolute ethanol. With the additional ethanol in the resuspending medium and incubation buffer, the final concentration was about 3.5%, or 40% more than the normal amount. The increase in specific enzyme activity over the next to highest response was approximately 9% for paraoxon production and 3% for aqueous metabolite production. Whether this maximal response is a function of altered solubility of parathion, enzyme activation or stabilization, altered enzyme-substrate affinity, or some other factor is not known.

Enzyme activity vs pH A study was made of the effects of pH on the metabolism of parathion by crude microsomes. The results are illustrated in Figure 19. An analysis of variance test (Chamberlain and Jowett 1968) was performed by computer on the data, the results of which are summarized in Table 18 (Appendix). This test indicated that a highly significant portion of the variance was contributed by the pH variable. A regression analysis (Chamberlain and Jowett 1968) of the data was performed, based on the model \( \hat{Y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1^2 \) where
Figure 19. The relationship of the mean specific enzyme activity values from incubations of parathion with crude microsomal enzymes and the pH of the incubation mixture. Incubation conditions are described in the text.
$X_1$ is pH, $X_2$ is microsomal concentration, and $X_3$ is the experiment or replication variable. The maximal, mean specific enzyme activity values for paraoxon production and aqueous metabolite production occurred at pH 7.5 as observed in Figure 19 and predicted by the regression model.

When the least significant difference test (LSD) previously described with $\pm 0.95$ value was applied to the treatment means, the specific enzyme activity values for paraoxon production were not significantly different over the pH range of 7.1 through 7.6 and 7.8. The mean response at pH 7.7 was significantly lower than the response at 7.5. If a $\pm 0.975$ value was used, all values from pH 7.1 through 7.8 were not significantly different.

When the LSD test with $\pm 0.95$ value was performed on the sample means of aqueous metabolite production, the specific enzyme activity values over the pH range of 7.3 through 7.8 were not significantly different. Thus, nearly identical responses were observed for both paraoxon and aqueous metabolite formation from parathion in vitro. The pH of rat liver tissue has been reported to range from 6.4 to 7.5 for the various cell types (Spector 1956). The optimal pH range in vitro appears to fall partially within the physiological range reported for intact liver cells with the maximal response coming at the upper end of the physiological range.

Nakatsugawa and Dahm (1967) observed an optimum pH of approximately 7 for the metabolism of parathion by rabbit liver microsomes, and Nakatsugawa et al. (1969) employed an incubation mixture of pH 7.2 to 7.3 for similar studies with rat liver microsomes. A wide optimum pH range around 7.5 was reported for parathion metabolism by fat body microsomes of the American cockroach (Nakatsugawa and Dahm 1965). Neal (1967a) employed
an incubation system for parathion metabolism by rat and guinea pig microsomes in which the pH of the phosphate buffer used was 8.0.

From the results of this study, an incubation mixture with pH around 7.5 is regarded as optimal for in vitro metabolism studies of parathion by rat liver microsomes.

**Temperature**

Most drug metabolism studies with hepatic microsomes or microsomal fractions have been performed at 37°C, oftentimes with shaking in an atmosphere of oxygen (Gram et al. 1967, Gram and Fouts 1967, and Fouts 1970). Metabolism studies of parathion with crude microsomes from livers of several small mammals have been performed at room temperature without shaking (Nakatsugawa and Dahm 1967, Nakatsugawa et al. 1968, and Nakatsugawa et al. 1969), at room temperature with shaking (O'Brien 1959), and at 37°C with shaking (Neal 1967a, 1967b). I chose to incubate parathion with liver cell fractions routinely at room temperature, approximately 23°C, without shaking.

The daytime rectal temperature of the white rat averages 37.3°C and ranges from 34.5 to 40.0°C (Spector 1956). It was anticipated that maximal conversion in vitro of parathion would occur at or near physiological temperatures. It was not known how temperature would affect the two pathways of parathion metabolism. Crude microsomes from the same preparation were incubated in replicate experiments with parathion at different temperatures ranging from 4 to 58°C, inclusively. The results are illustrated in Figure 20 together with predicted mean values based on a regression analysis performed on the raw data (Chamberlain and Jowett 1968). An analysis of variance was performed on the data (Chamberlain and Jowett
Figure 20. The relationship of the mean specific enzyme activity values from incubations of parathion with crude microsomal enzymes and the temperature of the incubation mixture. Incubation conditions are described in the text. Broken lines represent predicted values from regression analysis of the data (Chamberlain and Jowett 1968)
1968), and the results are summarized in Table 19 (Appendix).

The results of the analysis of variance test indicate that the influence of temperature is highly significant and the relationship between specific enzyme activity and temperature is curvilinear for paraoxon and aqueous metabolite production. The maximal mean specific enzyme activity value for paraoxon production occurred at 40 C and for aqueous metabolite production occurred at 34 C. The lsd test with a $t_{.95}$ value as previously described was applied to the mean values. It was found that over the temperature range of 34 through 45 C the specific enzyme activity values for paraoxon production were not significantly different. In the metabolism of parathion to aqueous metabolites, the specific enzyme activity values were not significantly different over 34 to 40 C, inclusively. These results argue for performing these experiments at approximately 37 C; less variation in results might occur.

The results of this study indicate a difference in the temperature at which maximal enzymatic activity occurs in the two pathways of parathion metabolism. Furthermore, a difference in the range of temperatures over which maximal activity occurs was observed, with paraoxon production having a broader range than aqueous metabolite production. These variations in response to temperature can be interpreted as evidence for separate enzyme systems involved in desulfuration and in hydrolysis of parathion. On the contrary, one can speculate as to a single enzyme involvement with a separate enzyme to hydrolyze paraoxon, with the latter enzyme being adversely affected by temperatures exceeding 37 C. Consequently, desulfuration continues at a near maximal rate up to approximately 45 C.
but enzymatic degradation of paraoxon is retarded above 37 C. Alternatively, perhaps separate enzyme systems are involved in desulfuration and hydrolysis of parathion, respectively, and yet a third enzyme system is responsible for the hydrolysis of paraoxon. Separate lines of evidence seem to favor the third hypothesis (Nakatsugawa and Dahm 1967, Nakatsugawa et al. 1968, Neal 1967a, 1967b, and Kojima and O'Brien 1968).

**Enzyme concentration**

Different quantities of a 20% crude microsomal suspension were incubated with parathion in a single experiment with duplicate incubations for each volume added, as described in the MATERIALS AND METHODS section. The absolute quantities of paraoxon and aqueous metabolites produced were measured and plotted against the concentration of TCA-precipitable nitrogen per incubation mixture (Figure 21). The relationship between specific enzyme activity values and nitrogen concentration is presented for paraoxon and aqueous metabolites in Figure 22. An analysis of variance test performed on the data (Chamberlain and Jowett 1968) is summarized in Table 20 (Appendix). The analysis of variance results indicate that the linear variable enzyme concentration accounts for a highly significant portion of the variance, but the quadratic function, concentration squared, does not. Consequently, a regression analysis of the data, based on the linear model \( \hat{Y} = \beta_0 + \beta_1 X_1 \) where \( X_1 \) is concentration, yielded the predicted mean values indicated in Figure 22 by broken lines. The data in Figure 22 show decreasing specific enzyme activity values for both paraoxon and aqueous metabolite production with increasing concentration of crude microsomes expressed as total TCA-precipitable nitrogen.
Figure 21. The relationship of the mean quantities of paraoxon and aqueous metabolites from incubations of parathion with crude microsomes and the enzyme concentration expressed as mg N/incubation mixture. Incubation conditions are described in the text.
Figure 22. The relationship of the mean specific enzyme activity values from incubations of parathion with crude microsomes and the enzyme concentration expressed as mg N/incubation mixture. Incubation conditions are described in the text. Broken lines represent predicted values from regression analysis of the data (Chamberlain and Jowett 1968).
I conclude that the products per mg N per unit time are decreasing with increasing nitrogen concentration at a uniform rate as evidenced by negative regression coefficients and the significance of the linear enzyme variable. The small variation between observed and predicted values over the 0.2 to 2.0 mg N/ml portion of the range tested suggests that these concentrations are optimal for this system, i.e., the predicted response and the observed response are closely related; the absolute quantities of products are easily measured over this range; and variation in specific enzyme activity responses was minimal. All crude microsomal concentrations employed in this study fell within this range (Table 2). When expressed as mg protein/ml of microsome suspension, the range of 0.2 to 1.0 mg N/ml becomes 1.2 to 6.2 mg protein/ml or 0.3 to 1.5 mg protein/ml incubation mixture in a 4 ml incubation. The rough and smooth microsome concentrations averaged less than 0.4 mg N/ml of 20% suspension. However, the comparable or slightly higher activities in these two fractions resulted in quantities of products readily quantified and generally commensurate with quantities yielded by the crude microsomes.

Recently Fouts (1970) studied the effects of microsomal protein concentration and other in vitro conditions on detection and quantitation of drug metabolism. He observed that higher enzyme activity occurred when microsomal protein concentrations were less than 2 mg/ml of incubation mixture. If in vitro conditions affecting drug metabolism are also influential in insecticide metabolism, the concentration range employed in this study is presumed optimal (according to the findings of Fouts). Fouts (1970) stated that, over the range of 1.0 to 4.0 mg protein/ml of
incubation mixture, specific enzyme activity was "usually fairly linear with respect to microsomal protein concentrations." The results in Figure 21 do not show a linear relationship with respect to crude microsomal nitrogen concentrations used in this study, but when specific enzyme activity values were plotted against enzyme concentration expressed as mg N/incubation mixture, a linear response with negative slope was observed over the range tested. This empirical evidence indicates that the amount of product formed from parathion is not directly proportional to the amount of microsomal protein present in the incubation mixture when the response is tested over the range of 0.6 to 12.5 mg protein/incubation mixture. Perhaps a direct comparison cannot be made between these two studies, as certain other incubation parameters were not equivalent.

Earlier studies have not quantified the metabolism of parathion in terms of amount of product/mg N or protein/unit time. Instead, parathion metabolism has been expressed as amount of product per unit time in a mixture containing a final microsomal concentration equivalent to 2.5% liver homogenate (Nakatsugawa and Dahm 1967, Nakatsugawa et al. 1969) or adding microsomes equivalent to 100 mg of liver and expressing specific enzyme activity as amount of metabolite/hr/g of tissue (Neal 1967a, 1967b). I have found that using the same preparative method results in considerable variation in N content/ml of 20% microsomes prepared at different times from different donors (Table 2). The specific activities of microsomal enzymes also vary from day to day as has been pointed out previously in the breakdown of variance where experimental replication was a variable. The effects of a given influence such as pH or resuspending medium can be
reproduced at will, but the absolute enzyme activities that delineate that effect may vary from one experiment to another. Fouts (1970) has reported similar findings. For these reasons, an expression of parathion metabolism based on a quantity of enzymatic material measured by a sensitive method provides a more refined definition of the enzyme activity for a given preparation under a particular set of conditions than a definition based on equivalent concentrations. Although the micro-Kjeldahl method for determining precipitable nitrogen is slow and a laborious method, it was used throughout this study for the following reasons: (1) it is one of the most sensitive methods commonly employed (Juchau and Fouts 1967); (2) it was readily used in conjunction with the method for RNA determination used (Schneider 1945); and (3) it was deemed wise to retain the same method used initially throughout the duration of this research.

**Parathion metabolism vs time** The progress of parathion metabolism with time by crude microsomes is presented in Figure 23. A regression analysis of the sample specific enzyme activity values based on the model \( \hat{Y} = \beta_0 + \beta_1 X_1 + \beta_2 X_1^2 \) where \( X_1 \) is incubation interval was performed (Chamberlain and Jowett 1968). The predicted mean values were close to the observed mean values and were not graphed. An analysis of variance test was performed on the sample data and is summarized in Table 21 (Appendix).

An examination of the pair of curves in Figure 23 suggests that the metabolism of parathion is not linear with time. Statistical treatment of the data produced highly significant linear and quadratic components with a negative quadratic regression coefficient, i.e., the metabolism of
Figure 23. The relationship of the mean specific enzyme activity values from incubations of parathion with crude microsomes and the length of the incubation period. Incubation conditions are described in the text.
parathion is most rapid initially and slows with time with a definite plateau being reached after 1 hr for paraoxon production, while aqueous metabolite formation continues at a decreasing rate. The metabolism of \(^{35}\text{S}^-\)parathion to paraoxon by rabbit liver microsomes was observed by Nakatsugawa and Dahm (1967) to occur in a similar manner. Neal (1967a) described the metabolism of \(^{32}\text{P}^-\)parathion by crude liver microsomes of adult male rats as follows: (1) An initially rapid formation of DEPTA and paraoxon occurred during the first 20 min; (2) A plateauing of paraoxon formation followed for the next 20 min; and (3) A decrease in paraoxon was observed after 40 min of total incubation time had elapsed. Neal (1967a) suggested that paraoxon continues to be formed but is broken down to DEPA and \(\text{p}^-\)nitrophenol thereby preventing further increase in paraoxon concentration.

The results of this study support Neal's hypothesis. DEPA was identified as one of the aqueous products. Also, the ratio of DEPTA:DEPA decreased with increasing incubation interval (Table 6). The leveling off of paraoxon formation observed can be explained by the increased hydrolysis of paraoxon to DEPA and \(\text{p}^-\)nitrophenol.

The ratio of DEPTA + DEPA:paraoxon of approximately 6 reported by Neal (1967a) for 1 hr incubations is considerably larger than a ratio of < 1 observed in this study (Table 6). However, the experimental conditions were not equivalent in these two independent studies. Neal employed a buffer of \(\text{pH} 8.0\) while the buffer used in this study gave an incubation \(\text{pH}\) of approximately 7.4. Kojima and O'Brien (1968) have observed a paraoxon-degrading enzyme in the crude microsomal fraction with an optimum \(\text{pH}\)
of 7.7. The hydrolysis of paraoxon may have occurred at a higher rate in Neal's study, thereby increasing the proportion of DEPTA plus DEPA to paraoxon. The ratio of approximately 1 observed in this study is in close agreement with the findings of Nakatsugawa and Dahm (1967).

**Microsomal storage** The metabolism of parathion by crude microsomes that were freshly prepared or that had been frozen at -20 C from 1 to 6 weeks was compared in order to assess any trends or changes in enzymatic activity with length of storage. The results are summarized in Figure 24. The specific enzyme activity values fluctuated from week to week for both paraoxon and aqueous metabolite production with the lowest response coming at 3 weeks of storage. An analysis of variance test performed on the data is summarized in Table 22 (Appendix) (Chamberlain and Jowett 1968). In an effort to identify trends in activity variation, a regression analysis was made employing the following model: \( \hat{Y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_2^2 + \beta_4 X_2^3 \), where \( X_1 \) is microsome concentration and \( X_2 \) is the treatment or storage period. The predicted mean values were nearly identical to the observed mean values and were not included in Figure 24.

A major portion of the sample variance is related to differences in microsomal concentration from week to week. The fluctuations due to treatment (storage period) were not significant for paraoxon formation but were significant for aqueous metabolite production. The inclusion of quadratic and cubic components in the statistical model explained a smaller but significant portion of the variance. The analysis of variance data favor the conclusion that variation in specific activity is more a function of differences in enzyme concentration and random, unexplained day to day.
Figure 24. The relationship of mean specific enzyme activity values from incubations of parathion with crude microsomes and length of microsomal storage at -20 C. Incubation conditions are described in the text.
day differences than a function of storage length. The significant F-test (Table 22) for parathion conversion to aqueous metabolites reflects what appears to be an increasing trend with more extensive storage periods (Figure 24).

O'Brien (1959) reported that mouse microsomes, after being frozen rapidly, deteriorated slowly and became inactive toward thionophosphates after 8 days. Neal (1967a) observed that rat liver microsomes maintained essentially full activity towards parathion for as long as 3 weeks. He later reported (1967b) that the total activity of rat liver microsomes toward parathion decreased from 10 to 20% during a 28-day period. Nakatsugawa et al. (1968) reported that mammalian microsomes could be stored frozen for a month or more. No specific data comparisons were reported. The decrease in specific enzyme activity reported by Neal (1967b) was not observed in this study over an interval 50% longer than that reported by him.

It was not possible to establish with certainty any definite trends during this six-week period of study. It can be stated that rat liver crude microsomes retain their enzymatic activity for a period of 6 weeks when pellets are drained and stored at -20 C. Similar data on the effects of freezing on activity have not been reported for rat liver microsomes. Epoxidase, hydroxylase, and demethylase specific activities in insect (Prodenia eridania) midgut microsomal preparations were retained over a 4-week period of storage at -15 C (Krieger and Wilkinson 1970) and showed fluctuations from week to week similar to those observed in this study.

Additional study with even longer storage periods is necessary to
establish whether the higher specific enzyme activity values for aqueous metabolite production at 4 and 6 weeks are significant, whether these are maximal levels that will show a subsequent decline, or whether the higher values are attributable to experimental variation with overall retention of activity for both metabolic pathways. It is concluded that under the conditions of this study, rat liver crude microsomes retain their enzymatic activity towards parathion for at least 6 weeks.

**Paraoxon hydrolysis**

The hydrolysis of paraoxon was studied by incubating crude, rough, and smooth microsomes with paraoxon. The metabolism of paraoxon, as estimated from the decrease in paraoxon extractable from the incubation mixture, never exceeded 13% of the initial concentration at the end of 1 hr of incubation. The specific enzyme activity values for these 3 cell fractions are summarized in Table 11. An analysis of variance was performed on the data (Mexas 1968) and the results are summarized in Table 23 (Appendix). An lsd test with $t_{.05}$ value was applied to the mean specific enzyme activity values. The specific enzyme activity value of the smooth microsomal fraction was significantly higher than values from either crude or rough microsomal fractions. The values for crude and rough microsomal fractions were not significantly different. After adjusting for nitrogen contributed by ribosomal nitrogen, smooth microsomes still showed the highest specific enzyme activity. Because these results are based on a single method for the quantitation of paraoxon metabolism, and the sample sizes are small, the results must be regarded as tentative. It has been shown previously (Table 6) that the largest mean quantity of
Table 11. A comparison of the specific enzyme activity data obtained from incubation of paraoxon with crude, rough, and smooth microsomes. Nitrogen values were determined by micro-Kjeldahl. Incubation conditions are described in the text.

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Exp. No.</th>
<th>Total TCA-precipitable N</th>
<th>Paraoxon&lt;sup&gt;a&lt;/sup&gt; converted to aqueous metabolites nmoles/mg N/hr</th>
<th>Adjusted TCA-precipitable N&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Paraoxon converted to aqueous metabolites nmoles/mg N/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Microsomes (4)</td>
<td>5</td>
<td>0.463</td>
<td>20.1</td>
<td>0.383</td>
<td>24.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.420</td>
<td>21.8</td>
<td>0.348</td>
<td>26.3</td>
</tr>
<tr>
<td>Rough Microsomes (5)</td>
<td>5</td>
<td>0.421</td>
<td>24.8</td>
<td>0.299</td>
<td>35.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.425</td>
<td>25.2</td>
<td>0.319</td>
<td>33.6</td>
</tr>
<tr>
<td>Smooth Microsomes</td>
<td>5</td>
<td>0.290</td>
<td>38.6</td>
<td>0.240</td>
<td>46.7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.302</td>
<td>40.4</td>
<td>0.288</td>
<td>43.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Paraoxon measured by GLC and quantity metabolized estimated from the decrease in paraoxon extractable from incubation mixture.

<sup>b</sup>Nitrogen adjustment described in Data Calculations of MATERIALS AND METHODS.
DEPA produced with parathion as the initial substrate occurred in smooth microsomal incubations.

Nakatsugawa et al. (1968) reported the in vitro metabolism of paraoxon by rat liver crude microsomes (5% final concentration) was approximately 21.6% of the initial paraoxon concentration when NADPH₂ was included. This is approximately twice the conversion observed in this study (Table II). The final concentration of microsomes in each incubation mixture in this study was about one half that found in the former study. If the enzyme concentrations had been equivalent, the disparity would likely be much less. The importance of cofactors was not included in this study.

The pH used in this study was 7.4, as in standard incubations with parathion. The significance of the pH optimum of 7.7 observed by Kojima and O'Brien (1968) for paraoxon metabolism by rat liver crude microsomes in relation to the results of this study is uncertain. They found 42% degradation of paraoxon by 20% crude microsomes when incubated at 37.5 C for 1 hr. Perhaps their 4X increase in degradation above the results in this study can be explained by their incubating at a higher temperature (37.5 C) and using a higher microsomal concentration (17 mg protein/ml of incubation mixture) than the conditions used in this study (23 C, <0.7 mg protein/ml of incubation mixture).

The results of this study support other reports that paraoxon is enzymatically degraded by enzymes found principally in the microsomal fraction of rat hepatic tissue and should be considered as a source of aqueous metabolites in studies of the metabolism of parathion.
Effects of solubilizing agents

The overall results of the treatments intended to dissociate enzymes from membranes when applied to rat liver cell fractions were marked reductions in the capacity to metabolize parathion in vitro. The enzyme activity was effectively destroyed or simply lacking in most of the supernatant fractions obtained from the 8 treatments tested. Supernatants obtained from treatments 2 through 6 described in MATERIALS AND METHODS were the only ones that gave 5 to 10% of the original enzyme activity. The amount of parathion metabolized by the pellets recovered by centrifugation after treatments varied and appeared to be inversely related to the severity of the treatment.

Surface active agents, sonication, autolysis, and lysozyme inactivated microsomal enzymes involved in the metabolism of parathion. The activity found in the supernatants was too low to be significant, and activity remaining in the pellet after centrifugation was reduced >50%. It has been reported that conventional methods of solubilization inactivate many of the drug-metabolizing enzymes in liver microsomes which also require $\text{NADPH}_2$ and oxygen (Shuster 1964).
SUMMARY

The centrifugation of rat liver homogenates yielded 6 fractions which were assayed for their capacity to activate parathion to its more toxic analogue paraoxon and to degrade parathion and paraoxon to water-soluble metabolites. Cytological examination by electron microscopy combined with biochemical analysis showed that the centrifugal procedure produced the four classical fractions; i.e., debris, mitochondrial, microsomal, and soluble, and that fractionation of the postmitochondrial supernatant or a microsomal suspension adequately separated the rough-surfaced membranes (rough microsomes) from the smooth-surfaced membrane component (smooth microsomes). RNA analyses of the latter two fractions supported the cytological picture that the RNA present in the smooth microsomal fraction originated primarily from a low level of contamination by free ribosomes.

A comparison of the metabolism of parathion by the homogenate, debris fraction, mitochondrial fraction, and crude, rough, and smooth microsomal fractions showed the highest specific enzyme activity in the smooth microsomal fraction. Statistical analyses of the data supported these observations. When analysis of variance and least-significant-difference tests were applied to data in two separate experiments in which the nitrogen levels were adjusted for the mitochondrial and crude, rough, and smooth microsomal fractions to remove the nitrogen contribution by ribosomes, the mean specific enzyme activity values for smooth microsomes remained significantly higher at the 5% level.

An analysis of variance of the activity response of crude microsomes
as a function of the medium used to resuspend the microsomes indicated that treatment differences were not significant for several solutions tested. The concentration of cesium, employed as CsCl in the fractionation of the postmitochondrial supernatant into rough and smooth microsomes, did not affect parathion metabolism by crude microsomes.

Several physiological parameters were assessed for the metabolism of parathion in vitro. A study of enzyme activity as a function of pH determined an optimal pH range of 7.1 to 7.8 for paraoxon production and an optimum pH range of 7.3 to 7.8 for aqueous metabolite production with maximal activity for both metabolic pathways occurring at pH 7.5. A highly significant effect of temperature on specific enzyme activity was observed with an optimal response ranging over temperatures of 34 to 45°C for paraoxon formation and a narrower optimal range of 34 to 40°C for production of aqueous metabolites. A comparison of specific enzyme activity values and different microsomal concentrations showed a decrease in response with increasing concentration. A linear model provided the best fit of specific enzyme activity data on concentration.

The metabolism of parathion was not linear with time but occurred most rapidly initially, reached a plateau for paraoxon production at 1 hr, and showed a gradual decrease in rate for aqueous metabolite production. Approximately 63% of the parathion had been metabolized after 2 hr of incubation.

The variation in enzyme activity of crude microsomes after storage as frozen pellets at -20°C was a function of experimental variation rather than an alteration in enzyme activity. Significant changes in crude
microsomal activity were not observed over a 6-week period of storage.

When paraoxon was substituted for parathion in the standard incubation mixture, 9 to 13% of the paraoxon was degraded in 1 hr by the microsomal fractions; smooth microsomes showed the greatest amount of paraoxon degradation. Efforts to solubilize microsomal enzymes and retain enzymatic activity towards parathion were unsuccessful.


Mexas, A. G. 1968. AARDVARK (Analysis of variance system, Algebraic method options, Residual and mean options, Data format options, Variate or covariate analysis, Analysis on means option, Requested pooled terms, Key statistical transformations): Reference Manual. Iowa State University, Statistical Laboratory, Numerical Analysis-Programming Series, No. 1.


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APPENDIX
Table 12. Analysis of variance on the specific enzyme activity data from incubations of parathion with homogenate and debris fractions. The Kennedy regression program was used (Kennedy 1970)

<table>
<thead>
<tr>
<th>Model (Source)</th>
<th>df</th>
<th>Mean square ((Y_1)^a)</th>
<th>Mean square ((Y_2)^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full (Experiment + Treatment + Treatment-Experiment (TE) interaction)</td>
<td>5</td>
<td>10.045**</td>
<td>13.050**</td>
</tr>
<tr>
<td>Residual</td>
<td>9</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>Experiment + Treatment</td>
<td>3</td>
<td>15.710**</td>
<td>20.830**</td>
</tr>
<tr>
<td>Residual</td>
<td>11</td>
<td>0.289</td>
<td>0.259</td>
</tr>
<tr>
<td>Experiment</td>
<td>2</td>
<td>17.872**</td>
<td>30.491**</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>1.214</td>
<td>0.363</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>22.500**</td>
<td>11.095</td>
</tr>
<tr>
<td>Residual</td>
<td>13</td>
<td>2.139</td>
<td>4.172</td>
</tr>
</tbody>
</table>

\(a_{Y_1}\) = nmoles paraoxon produced/mg N/hr.

\(b_{Y_2}\) = nmoles parathion converted to aqueous metabolites/mg N/hr.

** = \(P < 0.01\).
Table 13. Analysis of variance on the specific enzyme activity data obtained from incubations of parathion with mitochondrial (3), crude microsomal (4), rough microsomal (5), and smooth microsomal (6) fractions. The Kennedy regression program was used (Kennedy 1970). See footnotes in Table 12 for further explanation of table.

<table>
<thead>
<tr>
<th>Model (Source)</th>
<th>df</th>
<th>Mean square $(Y_1)^a$</th>
<th>Mean square $(Y_2)^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full (Experiment + Treatment + Treatment-Experiment (TE) interaction)</td>
<td>19</td>
<td>721.980**</td>
<td>1103.241**</td>
</tr>
<tr>
<td>Residual</td>
<td>32</td>
<td>1.917</td>
<td>1.248</td>
</tr>
<tr>
<td>Experiment + Treatment</td>
<td>7</td>
<td>1760.594**</td>
<td>2642.066**</td>
</tr>
<tr>
<td>Residual</td>
<td>44</td>
<td>33.064</td>
<td>56.979</td>
</tr>
<tr>
<td>Experiment</td>
<td>5</td>
<td>1843.565**</td>
<td>2272.841**</td>
</tr>
<tr>
<td>Residual</td>
<td>46</td>
<td>99.155</td>
<td>209.507</td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>1905.612**</td>
<td>2500.737**</td>
</tr>
<tr>
<td>Residual</td>
<td>47</td>
<td>130.990</td>
<td>234.012</td>
</tr>
</tbody>
</table>
Table 14. Analysis of variance on the specific enzyme activity data obtained from incubations of parathion with crude microsomes and rough and smooth microsomes prepared by two methods described in the text. See footnotes in Table 12 for further explanation of table.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square ($Y_1^a$)</th>
<th>Mean square ($Y_2^b$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>4</td>
<td>699.671**</td>
<td>640.522**</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>12.983</td>
<td>4.723</td>
</tr>
</tbody>
</table>

*Significant at the 0.01 level.
Table 15. Analysis of variance on the specific enzyme activity data obtained from incubations of parathion with mitochondrial, crude microsomal, rough microsomal, and smooth microsomal fractions for which the nitrogen values had been adjusted to exclude the nitrogen contribution of ribosomes. See footnotes in Table 12 for further explanation of table.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square ($Y_1$)</th>
<th>Mean square ($Y_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment (E)</td>
<td>1</td>
<td>356.267**</td>
<td>447.322**</td>
</tr>
<tr>
<td>Treatment (T)</td>
<td>3</td>
<td>557.562**</td>
<td>1965.874**</td>
</tr>
<tr>
<td>TE interaction</td>
<td>3</td>
<td>72.334**</td>
<td>57.288**</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>3.532</td>
<td>1.062</td>
</tr>
</tbody>
</table>
Table 16. Analysis of variance on the specific enzyme activity values obtained from incubations of parathion with crude microsomes as influenced by resuspending media (A) through (F) (Table 10). An AARDVARK (Mexas 1968) program was used. See footnotes in Table 12 for further explanation of table.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square $(Y_1)^a$</th>
<th>Mean square $(Y_2)^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td>1</td>
<td>34.082**</td>
<td>451.534**</td>
</tr>
<tr>
<td>Treatment</td>
<td>5</td>
<td>1.772</td>
<td>3.145</td>
</tr>
<tr>
<td>TE interaction</td>
<td>5</td>
<td>5.515*</td>
<td>4.257</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>1.372</td>
<td>10.705</td>
</tr>
</tbody>
</table>

* = $P < 0.05$. 

\[ Y_1 \]
Table 17. Analysis of variance on the specific enzyme activity values obtained from incubations of parathion with crude microsomes as influenced by resuspending media (G) through (M) (Table 10). An AARDVARK (Mexico 1968) program was used. See footnotes in Table 12 for further explanation of table.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square ($Y_1$)$^{a}$</th>
<th>Mean square ($Y_2$)$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>271.008**</td>
<td>547.228**</td>
</tr>
<tr>
<td>Residual</td>
<td>21</td>
<td>0.767</td>
<td>0.940</td>
</tr>
</tbody>
</table>
Table 18. Analysis of variance on the specific enzyme activity data obtained from a study of the effects of pH on the metabolism of parathion by crude microsomes. An OMNITAB program was used (Chamberlain and Jowett 1968). See footnotes in Table 12 for further explanation of table

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square ( Y_1 )</th>
<th>Mean square ( Y_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (pH)</td>
<td>1</td>
<td>801.000**</td>
<td>3127.276**</td>
</tr>
<tr>
<td>Enzyme concentration</td>
<td>1</td>
<td>122.050**</td>
<td>184.836**</td>
</tr>
<tr>
<td>Experiment</td>
<td>1</td>
<td>120.578**</td>
<td>18.865</td>
</tr>
<tr>
<td>Quadratic</td>
<td>1</td>
<td>335.353**</td>
<td>318.270**</td>
</tr>
<tr>
<td>Residual</td>
<td>31</td>
<td>7.355</td>
<td>13.484</td>
</tr>
</tbody>
</table>
Table 19. Analysis of variance on the specific enzyme activity data obtained from a study of the influence of temperature on the metabolism of parathion by crude microsomes. An OMNITAB program was used (Chamberlain and Jowett 1968). See footnotes in Table 12 for further explanation of table.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square ($Y_1$)</th>
<th>Mean square ($Y_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme concentration</td>
<td>1</td>
<td>245.252**</td>
<td>25.704</td>
</tr>
<tr>
<td>Treatment (temperature)</td>
<td>1</td>
<td>4280.746**</td>
<td>1927.228**</td>
</tr>
<tr>
<td>Quadratic</td>
<td>1</td>
<td>204.590**</td>
<td>1669.560**</td>
</tr>
<tr>
<td>Experiment</td>
<td>1</td>
<td>0.002</td>
<td>0.014</td>
</tr>
<tr>
<td>Residual</td>
<td>22</td>
<td>16.704</td>
<td>18.210</td>
</tr>
</tbody>
</table>
Table 20. Analysis of variance on the specific enzyme activity data obtained from a study of the effect of crude microsomal concentration, expressed as total TCA-precipitable nitrogen, on the metabolism of parathion. An OMNITAB program was used (Chamberlain and Jowett 1968). See footnotes in Table 12 for further explanation of table.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square ((Y_1)^a)</th>
<th>Mean square ((Y_2)^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration ((X))</td>
<td>1</td>
<td>834.423**</td>
<td>698.956**</td>
</tr>
<tr>
<td>Quadratic ((X^2))</td>
<td>1</td>
<td>0.680</td>
<td>1.962</td>
</tr>
<tr>
<td>Residual</td>
<td>15</td>
<td>1.092</td>
<td>0.908</td>
</tr>
</tbody>
</table>
Table 21. Analysis of variance on the specific enzyme activity data obtained from the incubation of parathion with crude microsomes for varying time intervals. An OMNITAB program was used (Chamberlain and Jowett 1968). See footnotes in Table 12 for further explanation of table.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square ((Y_1))^a</th>
<th>Mean square ((Y_2))^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation interval</td>
<td>1</td>
<td>2526.725**</td>
<td>9053.508**</td>
</tr>
<tr>
<td>(linear)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation interval</td>
<td>1</td>
<td>511.311**</td>
<td>545.025**</td>
</tr>
<tr>
<td>(^2) (quadratic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>13</td>
<td>11.295</td>
<td>8.975</td>
</tr>
</tbody>
</table>
Table 22. Analysis of variance on the specific enzyme activity data obtained from a study of the influence of crude microsomal storage at -20°C on the metabolism of parathion in vitro. An OMNITAB program was used (Chamberlain and Jowett 1968). See footnotes in Table 12 for further explanation of table.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square ($Y_1^a$)</th>
<th>Mean square ($Y_2^b$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme concentration</td>
<td>1</td>
<td>128.539**</td>
<td>64.704**</td>
</tr>
<tr>
<td>Treatment (storage)</td>
<td>1</td>
<td>0.010</td>
<td>26.337**</td>
</tr>
<tr>
<td>Treatment$^2$ (quadratic)</td>
<td>1</td>
<td>35.970**</td>
<td>10.119*</td>
</tr>
<tr>
<td>Treatment$^3$ (cubic)</td>
<td>1</td>
<td>15.518*</td>
<td>27.165**</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>1.861</td>
<td>1.558</td>
</tr>
</tbody>
</table>

* = P < 0.05.
Table 23. Analysis of variance on the specific enzyme activity data\(^a\) obtained from incubations of paraoxon with crude, rough, and smooth microsomes. An AARDVARK program was used (Mexas 1968)

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square (Y)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment replication</td>
<td>1</td>
<td>5.070</td>
</tr>
<tr>
<td>Treatment (fraction)</td>
<td>2</td>
<td>380.503**</td>
</tr>
<tr>
<td>Treatment-Experiment interaction</td>
<td>2</td>
<td>0.610</td>
</tr>
<tr>
<td>Residual</td>
<td>6</td>
<td>4.723</td>
</tr>
</tbody>
</table>

\(^a\)Based on total TCA-precipitable nitrogen.

\(^b\)\(Y = \text{nmoles of paraoxon converted to aqueous metabolites/mg N/hr.}\)