THE EFFECTS OF DIFFERENT LEVELS OF DIETARY PROTEIN
ON THE TOXICITY AND METABOLISM OF
MALATHION AND PARATHION AND THEIR OXYGEN ANALOGUES

by

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INTRODUCTION

With increasing concern about overpopulation and starvation among some of the world's countries, the necessity for optimum food production is obvious. The President's Science Advisory Committee of the Panel on the World Food Supply in 1969 underlined the importance of insecticides to present and future global food supply: "At the present time, about 120,000 metric tons of all types of insecticides are used each year by the developing world, excluding mainland China. If food production is to be double, the annual usage must be increased to over 700,000 metric tons" (1).

Recent concern over persistent residues which contaminate the environment has caused the popular chlorinated hydrocarbons to be publicly condemned. Two alternative classes of insecticides which do not leave persistent residues are the organophosphates and carbamates, which also have an advantage over chlorinated hydrocarbons in that they do not accumulate in the body fat of animals. In addition, the effects of organophosphates on mammals are more clearly understood than for any other type of synthetic organic toxicant (2).

The metabolism of an insecticide or any other "foreign" environmental chemical is carried out by enzymes (3) which are subject to the influence of diet. McLean and McLean (4) observed that "if the site of relevant interaction between a toxin and a cell is also a site altered by diet, then changes
of diet are likely to alter the toxic effect."

Malnutrition is prevalent in many of the developing countries of the world, especially from diets lacking in protein (1). Since many of these countries will probably use increasing amounts of insecticides in order to boost their food production, the effect of these insecticides on their populations obviously needs evaluation.

The studies described in this paper attempt to partially evaluate the toxicity and metabolism of two widely used organophosphate insecticides, malathion and parathion, on animals subjected to protein deprivation.
REVIEW OF LITERATURE

General Properties of Organophosphates

The organophosphates comprise a very large class of compounds; it was estimated in 1959 that there were 50,000 of them and new ones are reported regularly (5). Among this wide variety are three main groups of compounds: 1) those which are soluble in water with low chemical stability such as TEPP and mevinphos (Table I); in this group the applied compound appears to be the actual cholinesterase inhibitor; 2) those which are soluble in oil with low water solubility such as malathion and parathion (Table I); these compounds usually require in vivo activation for cholinesterase inhibition; 3) those which are soluble in both water and oil such as schradan (Table I); these compounds are systemic poisons (i.e., they can be translocated within the living system) with low activity in vitro or by direct contact but become activated in vivo (6).

The organophosphates can be considered esters of alcohols with phosphoric acid or anhydrides of phosphoric acid with some other acid (5).

Development

The history of the organophosphates dates back to 1820 when the first esterification of alcohols and phosphoric acids was reported (7). The major development, however, occurred during the 1930's and 1940's through the efforts
<table>
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<th>Chemical Name</th>
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<td>DFP</td>
<td>di-isopropyl phosphorofluoridate</td>
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<tr>
<td>dimefox</td>
<td>bis(diethylamido) phosphoryl fluoride</td>
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<tr>
<td>dioxathion</td>
<td>2,3-p-dioxanedithiol S,S'-bis (O,O-diethyl phosphorodithioate)</td>
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<td>EPN</td>
<td>O-ethyl O-p-nitrophenyl phenylphosphonothioate</td>
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<td>TEPP</td>
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<td>thiono-demeton</td>
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<td>TOCP</td>
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of two groups, one in England directed by Saunders and one in Germany headed by Schrader (7). Most of the studies of the English workers concerned DFP (Table I) and other fluorine containing compounds. The German group made many of the insecticidal phosphates in use today including, among many others, dimefox, schradan, and parathion (Table I) (7).

Mode of Action

A British group: Adrian, Feldberg, and Kilby, first discovered the anticholinesterase property of organophosphates in 1941 by observation of the similarity of muscle response to DFP and eserine (a potent anticholinesterase) (7). It is now widely accepted that organophosphates kill animals, both vertebrate and invertebrate, by inhibiting cholinesterase with consequent disruption of nervous activity caused by accumulation of acetylcholine at nerve endings (5).

Active compounds are almost entirely of the type $\text{R}_1\text{R}_2\text{P}(\text{O or S})\text{R}_3$ (2). $\text{R}_1$ and $\text{R}_2$ may be alkoxy, alkyl, aryloxy, aryl, or amino groups; the $\text{R}_3$ group can be extremely varied (2) and is often referred to as the leaving group (5).

In the reaction sequence of organophosphate and cholinesterase, the organophosphate attacks the OH-group of serine at the active site of cholinesterase, loses its leaving group, and forms a covalent bond with the enzyme, thus inhibiting the enzyme (5). The reaction rate is determined
by the nature of the particular cholinesterase and the particular organophosphate (5). There are two factors which affect the anticholinesterase activity of a compound: 1) affinity for the active site, and 2) ability to phosphorylate it (8).

Various physiological alterations which suggest the mode of action of organophosphate poisoning have been observed. Among them is a report of necrosis in the region of the motor end-plates in fibres of active striated muscle of rats, probably as a consequence of the presence of abnormal amounts of acetylcholine (9). Demyelination of nerve sheaths causing hind-limb paralysis has been observed in some species with some compounds, especially phosphorofluoridates and some substituted triphenyl phosphates. The mechanism of this action is unknown (5). Effects of organophosphates on the pituitary-adrenal system were recently considered (10). In addition to large early increases in blood glucose and blood lactate with intraperitoneal injections of parathion and guthion (Table I), increased levels of plasma corticosterone and depletion of adrenal ascorbic acid were observed. The author speculated that these effects were due to stimulation of secretion of pituitary adrenocorticotropic hormone (ACTH). Increased levels of the liver enzymes tyrosine transaminase, tryptophan pyrrolase, and alkaline phosphatase were also noted.
Metabolism of Organophosphates

The metabolism of organic insecticides may be divided into two distinct categories: (1) activation and (2) detoxication. Activation is defined as the metabolic reaction(s) that convert(s) an intrinsically inactive compound to an active compound or an active compound to another active compound. Detoxication is defined as the reaction(s) that lead(s) to nontoxic substances (11).

Activation

In 1951, Diggle and Gage observed that the anticholinesterase activity of impure samples of parathion was due almost entirely to contamination by the S-ethyl isomer (12). This observation was preceded by the suggestion by Gardiner and Kilby in 1950 of the "activation" of schradan, which they proved in 1952 (13). A similar activation of parathion by liver slices was shown by Diggle and Gage in 1951 (14) and the in vivo activation product identified as paraoxon by Gage in 1953 (15).

Activation of parathion by homogenates was demonstrated by Davison in 1955 using NAD as a cofactor (16). O'Brien in 1959 showed that microsomal enzymes effected the reaction and that NADPH was the best cofactor (17). "Thus, the enzymes that catalyze the activation are similar in nature to drug oxidizing enzymes" (3).

O'Brien in 1965 (18) reported that in vertebrates activation of phosphorothionates occurs in liver microsomes with
in vitro requirements like those of the microsomal drug oxidizing system (NADPH, Mg
tt, and 02). Since the microsomal drug degrading system is absent in aquatic vertebrates while they are just as competent as terrestrial vertebrates at activating phosphorothionates, he concluded that the phosphorothionate activating system differs profoundly in its distribution from the drug degrading system (18).

O'Brien further reported (5) that most (perhaps 75 per cent) of organophosphates are poor cholinesterase inhibitors in vitro, but are activated in vivo; the commonest activation is the conversion of P=S to P=O for which, technically, "desulfuration", not oxidation, is the correct term since no change in valence occurs. According to O'Brien (5), microsomal activation increases anticholinesterase potency about 10,000-fold for parathion, methyl parathion, thiono-demeton (Table I), and malathion. In vertebrates activating ability is found almost exclusively in liver; although lung, heart, and testes have a little activity (5). The precise mechanism of these activations has never been elucidated.

Nakatsugawa and Dahm in 1967 (19) in studies of the microsomal activation of 35S-labeled parathion by rabbit liver microsomes showed that a 35S-metabolite was bound onto microsomes probably as the result of desulfuration in the activation reaction. The system required NADPH and O2.

Although the actual level of paraoxon formed in the animal is low compared with that of other metabolic products-
for example, 4.5 per cent of the LD$_{50}$ dose administered to rats is converted to paraoxon in one hour - there is little doubt that the toxic action is caused by paraoxon, since no other anticholinesterase metabolite is formed (11).

The *in vivo* formation of malaoxon by rats and insects was shown conclusively by Kruger and O'Brien (20) and by Mitchell and Arthur (21) in 1959. Malaoxon production is considerably less in rats and mice than in insects (22).

**Detoxication**

Detoxication of organophosphates was first studied by Mazur in 1946 (7), who found a so-called "DFP-ase" enzyme, which occurred principally in liver. All the organophosphates can be hydrolyzed, in mammals, insects, and plants, by phosphatases; commonly phosphatase hydrolysis is the major metabolic route (5). There are two classes of phosphatases; the more common ones hydrolyze off the leaving group, the less common dealkylate (5). (See Figure 1).

Arthur (22) observed that parathion follows two metabolic detoxication pathways, hydrolysis of the P-0-phenyl bond and reduction of the p-nitro group forming amino parathion. The latter mechanism appears unique for p-nitrophenyl phosphates.

Dahm and Nakatsugawa recently reported (3) that the "phosphatases" that produce 0,0-dialkylphosphorothioic acids from parathion-like compounds in mammals are actually microsomal oxidases. They (3) also reported the finding of Fukami and Shishido that phosphatases that split alkyl phosphate
Figure 1. Major Metabolic Pathways of Parathion
Parathion

Paraoxon
bonds are really transferases that require reduced glutathione. "We must, therefore, take into consideration oxidative enzymes and transferases as well as hydrolases in considering the degradation of organophosphates in future research" (3). Phosphorothionates can also be hydrolyzed directly without prior "oxidation" of P=S to P=O as evidenced by the fact that diethyl phosphorothioate is a common metabolite of dioxathion in rats (5).

Among the early workers who studied the in vivo metabolism of parathion were Gardocki and Hazleton. In 1951 (23) they reported studies of parathion metabolism following intravenous administration to dogs. At a dosage of 10 mg/kg, they recovered two per cent from urine as p-aminophenol derivatives (aminoparathion or paraoxon). The nonphosphorus moiety was recovered from the urine at the 50 per cent level following a dose of 2 mg/kg or 81 per cent following a 4 mg/kg dose. They concluded that the principal metabolite of parathion in dogs is p-nitrophenol. These results are in contrast to those reported by Ahmed et al. in 1958 (24) on their studies of parathion metabolism by cows. They found that amino parathion constituted a major excretory metabolite along with diethyl phosphoric and phosphorothioic acids. They also found a similar rate of reduction of radioactive parathion by stagnating rumen juice in vitro. In the same study, using rats, they found a different metabolic pattern. With 10 mg/kg oral doses of parathion and amino parathion,
66 and 68 per cent, respectively, were excreted as metabolites in the urine during the first 24 hours, with an additional five per cent in each case during the second 24 hours. Radioactivity excreted in the urine within the first two days after parathion treatment was 99.5 per cent hydrolysis products with an indication based on partitioning that the remaining 0.5 per cent might be amino parathion. After amino parathion treatment, 96.3 per cent of excreted radioactivity in urine was hydrolysis products with the remaining 3.7 per cent amino parathion with a possible trace of acetylamino parathion.

In a study involving human volunteers exposed to two per cent parathion dust at different ambient temperatures, Funckes et al. (25) reported that maximum p-nitrophenol excretion in the urine occurred five to six hours following initiation of exposure and varied directly with temperature between 58° and 105°F. They observed neither depressed cholinesterase activity nor clinical symptoms.

Among the first researchers to study the degradative metabolism of parathion by an in vitro system was Aldridge (26, 27). He introduced the terms A- and B-esterase. A-esterase is the serum enzyme which hydrolyzes p-nitrophenyl acetate and paraoxon; B-esterase is the serum enzyme which hydrolyzes p-nitrophenyl acetate but is inhibited by paraoxon (10^-8 M). A-esterase activity varies with species and organ; it is about 17 times higher in rabbits than in most
organisms; rat liver is three to four times more active than rat serum.

Two studies in 1967 (19, 28) showed that microsomal enzymes requiring NADPH and \( O_2 \) can not only "oxidize" para-thion analogs to the corresponding phosphates, but can also effect cleavage at the aryl phosphate bond resulting in the liberation of p-nitrophenol. In addition to a \(^{35}S\)-metabolite bound to microsomes which was mentioned earlier, Nakatsugawa and Dahm (19), using rabbit liver microsomes, observed para-oxon, p-nitrophenol, and a \(^{35}S\)-acidic metabolite, which they identified as 0,0-diethyl phosphorothioic acid. Neal (28) in a similar experiment using \(^{32}P\)-parathion and microsomes from adult male and female rats, adult male mice, and adult male guinea pigs, observed paraoxon, p-nitrophenol, diethyl hydrogen phosphate, and diethyl hydrogen phosphorothionate as metabolites. He concluded that the paraoxon hydrolysis that he also observed was due to an enzyme similar to Aldridge's esterase enzyme. In addition, Neal found that the enzymatic activity toward parathion per unit weight of liver was increased 65 to 130 per cent by pretreatment of male rats with phenobarbital and 3,4-benzopyrene. He also found that the system was inhibited by incubation in an atmosphere of nitrogen, carbon monoxide, or pure oxygen, and suggested the possibility of a deficiency of oxygen for maximal activity as well as the lability of some component of the system to oxidation. In a later study, Nakatsugawa and Dahm (29)
reported the metabolism of several parathion analogs by microsomes from rat and rabbit livers and housefly abdomens. They again found that all P=S compounds were metabolized via two oxidative pathways, activation to P=O analogs and cleavage at the aryl phosphate bond, with a requirement for NADPH and O$_2$. The oxidative cleavage also occurred with one P=O compound, N-propyl paraoxon.

In 1968, Kojima and O'Brien (30) showed that $^3$H-paraoxon was degraded by at least four distinct enzymes in rat liver with different pH optima, reaction constants, and sensitivities to metallic ions, SH-reagents, and inhibitors. These enzymes were present in both soluble and particulate fractions of the liver. The specific activity was highest in the microsomes and lower in the washed mitochondria and the crude soluble fraction at the respective optimum pH's. The crude soluble fraction contained two different enzymes which were partially purified by ammonium sulfate fractionation. The washed mitochondrial, microsomal, and crude and partially purified soluble enzymes produced diethyl phosphate as the principal metabolite. A partially purified soluble fraction also produced 0-desethyl paraoxon. The enzymes in microsomes and mitochondria were solubilized by treatment with sodium deoxycholate.

The subcellular fractions more slowly degraded $^3$H-parathion but no attempt was made to show whether the enzymes responsible for parathion and paraoxon degradation were the
Schwark and Ecobichon reported in 1968 (31) that rat liver and kidney esterases were found primarily in the microsomal fraction of the cell with some esterase activity in soluble or cytoplasmic fractions. They measured subcellular distribution by differential centrifugation followed by quantitative titrimetric analysis, starch gel electrophoresis, and electron microscopy of subcellular fractions.

In 1968, Gram and Fouts (32) reported that drug metabolizing enzymes were associated with the microsomal membranes and not with the ribosomes. Data obtained with three different NADPH-generating systems indicated significantly more drug metabolizing enzyme activity in smooth microsomes than in rough. Microsomal UDP-glucuronyl transferase activity toward o-aminophenol and p-aminophenol was significantly (about 2:1) concentrated in rough microsomes but was evenly distributed toward phenolphthalein. The authors noted that the animal species used as well as the method employed to subfractionate microsomes can influence the results obtained. Fouts (33) had previously made the point "that the activity of drug metabolizing enzymes in liver microsomes may accurately reflect the rate of drug metabolism in the intact hepatic cell and in the animal".

Various authors have indicated that p-nitrophenol, an identified metabolic product of parathion, is excreted as a glucuronide conjugate in the urine (34, 35, 7). Other workers,
such as Gardocki and Hazleton (23) and Funckes et al. (25), preceded measurement of p-nitrophenol with acid hydrolysis and did not differentiate between free and bound forms.

Malathion can be hydrolyzed by two types of enzymes, phosphatases and carboxyesterases. Malathion metabolism in rats, cows, and poultry involves hydrolysis of the P-S and S-C bonds as well as carboxylic ester groups of the diethyl succinate moiety (22). (See Figure 2). Hydrolysis of the P-O-methyl bond forming desmethyl malathion or desmethyl malaoxon was suspected in 1959 based on indirect evidence (22) and later shown in metabolic studies with malathion upon observation that total urinary output contained 7, 11, and 21 per cent desmethyl malathion in the cow, rat, and dog, respectively (36). Studies by Fukami and Shishido in 1963, according to O'Brien (5), have shown that in rat liver, there is a soluble dealkylating phosphatase active against methyl parathion, methyl paraoxon, and sumithion (Table I) which is separable from mitochondrial and microsomal phosphatases. The fact that ethyl sumithion is poorly dealkylated suggests that the enzyme is a demethylase with little de-ethylase activity.

Malathion and malaoxon degrading activity is found in liver, kidney, spleen, serum, lung, ileum, muscle, and brain of all mammals studied. There is a variation among species, but only 10-fold at the most. The ratio of malathion to malaoxon hydrolysis varies greatly, suggesting that different
Figure 2. Major Mammalian Metabolic Pathways of Malathion
Malathion | Malathion α-monoacid | Malathion diacid

Malaoxon | Malaoxon diacid

Malaoxon monoacid
enzymes are involved (5).

Attainment of selective toxicity (i.e., killing insects and sparing higher animals) has been realized with very few chemicals, notably malathion and sumithion; and with these the differences appear quantitative rather than qualitative (3). Malathion degradation to nontoxic ionic products is much more extensive in the mouse than in insects with correspondingly lower malaoxon formation (18, 11). Investigations with the American cockroach and the mouse have shown substantially greater levels of malaoxon in the roach at any time after injection. The relative proportion of malaoxon indicates greater P=S to P=O activation in insects compared with mammals and/or faster malaoxon degradation in mammals compared with insects. Further, in mammals, degradation by carboxyesterase hydrolysis of the carboethoxy moiety predominates, leading to the ionic carboxylic acid, which is rapidly excreted (11). Thus, the metabolic specificity of malathion between insects and mammals may be explained by differences in rates and in the route by which malathion is metabolized in different animals (37, 11). The predominance of carboxyesterase over phosphatase activity in mammals is apparently well established; O'Brien (5) reported that in mice, 77 per cent of the total identified products were due to carboxyesterase. Further carboxyesterase action can cleave the second C\textsubscript{2}H\textsubscript{5} group from malathion to yield malathion diacid. The per cent of urinary products observed as
mono- and diacids are 63 and 17 in cow, 12 and 48 in rat, and 40 and 21 in dog (5). Other studies with rats and dogs have shown the presence of malaoxon in the blood and malathion mono- and diacids in the urine as well as monomethyl phosphate, dimethyl phosphate, and dimethyl thiophosphate (37). Arthur (22) reported that following oral administration of malathion to rats, monomethyl phosphoric acid was isolated from the urine. This acid was probably formed by hydrolysis of desmethyl malaoxon and not by further degradation of dimethyl phosphoric acid, since the dimethyl derivative was recovered from the urine of rats as the administered chemical. A study by Bourke et al. in 1968 (38) indicated that $^{14}$C-malathion is metabolized fairly rapidly by rats; they found that rats eliminated the bulk of ingested radioactivity in the urine within 24 hours with insignificant evolution of respiratory $^{14}$CO$_2$. Chen et al. (39) in 1969 showed, using nuclear magnetic resonance and infrared spectroscopy, that the biologically produced monoacid in rat urine is O,O-dimethyl-S-(1-carboxy-2-carbethoxy) ethyl phosphorodithioate, the $\alpha$-monoacid of malathion.

Toxicity

The toxicity of the esterase-inhibiting pesticides to mammals varies considerably with species, sex, age, route and frequency of administration, and previous history of exposure to other chemicals (2). Among other factors worthy of consideration are diet and rhythms in drug metabolizing
enzymes.

Murphy et al. (40) in 1968 reported results which suggest that for some insecticides species differences in the reactivities of brain cholinesterases with the oxygen analogs contribute significantly to species differences in susceptibility to poisoning. The relative quantities of oxygen analogs that accumulate and are available to inhibit cholinesterase in different species will be dependent (at least in part) upon the relative rates of their formation from the parent insecticides and the relative rates of metabolic degradation of the P=O or P=S compounds by tissue hydrolases or other mechanisms of inactivation. Murphy et al. (40) indicated that for some compounds species differences in the sensitivities of the cholinesterases to inhibition are sufficiently large to modify the influence of differences in rates of metabolism.

Lamanna and Hart (41) considered the relationship of lethal toxic dose to body weight of the mouse. After administration of a large number of compounds intraperitoneally to small, medium, and large male and female mice, they found that the most common relationship was a direct linear one characterized by a slope of unity when log of dose is plotted against log of body weight although a significant number of cases showed a variety of other relationships.

Brodeur and DuBois (42) considered, among other things, the influence of age and sex on the toxicity of malathion and
malaoxon in rats. They found that for male rats, malathion hydrolytic activity increased progressively and rapidly from the twelfth day of age and reached a maximum of 0.360 μg/mg tissue/10 minutes around the sixtieth day of age. For females, malathion hydrolytic activity paralleled that of males for the first weeks of life, increased slightly after the thirtieth day, and reached a maximum of 0.145 μg/mg tissue/10 minutes at 36 days of age. They also found that estradiol decreased and testosterone increased resistance to malaoxon.

**Influence of Nutritional Status on Metabolism**

The nutritional status of an animal can affect many of its living processes such as composition of body fluids, tissues, and subcellular organelles; protein metabolism; and incidence and severity of disease, cancer, and toxicity symptoms (43). Liver size, protein/DNA ratio, and RNA/DNA ratio increase with the quantity of casein consumed (43, 44). Freedland et al. (45) concluded that the effects of hormones on enzyme activity could not be properly evaluated without considering the nutritional condition of the animal. Kato et al. (46) reported results which indicate that the toxicities of drugs in rats fed high (18 and 50 per cent), low (5 per cent), or non-protein diets were closely related to rates of drug metabolism by liver microsomes and that the activities of androgen-dependent enzymes were closely related to the amount of dietary protein. Their results also indi-
cated that the drug-metabolizing activity of liver microsomes may be regulated at two steps in relation to the amount of dietary protein - first, the activity of the NADPH-linked electron transport system and second, the terminal oxidase activity.

Boyd (47) reported that rats fed 27 per cent protein were 5.9 times more resistant to orally administered parathion and 2.3 times more resistant to malathion when compared with rats fed 3.5 per cent protein. His laboratory further reported (48) that the acute oral toxicity of technical malathion (95 per cent) in young male albino rats is $1090 \pm 83$ mg/kg ($LD_{50} \pm$ S.E.) for rats fed laboratory chow, $1401 \pm 99$ for rats fed 26 per cent casein, and $599 \pm 138$ for rats fed 3.5 per cent casein. His values for rats fed laboratory chow and 26 per cent casein agreed well with values from other laboratories which he cited.

Read et al. (49) reported that a low protein diet had little effect on liver and kidney carboxyesterases. Addition of 10ppm parathion to the low protein diet caused a reduction of both parameters but only a reduction in liver carboxyesterase for control animals. Casterline and Williams (50) reported that brain microsomal protein and aliesterase and acetylcholinesterase were only slightly affected by dietary protein levels or by the length of time the animals were fed the diet. Liver microsomal protein and aliesterase and acetylcholinesterase activity, and serum acetylcholinesterase
activity were reduced at levels lower than 15 per cent dietary casein; serum protein and aliesterase activity (except for 5 per cent casein where serum aliesterase was unexplainably higher) were independent of the casein content of the diet. At all casein levels, a single intraperitoneal injection of parathion inhibited the serum aliesterase but affected liver microsomal and brain microsomal aliesterase slightly or not at all. In general, acetylcholinesterase activities of serum, liver microsomes, and brain microsomes were decreased by parathion. It was also observed that the lethal action of all three pesticides (banol, parathion, and chlordane) (Table I) was increased with lower dietary protein.

Several investigators have reported various physiological alterations associated with protein deficiency. Among them are nervous system damage (51), decreased liver protein (52), morphological and respiratory changes in liver mitochondria (53), enzyme changes in the jejunal mucosa (54), impaired utilization of glucose (55), kwashiorkor cachexia characterized by stunting, alopecia, tail dermatitis, and augmented caloric intake per kg body weight (56), and loss of one-fourth of liver cell RNA (57). Kirsch et al. (58) found no defect in amino acid absorption from the gut in protein-deficient rats.

McLean and McLean (59) have shown that liver microsomal enzymes performing demethylation and hydroxylation reactions are decreased approximately 80 per cent in activity by with-
holding dietary protein or feeding three per cent casein for four days and a 50 per cent decline for rats receiving diets with six per cent casein.

Influence of Age and Development on Metabolism

Studies in vitro showed that newborn guinea pigs lacked the conjugative system that forms phenolphthalein glucuronide and the oxidative systems which metabolize amidopyrine, phenacetin, and 0-hexobarbitone. The enzyme systems were absent 24 hours after birth, appeared during the first week, and increased in activity until the animals were about eight weeks old. In vivo studies on newborn mice showed that they lacked the oxidative enzyme systems but had developed them by seven days of age (60). The newborn rabbit is unable to metabolize hexobarbital, aminopyrine, l-amphetamine, acetyaminilid, chlorpromazine, or p-nitrobenzoic acid. By two weeks of age, the rabbit has developed 5 to 37 per cent of adult activity; three-week-old animals have even more activity; and activity in four-week-old animals approximately equals that of adults (61). Fouts and Hart sum up the situation: "Newborn rabbits, mice, and guinea pigs appear to lack the hepatic enzyme systems that in the adult are responsible for the metabolism of a wide variety of drugs" (62).

In their studies on rats, Brodeur and DuBois (42) found that malathion hydrolytic activity increased progressively and rapidly from the twelfth day of age and reached a maximum around the sixtieth day for male rats and that activity for
females paralleled males for the first few weeks of life, increased slightly after the thirtieth day, and reached a lower maximum at the thirty-sixth day of age.

**Influence of Drugs and Other Insecticides on Metabolism**

"Many types of synergism and antagonism have been demonstrated to involve a modification of the oxidative enzyme activity of liver microsomes" (63). Welch and Coon found that liver microsomal β-esterases that catalyze hydrolysis or organophosphate esters are induced by phenobarbital and other drugs (64). DuBois and Kinoshita (65) found that pretreatment with phenobarbital either decreased the toxicity or had no effect on 14 cholinergic organophosphates (malathion and parathion included) while toxicity of only one compound, OMPA (Table I) was increased in rats. They concluded that the toxicity of cholinergic organic phosphates more closely parallels changes in the microsomal detoxication enzymes than in the activation process; OMPA is an exception but its activation involves oxidation of an alkylamide linkage rather than desulfuration. Brodeur (66) investigated the mechanism of phenobarbital-induced protection against malathion and EPN (Table I). Pretreatment for two days provided maximum protection against EPN; five day pretreatment afforded maximum protection against malathion. Phenobarbital (50 mg/kg/day) increased resistance of adult female rats to the toxic effects of malathion as indicated by increased LD$_{50}$ values and decreased in vivo anticholin-
esterase activity and produced a rise in liver aliesterase activity. Administration of TOCP (Table I), an aliesterase inhibitor, abolished the protective effect of phenobarbital against malathion but had no effect on its protection against EPN.

In 1954, Ball et al. (67) observed that oral pretreatment of rats with chlorinated hydrocarbons (aldrin, chlordane, or lindane) (Table I) greatly increased their resistance to parathion and that the pretreatment is followed by a rise in the serum aliesterase level. Oral aldrin pretreatment offered the same degree of protection (seven-fold) against parathion administered by intraperitoneal injection. Triolo and Coon (68, 69) confirmed the protection of aldrin against several organophosphates and also observed that two days after aldrin, A-esterase increased 38 per cent in the liver but decreased 50 per cent in the plasma although plasma B-esterase increased 24 per cent. Aldrin had no effect on paraoxon's inhibition of plasma cholinesterase but reduced paraoxon's effect on brain cholinesterase. Simultaneous administration of ethionine blocked organochlorine antagonism of parathion and paraoxon toxicities, indicating an antagonism dependent upon protein synthesis.

The potentiation of malathion's toxicity by EPN is the classical example of inhibition of the synthesis or action of microsomal enzymes leading to greatly altered toxicant action (63). Knaak and O'Brien investigated this phenomenon
in 1960 (36). They found that in both the rat and the dog EPN resulted in a marked shift in detoxication site of the malathion molecule from carboxyester to thiolo-phosphate bond (indicative of carboxyesterase inhibition by EPN). Malathion levels in rat tissue were increased by EPN; malaoxon levels in blood were reduced. Their conclusion that potentiation results from increased persistence rather than increased concentration of malaoxon in the tissues has been confirmed by later experiments (18).

There is evidence that the development of resistance in insects is due to the development of carboxyesterase activity (70). EPN and malathion are among the few pairs of organophosphates for which synergism has been detected (2). This synergism is due to EPN's inhibition of carboxyesterase (18, 8). Insect resistance was thought to be due to development of a carboxyesterase system; synergism by EPN of malathion's toxicity to resistant but not to susceptible insects provided added evidence of the development of such a system (18).

There is also evidence that previous exposure to malathion is synergistic. In 1967, Murphy (71) reported that hydrolysis of malathion by rat, mouse, and guinea pig liver homogenates and by rat plasma was inhibited by previous treatment with malathion. He also reported that guinea pigs and rats that were pretreated with less than cholin-
esterase inhibiting quantities of malathion were more sensitive to subsequent doses. These observations led to the suggestion that an early biochemical event leading to poisoning by malathion is the inhibition of its own further hydrolytic detoxication by a metabolite (or, in cases of less pure samples, by a contaminant) of the parent insecticide. In a later study, Murphy and Cheever (72) reported that dietary concentrations of dioxathion (Delnav), ronnel, and malathion (Table I), which were less than those required to inhibit red blood cell or brain cholinesterase, inhibited liver and plasma carboxyesterase. Red blood cell cholinesterase and liver and plasma carboxyesterase were equally sensitive to inhibition by low dietary parathion. Oral pretreatment of rats with concentrations of dioxathion or ronnel which produced 50 per cent inhibition of liver carboxyesterase but no inhibition of brain cholinesterase increased susceptibility to brain cholinesterase inhibition by a single dose of 200 mg/kg of malathion. No potentiation of brain cholinesterase inhibition was detected in ronnel-fed rats given 15 mg/kg of EPN. Murphy's two studies suggest the possibility that the synergistic effect of previous malathion exposure may be due to carboxyesterase inhibition.
EXPERIMENTAL PROCEDURES

Experimental Diets

To evaluate the effects of the level of dietary protein on organophosphate toxicity and metabolism, diets were prepared with 5 or 20% vitamin-free casein. The basal diet (20% casein) consisted of the following: (in per cent) sucrose, 69.6; vitamin-free casein, 20.0; corn oil, 4.0; Jones-Foster Salt Mixture (73) 4.0; vitamins, 2.2; and dl-methionine, 0.2. The vitamin mix (Vitamin Diet Fortification Mixture) and salt mixture were obtained from Nutritional Biochemical Corporation. In preparing the 5% casein diet, casein was replaced by additional sucrose. In addition, magnesium oxide was added to all diets (0.0332 g/100 g diet) because magnesium deficiency symptoms were observed in rats during the first malathion LD$_{50}$ study. Analysis of a sample of diet by atomic absorption spectrophotometry revealed Mg level to be low (about 200 ppm); magnesium oxide was added to give about 400 ppm dietary Mg.

Feeding Regimen

All test animals were maintained on the test diets for 10 days, with groups receiving 5% and 20% casein diets ad libitum and a third group receiving 20% casein pair-fed to the 5% casein group. The rats had previously been divided
into three groups with animals paired according to body weight (≤ 1g) distributed among the three groups, placed in individual cages, and fed 20% casein diet ad libitum for 48 hours to allow adjustment to laboratory conditions. Food consumption was recorded daily and body weights of test animals observed at the beginning, once during, and at the end of experimental feeding periods.

Toxicity Studies

Male weanling Sprague-Dawley derived rats (Flow Research Animals, Dublin, Virginia) weighing 45-55 g were placed in individual stainless steel cages and fed the experimental diets as described above with distilled water provided ad libitum. Animals were housed in an air-conditioned room equipped with a light-control switch providing alternate 12 hour periods of light and darkness. Following pilot studies to determine approximate LD$_{50}$ values at least 50 animals were fed according to each dietary regimen and malathion (or malaoxon) was administered intraperitoneally on the tenth day using five levels of the toxicant with approximately 10 animals per treatment level. The malathion (or malaoxon) was administered in a solution of ethanol: propylene glycol (20:80) with each rat receiving 1.0 ml/100g body weight. Mortality was noted and recorded at 24 hours, 48 hours, and 72 hours after injection. The LD$_{50}$ values were
calculated by probit analysis using the BMD 03S computer program compiled by Dixon (74).

Similarly designed experiments were conducted to determine the acute toxicities of parathion and paraoxon, except that rats received 0.5 ml/100g body weight of the toxicant solution. Solution volume was reduced because the larger volume previously used had caused sluggishness in control animals. Control studies revealed that the vehicle itself was not lethal to the rats.

**Metabolism Studies**

Male weanling rats were fed according to the protocol used in the toxicity studies. On the tenth day, five rats from each dietary regimen were injected intraperitoneally with parathion (0.3429 mg/ml), paraoxon (0.0177 mg/ml), or p-nitrophenol (0.0950 mg/ml) in solutions of ethanol: propylene glycol (20:80) with each rat receiving 0.5 ml/100g body weight. Rat weights, time of injection, and volume of injection solution were recorded. Urine was collected at 12 hours and 24 hours postinjection in graduated centrifuge tubes including minimal washings of metabolism cage collection funnels with distilled water. Urine volumes were recorded at each collection and samples from the five rats on each dietary regimen were pooled for animals injected with parathion, paraoxon, and p-nitrophenol, respectively. Urine
samples were treated in three different manners before assay: untreated, acidified by addition of concentrated HCl (1:1), or acidified and boiled gently for one hour (acid hydrolyzed) using one-fourth of each pooled sample for each treatment. Samples were then assayed using a modification of the method of Elliot et al. (75). Samples were extracted by shaking 10 minutes in 125 ml separatory funnels on a mechanical shaker with 60 ml of ethyl ether: benzene (20:80). Fifty ml of the ethyl ether: benzene phase was transferred to a second 125 ml separatory funnel and extracted by shaking 10 minutes on a mechanical shaker with 20 ml of 20% NaOH. The NaOH phase was quantitatively transferred to a 60 ml separatory funnel and extracted by shaking 10 minutes on a mechanical shaker with 10 ml of acetonitrile. The acetonitrile phase was quantitatively transferred to a 15 ml centrifuge tube and evaporated to dryness under a stream of air. The residue was taken up in 5 ml of acetonitrile. Five ml of 3N NH₄OH and 1 ml of o-cresol reagent (2% in 0.2N NaOH) were added and the solution was mixed. One ml of freshly prepared titanium trichloride reagent (1 ml of 20 per cent stock solution plus 16 ml H₂O) was added and tubes were shaken until the dark precipitate disappeared. Samples were then allowed to sit thirty minutes for color to develop, were centrifuged until optically clear, and the absorbance read against a reagent blank at 620 μm using a Bausch and Lomb Spectronic 20 colorimeter.
Quantities of p-nitrophenol were estimated by comparison with a standard curve prepared by assaying samples with known amounts of p-nitrophenol. In addition, two known levels of p-nitrophenol were assayed with each day's samples to check for daily variation.

Malathion secondary standard (95%), malaoxon (>95%), parathion, technical, (>98.5%), and paraoxon (>99%) used in these studies were gratis research samples supplied by American Cyanamid Company, Princeton, New Jersey.

Urine from untreated animals and from animals injected with the ethanol: propylene glycol solvent gave no absorbancy when compared with a reagent blank.

Data from the metabolism studies were obtained by individual determinations upon aliquots from pooled samples.
RESULTS

Gross Effects of Experimental Diets

Feeding the experimental diets resulted in different patterns of weight gain and food efficiency in the test animals. Table II shows that the 5% casein-fed rats gained very little weight during the 10-day feeding period and showed only a 10 per cent food efficiency (weight gain (g)/food eaten (g)) while both groups fed 20% casein gained weight and showed food efficiencies greater than 50 per cent. Rats fed 5% casein also showed a lower protein efficiency (weight gain (g)/protein eaten (g)).

Toxicity Studies

The evaluations of acute toxicity of malathion, malaoxon, parathion, and paraoxon were based on LD_{50} determinations. Since LD_{50} data for intraperitoneally administered compounds were not available, pilot studies were conducted to determine the general toxicity range. Brodeur and DuBois (42) had reported intraperitoneal administration of malathion in a solution of 20 per cent ethanol in propylene glycol and had estimated the LD_{50} of 42 day old rats to be greater than 800 mg/kg. There was also a report of a parathion LD_{50} intraperitoneally of 5.5 mg/kg (76). These values were used as guides for the pilot studies.

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Table II. Weight gain and food efficiency of rats fed the experimental diets for ten days

<table>
<thead>
<tr>
<th>Percent Casein in Diet</th>
<th>Number of Rats</th>
<th>Ave. Wt. Gain (g)</th>
<th>Ave. Food Eaten (g)</th>
<th>Food Efficiency</th>
<th>Protein Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (ad libitum)</td>
<td>216</td>
<td>5.90</td>
<td>57.37</td>
<td>0.1028</td>
<td>2.06</td>
</tr>
<tr>
<td>20 (pair-fed)*</td>
<td>213</td>
<td>29.88</td>
<td>57.99</td>
<td>0.5152</td>
<td>2.58</td>
</tr>
<tr>
<td>20 (ad libitum)</td>
<td>211</td>
<td>45.94</td>
<td>88.39</td>
<td>0.5197</td>
<td>2.60</td>
</tr>
</tbody>
</table>

*Animals were pair-fed to the 5% ad libitum group.
After injection, animals showed the usual symptoms of organophosphate poisoning: lachrymation, salivation, twitching, and convulsions followed by death. Death occurred rather quickly especially upon administration of parathion and paraoxon. The rates of mortality occurrence are shown in Table III.

The acute toxicity studies revealed that dietary protein level affected not only the parent compounds, malathion and parathion, but also their oxygen analogues, malaoxon and paraoxon. The LD$_{50}$ values are shown in Tables IV and V.

**Metabolism Studies**

Modifications of the method of Elliot et al. (75) included increasing NaOH volume from 5 ml to 20 ml, increasing acetonitrile volume from 5 ml to 10 ml, and evaporation of acetonitrile to concentrate sample. These modifications increased the per cent recovery of the method from approximately 50 per cent to approximately 80 per cent. (According to the original publication, per cent recovery of the method should be about 75 per cent but tests in our laboratory gave lower recovery.) The method is reported to be sensitive for 10 µg of p-nitrophenol; our results indicated sensitivity of 5 µg or perhaps less.

Results of urinary excretion of p-nitrophenol as indicated by acid hydrolysis of urine samples are shown in Table
Table III. Rate of mortality occurrence after organophosphate administration (% of total deaths)

<table>
<thead>
<tr>
<th>Percent Dietary Casein</th>
<th>Malathion</th>
<th>Malaoxon</th>
<th>Parathion</th>
<th>Paraoxon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First 24 hr.</td>
<td>Second 24 hr.</td>
<td>Third 24 hr.</td>
<td>First 24 hr.</td>
</tr>
<tr>
<td>5 (ad libitum)</td>
<td>79.1</td>
<td>20.9</td>
<td>0.0</td>
<td>66.7</td>
</tr>
<tr>
<td>20 (pair-fed)</td>
<td>79.4</td>
<td>11.8</td>
<td>8.8</td>
<td>76.7</td>
</tr>
<tr>
<td>20 (ad libitum)</td>
<td>59.5</td>
<td>35.1</td>
<td>5.4</td>
<td>76.7</td>
</tr>
<tr>
<td>5 (ad libitum)</td>
<td>89.7</td>
<td>6.9</td>
<td>3.4</td>
<td>81.0</td>
</tr>
</tbody>
</table>
Table IV. Comparative LD$_{50}$ values (± S.E.) for parathion and paraoxon (mg/kg body weight)

<table>
<thead>
<tr>
<th>Percent Dietary Casein</th>
<th>Number of Rats</th>
<th>Parathion LD$_{50}$</th>
<th>Number of Rats</th>
<th>Paraoxon LD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (ad libitum)</td>
<td>50</td>
<td>1.24±0.0595$^+$</td>
<td>50</td>
<td>0.385±0.031$^+$</td>
</tr>
<tr>
<td>20 (pair-fed)</td>
<td>48</td>
<td>1.65±0.1439</td>
<td>47</td>
<td>0.537±0.035*</td>
</tr>
<tr>
<td>20 (ad libitum)</td>
<td>50</td>
<td>2.04±0.1485</td>
<td>48</td>
<td>0.666±0.020$'$</td>
</tr>
</tbody>
</table>

* significantly different from 20% ad libitum (P < 0.05)
+ significantly different from 20% ad libitum (P < 0.01)
' significantly different from 20% pair-fed (P < 0.05)
Table V. Comparative LD$_{50}$ values (± S.E.) for malathion and malaoxon (mg/kg body weight)

<table>
<thead>
<tr>
<th>Percent Dietary Casein</th>
<th>Number of Rats</th>
<th>Malathion LD$_{50}$</th>
<th>Number of Rats</th>
<th>Malaoxon LD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (ad libitum)</td>
<td>59</td>
<td>151.1±15.70$^+$</td>
<td>57</td>
<td>7.75±0.3464$^+$</td>
</tr>
<tr>
<td>20 (pair-fed)</td>
<td>60</td>
<td>273.6±17.07$^+$</td>
<td>58</td>
<td>14.33±0.7308</td>
</tr>
<tr>
<td>20 (ad libitum)</td>
<td>59</td>
<td>418.6±11.09$^o$</td>
<td>50</td>
<td>15.23±1.0330</td>
</tr>
</tbody>
</table>

$^+$ significantly different from 20% ad libitum (P< 0.01)

$^o$ significantly different from 20% pair-fed (P< 0.01)
VI. Total urinary recovery of injected parathion and paraoxon 24 hours after injection appears less for the animals fed 20% casein *ad libitum* when compared with other groups. Rats pair-fed 20% casein excreted greater amounts of injected parathion and paraoxon during the first 12 hours after injection, with rats fed 5% casein "catching up" during the second 12 hours. Rats pair-fed 20% casein also appeared to excrete injected p-nitrophenol more quickly and to a greater extent than other groups but the difference may not be great enough to be meaningful.

The distribution of excreted p-nitrophenol between free and bound forms was studied by varying pretreatment of assayed samples. Acid hydrolysis of urine samples before assay indicates the total amount of p-nitrophenol present in all forms, whether as parathion, paraoxon, p-nitrophenyl glucuronide, p-nitrophenyl sulfate, free p-nitrophenol, or other metabolites containing the p-nitrophenyl or p-aminophenyl groups. Acidification of samples before assay indicates "free" p-nitrophenol plus p-nitrophenol "loosely bound" to groups such as sulfate with only about 5 per cent recovery from p-nitrophenyl glucuronide and no apparent recovery from parathion or paraoxon. Extraction of samples without pretreatment indicates primarily "free" p-nitrophenol with some (about 10 per cent) response from p-nitrophenyl sulfate and no detectable response from more tightly-bound forms. The distribution of recovered urinary p-nitrophenol is shown in Table VII. The percent of recovered parathion, paraoxon, and p-nitrophenol excreted
Table VI. Urinary excretion of p-nitrophenol (% of intraperitoneally injected compound recovered by acid hydrolysis)

<table>
<thead>
<tr>
<th>Compound Injected</th>
<th>Percent Dietary Casein</th>
<th>Time after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-12 hr.</td>
</tr>
<tr>
<td>Parathion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (ad libitum)</td>
<td>45.91</td>
<td>16.48</td>
</tr>
<tr>
<td>20 (pair-fed)</td>
<td>60.45</td>
<td>4.88</td>
</tr>
<tr>
<td>20 (ad libitum)</td>
<td>39.83</td>
<td>9.32</td>
</tr>
<tr>
<td>Paraoxon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (ad libitum)</td>
<td>76.76</td>
<td>20.03</td>
</tr>
<tr>
<td>20 (pair-fed)</td>
<td>91.95</td>
<td>0.00</td>
</tr>
<tr>
<td>20 (ad libitum)</td>
<td>57.97</td>
<td>14.72</td>
</tr>
<tr>
<td>p-nitrophenol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (ad libitum)</td>
<td>70.07</td>
<td>4.82</td>
</tr>
<tr>
<td>20 (pair-fed)</td>
<td>75.54</td>
<td>7.02</td>
</tr>
<tr>
<td>20 (ad libitum)</td>
<td>69.48</td>
<td>3.60</td>
</tr>
</tbody>
</table>
Table VII. Distribution of recovered urinary p-nitrophenol (values expressed as % of total recovered p-nitrophenol)

<table>
<thead>
<tr>
<th>Compound Injected</th>
<th>Percent Dietary Casein</th>
<th>% &quot;free&quot; (untreated urine)</th>
<th>0-12 hr.</th>
<th>12-24 hr.</th>
<th>0-24 hr.</th>
<th>% &quot;free&quot; + % &quot;loosely bound&quot; (acidified urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0-12 hr.</td>
<td>12-24 hr.</td>
<td>0-24 hr.</td>
<td>0-12 hr. 12-24 hr. 0-24 hr.</td>
</tr>
<tr>
<td>parathion</td>
<td>5 (ad libitum)</td>
<td>4.25</td>
<td>0.00</td>
<td>4.25</td>
<td>60.85</td>
<td>20.28 81.13</td>
</tr>
<tr>
<td></td>
<td>20 (pair-fed)</td>
<td>4.67</td>
<td>0.00</td>
<td>4.67</td>
<td>85.36</td>
<td>5.92 91.28</td>
</tr>
<tr>
<td></td>
<td>20 (ad libitum)</td>
<td>12.61</td>
<td>0.00</td>
<td>12.61</td>
<td>85.08</td>
<td>14.50 99.58</td>
</tr>
<tr>
<td>paraoxon</td>
<td>5 (ad libitum)</td>
<td>13.79</td>
<td>0.00</td>
<td>13.79</td>
<td>72.41</td>
<td>24.14 96.55</td>
</tr>
<tr>
<td></td>
<td>20 (pair-fed)</td>
<td>27.27</td>
<td>0.00</td>
<td>27.27</td>
<td>97.73</td>
<td>2.27 100.00</td>
</tr>
<tr>
<td></td>
<td>20 (ad libitum)</td>
<td>41.77</td>
<td>1.27</td>
<td>43.04</td>
<td>83.54</td>
<td>15.19 98.73</td>
</tr>
<tr>
<td>p-nitrophenol</td>
<td>5 (ad libitum)</td>
<td>8.15</td>
<td>0.21</td>
<td>8.37</td>
<td>67.60</td>
<td>3.86 71.46</td>
</tr>
<tr>
<td></td>
<td>20 (pair-fed)</td>
<td>14.38</td>
<td>1.50</td>
<td>15.88</td>
<td>95.00</td>
<td>4.75 99.75</td>
</tr>
<tr>
<td></td>
<td>20 (ad libitum)</td>
<td>19.17</td>
<td>0.88</td>
<td>20.04</td>
<td>86.53</td>
<td>3.07 89.59</td>
</tr>
</tbody>
</table>
"free" is greater for animals fed 20% casein ad libitum than for animals pair-fed 20% casein which is greater than for animals fed 5% casein ad libitum. The per cent of recovered parathion, paraoxon, and p-nitrophenol excreted as "free" plus "loosely bound" is greater for animals fed 20% casein than for animals fed 5% casein. This analysis of the distribution of the various forms of p-nitrophenol, while not entirely selective, does give an indication of the various forms in which the compound is excreted.
DISCUSSION AND CONCLUSIONS

In a 1960 study, Gaines (77) reported LD$_{50}$ values for 42 pesticides and two metabolites of DDT administered in a single dose by the oral or dermal route to Sherman strain adult rats. Among the values were those for malathion (oral route: male = 1375 mg/kg, female = 1000 mg/kg, dermal route: male > 4444 mg/kg, female > 4444 mg/kg) and parathion (oral route: male = 13 mg/kg, female = 3.6 mg/kg; dermal route: male = 21 mg/kg, female = 6.8 mg/kg). Among other reported LD$_{50}$ values are 5.5 mg/kg for intraperitoneally injected parathion (76), and 925 mg/kg in adult rats orally and 124 mg/kg in newborn rats intragastrically for malathion (78).

The reported values for both malathion and parathion are higher than those obtained in this study when compounds were administered intraperitoneally to young male rats fed 20% casein diet ad libitum. The differences observed could be due to the use of younger animals, different modes of administration and/or solvent systems, variations in laboratory conditions, or various other factors.

There is little doubt that dietary protein intake is of major importance in determining the toxicities of malathion and parathion and their oxygen analogues to young rats. The toxicity of all four compounds is significantly greater ($P < 0.01$) in rats fed 5% casein ad libitum when compared with rats fed 20% casein ad libitum. It is unlikely that the
differences seen are due to differences in caloric intake since the toxicities are also significantly greater (malathion and malaoxon, $P < 0.01$; parathion and paraoxon, $P < 0.05$) in rats fed 5% casein *ad libitum* when compared with rats pair-fed 20% casein. The observation that the toxicities of malathion ($P < 0.01$) and paraoxon ($P < 0.05$) are significantly greater for rats pair-fed 20% casein when compared with rats fed 20% casein *ad libitum* is difficult to explain.

Since dietary protein level affected the toxicity of both the inactive parent compounds and their active metabolites, the oxygen analogues, the effect of protein is apparently manifested in the detoxication, not the activation, of the compounds. These results are in contrast to those reported by Weatherholtz et al. (79) for heptachlor, another enzymatically activated insecticide. They found a protein effect on the inactive parent compound but no effect on the active metabolite. However, heptachlor differs from the organophosphates in that its active metabolite is not known to undergo further degradative metabolism.

The organophosphate compounds used in this study produced their effect rather quickly (Table III). Approximately 75 per cent of the total mortality occurred during the first 24 hours postinjection with most of the additional during the second 24 hours and from 0 to 10 per cent (except in one case) during the third 24 hours. Mortality was observed as early as 10 to 15 minutes after injection and a large number of the
deaths within the first 24-hour period occurred within two or three hours. This rapid rate of mortality would be expected since the compounds are apparently metabolized quite rapidly. As mentioned earlier, Bourke et al. (38) reported that rats eliminated the bulk of ingested radioactivity from malathion-\(^{14}\)C within 24 hours.

In the metabolism studies, it is interesting to note that the animals fed 20% casein \textit{ad libitum} appeared to excrete less of the injected parathion and paraoxon as p-nitrophenol than other groups (Table VI). The reasons for this excretion pattern are not obvious. If the lower toxicity of these compounds is due to an increased rate of metabolism, then a greater, not a smaller, degree of excretion would be expected in the 20% \textit{ad libitum} group. The group fed 20% casein \textit{ad libitum} also appeared to excrete injected p-nitrophenol more slowly and to a lesser extent although the difference in this case may not be great enough to be meaningful.

It is also interesting to note that animals which were pair-fed 20% casein appeared to excrete the injected compounds more quickly than either of the \textit{ad libitum} fed groups. This anomaly may be related to the diurnal variations in drug metabolizing activity which have been reported (80, 81). These rhythms are altered by various conditions such as adenalec- tomy, corticosterone administration, and phenobarbital treatment (81), and may possibly be affected by the feeding pattern of the animals. Since rats on the pair-feeding
regimen ate their food as a "meal" once every 24 hours, and since food was offered in the morning instead of at night when an *ad libitum* -fed animal would choose to eat, rats on the pair-feeding regimen were subjected to the additional stress of an unnatural feeding pattern. Such a stress could conceivably induce the enzymes which metabolize parathion, paraoxon, and possibly even p-nitrophenol resulting in an increased rate of excretion of p-nitrophenol in the pair-fed group.

This factor of diurnal rhythms might also be important in the toxicity difference between the 20% pair-fed and 20% *ad libitum*-fed groups which was mentioned earlier. It is possible that in the cases where this difference was seen that the compound was administered immediately preceding the peak or the trough of enzyme activity, thus resulting in an altered pattern of metabolism in the pair-fed groups. Since this variable was not considered in the planning of the toxicity studies, care was not taken to insure constant feeding and injection times for the various studies.

The observation that 50 to 65 per cent of injected parathion was recovered as p-nitrophenol while 73 to 97 per cent of injected paraoxon was recovered is difficult to explain since the method used was for the p-nitrophenol or p-aminophenol group and any compound containing the group should have been detected.

Results from the studies on distribution of excreted
p-nitrophenol do not agree very well with the results of Robinson et al. (35) from their studies of the detoxication of nitrophenols by rabbits. They found less than one per cent of administered p-nitrophenol excreted free; glucuronides accounted for about 70 per cent of the dose; ethereal sulfates were also detected. They also found a 14 per cent reduction of the nitro group of p-nitrophenol. In contrast, our results indicated from 8 to 20 per cent of the p-nitrophenol recovered from urine samples was unconjugated when p-nitrophenol was injected intraperitoneally. From 14 to 43 per cent of the recovered p-nitrophenol was unconjugated when paraoxon was injected and from 4 to 13 per cent when parathion was injected. Although no analysis for aminophenol, the product of nitro group reduction, was performed, any aminophenol present would have given a blue color prior to the addition of titanium trichloride reducing reagent during the assay procedure. In none of the samples was any blue color observed at this time; therefore, there was no indication of the presence of p-aminophenol. Perhaps the differences seen are due to the use of a different animal species.

Also of interest is the finding that the per cent of recovered parathion, paraoxon, and p-nitrophenol excreted as "free" plus "loosely bound" is greater for animals fed 20% casein than for animals fed 5% casein. This situation might be due to a lack of glucuronide conjugating enzyme(s) in the protein deficient animals.

Researchers concerned with the metabolism of xenobiotics
have concentrated on liver activity. There are indications, however, in the case of organophosphates, that enzymes in the blood may make a significant contribution to metabolism. Several workers have reported a rise in serum aliesterase (B-esterase) activity upon administration of compounds which have a protective effect against organophosphate toxicity (66, 67, 82, 83, 69, 64) and have found that this protective action is blocked by ethionine (69, 84) or TOCP (66), an aliesterase inhibitor. The most often proposed mechanism for the protective action is provision of a less vital enzyme with which the cholinesterase inhibitors can react, thus sparing cholinesterase. The mechanism most recently proposed, however, was based on in vitro studies using mouse plasma and paraoxon. Triolo et al. (85) in these studies, found inactivation of paraoxon by a nonenzymatic binding process and found that pretreatment of the animals with organochlorine insecticides increased this binding and lowered mortality. Since these results were based on "free paraoxon" available for reacting with acetylcholinesterase after incubation of paraoxon with plasma and since paraoxon is not hydrolyzed by aliesterase (B-esterase), this inactivation was probably not due to an increased B-esterase such as has been observed by other workers after organochlorine pretreatment.

Another interesting phenomenon which may be important in organophosphate metabolism is lowered serum and elevated liver A-esterase often seen after pretreatment of animals with
compounds which protect against organophosphate toxicity (64, 69, 83, 82). This enzyme associated protection is commonly seen when organophosphates are administered orally; Main (83), however, observed that although aldrin pretreatment decreased the oral toxicity of paraoxon three-fold, it increased the intravenous toxicities of parathion and paraoxon. He concluded that the A-esterase activity of liver mediates oral toxicity and that serum activity influences intravenous toxicity. Thus, it appears that route of administration is influential in the determination of metabolic pathway. In the case of intraperitoneal administration, as in these studies, the selected metabolic pathway is apparently undetermined but the possible role of serum esterases cannot be ignored.

There is some doubt as to whether the results from our toxicity and metabolism studies can be legitimately compared. Since an animal's response to a toxicant in terms of routes and extents of degradation is dose-dependent (8), the metabolic responses to lethal and sub-lethal doses may be different. This situation may explain our inability to find any correlation between the toxicity and metabolism studies.

This study is very broad. It involves administration of chemicals known to be toxic, gross observation of their effects on maturing rats on various dietary treatments, and inspection for excretion of a metabolite from two of the compounds. It makes no attempt to study the complex series
of reactions that occur between administration of the compounds and their excretion. Further studies in this area should include investigation of the serum enzymes involved in organophosphate metabolism and the effect of protein deficiency on these as well as relevant liver enzymes. The effect of feeding patterns and the importance of age and development on the enzymes involved in organophosphate metabolism also need to be studied. There is also a need for more work on identification and characterization of the "non-specific esterases" in liver and serum which are important in organophosphate metabolism.

Despite their weaknesses, the studies reported in this paper do lead to some basic conclusions concerning the effect of dietary protein deficiency in rats especially in relation to the toxicity of two widely used organophosphates. Among the conclusions that can be drawn are the following:

1. Rats fed adequate dietary protein utilize their food and their protein more efficiently.

2. Dietary protein intake is of major importance in determining the toxicities of the organophosphates malathion and parathion.

3. Dietary protein intake is also of major importance in determining the toxicities of the oxygen analogues of malathion and parathion, i.e. malaoxon and paraoxon.
4. The major effect of dietary protein intake appears to be on the degradation and not the activation of malathion and parathion.

5. Populations on low protein intake may be in greater danger than their well-fed counterparts when exposed to acute levels of malathion and parathion and possibly other similar compounds.


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THE EFFECTS OF DIFFERENT LEVELS OF DIETARY PROTEIN ON THE TOXICITY AND METABOLISM OF MALATHION AND PARATHION AND THEIR OXYGEN ANALOGUES

Catherine Carico Bloomer

Abstract

The toxicities of two widely used organophosphate insecticides, malathion and parathion, and their oxygen analogues were investigated in rats fed different levels of dietary protein. Male weanling rats were pair-fed either 5 or 20% casein diets for 10 days and control animals received a 20% casein diet ad libitum.

Toxicity studies showed that rats which received 5% casein diets were more susceptible to malathion and malaoxon \( \text{LD}_{50} = 151.1 \text{ mg/kg and 7.75 mg/kg} \) when compared with rats pair-fed 20% casein \( \text{LD}_{50} = 273.6 \text{ mg/kg and 14.33 mg/kg} \) or with rats fed 20% casein ad libitum \( \text{LD}_{50} = 418.6 \text{ mg/kg and 15.23 mg/kg} \). Rats which received 5% casein diets were similarly more susceptible to parathion and paraoxon \( \text{LD}_{50} = 1.24 \text{ mg/kg and 0.385 mg/kg} \) when compared with rats pair-fed 20% casein \( \text{LD}_{50} = 1.65 \text{ mg/kg and 0.537 mg/kg} \) or with rats fed 20% casein ad libitum \( \text{LD}_{50} = 2.04 \text{ mg/kg and 0.666 mg/kg} \). Toxicities of both the inactive parent compounds and their
active analogues were affected by dietary protein intake.

Metabolism studies based on excretion of p-nitrophenol, a metabolite of parathion and paraoxon, were largely inconclusive.

It is concluded from these studies that dietary protein intake is of major importance in determining the toxicities of malathion and parathion and their oxygen analogues and that the major effect of dietary protein is probably on the degradation and not the activation of the parent compounds.

It is further concluded that populations on low protein intake may be in greater danger than their well-fed counterparts when exposed to acute levels of these organophosphates and possibly other similar compounds.