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Ce travail est affectueusement dédié

à mon épouse Pauline.

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ABSTRACT

The first purpose of this work was to determine the teratogenic effects of organophosphates on fish embryos. This appeared justified by the following facts: a) the organophosphates are potential hazards to aquatic life; b) the effects of organophosphates on the embryonic development were unknown; c) embryos offer a living system where biochemical differentiation is incomplete and thus permit to study effects other than cholinesterase inhibition. The second purpose of this work was to make comparative toxicological, histological, histochemical and biochemical studies of larvae treated with organophosphates in order to elucidate some of the drawbacks of the anticholinesterase explanation of the mode of action of the organophosphate insecticides in fishes. Fish embryos and larvae were treated with two water-soluble organophosphates.

Treatment of Brachydanio rerio (zebra fish) embryos with concentrations of Phosphamidon ranging from 100 to 1000 ppm for various lengths of time and during various stages of development showed that Phosphamidon is toxic at a concentration of 600 ppm mainly during the gastrulation and the prehatching periods. Gross teratisms such as very short and crooked tail, underdevelopment of head structures, large cardiac vesicles and yolk sac distortion, resulted only when treatment occurred during the gastrulation period, even if it was of short duration ($5\frac{1}{2}$ hr). These anomalies partly resulted from a kind of exogastrulation. Histological studies revealed a lack of differentiation and of organization of the nervous and muscular structures and the presence of large blood-filled vesicles. Some experiments with Dylox showed

that it was slightly less toxic and that it had a more delayed action than Phosphamidon. Young larvae were more sensitive than embryos: 100% mortality resulted after a 24-hr treatment with 300 ppm of Phosphamidon.

Salvelinus fontinalis (speckled trout) embryos were treated for three or six days during the first half of their development with 30, 100, 200 ppm of Phosphamidon or with 100 and 200 ppm of Dylox. Treatment with 30 ppm of Phosphamidon had a negligible effect, but treatment with 100 or 200 ppm, especially when given during gastrulation, resulted in high percentages of mortality and of gross teratisms and in a decreased hatching. The action of Dylox was more delayed and less severe during the gastrulation period but more important in later stages. Abnormal embryos were characterized by a very small size, aberrant head and brain morphology, poor differentiation of nervous tissue, and absence of muscle in the head. Body muscle differentiation was also greatly impaired but differentiation of the digestive tract was normal. Anomalies resulting from gastrulation treatment were associated with a delay or failure of the closure of the blastopore. The inhibition of phosphorylase is suggested as a possible cause of abnormal gastrulation. At 26 days of development, the activity of the enzyme malic dehydrogenase was not affected by treatment, but the activity of cholinesterase (ChE) was severely inhibited, even by treatment given at a time when no ChE could be detected. Although good recovery of ChE, mainly due to the de novo synthesis, had occurred at day 32, the activity was still below normal. Treatment prior to hatching reduced growth and advanced the onset of hatching, but it did not lower the hatching ability.

Young and old Salmo gairdneri irideus (rainbow trout) larvae were treated for 16 hr with 10 to 100 ppm of Dylox, or for 40 hr with

5 ppm. Young and old Salvelinus fontinalis larvae were treated with 5 to 100 ppm of Phosphamidon or Dylox for 24 hr. Young larvae of both species were more resistant than old larvae, and Dylox was more toxic than Phosphamidon. Treatment produced a marked ChE inhibition that was reflected by their abnormal behaviour patterns. Histochemical staining showed no difference in the activity of lactic and malic dehydrogenases, NADH diaphorase, cytochrome oxidase, ATPase, acid and alkaline phosphatases; but the activity of phosphorylase was considerably decreased, especially in the Dylox-treated larvae. Histological examination revealed pathological changes in the heart, liver, blood cells, pseudogills and muscular tissues. Electron microscope study of the muscle showed in the abnormal fibers an extensive vacuolization associated with a swelling of the longitudinal tubular system, and a clumping of abnormal mitochondria. Mortality of the larvae was more related to the extent of the pathology than to the degree of ChE inhibition. The extensive histological damage and the inhibition of phosphorylase, both reported for the first time in fish, appear as important factors in causing death of the larvae.

ABREGE

Le premier but de ce travail était de déterminer les effets tératogènes des organophosphates sur les embryons de poisson. Les faits suivants justifiaient ce travail: a) les organophosphates présentent des dangers possibles pour la faune aquatique; b) leurs effets sur le développement embryonnaire des poissons étaient encore inconnus; c) la différentiation biochimique incomplète des embryons permet d'étudier les effets autres que l'inhibition de la cholinesterase. Le second but du travail était de faire une étude comparée des points de vue toxicologique, histologique, histochimique et biochimique chez les alevins de poissons afin de résoudre certaines difficultés que présente l'explication généralement acceptée du mode d'action des organophosphates, à savoir l'inhibition de la cholinesterase. Nous avons donc soumis des embryons et des alevins de poissons à divers traitements d'organophosphates solubles dans l'eau.

Chez les embryons de Brachydanio rerio (poisson zébré), des traitements de durée variable et pendant divers stages de développement avec des concentrations de Phosphamidon allant de 100 à 1000 ppm ont démontré qu'une concentration de 600 ppm est toxique pendant la période de la gastrulation et la période précédant l'éclosion. Les traitements pendant la gastrulation, même s'ils étaient de courte durée ($5\frac{1}{2}$ h.), ont produit des malformations tels que raccourcissement considérable et crochissement de la queue, sous-développement de la tête, présence de grosses vesicules cardiaques et distortion du sac vitellin. Ces monstruosités furent causées en partie par une exogastrulation. L'étude histologique a montré que le système nerveux et les fibres musculaires

étaient désorganisés et peu différenciés. Ces tissus contenaient aussi des vésicules remplies de cellules sanguines. Quelques expériences avec le Dylox ont montré que celui-ci était moins toxique et qu'il avait une action moins immédiate que le Phosphamidon. Les alevins montrèrent une susceptibilité beaucoup plus grande que les embryons; une dose de 300 ppm pendant 24 h. fut mortelle.

Nous avons aussi traité des embryons de Salvelinus fontinalis (truite mouchetée) pendant trois ou six jours avec des concentrations de 30, 100 et 200 ppm de Phosphamidon et de 100 et 200 ppm de Dylox. A la dose de 30 ppm, Phosphamidon a eu très peu d'effet. Mais les doses plus fortes, surtout si le traitement coincidait avec la période de gastrulation, causèrent de forts pourcentages de mortalité et de monstruosité et diminuèrent le pourcentage d'éclosion. D'autre part, l'action du Dylox fut moins marquée et moins immédiate pendant la période de la gastrulation; elle fut cependant plus importante pendant les stages ultérieurs. Les embryons anormaux étaient petits. La tête et le cerveau étaient très difformes. Les muscles de la tête étaient absents tandis que ceux du tronc et de la queue étaient peu différenciés. Bon nombre de difformités causées par un traitement durant la période de gastrulation semblaient provenir d'un retard de fermeture ou de la fermeture incomplète du blastopore. L'inhibition de l'enzyme phosphorylase serait possiblement la cause de la gastrulation incomplète. Nous n'avons remarqué aucun changement dans l'activité de la déshydrogénase malique au 26ième jour du développement. D'autre part, la cholinesterase était fortement diminuée, même chez les embryons qui avaient été soumis aux organophosphates à un moment où l'enzyme ne pouvait pas encore être décelé. Au 32ième jour, l'activité de la ChE

avait augmenté considérablement par suite de la synthèse de novo de l'enzyme, mais elle demeurait quand même inférieure à celle des témoins. Un traitement pendant la période précédant l'éclosion a diminué la croissance des embryons mais il n'a pas diminué le taux d'éclosion. Toutefois, les embryons traités ont éclos avant les témoins.

De jeunes alevins et des alevins plus âgés de Salmo gairdneri (truite arc-en-ciel) ont été soumis à des concentrations de Dylox variant de 10 à 100 ppm pendant 16 h. et à une concentration de 5 ppm pendant 40 h. Des alevins de Salvelinus fontinalis (truite mouchetée) de deux âges ont également été soumis à des concentrations de Phosphamidon et de Dylox allant de 5 à 100 ppm pendant 24 h. Les alevins plus jeunes de chaque espèce se montrèrent plus résistants que les alevins plus vieux. D'autre part, le Dylox fut plus toxique que le Phosphamidon. Les divers traitements produisirent une inhibition marquée de la ChE illustrée par le comportement anormal des alevins. Des études histochimiques n'ont montré aucun changement dans l'activité de plusieurs enzymes: les déshydrogénases lactique et malique, la diaphorase NADH, la cytochrome oxidase, l'ATPase, les phosphatases acide et alcaline; ils ont cependant révélé une forte baisse de l'activité de la phosphorylase, surtout chez les alevins traités au Dylox. Des coupes histologiques montrèrent que le coeur, le foie, les erythrocytes, les branchies, les pseudobranchies et en particuliers les muscles avaient subi des changements pathologiques importants. Le microscope électronique a de plus montré dans les fibres musculaires la présence de groupes compacts de mitochondries anormaux ainsi que la présence d'un nombre considérable de grosses vacuoles provenant d'un renflement du système de tubules longitudinales. La mortalité des alevins était davantage en relation

avec l'étendue des dommages tissulaires qu'avec l'inhibition de la ChE. Les dommages tissulaires et l'inhibition de la phosphorylase, tous deux inconnus jusqu'à date chez les poissons, apparaissent donc comme des facteurs importants dans les causes de la mort des alevins.

CHAPTER ONE

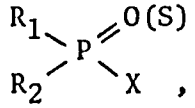
INTRODUCTION

I. HISTORY OF THE DEVELOPMENT OF ORGANOPHOSPHATE.

Although most of the work for the synthesis of organophosphates has been done since the beginning of the Twentieth Century, the first esterification of alcohols and phosphoric acids was achieved a hundred years earlier by Lassaigne in 1820 (O'Brien, 1960). In 1834, another French organic chemist, Philippe de Clermont, synthesized TEPP (tetraethylpyrophosphate). He tasted the compound but apparently did not suffer any discomfort since the toxicity and mode of action were discovered only several years later (Holmstedt, 1963). During the last part of the Nineteenth Century and the first part of the Twentieth Century, several new organophosphates were synthesized in Russia, Germany, England and France.

Interested in developing new types of organic insecticides, W. Lange started to work on the organophosphorus compounds and in 1932, he synthesized compounds containing the P-F bond such as DFP (diisopropyl phosphorofluoridate). During his work, Lange and his collaborators suffered discomfort and noticed the toxic effect of the vapors on themselves. This was probably the first hint into the recognition of the poisonous properties of organophosphates since very intensive work on these compounds as potential chemical warfare agents was then conducted during the last war in Germany and in England (Holmstedt, 1963).

In Germany, the group, headed by Schrader, made several synthesis of organophosphates of the general formula:



, where R_1 and R_2 are usually alkyl (CH_3-), alkoxy (CH_3O-), alkyl amido ($(CH_3)_2N$-) radicals, and where X is usually a halogen (F), a CH, or an alkyl,

alkoxy or phenoxy. Among the organophosphates synthesized were DFP, TEPP, OMPA (octamethyl pyrophosphortetramide), Parathion and Sarin. In 1944, this group had synthesized not less than 2,000 organophosphates. They were not only interested in the synthesis of new compounds, but also in their biological activity. They realized the insecticidal property of several organophosphates and by the end of the war, compounds such as Dimefox, Schradan and Parathion were patented as contact insecticides.

Eberhard Gross is recognized as the first scientist to discover the anticholinesterase property of the organophosphate (TEPP in 1939). One year later, H. Gremels discovered that Sarin behaved similarly. In 1941, Adrian, Feldberg and Kilby compared DFP to eserine and found that DFP had an anticholinesterase action. In 1942, Dixon, Machworth and Kilby found that DFP was a strong inhibitor of horse serum cholinesterase (Holmstedt, 1963). After the war, all the knowledge about organophosphates acquired by the German scientists became known to the allies. This constituted a basis for an enormous amount of scientific research in England and the United States to further study the chemical, biochemical and biological properties of the organophosphates. R. L. Metcalf and J. E. Casida were two of the most active and productive workers in this field. The cholinesterase inhibitors were also extensively used as a tool for the study of the biological function and of the biochemical structure and mode of action of the cholinesterase enzymes.

II. ADVANTAGES OF ORGANOPHOSPHATES AS INSECTICIDES.

Because of their many advantages, organophosphates are gradually

replacing the chlorinated hydrocarbon insecticides such as DDT which were considered to be the universal and miracle insecticides. The acquired resistance of several species of insects to compounds such as DDT have suggested the use of other insecticides (O'Brien, 1960, 1967).

Some organophosphates have a higher toxicity than DDT to mammals (Anonymous, 1967), however, most of the organophosphates used widely as insecticides do have a lower mammalian toxicity (O'Brien, 1960; Casida, 1964). The lower toxicity to several species of fish (Kerswill and Edwards, 1967; Nizhegorodov et al., 1967) is probably a more important advantage since insecticides are easily drained by rain waters into rivers and become a serious hazard to aquatic organisms. Probably the most important advantage of organophosphate insecticides over chlorinated insecticides is the fact that organophosphates are much less stable compounds (O'Brien, 1967). Organophosphates undergo self-degradation rather rapidly by hydrolysis or by isomerization (O'Brien, 1960, 1967) with the immediate loss of their actual or potential biological activity (Heath, 1961). The building up of dangerous quantities of insecticides in soil, water and food chain, coming from repeated applications have become a major concern of people and governments. The rapid self-degradation of organophosphates and the great ability of mammals to rapidly metabolize these compounds make them less hazardous than chlorinated hydrocarbons, which are much more stable, less easily metabolized and have the tendency to accumulate in the fats of insects, mammals and fishes (O'Brien, 1967).

III. BIOLOGICAL ACTION OF ORGANOPHOSPHATES.

A. Symptoms and causes of death.

The precise way in which symptoms occur following poisoning of several species by different organophosphates has been described by several authors (O'Brien, 1960, and Heath, 1961, give several references on this subject). Two types of effects result from organophosphate poisoning: the muscarinic and the nicotinic effects. The first type includes salivation, lachrymation, defecation, urination, contraction of the pupil, brachycardia and fall in blood pressure. These are all symptoms of the stimulation of the parasympathetic systems. The nicotinic effect involves the neuromuscular junction: muscle twitching first appears, followed eventually by paralysis. Convulsions are also common. Death of the organism would result from asphyxia which can result from one or a combination of the following factors: bronchoconstriction, lowered blood pressure, neuromuscular block of the respiratory muscle or failure of the respiratory center of the brain. The "killing factor" varies with the species and the compounds. Thus for instance, rabbits, poisoned by Paraoxon, die from paralysis of the respiratory muscles while cats die from bronchoconstriction when poisoned by DFP and usually from failure of the respiratory center when poisoned with TEPP (Heath, 1961).

Abnormal functioning of the nervous system would be due to the blockade produced by the accumulation of the acetylcholine resulting from the inhibition of cholinesterases (ChE). Acetylcholine is known to rise two or three fold during acetylcholinesterase (AChE) poisoning (O'Brien, 1967).

In non-mammalian vertebrates, the symptomology is less well-

known. Mulla et al. (1967) reported that carp and largemouth bass fingerlings, poisoned by Parathion, stiffened and became paralyzed in a slow non-violent way. Hoff and Westman (1965) observed the following sequence of symptoms in bluegills, pumpkin-seed fish and largemouth bass fingerlings poisoned by a mixture of Dibrom-Malathion: darkening of the skin, extreme forward positioning of the pectoral and pelvic fins, high excitability and body tremors followed by loss of equilibrium and finally, non-violent death. The time of death after the loss of equilibrium was dependent upon the temperature.

Organophosphate poisoning and death have been associated with ChE inhibition in fish (Weiss, 1958, 1959, 1961, 1964, 1965; Williams and Sova, 1966).

B. Factors affecting toxicity of organophosphates.

The toxicity of organophosphates depends upon several factors. The chemical structure of the compound is an important factor, since it determines in some way the ability of the organophosphate to phosphorylate the enzyme AChE. Thus, for instance, esters of ethyl alcohol are more toxic than esters of methyl alcohol (Schrader, 1965). The nature of the leaving group X is also important (Stevanovic, 1965).

The toxicity of one compound varies in different species and important differences have been attributed to the structure of the active site of the ChE enzyme which is phosphorylated. The structure of the active site of ChE (distance between the anionic and esteratic sites) would be slightly different in insects than in mammals and would thus be the reason why organophosphates are more toxic to insects than to mammals (Hollingworth et al., 1967). The high resistance of

amphibians to organophosphates, compared to the resistance of mammals, is also explained on these grounds (Edery and Schatzberg-Porath, 1960).

Another important factor influencing the toxicity is the ability of the organism to degrade the organophosphate and the rapidity with which this degradation is carried out. Thus, the low toxicity of Dipterex in mammals is explained by its rapid detoxification by liver enzymes (Dubois and Cotter, 1955). In this regard, the mode of administration of a compound to an animal has a large influence: the toxicity will be largely decreased if the compound goes through the liver before reaching the central nervous system, as shown by the experiments of Gaines et al. (1966).

The toxicity of a compound is enhanced when the process of degradation or detoxification is interfered with. The synergistic action of one organophosphate on a second insecticide would be explained by this mechanism (O'Brien, 1967; Murphy and Dubois, 1958). On the other hand, any factor such as phenobarbital or organochlorine, which directly or indirectly stimulates the activity of detoxifying enzymes decreases the toxicity (Menzer and Best, 1968; Triolo and Coon, 1966).

The ability to detoxify an organophosphate could be a key factor in the toxicity of these compounds to fish. Although it is extremely difficult to compare the mammalian toxicity with the piscine toxicity since the modes of administration differ so much, it is generally thought that organophosphates are more toxic to fish than to mammal. Several authors have suggested that in fish there is very little drug metabolism (Brodie and Maickel, 1962; Boni, 1965; Terriere, 1968; Salseduc, 1968) but Lotliker et al. (1967) have shown that this statement is not true for all drugs. According to Smith et al. (1966) the

organophosphate Dursban is rapidly metabolized by fish, but several authors (Potter and O'Brien, 1964; Murphy, 1966a; Hitchcock and Murphy, 1967) believed that organophosphates are less rapidly metabolized in aquatic than in terrestrial vertebrates. This reduced metabolism would be due to the fact that fish may be deficient in liver esterases (Terriere, 1968).

C. Histological effects.

The most important histological damage produced by organophosphate poisoning is the demyelination associated with axon damage in the medulla, spinal cord and sciatic nerve (Davies, 1963). Demyelination is produced by only a few compounds and in a few species such as man, chick, calf, lamb and rabbit. The similarity of this syndrome to the vitamin E deficiency syndrome had suggested that demyelination could be caused by interference of the organophosphates with vitamin E metabolism (O'Brien, 1967). Dystrophic and necrobiotic changes in liver, kidneys, myocardium and endocrine glands produced by lethal doses of mercaptophos and disistone in rats and cats have been reported by Kagan and Makovskaya (1960). These were not considered as specific for organophosphates, but were believed to result from disturbances in acetylcholinesterase metabolism or hypoxia. Vacuolization of the acinar cells of salivary glands and pancreas were attributed to excess acetylcholine (Denz, 1951). Reduction of red and white blood cells accompanied by a change in the proportions of the different types of white blood cells was observed in the frog following treatments with several organophosphates. Excessive shedding of the skin was also noted (Kaplan and Glaczenski, 1965). Both DDT and Parathion induced dystrophic changes in abdominal muscles of the

insect Drosicha stebbingi: loss of striations, appearance of vacuoles between the myofibrils, breakage of the sarcolemma and clumping of chromatin (Ashraf, 1964). Similar changes were also observed in other insects poisoned by pyrethroid insecticides.

Two months of sublethal doses of Dipterex to rats produced a slower growth rate, reduction of the number of red blood cells and changes in the internal organs (Kirichek et al., 1964). Khera et al. (1966) have shown, in ducks, that EPN produced foot deformity which was associated with muscle fiber fragmentation and proliferation and central displacement of the nuclei. Legs and beak deformities were observed in chick embryos after injection of Malathion (Greenberg and LaHam, 1969) and of other ChE inhibitors such as eserine and pilocarpine (Landauer, 1954).

Buttler (1963) reported that chronic exposure (for six days) of Leiostomus xanthurus to sublethal concentration of several organophosphates, among which were DDVP, Dipterex and Phosphamidon, produced a thickening of the gill lamellae. Except for this case, no report of histological lesions produced by organophosphates in fish has been found in the literature. It is true, however, that these have been very seldom looked for in fish. Most studies were aimed at establishing the lethal concentrations and the ChE levels.

IV. BIOCHEMICAL EFFECTS.

A. Inhibition of cholinesterase.

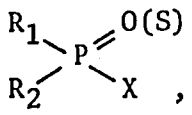
Since Gross and Gremels discovered in 1939 and 1940 that organophosphates were powerful inhibitors of the cholinesterases, a very

imposing amount of work has been done to characterize the in vivo and the in vitro inhibition of both specific (acetyl-) and non-specific (pseudo-) cholinesterases; the kinetics and the way by which this inhibition occurs has also been worked out, so that the relationships between the inhibitor and the enzyme is probably one of the best known in enzymology.

The active center of the enzyme acetylcholinesterase contains two sites: a) the anionic site (ω-carboxyl groups of glutamic acid) which holds the substrate (containing a cationic group) by electrostatic forces; b) the esteratic site containing the amino acid serine with its OH group which catalyzes the hydrolysis of the substrate. Activation of serine by other amino acid groups, such as the imidazole group of histidine, would be necessary for the catalytic function of serine to take place.

The hydrolysis of acetylcholine involves the nucleophilic displacement of the choline part of the substrate and the acetylation of the serine, followed by the deacetylation of the serine and formation of acetic acid.

In the general chemical structure of organophosphorus compounds:



R_1 and R_2 are considered as basic groups while X is the acidic group. Thus P-X forms an acid anhydride bond.

During the inhibition of AChE by an organophosphate, two processes take place. The organophosphate first becomes attached to the enzyme. Thus, the chemical configuration of the inhibitor is important. The introduction of a cationic group in the organophosphate will

increase the affinity of the inhibitor for the enzyme (because of its anionic site). Secondly, the esteratic site becomes phosphorylated. The OH group of serine displaces the X group of the organophosphate. The P-X bond is thus broken: X is liberated as an anion(which may later react to give a neutral compound) and P becomes attached to the serine, producing the phosphorylation of the enzyme (Engelhard, 1967). The inhibition is irreversible: the complex inhibitor-enzyme is not separated by dialysis and when separation of the complex occurs, the inhibitor is chemically different (it has been hydrolyzed) and has lost its inhibiting capacity.

The phosphorylating or inhibiting ability of a compound is influenced by its structure: both the acidic (X) and basic (R_1 and R_2) groups have a role. The influence of the acidic group (X) depends mainly on its readiness to accept electrons or, in other words, on the strength of the acid HX: the more acidic the X, the faster the rate (Heath, 1961).

The influence of the R_1 and R_2 groups is somewhat more complex. Alkyl groups have a slight tendency to donate electrons, thus making them less electrophilic and less reactive. According to the electronic theory, the rate of alkaline hydrolysis will decrease with lengthening of the chains. When the rates are measured for a series of compounds, this is found to be true. The same thing should hold also, according to the electronic theory, for the rates of phosphorylation (Stevanovic, 1965) but it is not the case. For the inhibition of AChE, the rates vary slightly with the source of the enzyme, but generally the rates decrease in the following order: $(EtO)_2 > (MeO)_2 \simeq (PrO)_2$. The discrepancy with the theoretical order $(MeO)_2 > (EtO)_2 > (PrO)_2$ is

explained in terms of "fit" of the inhibitor on the enzyme surface (Heath, 1961; Ooms, 1961). Babers and Mitlin (1955), however, have found that the toxicity of homologs and derivatives of Dipterex was decreasing with the lengthening of the R_1 and R_2 chains.

B. Anticholinesterase activity of Dylox, DDVP[1] and Phosphamidon.

Hassan et al. (1965a) have shown that Dipterex produces a progressive and irreversible inhibition and that this inhibition is competitive: Dipterex attaches itself to the binding groups of the active center; Braid and Nix (1969) have shown for DDVP and Phosphamidon that the inhibition involves a reversible complex formation which is followed by the phosphorylation of the enzyme. As previously stated, the phosphorylating ability varies with the compound. Table 1.1 lists the pI_{50} (inverse logarithm of the concentration inhibiting 50% of the enzyme) for Dipterex, DDVP and Phosphamidon.

TABLE 1.1 Inhibition (pI_{50}) of ChE by Dipterex, DDVP and Phosphamidon.

ORGANOPHOSPHATE	ENZYME SOURCE	pI_{50}	REFERENCE
Dipterex	rat brain	5.7	Holmstedt, 1963.
	rat brain	5.2	Hassan et al., 1965a.
	fly brain	7.8	Metcalf et al., 1959.
DDVP	bovine RBC	5.66	Durham et al., 1957.
	fly brain	8.2	Metcalf et al., 1959.
Phosphamidon	rat brain	6.7	calculated from Anonymous, 1967.

[1] 0,0-Dimethyl 2,2-dichlorovinyl phosphate.

As seen from the table, Phosphamidon is a stronger inhibitor than both DDVP and Dipterex. Since all three compounds have identical basic groups (MeO-) the greater reactivity would be due to the acidic group of Phosphamidon.

The rate of inhibition of mammalian ChE is similar for Dipterex and DDVP. Dipterex has a peculiar structure in that it does not have an acid anhydride bond. Because the rate of inhibition by Dipterex is pH dependent while the rate of inhibition by DDVP is not, Metcalf et al. (1959) believed that the anti-ChE activity of Dipterex would be due to the DDVP formed by dehydrochlorination. Holmstedt (1963), however, in discussing the structure and activity affirmed that "a cleavage of the carbon-phosphorus bond occurs instead of a dehydrochlorination". According to this view, the anti-ChE activity would then be due to the intact Dipterex molecule.

With both DDVP and Dipterex, there is at least a hundred-fold difference in the rate of inhibition between the inhibition of mammalian ChE and fly ChE. This could be explained by the observations of Van Asperen and Dekhuijzen (1958) that the inhibition of mouse brain ChE by DDVP is slowly reversible while it is irreversible in the fly, and that DDVP has a much greater affinity for fly ChE than for mouse ChE. Furthermore, they have shown that the effective concentration of DDVP is considerably reduced by a binding to some proteinous material of the brain homogenate. Boyer (1967) has also shown that the free concentration of vinyl phosphate insecticide was reduced by its binding to plasma proteins. Organophosphates are irreversible inhibitors of ChE since the activity of the enzyme does not readily return with dialysis. This, however, does not mean that a phosphorylated enzyme is an extremely

stable compound. Phosphorylated enzymes are phosphate esters which can be hydrolyzed by nucleophilic reagents such as water; the activity of the enzyme is then restored and this phenomenon is called reactivation.

The rates of reactivation depend upon the source of the enzyme (species) and upon the basic groups on the phosphorus, but do not depend upon the acidic group of the original inhibitor. The rates of reactivation decrease with increasing length of the chain of the R_1 and R_2. Dimethyl phosphorylated enzymes are reactivated much more rapidly than others: 25% of the activity returns after one hour and twenty minutes when the source of the enzyme is coming from rat brain. Enzyme phosphorylated by Dylox or Phosphamidon should then be reactivated at the same rate.

C. Inhibition of other enzymes.

Soon after the discovery of the anticholinesterase action of organophosphates, it was discovered that organophosphates could also inhibit other enzymes both in vitro and in vivo.

Heath (1961) has reviewed this subject and gave the pI_{50} of the most active organophosphate for at least fourteen different enzymes. Among these are trypsin and chymotrypsin, proteinase, acid phosphatase, pyrophosphatase, succinoxidase, dehydrogenases, lipases and aliesterases. Villeneuve et al. (1969) have also reported the inhibition of beef liver aliesterases by several organophosphates; Welch et al. (1967) have also noted the inhibition of rat steroid hydroxylases. The lipases, aliesterases and beef liver hydrolases excepted, the concentration necessary for a 50% inhibition is usually at least one hundred times stronger than that necessary to produce a similar inhibition of specific or non-

specific cholinesterase. Although rather high concentrations of organophosphates (10^{-3} M) were necessary to inhibit dehydrogenases, Michaelis et al. (1949) claimed that their ability to alter physiological behaviour of nerve and brain tissue follows their action on respiratory enzymes more closely than their action on ChE.

Fewer enzymes have been found to be inhibited in vivo. In rats, some aliesterases are inhibited by TOCP and DFP (Heath, 1961), EPN and Delnav (Dubois et al., 1968). In these cases and in others (Murphy and Cheever, 1968), the aliesterases are either more inhibited than ChE or are inhibited by doses which do not have any effect on ChE. Van Asperen (1958) has shown that in the fly poisoned by DDVP, at the time of knockdown, 83% of the aliesterase activity was inhibited, while the inhibition of ChE was only half as much inhibited. From these results, the author concluded that aliesterase inhibition was the determining factor. Rucinski et al. (1966) have observed that doses of Parathion that can be lethal to rats produced a prolonged and significant decrease of alkaline phosphatase in survivors. But Murphy (1966b) has observed the reverse situation after acute poisoning with Dipterex and Malathion: alkaline phosphatase had an increased activity. Tyrosine α-ketoglutarate transaminase was also increased. It seems that these effects would be mediated through the effect of excess acetylcholine on the adrenals. Sasinovich (1967) has also noted, after DDVP treatment, increased activity of glutamino-pyruvic and glutamino-oxalacetic transaminase; disturbance of the carbohydrate metabolism in the liver was also apparent. Much more work is needed before the significance of these results can be estimated and correlated with the toxic action of these compounds.

Some work has been done to find the influence of the structure of organophosphates on inhibition of enzymes other than ChE. Ooms et al. (1966b) and Becker (1967) have shown that the rate of inhibition of trypsin, chymotrypsin and of ChE was influenced by the strength of the P-X bond; the rates of inhibition, however, are not parallel to the reactivity of the basic groups R_1 and R_2. The inhibition of citrus acetyl esterase is influenced in a way which parallels the rates of alkaline hydrolysis (Ooms et al., 1966a). The basic groups have little influence on the inhibition of horse liver aliesterase, while the strength of the P-X bond has more influence, although the correlation is not perfect (Ooms and Breebart-Hansen, 1965). The inhibition of milk xanthine dehydrase produced by phosphoric and thiophosphoric acid esters is due only to the absorption of the compound on the enzyme and the inhibition is stronger when the basic groups are shorter (Wildbrett et al., 1967).

The only conclusion that can actually be drawn, is that the structure of the acid or basic groups have an influence which is different with each enzyme.

D. Organophosphates as killing agents.

In trying to find the cause of death produced by a poison, one is normally inclined to look at the molecular level, and tries to find which vital biochemical reaction is disrupted by the presence of the poison. There is very little doubt that the death produced by organophosphates is a "chemical death", but the question can be asked "what is the target molecule: cholinesterase or any other enzyme?".

The view accepted by most authors, among which are Heath

(1961), Davies (1963), O'Brien (1967), is that organophosphates kill animals by inhibiting cholinesterase with the consequent disruption of nervous activity leading to asphyxia. The main arguments brought in favour of the anticholinesterase theory are the following: a) acetylcholinesterase is a vital enzyme; its severe inhibition is usually associated with death, and AChE is always inhibited by toxic doses of organophosphates; b) in general, very good inhibitors are very toxic and very bad inhibitors are not: there is also a general correlation between the AChE I_{50}'s and the LD_{50}'s of several compounds; c) AChE is the only enzyme which is constantly inhibited and organophosphates are usually poor inhibitors of other vital enzymes which have been studied; d) animals can be protected by reversible inhibitors; e) recovery is usually associated with some reactivation of AChE and compounds which reactivate phosphorylated AChE are therapeutically effective.

These arguments seem very strong, but they do not "explain why the level of AChE is not reduced to the same extent by lethal doses of different compounds in the same species" (Heath, 1961). If the level of AChE was critical, one would expect that doses would be lethal when they lower the AChE to a certain level. But such is not the case. Some compounds are lethal at doses which reduce the AChE to 60% of the normal while others are lethal only at doses reducing the AChE to 20%. Chadwick (1963), a leading scientist in this field, believed that, at least in invertebrates, "the toxicity is rarely exercised through what is ordinarily understood as a cholinergic mechanism". The suggestion of Van Asperen (1958) that toxicity could be due to aliesterase inhibition has already been mentioned. More recently, Morallo and Sherman (1967) have studied the effect of four organophosphates on four species of fly.

Their results confirm Chadwick's idea since they have found that the most toxic compounds are not always the most effective ChE inhibitors and that toxicity may or may not be related to either in vivo or in vitro inhibition. It could be that aliesterases are important enzymes. Dubois et al. (1968) do not reject the suggestion put forward by Myers, that aliesterase may have a role in protein metabolism since they have the ability to hydrolyze amino acid esters and amides. Inhibition of aliesterase as the cause of death is an interesting hypothesis but until the physiological function of aliesterase enzymes in normal metabolism will be identified, the suggestion of Van Asperen probably has little chance to be accepted.

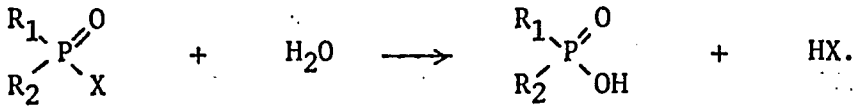
V. METABOLISM OF ORGANOPHOSPHATES.

A. General metabolism.

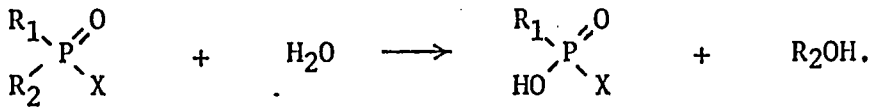
A considerable amount of work has been done on the metabolism of organophosphates. Excellent reviews of this work can be found in O'Brien (1960, 1967), Heath (1961) and Mounter (1963). Only the main important points will be summarized here.

The principal reactions occurring in living organisms are reaction of activation and reaction of degradation. Activation is the mechanism by which a weak inhibitor of ChE is converted to a stronger inhibitor of ChE. The major activative reactions are conversion of P=S to P=O of thiophosphates, hydroxylation of phosphoro amides, thioether oxidation and cyclization. In fishes (Murphy, 1966a) and in mammals (O'Brien, 1967; Terriere, 1968), these reactions are believed to be catalyzed by the liver microsomes and by the whole body microsomes of

insects (O'Brien, 1967). By activation, the anticholinesterase property of a compound such as Parathion and Malathion, can be increased up to ten thousand-fold. By degradation, the organophosphates lose their ability to inhibit ChE. Degradation occurs primarily by hydrolysis of the leaving group (acid anhydride bond) or of one or two of the basic groups. The hydrolysis of the acid anhydride bonds yields two acids:



The anionic group attached to the phosphorus atoms reduces its positivity with the consequent loss of its anticholinesterase action. The hydrolysis is catalyzed by phosphatases in mammals, insects and plants (O'Brien, 1967). In mammals the enzyme is present in several tissues. Thus for instance, the phosphatase hydrolyzing DFP is abundant in liver and found in a decreasing order in the following organs: spleen, serum, kidney, heart and muscle (Aldridge, 1953a, 1953b). Most of the hydrolysis products of organophosphates are polar compounds and can be excreted by the kidneys. Although less common and less important as a detoxification mechanism, hydrolysis of the alkyl group can also occur. Dealkylation would be catalyzed by phosphatases in the following manner:



In mammals, the enzyme is found mainly in liver.

B. Metabolism of Phosphamidon, Dipterex and DDVP.

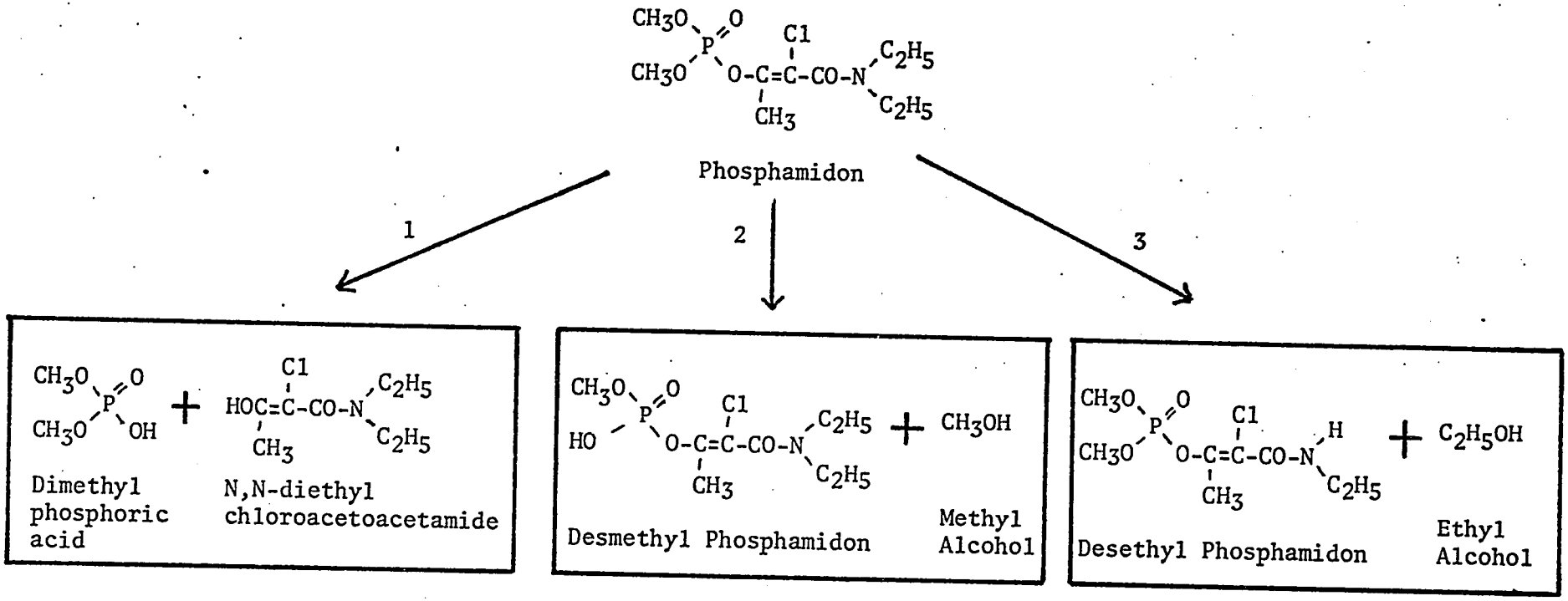
i - Phosphamidon.

The main metabolic route of Phosphamidon is the hydrolysis of the leaving group (X) with the consequent production of dimethyl

phosphoric acid and N,N-diethyl chloroacetoacetamide (table 1.2, reaction 1). Bull et al. (1967) reported that after 24 hr, 60% of the Phosphamidon given to rats was recovered in urine, mainly as dimethyl phosphate. Only traces of the toxic compounds were found. In rats and goats, Clemons and Menzer (1968) have found that 90% of the oral dose is recovered in urine as non-toxic polar metabolites. The N,N-diethyl chloroacetoacetamide is rapidly broken down into smaller components which are also found in urine.

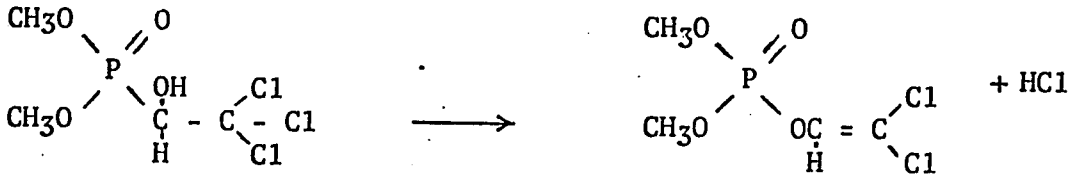
The second type of reaction of Phosphamidon involves the dealkylation of one of the methyl groups, resulting in the formation of desmethyl Phosphamidon (table 1.2, reaction 2). Only small concentrations of desmethyl Phosphamidon is found in the urine of rats (Bull et al., 1967). Metabolism can also involve one of the ethyl group of the leaving group; oxidative N-dealkylation would occur as suggested for Phosphamidon and similar compounds by Sun and Johnson (1965) and by Bull et al. (1967) with the formation of desethyl Phosphamidon (table 1.2, reaction 3). Only traces of this compound, which is as toxic as Phosphamidon (Clemons and Menzer, 1968) are found in urine. Desethyl Phosphamidon is further broken down by the hydrolysis of the acid anhydride bond resulting in the formation of dimethyl phosphoric acid and N-ethyl chloroacetoacetamide. These three metabolic pathways are also present in plants (Anliker et al., 1961; Anonymous, 1967; Bull et al., 1967) and their rate is affected both by light and temperature (Menzer and Ditman, 1963).

TABLE 1.2 Metabolic pathways of Phosphamidon.



ii - Dipterex.

Dipterex is a phosphonate and differs from phosphates by the fact that it does not have an acid anhydride bond between the phosphorus atom and the leaving group. Several workers have found that it is easily converted in slightly alkaline solution to DDVP (Lorenz et al., 1955; Mattson et al., 1955; Barthel et al., 1955; Van Die, 1957; Miyamato, 1959).



Dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate

Dipterex

Dimethyl 2,2-dichlorovinyl phosphate

DDVP

The question can be asked whether, when metabolized, Dipterex is first converted to DDVP. The metabolism of Dipterex has been studied in the rat by Dubois and Cotter (1955) and by Hassan et al. (1965a, 1965b, 1965d, 1965e), in the house fly by Babers and Mitlin (1955) and by Metcalf et al. (1959), in the cow by Robbins et al. (1956), in the dog by Arthur and Casida (1957), in the cotton leaf worm by Zayed and Hassan (1965) and Hassan et al. (1965c) and in the microorganisms by Hassan et al. (1965e).

In all mammals studied, no DDVP is found and Dipterex is very rapidly metabolized. Excretion of the metabolites occurs mainly in the urine and no Dipterex or very little is excreted unchanged in the urine or the feces. The main route of metabolism varies with the species. In the dog, hydrolysis of the phosphonate bond would occur and the main metabolites are dimethyl phosphate, dimethyl phosphite and

trichloroethyl glucoronide (which comes from the reaction of trichloroethanol, liberated by hydrolysis, with glucoronic acid). In the cow, about 17% of the original Dipterex is excreted as dimethyl phosphate while 76% is excreted as an unknown metabolite (possibly resulting from dealkylation). In rats, hydrolysis of the phosphonate bond and excretion of metabolites similar to the ones found in dog, would be as important as dealkylation. Part of the methanol resulting from dealkylation would be excreted eventually as carbon dioxide while the desmethyl-Dipterex can have its phosphonate bond hydrolyzed like Dipterex.

In the cotton leaf worm, hydrolysis of the O-methyl ester bond is much more important than the hydrolysis of the phosphonate bond. Demethylation is also the main pathway in microorganisms.

In the house fly however, the metabolism would be quite different and would occur after transformation of Dipterex to DDVP (Metcalf et al., 1959).

iii - DDVP.

Since Dipterex is rapidly converted to DDVP and could be of importance in the metabolism of Dipterex, its metabolism will be summarized. In the house fly, 50% of the compound is degraded within 4 hr (Arthur and Casida, 1957). In rats, Gaines et al. (1966) have shown that the liver rapidly metabolizes DDVP, since the compound injected in the hepatic artery is at least three times less toxic than when it is injected in the femoral artery. DDVP is degraded by phosphatases found in several organs, with decreasing activity in the following order: liver, kidney, spleen, serum (Hodgson and Casida, 1962). The degradation in liver is due to three enzymes: one is found in mitochondria, is stimulated by Ca++ and cleaves only the P-O-methyl bond

(dealkylation). A second enzyme also cleaves the same bond but is a soluble enzyme and is stimulated by Mn++ ions. The third enzyme is also soluble but is not influenced by either Ca++ or Mn++ : it cleaves the P-O-vinyl bond (hydrolysis of the acid anhydride bond). In rats and rabbits (Hodgson and Casida, 1962; Akintonwa and Hutson, 1967) and in cows and goats (Casida et al., 1962), in fishes (Hogan and Knowles, 1968) as well as in insects (Metcalf et al., 1959), the main pathway is the cleavage of the acid anhydride bond and dealkylation would play a very minor role.

VI. ORGANOPHOSPHATES AND FISHES.

A. Toxicity of organophosphates.

Compared to the number of studies performed on mammals, the number on fishes is very small. Partial review of the literature can be found in Edson and Sanderson (1965), Tarzwell (1965), Katz et al. (1966), Johnson (1968). Reviewing the literature about toxicity of organophosphates is not a simple task for several reasons. First, the species studied varies widely with different workers. The kind of pesticides assayed is also quite different. Finally, there is also a wide variation in the way the results are given, some using as a parameter the 50% tolerance limit for 24 hr, others for 36, others for 48 or 96 hr, while in some reports, 90% or 100% tolerance limits are used. The main toxicity study can be found in the literature cited in this section and in the following authors: Henderson and Pickering (1957), Gagnon (1958), Keenlyside (1958), Katz (1961), Srivastava and Konar (1965a, b), Anonymous (1966), Marking (1966), Pickering and Henderson (1966), Von Windeguth

and Patterson (1966), Sreenivasan and Swaminathan (1967).

Although fishes have different sensitivities to different organophosphates, minnows and goldfishes appear to be generally more resistant than guppies, bluegills, salmons, trouts and bass. Salmonidae are quite sensitive to organophosphates.

There is a rather wide variation in the toxicity of organophosphates. Guthion is much more toxic than compounds such as Paraoxon, Parathion, TEPP, Chlorothion, Systox, Malathion, Dipterex and Disyston. Guthion is even more toxic than several organochlorines, such as DDT and Dieldrin. Most organophosphates are at least 50 times less toxic than organochlorines and several are 200 or even 1,000 times less toxic. Both Phosphamidon and Dylox are among the least toxic organophosphates to fish. From studies done by the Fisheries Research Board of Canada (Anonymous, 1962), Pickering et al. (1962), Buttler (1963), Srivastava and Konar (1965a, 1965b, 1966), Schouwenburg and Jackson (1966), CIBA Limited (Anonymous, 1967) and Willford (1967), it appears that Phosphamidon is less toxic than Dylox. DDVP is slightly more toxic than Dylox. The fact that the toxicity of Dylox increases with the hardness of the water (Pickering et al., 1962) lends support to the idea that the toxic effect of Dylox would be due to its transformation into DDVP. The results found in the literature do not permit strict comparison of the sensitivity of Salmonidae to these three compounds with the sensitivity of other species; there is an indication, however, that they are more sensitive than other species.

Organochlorines like DDT or Heptachlor are known to produce mortality among trout fry coming from treated parents (Allison et al., 1963) and to retard growth in bluegills (Andrews et al., 1966). Both

growth and reproduction were not affected by periodic or continuous exposure to concentrations of Dylox or Malathion equal to or smaller (1/45) than the 96 hr LC_{50} (Anonymous, 1965; Clemens et al., 1966; Holland and Lowe, 1966). But Matsue et al. (1957) reported that a concentration of Parathion 1/30 of the 48 hr LC_{50} was harmful to fishes and reduced their growth.

Although very few studies have been done, it appears that fish can acquire resistance to organophosphate insecticide (Ferguson et al., 1966; Ferguson, 1967). This resistance however, is not as important as the resistance acquired for organochlorine pesticides (Ferguson and Bingham, 1966a, 1966b).

B. Organophosphates and cholinesterases in fish.

As expected from mammalian studies, ChE is affected in fishes if they are exposed to organophosphates.

Both blood (Hayama and Kuwabara, 1962) and brain ChE are affected (Weiss, 1958). Weiss (1958, 1959) has proposed that fish brain ChE measurements could be used as an indication of organophosphate water pollution. He claims (1965) that concentrations of Guthion and Parathion as low as 0.1 ppb (part per billion) could be detected by this technique. Holland and Lowe (1966) have shown that exposure to low concentration of Malathion (10 ppb) for six months apparently did not result in any ill-effect although there was a decrease of 30% in brain ChE. Field studies performed by Holland et al. (1967) have shown that the activity of fish brain ChE from waters which could possibly be polluted was approximately 25% less than activity found in fishes from unpolluted areas. Williams and Sova (1966) found that distressed fishes from

polluted areas had a 50% reduction of brain ChE activity. According to Weiss (1958) death of fish exposed to organophosphates occurs when ChE has been reduced to 30-60% of the normal activity. The same author (1959) has also shown that the degree of the ChE inhibition is dependent on the concentration of the organophosphate, on the extent of exposure, on the nature of the compound and on the species of fish. Regeneration of the ChE depends also upon the nature of the compound and upon the species tested, but also upon the extent of the initial inhibition. Complete regeneration of ChE activity can take as long as 40 days in some cases. Weiss has also shown that the rapidity with which the ChE of one species is inhibited with different compounds, is parallel to the final degree of inhibition, that is, the compound which produces the fastest observable ChE inhibition also produces, at the end of a given period of time, the greatest ChE inhibition. Weiss (1961) has also demonstrated that the pH, especially for compounds such as DDVP and Dipterex, has an influence on the ChE inhibition: ChE is inhibited at least 10% more at pH 8.0 than at pH 6.5. Although Weiss, in any of his papers, never clearly stated that in fish the inhibition of ChE is the cause of death, it seems that he believed so since he said (1961): "The development of the organic phosphorus insecticides has had an unusual history in that the mode of action had an early elucidation and has been an extremely fruitful area of investigation for toxicological studies. The toxic action in insects results from inactivation of the enzyme cholinesterase. This inactivation has been shown to occur in the brain of fish following exposure to the organic phosphorus compounds."

When one closely examines the results of some of his experiments and those of other workers, one discovers that several results not

only do not support the idea that death would be due to ChE inhibition, but seem to contradict it.

i - Twenty-four hour treatment with 1.1 ppb of cyclohexyl methyl phosphono fluoridate results in death of 40% of the minnows exposed to it, and the survivors have only 31.7% of normal ChE activity. On the other hand, a dose which is almost double, produces only 10% death in goldfish, while the ChE activity of the survivor is only 28.8% of the normal (Weiss, 1958). Thus, between these two species, there is no correlation between the mortality and the ChE activity.

ii - In largemouth bass, exposure during $1\frac{1}{2}$ hr to 1 ppm (part per million) of Malathion lowers the ChE activity to less than 10% of the normal and no deaths are reported. A similar treatment with Parathion produced a similar but delayed ChE inhibition and also a delayed lethal action (Weiss, 1959). So that the total ChE inhibition produced by the two compounds is not related to their lethal action.

iii - The 96-hr TLm (median tolerance limit) values for Guthion and Parathion to bluegills are respectively 0.0052 and 0.095 ppm. So that Guthion is about 20 times as toxic as Parathion. When bluegills are exposed for 8 hr to a dose of Guthion which is twice the 96-hr TLm and to a dose of Parathion which is about equal to the 96-hr TLm, Guthion reduces the ChE activity to 35% while Parathion reduces the activity to about 18% (Pickering et al., 1962). Since Guthion was used in a dose double that of the 96-hr TLm, one would expect ChE inhibition to be more important with Guthion than with Malathion, if the lethal action was linked with the ChE inhibition capacity of the compound.

iv - Forty minutes exposure of largemouth bass and fathead minnows to Guthion produces 50% mortality but the ChE activity is reduced to 5% in

bass and to 50% in minnows (Weiss, 1965). It is hard to see how the ChE activity could differ so widely if it was the cause of death.

v - Finally, in largemouth bass, Guthion, EPN and DDVP kill 50% of the fishes after respectively 40 min, 9 hr and 30 min and 48 hr. At these times, the ChE activity in the dead fishes is respectively 3.8, 60.2 and 18.8%. Moreover, with EPN treatment, the ChE activity is about 20% more in dead fishes than in surviving fishes at the same period (Weiss, 1961). So that there is no correlation between the lethal action of a compound and the ChE inhibition. Moreover, dead fishes can even show a ChE activity greater than the one found in live fishes.

Such variation between the response of the same species to various compounds or of several species to the same compounds casts some doubt about ChE inhibition as being the cause of death in fishes.

VII. NATURE OF THE PROBLEM.

Among all the work done with organophosphates, the number of studies dealing with the effect of these compounds on the development are surprisingly very few. This is possibly due to the interest and to the promises offered by the anticholinesterase action of these compounds.

The ontogenetic effects of cholinesterase inhibitors have been reviewed by Karczmar (1963). In hen eggs, the teratogenic activity of cholinesterase inhibitors such as eserine and pilocarpine has been studied and reviewed by Landauer (1954); organophosphates have also been found to be teratogenic by Marliac and Mutchler (1963), Marliac (1964), Roger et al. (1964), Khera et al. (1966). The main teratisms that

were observed included deformation of the beak, bending and shortness of the long bones, syndactylism; these were obtained by treatment occurring both prior and after the beginning of neurogenesis and neural function. These defects are similar to those produced by insulin and boric acid. The suggestion has been made that such defects could be due to interference with carbohydrate metabolism (Landauer, 1954).

In amphibian embryos, concentrations of 10^{-10} M DFP produced exogastrulation of the blastulae while stronger concentrations killed the embryos (Karczmar and Koppanyi, 1953). Exposure of the larvae during premotile and motile stages produced shortening and bending of the trunk and a decrease in the size of the head. A similar effect was observed with physostigmine. Treatment of more advanced larval stages by DFP resulted in the death of the embryos, preceded by convulsions and paralysis. However, death was more related to DFP concentration than to ChE inhibition. It was then concluded that the action of DFP was not entirely cholinergic in nature since atropine could prolong life while it did not effect the convulsions produced by DFP and the time of their onset.

Only one study of the effects of anticholinesterase agents on fish embryos has been done. Interested in finding the relationship between the behaviour and the AChE levels, Sawyer (1944) treated Fundulus heteroclitus eggs with eserine. Exposure from the blastula stage to a concentration of 100 ppm did not affect the spontaneous somatic movements, but slowed the relaxation of induced contractions (flexure and coil response). Paralysis and death occurred before the swimming stage was attained.

Information about the antigrowth, toxic and teratogenic

action of organophosphates in fish embryos was entirely lacking; it thus seemed desirable to undertake the study of the effect of organophosphates on fish embryos for three reasons.

First, the wide use of organophosphate insecticides represents a potential hazard to the development and reproduction of fish, especially for game fish such as trout. Second, organophosphates are poisons with teratological property. Their study as teratological agents could yield some information about the mode of formation of teratisms. In this regard, it seems desirable to make a detailed histological and histochemical study and to compare their action on eggs developing rather rapidly at high temperature such as zebra fish, with their action on eggs developing more slowly at low temperature such as trout eggs. Third, embryos offer a system where biochemical differentiation is incomplete (cholinesterase is absent during the early stages). They thus permit the study of the effects produced by organophosphates, other than their anticholinesterase action.

A detailed histological study of the fish following organophosphate poisoning was also lacking in our knowledge of the effect of organophosphates. The suggestion of Karczmar and Koppanyi (1953) that the DFP toxicity in the Amblystoma larvae was not entirely cholinergic in nature and the lack of correlation between the toxicity of a compound and its effect on ChE suggested that a detailed comparative study on the toxicological, histological, histochemical and biochemical effects of organophosphates should be undertaken. Since young or larval fishes are usually more sensitive to poisons than adults, they appeared particularly suitable for such a study: trout larvae were then chosen.

The experimental work is divided into four parts, each

constituting a separate chapter.

i - The effects of Phosphamidon (and Dylox) on Brachydanio rerio (zebra fish) embryos and larvae: toxicological, morphological and histological studies.

ii - The effects of Phosphamidon and Dylox on Salvelinus fontinalis (speckled trout) embryos: toxicological, morphological, histological and histochemical studies.

iii - The effects of Dylox on Salmo gairdneri irideus (rainbow trout) larvae: toxicological, histological and histochemical studies.

iv - The effects of Phosphamidon and Dylox on Salvelinus fontinalis larvae: toxicological, histological, histochemical, biochemical and electron microscopic studies.

In the description of the results, the tables have been included in the text. However, all the figures referred to in one chapter have been placed at the end of the said chapter. In each chapter, the numbering of the figures starts at one.

CHAPTER TWO

MATERIALS AND METHODS

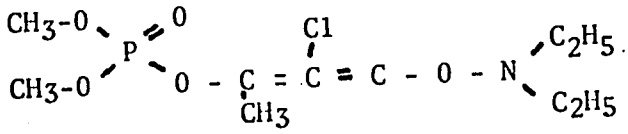
I. ORGANOPHOSPHATE INSECTICIDES.

In this study two organophosphate insecticides were used: Phosphamidon and Dylox.

A. Phosphamidon.

Phosphamidon is the common name for an insecticide which was synthesized in 1955 by E. Beriger in the laboratories of CIBA Limited, in Basle. It has several chemical names, but the most extensively used is 0- 2-chloro-2-(diethyl-carbamoyl)-1-methyl-vinyl -0,0 dimethyl-phosphate.

The structural formula is:



giving a molecular weight of 299.7 g.

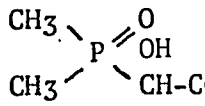
Technical grade Phosphamidon is a dark brown viscous liquid, miscible with water and most organic solvents and is chemically stable. It contains 89% of a mixture of α and β isomers (3:7) of Phosphamidon, 1% of deschloro Phosphamidon, 2% of γ-chloro Phosphamidon and 8% of inert by-products of the technical synthesis. When stored in closed glass or polyethylene vessels in the dark at 20 to 25 C no loss of activity is detectable after three years. In our laboratory, fresh technical grade Phosphamidon was obtained from CIBA Limited every six months and kept at room temperature (average 24 C). The purity of the compound was periodically checked by infra-red spectrography and the tracing compared to a standard curve furnished by CIBA Limited. In an acid or alkaline medium, Phosphamidon hydrolyzes to dimethyl phosphoric acid and

N,N-diethyl chloroacetamide. According to CIBA Limited (Anonymous, 1967), a 0.1% Phosphamidon aqueous solution at pH 7 kept at 25 C has a half-life of 13.8 days but at a higher pH and temperature it hydrolyzes more rapidly.

From the rates of hydrolysis given for various pH's and temperatures, it was calculated that at 26 C, not less than 92% of the original compound would remain and that at 10 C at least 98% is intact 24 hours after being put in solution.

B. Dylox.

Dylox (Chemagro Corporation) is an agricultural insecticide. Its chemical name is 0-0-dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate and has the following chemical structure:



It is a white crystalline compound having a molecular weight of 257.6 g and is 12% water soluble at 26 C. According to Lorenz et al. (1955), Dylox is stable at room temperature in aqueous solution. In 5M HCl, only 1% is hydrolyzed after 24 hours at 28 C (Arthur and Casida, 1957). In alkaline solutions however, a molecular rearrangement takes place and 0-0-dimethyl 2,2-dichlorovinyl phosphate (DDVP) is rapidly formed by dehydrochlorination (Lorenz et al., 1954; Arthur and Casida, 1957; Van Die, 1957).

A water soluble powder consisting of 50% Dylox and 50% inert ingredient (supplied by Chemagro Corporation, Latham, N.Y.) was used in our experiments.

Trichlorfon, Neguvon, Bayer 13/59 and Dipterex designate

formulations prepared with the same active ingredient.

II. PREPARATION OF SOLUTIONS.

The strength of the solutions of Phosphamidon and Dylox used was determined on the basis of their biological activity per milligram per volume of solution. Most toxicity studies on fishes and mammals use concentrations of insecticides given in parts per million while enzyme inhibition studies use molar solutions. Table 2.1 gives the molarity of solutions calculated from parts per million concentrations.

TABLE 2.1 Relationships between concentrations given in parts per million (ppm) and molar solutions.

PPM	MILLIMOLARITY	
	Phosphamidon	Dylox
5	0.016	0.019
30	0.100	0.114
100	0.333	0.388

III. EXPERIMENTS WITH BRACHYDANIO RERIO.

The zebra fish Brachydanio rerio (Hamilton-Buchanan) was chosen

because of its many advantages as an embryological tool.

First, a large number of eggs can be obtained throughout the year; second, the development of the embryo is rapid (four days from the fertilized egg to the hatching stage); third, the eggs can withstand careful manipulation for observation during development; fourth, the transparent membrane covering the egg allows for detailed observations and facilitates photomicrography of normal and abnormal stages of development on the same egg without sacrificing the specimen; and fifth, the living tissue of the embryo is transparent, becoming opaque as death ensues. One can follow therefore, the progressive degeneration that occurs following treatment.

Male and female fishes were kept in separate tanks until eggs were needed; they were fed tropical fish food twice daily and a ground mixture of oatmeal and beef liver three times a day. Fertilized eggs were obtained according to Legault's (Legault, 1958) or Hisaoka's and Firlit's (Hisaoka and Firlit, 1960) method. Following fertilization, the eggs were removed from the tank by gentle pipetting which also served to wash away any foreign material. Groups of twenty were placed in covered watch-glasses containing 15 ml of water or of test solution and incubated at 26 C.

Treated tap water was used throughout the experiments and was prepared by boiling for five minutes and then cooling to room temperature. Distilled water was added to restore the original volume and the pH adjusted to 7.0 with 0.1 N HCl or NaOH.

For each experiment development of the controls was checked at least every eight hours. If any abnormalities of development occurred in the control groups the experiment was aborted; likewise, if

more than 5% death occurred; if hatching did not reach at least 85%; or if important mold growth was found in the control or treated lots.

Every 12 hours dead embryos were counted and removed with an eye dropper. Water and test solutions were replaced every 24 hours. Embryos were removed periodically, fixed in Bouin's, dehydrated in alcohol and cleared in methyl salicylate. Following embedding in Tissuemat 6μ sections were stained with Hematoxylin, Phloxine and Orange G.

It was necessary to design a small chamber to orient the specimens for photomicrography. Two pieces of plastic approximately 0.9 mm high were glued one centimeter apart to a glass slide. Eggs were placed in the water-filled chamber and covered with a cover slip. The chamber was so designed that the cover slip rested gently on the capsule of the eggs. Thus, slight pressure on the cover slip permitted orientation of the egg to any desired place.

IV. EXPERIMENTS WITH TROUT.

A. Source of the eggs.

Experiments were performed on two species: Salvelinus fontinalis (Mitchill), commonly called speckled trout, and Salmo gairdneri irideus (Gibbons), the rainbow trout. Eggs of both species, either freshly fertilized or "eyed", were obtained from the Coaticook-Baldwin Hatchery through "Le Ministère du Tourisme de la Chasse et de la Pêche de la Province de Québec". "Eyed" rainbow trout eggs were also supplied by Mr. J.P. Cuerrier from a private hatchery in Ripon, Québec.

"Green" eggs of speckled trout for experiments during the

early part of development were obtained on October 29 and November 11, 1966 and on October 31, 1967.

The eggs were fertilized according to established artificial spawning techniques (Davis, 1953). Following a one-hour period of hardening, the eggs, in lots of 5,000, were placed in one-gallon glass jars two-thirds filled with water. The jars were packed in a mixture of peat moss and ice and transported immediately to Ottawa by automobile. Water temperature on arrival varied between 8 and 10 C. Unfertilized or dead (white) eggs (3.5-4.1%) were removed on arrival at the laboratory and the living eggs divided into experimental lots of approximately 570. Incubation was started eight hours after fertilization. From this point on, the eggs were undisturbed except for treatment or the removal of dead specimens.

B. Experimental design.

The eggs were incubated in a cold room at 10 C ± 0.5 C. During 1966 the eggs were placed in small plastic trays with nylon-screen bottoms. These small trays, each containing about 570 eggs, were placed in trout hatching trays and coupled to a closed circuit of water running at a rate of two gallons per minute.

During the year 1967, a slightly different and more practical system was developed. It consisted of a water-filled box (fig. 1 and 2), over which a column was mounted. Small wooden trays with nylon-screen bottoms were fitted into the opening of the square column. The column was built so that the trays could be placed at right angles to each other; one tray thus served as a cover for the tray placed underneath. A small lining of foam on the lower exterior edge of the tray

formed a tight seal so that no mixing of larvae from one tray to another could occur. The wooden trays were maintained four inches below the surface by small L-shaped wedges stuck in small openings at the top of the column. The water, recirculated by a pump, entered through the bottom of the column, flowed up through the trays and then overflowed into the box through two $1\frac{1}{4}$ inch diameter hoses. Each unit contained 10 gallons of water.

The same system was used for experiments on larvae at room temperature. For this purpose, a condenser was introduced between the pump and the hose bringing the water to the column. Tap water continuously flowed into the external part of the condenser while the water of the unit flowed into the inner part. The desired temperature could be maintained without difficulty by adjusting the flow of the tap water.

These columns had the main advantages that no intermixing of larvae from different trays took place and large number of trays could be handled in a rather small space.

Five units were used for treatment and one unit served for controls (fig. 2). Before or after treatment, the trays were placed in similar but higher columns placed over a large box containing 60 gallons of water.

During all experiments, experimental solutions or water were replaced every day using water which had aerated in the cold room for 24 hours. The physical characteristics of the water after aeration were as follows:

pH: 7.4-7.5;

dissolved oxygen: 11-12 ppm (Winkler's method, Lagler, 1952);

total hardness: 51 ppm (Lagler, 1952);

alkalinity (bicarbonate): 43 ppm (Lagler, 1952).

These properties are in the same range as those of the Coaticook Hatchery water and are considered suitable for fish (Lagler, 1952). The dissolved oxygen remained the same and the pH did not vary by more than 0.2 units during the 24-hour period.

No serious problems of mold growth were encountered. Mold growth was prevented or stopped by treatment with 1 ppm malachite green for one hour every three days, followed by thorough rinsing.

"Eyed" embryos for experiments during later embryonic life and during larval stages were incubated in a cold room at 7 C until four or five days before needed. At this time, the temperature of the cold room was raised one degree on three consecutive days. Experiments on larvae were carried out at 10 C; other conditions were as described above.

Handling of the trays was reduced to a minimum and, until the embryos reached the "eyed" stage, great care was taken when moving the small trays so that the eggs would not be displaced. Dead eggs or embryos were counted and carefully removed with a wide-mouth pipette, without touching the neighbouring eggs. In the controls, mortality was only 18% at the "eyed" stage. This procedure appears to have been successful if we compare our losses to those at the Coaticook Hatchery where their losses in 1965 were 13%.

C. Observations and samples.

Depending upon the histochemical methods to be used, samples were fixed in Bouin's or 10% formol calcium (Pearse, 1960) at 10 C or in

5% gluteraldehyde prepared with 0.1M phosphate buffer with a final pH of 7.2. Unfixed samples were also taken for histochemical studies. Eggs fixed in Bouin's are easily decapsulated with fine forceps.

Careful examination and photography of embryos at various stages were done on either unstained or on specimens which had been stained in stock Harris Hematoxylin, diluted 100 times, for 10 minutes. Serial sections were cut from Bouin's fixed samples.

Eggs, fixed in formol calcium, required at least 45 minutes of fixation before the capsule could be opened; otherwise, internal pressure when the capsule was broken ruptured the yolk and the embryo. After decapsulation, embryos were left in fixative for another 15 minutes followed by 20 minutes rinsing in water and then transferred to cold sucrose-gum acacia (Pearse et al., 1963) until the embryos sank to the bottom of the vial. Unfixed eggs were placed in cold sucrose-gum acacia for 15 minutes, the time required to crenate the capsule, which could then be easily removed without any damage to the yolk and embryo. Embryos were then dissected from the yolk sphere and left in cold sucrose-gum acacia for an additional 15 minutes prior to embedding and quick freezing.

Cryostat sectioning of embryonic tissues presents several difficulties because the samples are small and especially because the tissues are very soft and contain a large amount of water. The problem of ice-crystal formation during quick freezing of embryonic tissue has been solved by removing the excess water with sucrose-gum acacia. Pearse et al. (1963) placed small soft tissues between liver slices to serve as a support. However, when one needs to section several embryos, this is a very long and tedious process. The advent of embedding

material such as O.C.T. (Labtek) afforded a great advantage, since the frozen resin offers an excellent matrix, supporting the embryo equally from all sides. At first we used gelatin capsule to contain the resin and the tissue during freezing. However, there always remained the problem of quick and proper orientation of the tissue. This problem was easily solved by embedding embryos first in a small scotch tape rectangular box filled with O.C.T., no. II (-15 C to -30 C). The embryo could be placed horizontally and was not liable to fall during freezing in liquid nitrogen as it did in the gelatin capsules. A large number of small rectangular boxes of any size could be quickly made from scotch tape with the aid of a template: a steel bolt shaped to the desired size, secured in a wooden block. To make the molds, small pieces of scotch tape were folded over the template, moistened with glycerine to avoid sticking of the tape to the bolt, and the corners of the scotch tape were simply pressed together. After freezing in liquid nitrogen, the molds were simply peeled off, and the small blocks placed in sealed plastic bags and stored in an ultra-low temperature (-82 C) refrigerator. These blocks could be oriented as desired by freezing them to cork discs and then to a chuck.

Sections of 8μ or 15μ were cut in a cryostat; the optimal cutting temperature was found to be -28 C.

Table 2.2 summarizes the procedure used for several histochemical tests.

TABLE 2.2 Histochemical technique for localization of various enzymes.

ENZYME	STAGE	FIXATION	TECHNIQUE[1,2,3]	INCUBATION AT ROOM TEMP.
LDH, MDH, NADH	embryos	Gluteraldehyde 45 min	Hess, Scarpelli and Pearse (modified)	60 min
	larvae	Formol calcium 60 min		40 min
Cytochrome oxidase	larvae	none	Burnstone	60 min
Phosphorylase	larvae	none	Takeuchi (iodine staining[4] and PAS staining)	60 min
AChE	embryos	Gluteraldehyde 45 min	Karnovsky and Roots (1964)	75 min
	larvae	Formol calcium 60 min	Koelle, Karnovsky and Roots (1964)	45 min
Alkaline Phosphatase	larvae	Formol calcium 60 min	a) Gomori b) Azo dye (Fast violet B)	30 min 45 min
5'Nucleotidase	larvae	Formol calcium 60 min	Wachtein and Meisel (modified)	30 min

TABLE 2.2 (continued)

ENZYME	STAGE	FIXATION	TECHNIQUE[1,2,3]	INCUBATION AT ROOM TEMP.
ATPase	larvae	none	Padykula and Herman	20 min
Acid Phosphatase	larvae	Formol calcium 60 min	Rutenberg and Seligman (Fast red TR)	75 min

[1]. Tissues were mounted in glycerine jelly. If the tissue was not prefixed, a 5-minute post incubation fixation in 10% formol calcium was used.

[2]. All techniques, except for Takeuchi's and Karnovsky's and Roots' were taken from Pearse (1960). Takeuchi's technique was taken from Lillie (1965).

[3]. 8μ sections were used for all enzymes, except for phosphorylase where 15μ sections were used.

[4]. Tissues were mounted in Takeuchi's iodine mounting medium.

D. Tissue preparation for electron microscopy.

Speckled trout larvae were killed by placing them in cold (4 C) 5% gluteraldehyde. Following fixation (3-5 min), the masseter muscle was removed. The technique of Sabatini et al. (1963) was used for fixation and post fixation processing and Luft's (1961) epoxy resin as embedding medium. It consisted of 3.5 parts of the Epon 812-DDSA mixture to 6.5 parts of the Epon 812-MNA mixture. Gold sections (90-150 mμ) were cut on a MT-1 Sorvale ultramicrotome and stained for 15 minutes with lead hydroxide (Karnovsky, 1961). The electron microscope used was an Associated Electrical Industries, Model IM6B.

E. Biochemical analysis of acetylcholinesterase activity.

Speckled trout larvae, frozen and stored at -20 C, were thawed and the heads cut at the level of the hindbrain. Five heads were pooled from each sample and quickly weighed in a tared-glass homogenizing tube. Cold (4 C) 0.2M phosphate buffer of pH 7.2 was added to adjust the weight to one gram. The tube was then placed in crushed ice and the heads were homogenized for five minutes. Enough cold buffer was then added to give a final concentration of 60 mg of head/ml. This will be referred to as the stock solution of head homogenate.

The ChE activity of speckled trout larvae was measured by two methods, each based on a different principle.

i - Hestrin's method.

Hestrin's method (1949) is based on the reaction of acetylcholine with hydroxylamine. A known amount of acetylcholine is added to the medium and after hydrolysis by AChE has taken place during a

certain time, the reaction is stopped. The amount of residual acetylcholine is then estimated from the measurement of the intensity of the color formed by the reaction of acetylcholine with hydroxylamine.

A standard curve for various known amounts of acetylcholine was first run to verify the precision of the technique. The final solution had the following composition:

0.2M phosphate buffer pH 7	1 ml
Acetylcholine iodide in phosphate buffer (0 to 5 mM)	1 ml
2M sodium hydroxylamine and 3.5 N NaOH (1:1)	4 ml
Conc. Hcl (diluted 1:2)	2 ml
0.37M $Fecl_3$ in 0.1 NHcl	2 ml.

A blank tube containing no acetylcholine was also prepared and used to set the instrument at zero optical density (OD). The reaction ran for 20 minutes before OD was read on an infra-red spectrophotometer at 540 mμ.

As seen in figure 3, the OD is proportional to the amount of acetylcholine, with the average deviation from the optimal straight line being 10%.

Hestrin's method, as modified by Holland et al. (1967) was used for measurements of cholinesterase activity. Before doing the analysis of treated and control larvae, the technique was further tested by measuring the ChE percentage activity of dilutions of the stock solution. Each 1 ml dilution contained respectively 0, 6, 15, 30, 45 mg of head homogenate per ml and was incubated with 1 ml of 4 mM acetylcholine iodine for 45 minutes at room temperature. The reaction was stopped by adding 4 ml of a mixture of NaOH and hydroxylamine. The

mixture was immediately filtered, and to a 3 ml aliquot, 1 ml of HCl and 1 ml of $FeCl_3$ were added, each at two minutes' intervals. After 20 minutes of color development, the OD was read at 540 mμ. A blank containing 1 ml of stock homogenate, but no acetylcholine represented zero OD.

The tube with 1 ml of stock homogenate was considered as having 100% activity. Since the OD reading represented the residual amount of acetylcholine, that is, the amount which was not hydrolyzed, the percentage activity of a given solution was found by the following formula: $\% \text{ activity} = \frac{W\text{-OD of } X}{Y}$, where W is the OD of the tube containing no head homogenate and Y is the difference between the OD of the tube containing no head homogenate and the OD of the tube containing 100% head homogenate.

Figure 4 gives the percentage activities calculated in this fashion. The slope of the line is very near 45°, which is what one would expect, if the method and the manipulations were precise.

ii - Abou-Donia's and Menzel's method (1967).

This method is based on the fact that the products of hydrolysis of acetylthiocholine (AcThCh) resulting from the AChE, reacts with 5,5,-dithiobis-(2-nitrobenzoic acid) (DTNB) and forms a color which is determined spectrophotometrically at 412 mμ.

The method was slightly modified: the incubation media had the following composition:

COMPOUND	CONCENTRATION	QUANTITY	TOTAL AMOUNT
AcThCh	4 mM	.5 ml	2 mM
DTNB	1 mM	2.0 ml	2 mM
Head homogenate	1.2 mg/ml	2.5 ml	3 mg

After a 20-minute incubation period, the mixture was filtered and readings of the OD were taken exactly 30 minutes from the start of incubation. A blank containing no head homogenate was used to set the instrument at zero OD. Before assaying the ChE activity of control and treated samples, the proportionality between the OD and the amount of active enzyme was verified by measuring the OD of several dilutions of head homogenate containing respectively a total of 0, 0.6, 1.2, 1.8, 2.4 and 3 mg in 2.5 ml. Figure 5 shows a straight line indicating that the OD was proportional to the amount of active enzyme.

If the OD of the solution containing 0 mg and 3 mg of homogenate are respectively considered as 0% and 100% ChE activity, percentage activities of other dilutions are easily calculated. A straight line is obtained having a 45^{o} slope, showing that variations of OD can be correlated to total enzyme activity.

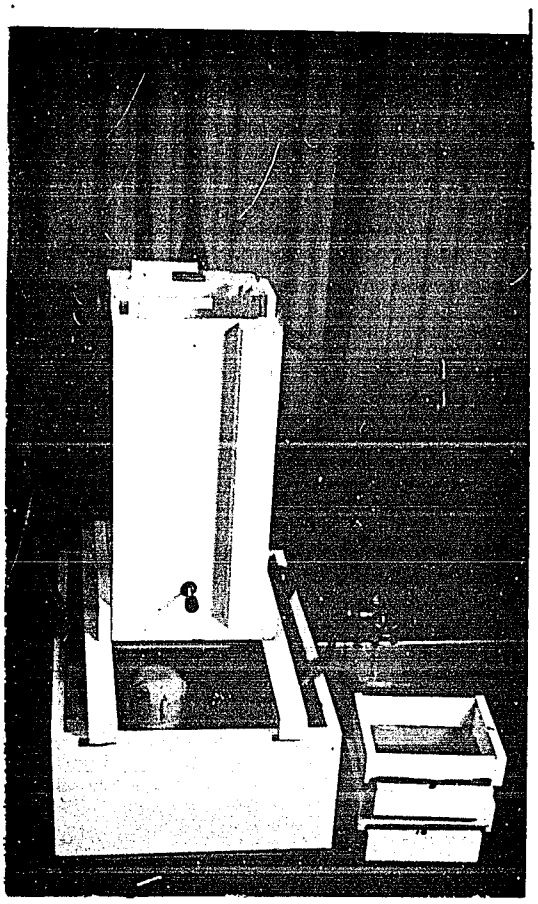


Fig. 1. Recirculating water unit consisting of a box containing a pump, a condenser and a column in which are placed small interlocking trays.

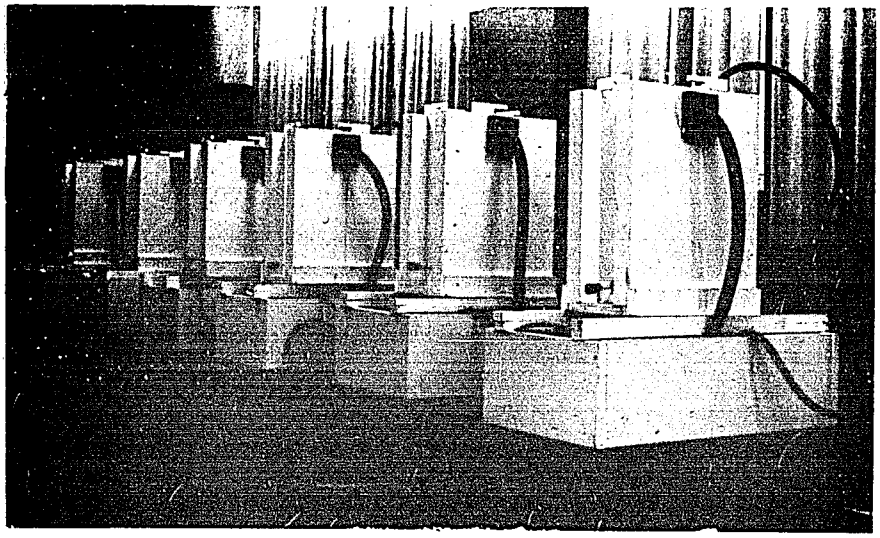


Fig. 2. Six parallel units making the experimental system.

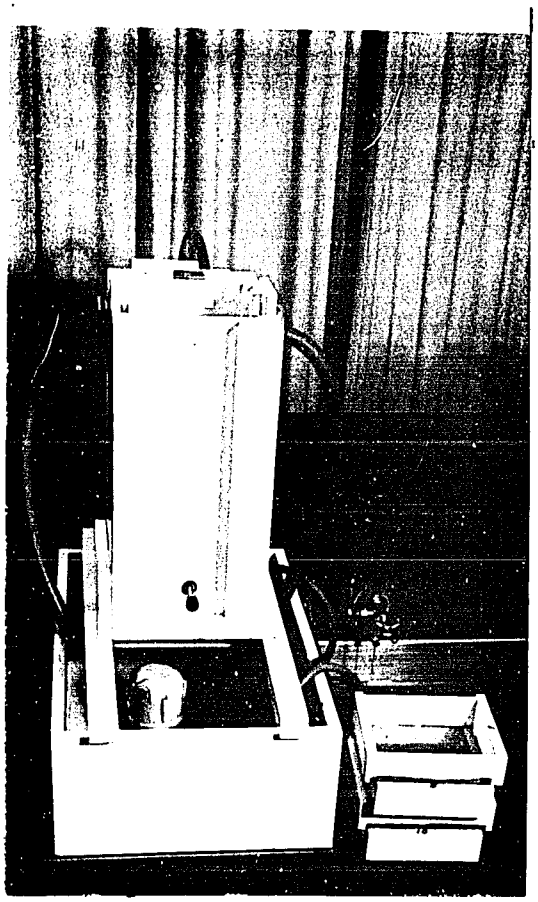


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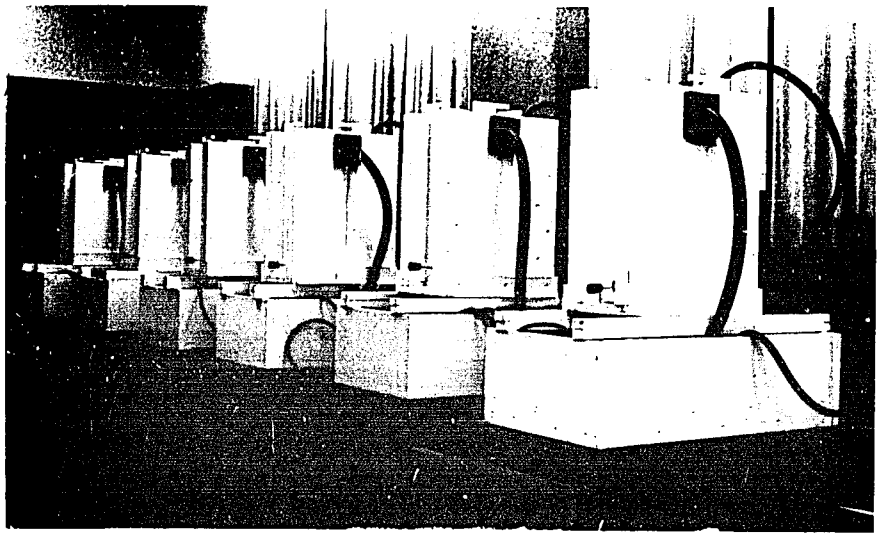


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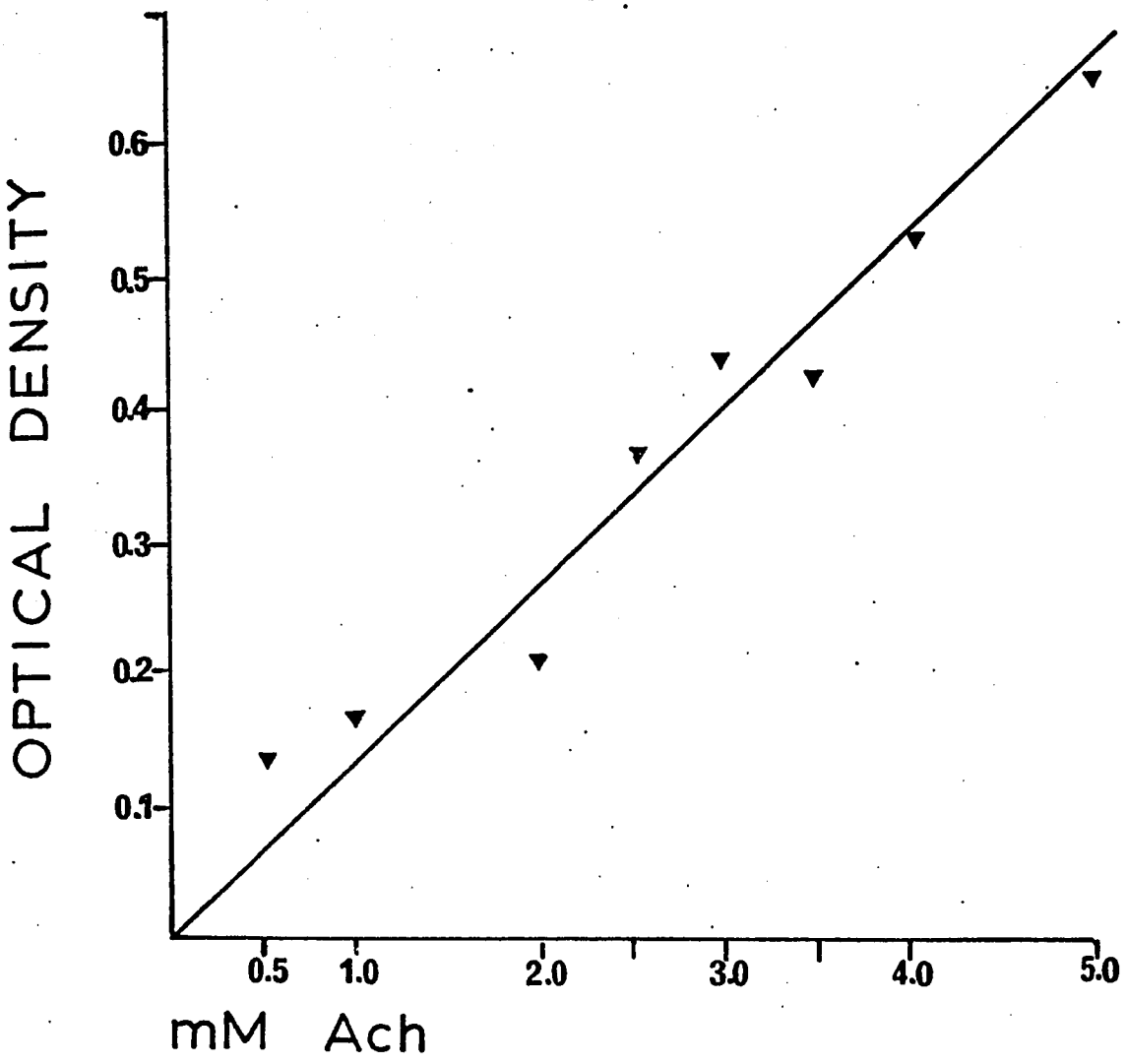


Fig. 3. Relationship between the optical density and the amount of acetylcholine (millimoles), in the Hestrin's method.

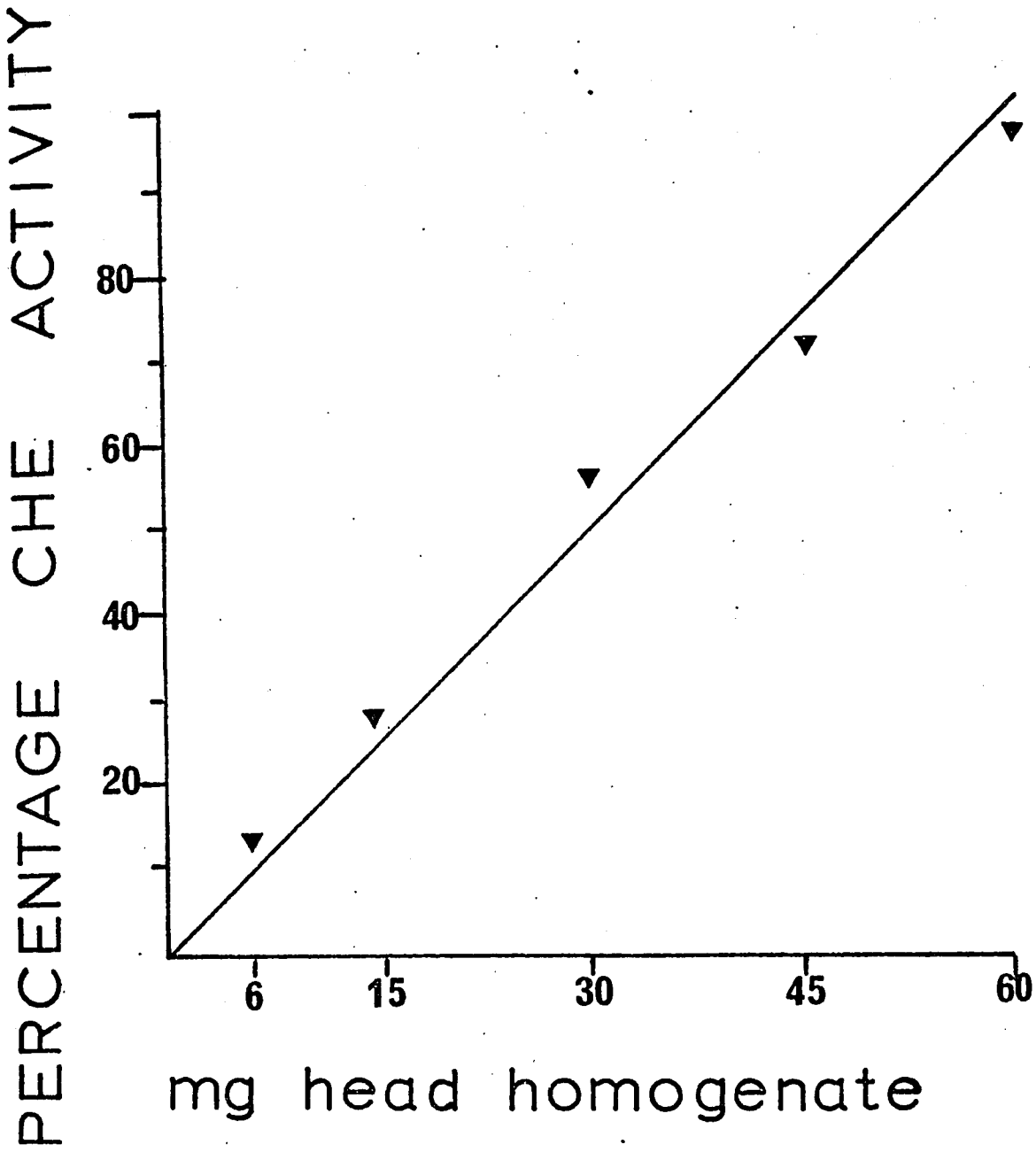


Fig. 4. Percentage activity of cholinesterase, calculated from optical density values obtained with varying dilutions of the stock solution of head homogenate (Hestrin's method).

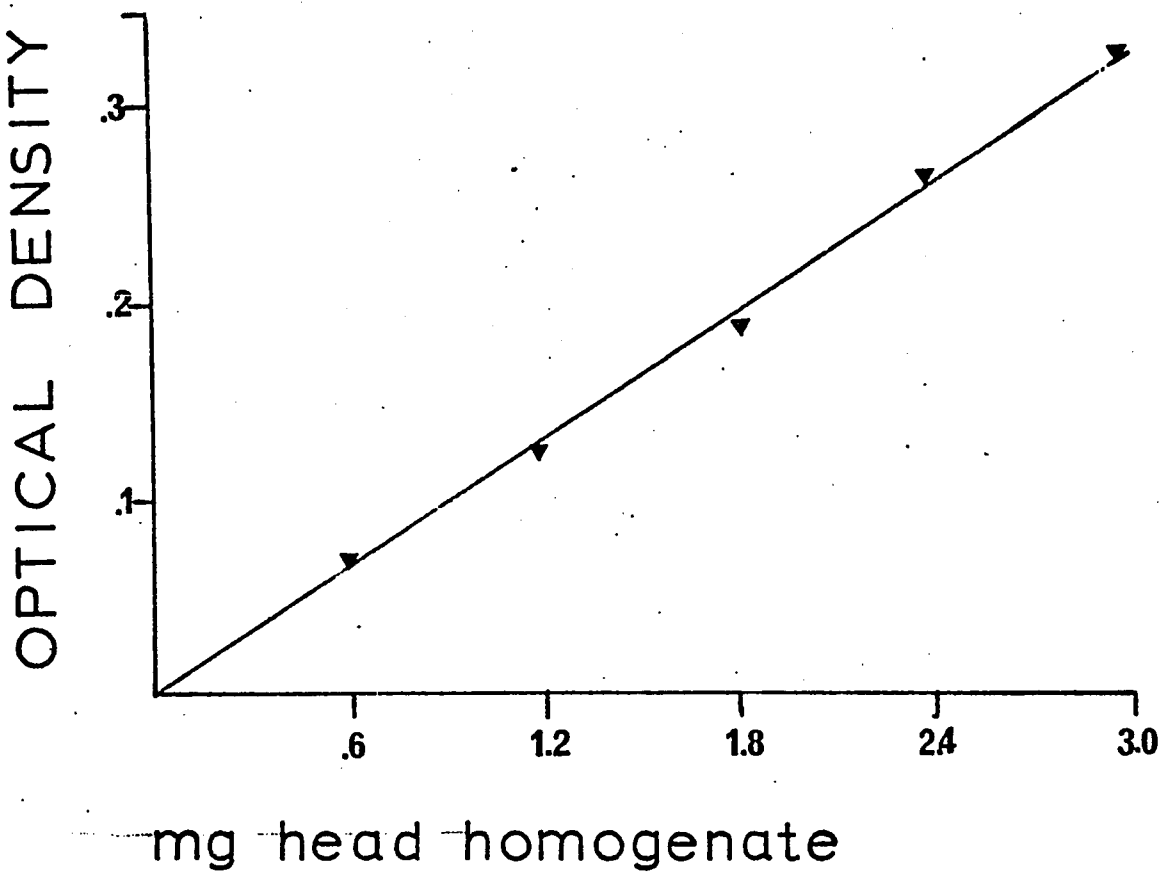


Fig. 5. Optical density following incubation with different quantities of head homogenate (Abou-Donia's and Menzel's method).

CHAPTER THREE

EXPERIMENTS WITH

BRACHYDANIO RERIO

EMBRYOS AND LARVAE

I. EXPERIMENTALS.

A. Normal development of the zebra fish Brachydanio rerio.

Hisaoka and Battle (1958) have described in detail and have assigned numbers to the different stages of the normal development of the zebra fish. Since in the experiments to follow, treatment lasted for different lengths of time and was started at different periods of development, it seems desirable to give the main features of its development. My observations are in perfect agreement with Hisaoka's and Battle's description.

a) Stages 1 to 8 (cleavage). Repeated oriented divisions of the cytoplasm at the animal pole, resulting in 256 cells, 110 min after fertilization (fig. 1 and 2).

b) Stages 9 to 12 (blastula). As cell division continues, the mass of cells first spreads over the surface of the yolk, becomes more compact and elevated (fig. 3) and eventually (4 hr and 7 min) rounded so that the margin of the blastoderm blends into the adjacent yolk surface (fig. 4).

c) Stages 13 to 16 (gastrula, fig. 5, 6 and 7). The blastoderm becomes more flattened and overgrows the yolk mass eventually enveloping it. When the blastoderm has enveloped three quarters of the yolk mass a distinct neural keel is present (9 hr and 42 min) (fig. 7).

d) Stage 17 (closure of the blastopore, 2 somites, fig. 8). The axis of the embryo can be seen as a translucent ridge which almost encircles the yolk (10 hr).

e) Stage 18 (optic vesicle, 5 somites, fig. 9). Lateral expansions of the prosencephalon form the optic anlagen. The neurocoele and the

notochord are present (14 hr).

f) Stage 19 (auditory placode, fig. 10). The posterior portion of the yolk mass begins to constrict; 15 somites have differentiated and the auditory placodes are invaginating (20 hr).

g) Stage 20 (optic cup, fig. 11). Constriction and elongation of the yolk mass is well-defined; the optic cup and the lens have developed; the body has elongated and the notochord extends to the tip of the tail which is elevated from the yolk. Twenty somites are present and muscular movement begins (24 hr).

h) Stage 21 (otolith formation, 30 somites, fig. 12). The tail is much longer, the pectoral fin buds appear and the heart begins to beat (27 hr).

i) Stage 22 (retinal pigmentation, 32 somites, fig. 13). The head becomes detached from the yolk and the caudal fin is formed; circulation is well-established; the ventricles of the brain are evident and there is vigorous movements of the pectoral fins (37 hr).

j) Stage 23 (body pigmentation-melanophores, 33 somites, fig. 14). The olfactory placodes have developed and the gut, air bladder and liver anlagen appear (49 hr).

k) Stage 24 (xanthophore development, 34 somites, fig. 15). Yellow pigment appears; precartilage can be seen in the jaws; the heartbeat is regular and averages 191 beats per minute; and frequent violent movements of the embryo occur (72 hr).

1) Stage 25 (hatching, fig. 16). The yolk mass is much smaller; body pigmentation is more intense; rapid movements of the eye are visible; liver, gut and air bladder are clearly visible through the semi-transparent body wall. The lengths of the larvae average 3.2 mm (96 hr).

B. Types of experiments.

During preliminary experiments, meant to find the effective dose range, eggs were exposed from Stage 12 (very late blastula) to Stage 25 (hatching) to concentrations of 1, 10, 100 and 1,000 ppm of Phosphamidon. In the next series of experiments, zebra fish eggs were exposed to Phosphamidon from different stages and for various lengths of time. Figure 17 summarizes these different types of exposure. The concentration of Phosphamidon in the various experiments varied from 100 to 600 ppm. Samples (5) for histological study of the interesting types were taken at 32 and 108 hr. In a second type of experiment, the egg capsules of both treated and control embryos were pierced with a fine glass needle to verify that the capsule was freely permeable to Phosphamidon. The third series of experiments, though less extensive, were similar to the foregoing except that the organophosphate Dylox replaced Phosphamidon. Stage 12 embryos were treated for 24 hr with doses ranging from 100 to 1,000 ppm.

Finally, in order to compare the susceptibility of larvae with the susceptibility of embryos, 50 young larvae (24 hr old) were exposed for 48 hr to each of five concentrations of Phosphamidon ranging from 100 to 600 ppm. Mortality was determined and behaviour was observed after 8, 16, 24, 36 and 48 hr.

Each type of treatment was usually repeated twice or several times; the similarity of the various results for one type of treatment was verified by the homogeneity test. Significant difference between control groups and various treated groups was tested by the chi-square test. In order to facilitate comparison between the different groups, the various results such as mortality, abnormality or hatching will be

expressed in percentages. The numerical values for each experiment as well as some of the statistical data will be found in the Appendix I (p. 321-328).

II. RESULTS.

A. Exposure of embryos to Phosphamidon.

Preliminary experiments with 1, 10, 100 and 1,000 ppm treatments from Stage 12 indicated that the toxicity level was between 100 and 1,000 ppm. Absolutely no effect was seen with 1 or 10 ppm, while 1,000 ppm was lethal to all embryos after 20 hr of treatment.

Type I - Continuous exposure.

Table 3.1 gives the results of experiments during which embryos were exposed from Stage 12 (very late blastula) to Stage 25 (hatching) to concentrations ranging from 100 to 800 ppm.

Exposure to 100 and 200 ppm had only a slight effect. There was a 12 to 18 hr delay in development and a slight decrease in body length but no significant difference in viability or hatching.

With 400 ppm, the shortening of the tail was more pronounced and hatching was delayed. Fifty per cent of the larvae that had hatched before the end of the 96-hr treatment period and which were thus still in Phosphamidon died within 10 to 12 hr. Continuous exposure to higher doses (600 and 800 ppm) had far-reaching effects and no hatching occurred. With concentrations of 800 ppm, the development of all embryos still alive was about 7 hr behind expected development at 27 hr. The following abnormalities were evident: elongated body, some with open blastopore and underdevelopment of head structures (fig. 37). At 48 hr,

80-90% had died; the others were abnormal and their development was 15 hr behind that of the controls. Mortality reached 100% at 96 hr.

TABLE 3.1 Mortality and hatching percentages of Brachydanio rerio eggs treated with Phosphamidon from Stage 12 (very late blastula) to Stage 25 (hatching).

Dose (ppm)	Total no. of eggs	D	Hn	Hct	uH
Control	200 (5)*	2	92	0	6
100	120 (3)	3	90	0	7
200	120 (3)	3	89	0	8
400@	100 (3)	23	64	13	0
600@	160 (4)	100	0	0	0
800@	100 (3)	100	0	0	0

D: mortality: Hn: hatched, normal; Hct: hatched with curved tail; uH: unhatched at 120 hr.

*Number of experiments.
@Significant difference from control (see Appendix I, p.321).

Type II - 48 hr exposure.

Similarly, exposure to a low dose (100 and 200 ppm) of Phosphamidon for 48 hr had almost no effect. The effects of exposure to higher doses are given in Table 3.2 (p. 61). All groups differed significantly from the control group (see Appendix I, p.322).

Treatment from the very late blastula (Stage 12) caused high mortality during the first 24 hr. When treatment started at the optic cup stage (Stage 20), death of the embryo occurred mainly during the second half of treatment. If treatment began at Stage 22 (retinal pigmentation, 36 hr) or at Stage 23 (body pigmentation, 48 hr), the embryos were able to survive the treatment period, but nonetheless died prior to hatching. Death was foreshadowed by a marked decrease in the rhythm and rate of heartbeat (approximately half of normal rate). Haemostasis accompanied the irregular heart action.

Type III- 36 hr exposure.

Treatment with 400 ppm (between 22 and 58 hr) had no effect on the viability of the embryos ($p>.20$) but hatching was 10% less than in the controls ($p<0.01$). With 600 ppm however, mortality rose as high as 63% ($p<.01$) and occurred mainly from the end of treatment to hatching (84 to 96 hr).

TABLE 3.2 Mortality, abnormality and hatching percentages of Brachydanio rerio embryos treated with Phosphamidon for 48 hr.

Dose (ppm)	Period of Treat.	Total no. of eggs	D	N	Mi	Ma[1]	H	uH
Control		120 (3)[2]	4	95	1	0	94	6
600	12-23[3] (4-52)[4]	80 (2)	100	0	0	0	0	0
600	20-24 (24-72)	80 (2)	85	0	5	10	5	95
400	22-24.5 (36-84)	100 (2)	25	0	75	0	65	35
600	22-24.5 (36-84)	80 (2)	48	0	52	0	52	48
400	23-25 (48-96)	80 (2)	40	0	60	0	54	46
600	23-25 (48-96)	100 (3)	94	0	6	0	2	98

D: mortality; N: normal; Mi: minor abnormality (see p. 64); Ma: major abnormality (see p. 65); H: hatched; uH: unhatched.

1. D + N + Mi + Ma equals 100%. H + uH equals 100%.
2. Number of experiments.
3. Stage number of beginning and end of treatment.
4. Time (hr) of beginning and end of treatment.

Type IV - 24 hr exposure.

Treatment with 400 ppm had an effect in only two cases. If exposed during the 72-96 hr period 14% of the embryos died ($p < .01$). Exposure during the gastrulation period manifested more drastic effects: 19% died, 16% had minor abnormalities ($p < .01$) and only 34% hatched ($p < .01$). In several embryos (fig. 19) the cervical curvature, which is normally present between 48 and 72 hr, persisted; the number of somites was reduced and less sharply defined than those of the controls (fig. 18 and 21). In some, the tail was also dorsally curved.

Treatment with 600 ppm had a more important effect than treatment with 400 ppm, but the stages of development which were the most sensitive were the same: the last 24-36 hr of development and the gastrulation period.

Table 3.3 shows the results of 24 hr of exposure to 600 ppm during several periods of development.

TABLE 3.3 Mortality, abnormality and hatching percentages of Brachydanio rerio embryos treated with 600 ppm of Phosphamidon for 24 hr.

Period of Treat.	Total no. of eggs	D	N	Mi	Ma[1]	H	uH
Control	297 (5)[2]	1.5	98	0.5	0	90	10
12-21[3] (4-28)[4]	290 (5)	80	4	9	7	15	85
17-22 (10-34)	240 (3)	5	85	10	0	75	25
18-22 (14-38)	240 (3)	1	96	3	0	90	10
20-23 (24-48)	240 (3)	1	99	0	0	89	11
22-23.5 (37-61)	240 (3)	6	93	1	0	80	20
23-24 (48-72)	100 (2)	7	93	0	0	69	31
23.5-24.5 (60-84)	198 (2)	24	73	3	0	50	50
24-25 (72-96)	50 (2)	24	76	0	0	70	30

D: mortality; N: normal; Mi: minor abnormality; Ma: major abnormality; H: hatched; uH: unhatched.

1. D + N + Mi + Ma equals 100%. H + uH equals 100%.
2. Number of experiments.
3. Stage number of beginning and end of treatment.

In larvae of all groups (hatched from treated eggs), there was a reduction of the body axis. Measurements of 30 control larvae gave a mean length of 3.2 mm (± sd 0.07) while the larvae treated between 48 and 72 hr averaged 2.9 (± sd 0.08, $p < .01$) (9% reduction). The reduction of the body axis was more important (12%) (2.8 mm, ± sd 0.08, $p < .01$) when treatment occurred shortly after gastrulation (14-38 hr). Deformed yolk pouch and enlarged cardiac vesicles were also frequent (fig. 22). Usually at the end of the 24-hr treatment period, the heartbeat was only one half the normal rate and after hatching the larvae were far less active than the controls. Hatching occurred simultaneously in treated and untreated groups, except in the group where the eggs were treated between 72 and 96 hr where it occurred 6 to 8 hr prematurely.

When treatment included the gastrulation period the effects were more drastic: high mortality and very few normal embryos. In addition to the types of abnormalities already described, two main types of minor abnormalities and two types of major abnormalities resulted from treatment during the gastrulation period with 600 ppm.

Minor abnormalities.

Figure 23 is a good example of one type of minor abnormality involving a 25% reduction in body size. The head and eyes were smaller than that of the control (fig. 21) while the yolk pouch was bigger. A cervical curvature partially attached the head to the yolk and a caudal curvature lifted the tail dorsally. Ventrally-directed curvatures were never seen. The somite arrangement was somewhat distorted and the number was reduced: only 12 to 13 somites posterior to the vent are present instead of the usual 19. Blood circulation through the yolk sac

was not as abundant and the melanophore distribution on the body did not have the linear arrangement seen in the control (fig. 21).

In the other type of minor abnormality, the head, body and yolk sac were of approximately normal size and normal morphology (fig. 20 and 24) but the tail was much shorter and the number of somites posterior to the vent was either 6 or 7. The short tail was also permanently curved sideways or dorsally. Those that hatched from this group could not swim normally. They either swam in circles or forward by a quivering movement of the whole body. Usually they died 12 hr after hatching.

Major abnormalities.

a) Blobby tail syndrome.

The embryos' length was much reduced so they did not appear slender and fish-like. They were bulky (fig. 25, 27 and 28) or ventro-dorsally flattened (fig. 29) over the yolk mass. Relative to the size of the embryo the yolk mass seemed enormous. The notochord was unrecognizable and individual somites could not be identified; pectoral fins were absent and the tail was either completely absent (fig. 29, 30 and 31) or extremely short and twisted (fig. 26 and 27) or blobby (fig. 28 and 32). When it was slightly longer (fig. 25), it was still attached to the yolk. It was not surprising to see that body movements were reduced to an antero-posterior contraction. Most of the embryos with this syndrome could not hatch but some were able to do so by bumping their heads against the capsule to break it. The head was much more compact (fig. 25, 29 and 30) and the eyes, sometimes absent (fig. 31), were smaller but in proportion to the size of the head (fig. 26). Very often though, they were abnormal and the lens was absent or displaced

(fig. 29 and 30). Most of the time the choroid fissure had not closed (fig. 25, 26 and 32). The otic vesicle was always present but usually displaced and distorted. The head usually failed to differentiate normally and if not totally fused to the yolk mass (fig. 25 and 30), it was still partially retained by a greatly swollen cardiac vesicle (fig. 26, 27, 28, 29, 31 and 32). The heart was tubular (fig. 31 and 32) and had not undergone the normal twist pattern. The heart beat more slowly (about half the normal rate) and irregularly resulting in reduced blood flow; sometimes blood flow was completely halted. The vitelline circulation, when present, was also much reduced.

b) Amorphous.

The other type of major abnormality was embryos with no recognizable morphology. It was only a disorganized pigmented mass of living tissue on the top of the yolk sphere. Epiboly appeared to be incomplete. Surprisingly, several of these monsters lived beyond the normal hatching time (fig. 33).

Type V - $5\frac{1}{2}$ hr exposure.

Treatment during the gastrulation period with 400 ppm had no effect (Table 3.4). Treatment during the last part of gastrulation produced some minor abnormalities ($p < .01$) but had no effect on the viability and hatching of the embryos. However, a very short exposure could have severe effects if it occurred during early gastrulation (Stages 12 to 16) (Table 3.4). Usually death of the embryos occurred 3 to 15 hr after treatment ended. If the embryo could survive this critical period, very often it would remain alive, even if very abnormal, until hatching time. If death occurred after 24 hr of development in embryos with less severe abnormalities, the tail was the first area to show necrosis.

TABLE 3.4 Mortality, abnormality and hatching percentages of Brachydanio rerio embryos treated with 400 or 600 ppm of Phosphamidon for $5\frac{1}{2}$ hr during the gastrulation period.

Dose (ppm)	Period of Treat.	Total no. of eggs	D	N	Mi	Ma[1]	H	uH
Control		297 (5)[2]	1.5	98	0.5	0	90	10
400	12-16[3] (4-9.5)[4]	180 (3)	4	96	0	0	88	12
400	15.5-18 (8.5-14)	180 (3)	3	97	0	0	91	9
600	12-16 (4-9.5)	390 (7)	25	24	21	29	32	68
600	15.5-18 (8.5-14)	200 (4)	4	92	4	0	89	11

D: mortality; N: normal; Mi: minor abnormality; Ma: major abnormality; H: hatched; uH: unhatched.

[1]·D + N + Mi + Ma equals 100%. H + uH equals 100%.
[2]·Number of experiments.
[3]·Stage number of beginning and end of treatment.
[4]·Time (hr) of beginning and end of treatment.

In several embryos with white tail (the transparent tissue became opaque white), the heart could still be seen beating and the blood circulating. Usually death of the embryos occurred 5 to 6 hr after the onset of tail necrosis.

This short treatment from the beginning of gastrulation was interesting since the mortality rate was moderate whereas the percentage of major abnormalities was high. Thus it was possible to study the progressive development of major abnormalities.

Mode of formation of major abnormalities.

Embryos which have been treated since the beginning of gastrulation were already delayed in development at the end of the $5\frac{1}{2}$-hr period of treatment. In the control, epiboly had enveloped at least three quarters of the yolk sphere. In the treated, epiboly had reached only the equator of the egg. Four hours later, in the untreated eggs, the yolk plug was completely closed and the embryonic axis was visible (fig. 34). In contrast, the antero-posterior axis of the treated embryos was elongated and epiboly was not yet completed. As the germ ring advanced, it constricted the yolk mass (fig. 35) and produced a kind of exogastrulation often resulting in an open blastopore, which could remain open until 27 hr (fig. 37). If by 27 hr the embryo was entirely lacking organization the end result was a disorganized mass which persisted until death (fig. 33). Even at 37 hr a remnant of the open blastopore could still be present (fig. 39). When it eventually closed part of the yolk material was included in the tail. Figure 40 shows an embryo at 49 hr with a blobby tail; the posterior portion of the body was partially embedded in yolk. Eye pigment developed but the eye was very small and abnormal. The shape of the tail at hatching

could be predicted at 48 hr. The embryo shown in Figure 42 had a very short crooked tail at 96 hr.

Histology of 32-hr embryos.

Compact and poorly differentiated tissues characterized the histology of the abnormal embryo such as the one shown in Figure 39.

In the extreme situation the embryo was a mass of cells showing almost no differentiation between nervous, muscular or notochordal cells (fig. 44 and 45). In the brain, cells with a moderate amount of cytoplasm formed large islands within the rest of tissue which was composed of more compact cells (fig. 45). Mitosis was seen in this tissue.

In other embryos, nervous structures such as the optic vesicles, were outlined and elongated cells in the brain were more or less perpendicular to a slit-like cavity (fig. 48).

Compact tissue with no brain cavities and absence of any mesenchymal cells contrasted sharply with the tissue of untreated embryos, in which a single layer of dense columnar cells surrounded the well-formed cavities of the brain and where rows of small cells, perpendicular to cavities, formed the bulk of brain tissue; mesenchyme tissue was also present (fig. 43).

In the neck region, where differentiating muscle should be found, only round undifferentiated cells were present. At the lateral margin of the tissue, usually surrounded by a large edemal vesicle (fig. 48), some very dense cells, looking like necrotic blood cells, formed a loose fringe (fig. 49).

In the body region, somite differentiation was outlined by a parallel arrangement of nuclei (fig. 47) and the tail was a big mass of

large undifferentiated cells (fig. 47).

Control embryos showed a marked difference between nervous and muscular tissues. In the somites, cells were already quite elongated and nuclei, still centrally located, were found near the future myotomal septa (fig. 46).

The notochord of the treated embryo, though far from being a clearly defined structure as in the untreated, was the tissue which had differentiated best. It was formed of parallel columnar cells with clear cytoplasm (fig. 50). The notochord of the untreated embryos was well-differentiated into a tube-like structure and was made out of large vesicular cells surrounded by a sheath of small cells.

Histology of 105-hr larvae.

Those larvae, showing minor abnormalities such as curved or reduced tail, had normal mouth, gills, notochord, digestive tract, liver and brain morphology. However, foci of necrotic cells could be found in the caudal part of the neural tube. Muscle was also quite abnormal. Differentiating fibers with central nuclei and no cross striations could have a normal arrangement on one side and be a completely disorganized mass of myoblasts on the other. Fibers were not grouped into bundles, but each fiber appeared to follow a random pattern (fig. 52 and 53). Muscles of untreated larvae had almost completed differentiation: the fibers were long with clear cross striations and elongated peripheral nuclei (fig. 51).

Embryos with major abnormalities had very abnormal tissues. Partial differentiation of nervous tissue accompanied an irregular morphology. Brain cavities could be absent and cells lacked the normal orientation and distribution, while numerous necrotic cells were

present, especially at the lateral margin of the tissue.

In several cases, brain and neural tube were fused into a large irregular mass of poorly differentiated nervous tissue extending from the anterior to the posterior end (fig. 57). A mass of small densely-arranged cells with scattered pigmented cells among them formed the eye which normally was differentiated into a pigmented layer and a sensory layer with three distinct zones.

The notochord usually contorted (fig. 57) and sometimes partially split had attained full differentiation. On the other hand, muscle fibers were not fully differentiated and lacked normal orientation (fig. 53) like the abnormal muscular tissue of embryos with minor abnormalities. Muscular and nervous tissues contained irregular vacuoles (fig. 57 and 59) often filled with blood cells. These blood-filled vacuoles were found mostly in the tail (fig. 59). In some cases, the morphology of muscle and neural tube were so abnormal that the tissues could not be identified. Small cells with pycnotic nuclei and strongly eosinophilic material separated large cells with vacuolar cytoplasm (fig. 54).

Gross abnormalities were also found in the digestive tract. The mouth was small and distorted and the gill arches were absent; undifferentiated tissue replacing the normal epithelium, muscle and cartilage, formed the jaws; the gut was also absent and no liver anlagen was seen.

Distinct thin-walled auricle and thick-walled ventricle, usually filled with blood and bent in the typical fashion, formed the heart of untreated normal larva (fig. 58). Some abnormal embryos did not have a heart but others had a thin tubular heart made of a single

layer of narrow cells (fig. 60). It ran through an extremely large, probably liquid-filled cavity. This cavity was lined by a thin cellular membrane containing groups of highly refractile red cells called "rosette cells". These cells, scattered in normal larvae on the ventral and lateral surfaces of small cardiac vesicle and yolk sac, were situated mainly on the anterior surface of the cardiac vesicle. Very little blood filled the heart but abundant blood cells were found in wide cavities located between the somatopleure and splanchnopleure surrounding the yolk mass (fig. 56). Ventral aorta and blood vessels, numerous in the gill region of normal embryos, were absent.

B. Permeability of the capsule to Phosphamidon.

Table 3.5 gives the results of one experiment comparing the action of Phosphamidon on normal eggs and on eggs whose capsules have been pierced with a glass needle.

TABLE 3.5 Number of dead, abnormal and hatched in normal and pierced Brachydanio rerio eggs exposed to 600 ppm of Phosphamidon for $5\frac{1}{2}$ hr from Stage 12.

Treatment	No. of eggs	D	N	Mi	Ma	H	uH
Normal 600 ppm	20	6	4	5	5	5	15
Pierced 600 ppm	20	7	4	4	5	6	14
Normal Control	10	0	10	0	0	9	1
Pierced Control	10	1	9	0	0	8	2

D: mortality; N: normal; Mi: minor abnormality; Ma: major abnormality; H: hatched at 96 hr; uH: unhatched.

There is no significant difference between the two groups treated with 600 ppm whether the capsules were pierced or not (homogeneity: $p > .40$). Furthermore, during all previous experiments, accidental breakage of capsules in controls or in treated, although not frequent, was not uncommon. It was observed that embryos in broken capsules were no more adversely affected than those in unbroken capsules. Several times, embryos in broken capsules have been seen to survive prolonged exposure, even when exposed during early stages of development.

C. Exposure of zebra fish eggs to Dylox (24 hr from Stage 12 to Stage 21).

Treatment with 100 and 200 ppm had no apparent effect (see Appendix I, p.328). Hatching was normal and identical to the controls. With higher doses (400, 600 and 800 ppm), all embryos were normal at the end of treatment (28 hr) but at a level of 1,000 ppm, 60% of the embryos showed some retardation. Embryos treated with 400 ppm began to show reduction of the tail at 49 hr and a tetanus-like shaking replaced the normal intermittent strong contraction of the body. The tail stopped growing but remained attached under the yolk and became blobby or short and crooked; embryos so affected (45%) did not hatch.

With 600 and 800 ppm, signs of tail abnormalities were already evident at 37 hr in all embryos. With 600 ppm, mortality gradually rose to 50% and only 35% hatching occurred. With 800 ppm, the yolk became glitteringly granular after 49 hr and all embryos were dead by 72 hr. With 1,000 ppm growth was almost normal till the end of treatment (28 hr), but after treatment it slowed down considerably and several embryos died. Mortality reached 100% at 72 hr.

Delayed effects observed were permanent attachment of the tail under the yolk and transformation of the yolk into a bright granular material. These appeared to be characteristic of the action of Dylox.

D. Exposure of zebra fish larvae to Phosphamidon.

Table 3.6 shows the mortality rate of larvae exposed to Phosphamidon.

TABLE 3.6 Mortality percentage in Brachydanio rerio larvae exposed to Phosphamidon. Each group contained 50 larvae.

DOSE (ppm)	TIME (hr)				
	8	16	24	36	48
100	0	0	0	0	10
200	0	0	0	0	50
300	8	30	100	-	-
400	10	50	100	-	-
600	100	-	-	-	-

Periodic observations revealed that death of larvae in each group occurred at almost the same time. For a long period before dying, the larvae had a reduced heart rate, did not have any spontaneous activity and were lying on their sides.

III. DISCUSSION.

Phosphamidon at a concentration of 600 ppm does have a definite lethal and teratogenic effect on zebra fish embryos. Prolonged and short periods of treatment show that, contrary to its action on larvae which is proportional to the dose, the deleterious action on embryos is not proportional to concentration but starts to be highly toxic at a "seuil" concentration. This level is slightly less than the LD_{50} of chloramphenicol (Anderson and Battle, 1966). It is also less toxic than 2-acetylaminofluorene (Hisaoka, 1958) but is more toxic than ethyl carbamate (Battle and Hisaoka, 1952) and barbituric and diethyl barbituric acid (Hisaoka and Hopper, 1957).

Exposure to Phosphamidon during the last 24-hr period of development results in appreciable mortality. Sudden death also occurs if larvae remain in the insecticide after hatching. This higher sensitivity may be explained by the fact that the Phosphamidon was in direct contact with the larvae or that it diffused more readily through the more fragile capsule. The experiment with pierced capsules and observations on embryos with accidentally broken capsules, however, indicate that Phosphamidon can easily reach the embryo at all times. A change in metabolism during the prehatching stage seems more likely to be responsible for this increased sensitivity. Thus for instance, gill breathing is much more important during and after hatching, while body surface and vitelline circulation are the main sources of gaseous exchange during the earlier stages.

The lethal action of Phosphamidon during prolonged exposure either before or during the last stages could possibly be explained by

the fact that Phosphamidon, like other organophosphates, inhibits acetylcholinesterase thus producing muscular paralysis and slower and weaker heartbeat.

The absence of teratogenic effects, when treatment occurs in later stages, is not too surprising since at this time the morphology is already established. However, in several experiments, embryos were exposed to Phosphamidon while tissues were differentiating and apparently Phosphamidon did not interfere with this differentiation, possibly with the exception of muscle.

Important lethal and teratogenic effects result if the early gastrulation period is involved. Several of the abnormalities are similar to abnormalities produced either by other chemical or physical agents. Hisaoka and co-workers (Battle and Hisaoka, 1952; Hisaoka and Hopper, 1957; Hisaoka, 1958) have found that treatment of zebra fish eggs with two carcinogenic substances (Urethan and 2-acetylaminofluorene) and with diethyl barbituric acid produces large edemal pouches in the cardiac region, similar to what is found in other teleosts eggs subjected to acidified water, extremes of temperature and salinity (Stockard, 1907a; Battle, 1929) and to x-ray irradiated frog tadpoles (Rugh, 1950). The absence of heart flexures, a shortening of the body axis, curvature of the tail, and yolk sac distortion are other abnormalities. Lack of nervous system differentiation and retardation of development of the digestive tract were also reported.

However, cyclopia and hyperplasia, which are characteristic of the effects of carcinogenic substances in fish, were not found in my experiments. Furthermore, major abnormalities were almost never found in my experiments if exposure did not include gastrulation. Only

shorter tails or curved tails were found. The first defect is associated with a lack of somite differentiation into muscle while the curved tail is due to an asymmetric differentiation of somites, or would be due, when it occurs just prior to hatching, to a kind of permanent tetanus (Oppenheimer, 1950).

It seems quite normal to find that the gastrulation period is a very susceptible one. Gastrulation has been observed to be a critical period in several cases (Holtfreter and Hamburger, 1955). Furthermore, gastrulation has a biochemical as well as a morphological importance. Protein synthesis in the embryo is enhanced at this stage and the different germ layers are established and almost concomitantly the central nervous system development is initiated, thus any factor affecting this process will result in an abnormal morphology. The description of the development of grossly abnormal embryos has shown that it begins by a kind of exogastrulation and constriction of the yolk sphere by the advancing germ ring. Exogastrulation in frogs, sea urchins, and teleosts results in severe abnormalities (Cohen, 1938; Hall, 1942; Pasteels, 1945; Child, 1940). Since exogastrulation produces abnormal mesoderm distribution, it is not surprising to see that the tail is so much affected.

Some of the abnormalities are strikingly similar to the anomalies produced by chloramphenicol (Anderson and Battle, 1966): failure of the sensory layer of the eye to differentiate, presence of large blood sinuses containing blood cells, absence of blood in the heart, lack of differentiation of myoblasts and their replacement by large cells with vesicular nuclei mixed with strongly acidophilic material (which probably comes from the yolk). Lack of orientation of

myoblasts, lack of organization of myoblasts into myotomes, and especially the formation of strongly abnormal embryos from partially exogastrulated eggs are other similarities. There is, however, one important difference: chloramphenicol produces important abnormalities only if the eggs are exposed to it before gastrulation, while Phosphamidon treatment starting with the onset of gastrulation does induce gross abnormalities. This would indicate that Phosphamidon is a fast-acting agent having its main effect on the cytoplasmic rather than nuclear structures.

Whether or not the abnormal gastrulation process caused by Phosphamidon is associated with inhibition of protein synthesis is uncertain. Organophosphates are not known to inhibit protein synthesis and furthermore, several chemical and physical agents, other than protein inhibitors, can cause abnormal gastrulation producing malformed embryos.

Two different mechanisms of action could explain the teratogenic action of Phosphamidon. It could alter metabolic processes and thus render gastrulation abnormal: abnormalities would result as a direct consequence of the abnormal gastrulation. Abnormal gastrulation could also be a secondary effect while the accumulation of the compound or of some of its breakdown products in the tissues or in the yolk could have at a later stage, after the solution has been replaced by aquarium water, a direct influence on the differentiation of tissues. This hypothesis would be supported by the fact that Dylox was seen to produce numerous major abnormalities which were not preceded by anomalous gastrulation. However, both factors are most probably involved and play a role in the production of anomalies.

Dylox is also lethal and teratogenic to zebra fish embryos, but its action differs from that of Phosphamidon in two ways. First, it is less toxic than Phosphamidon since a 24-hr treatment with 600 ppm produced only 50% mortality while a similar treatment with Phosphamidon produced 80% mortality. Second, Dylox does not produce exogastrulation and the effects of treatment take some time before being apparent. The delayed action may be indicative that the toxic effect is not exerted by Dylox but by its derivative DDVP. The difference of action between Phosphamidon and Dylox may indicate that the toxic action of these compounds is not dependent upon their anticholinesterase properties. Karczmar and Koppanyi (1953) have suggested that DFP had both a cholinergic and a non-cholinergic action. Comparison of the specific anomalies produced by a large number of organophosphates in zebra fish embryos could help elucidate this point.

PLATE 3.1

Main developmental stages of Brachydanio rerio (reproduced from Hisaoka and Battle, 1958, with the permission of Dr. H. I. Battle).

All embryos reared at 26 C. Time intervals are given from fertilization.

Photomicrographs, phase contrast microscope x 42.

Fig. 1. Stage 1 - Recently fertilized ovum.

Fig. 2. Stage 4 - Four-celled; 43 min.

Fig. 3. Stage 9 - Early high blastula; 131 min.

Fig. 4. Stage 12 - Very late blastula, small blastomeres; 4 hr, 7 min.

Fig. 5. Stage 13 - Early gastrula, epiboly initiated; 4 hr, 56 min.

Fig. 6. Stage 15 - Blastoderm enveloping one half of yolk sphere; lateral view; 6 hr, 45 min.

Fig. 7. Stage 16 - Blastoderm enveloping three quarters of yolk sphere; 9 hr, 42 min.

Fig. 8. Stage 17 - Closure of blastopore; 1-2 somites; termination of gastrulation; 10 hr.

Fig. 9. Stage 18 - Optic vesicle; 5 somites; embryo almost encircling the yolk; 14 hr.

Plate 3.1

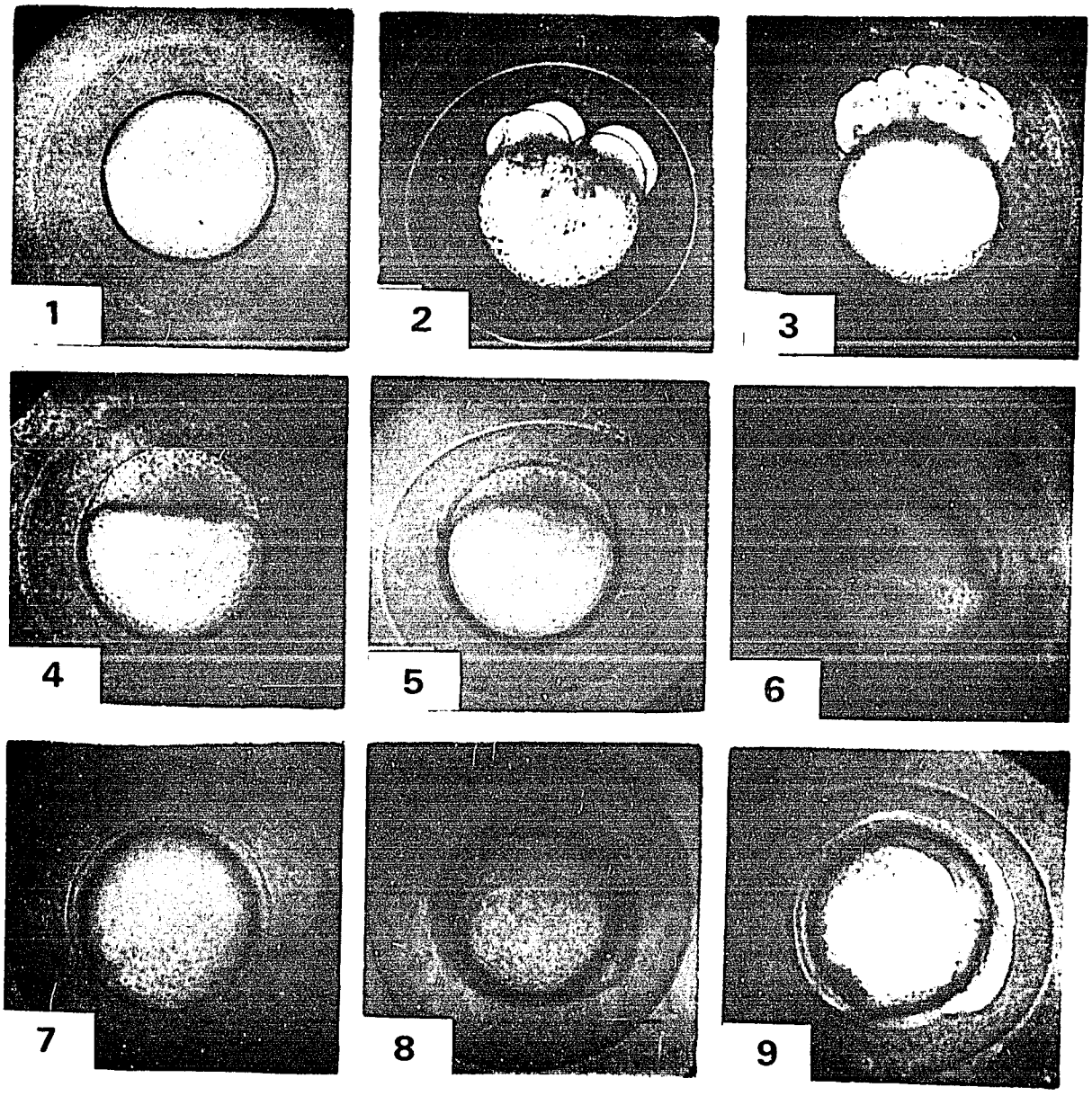


Plate 3.1

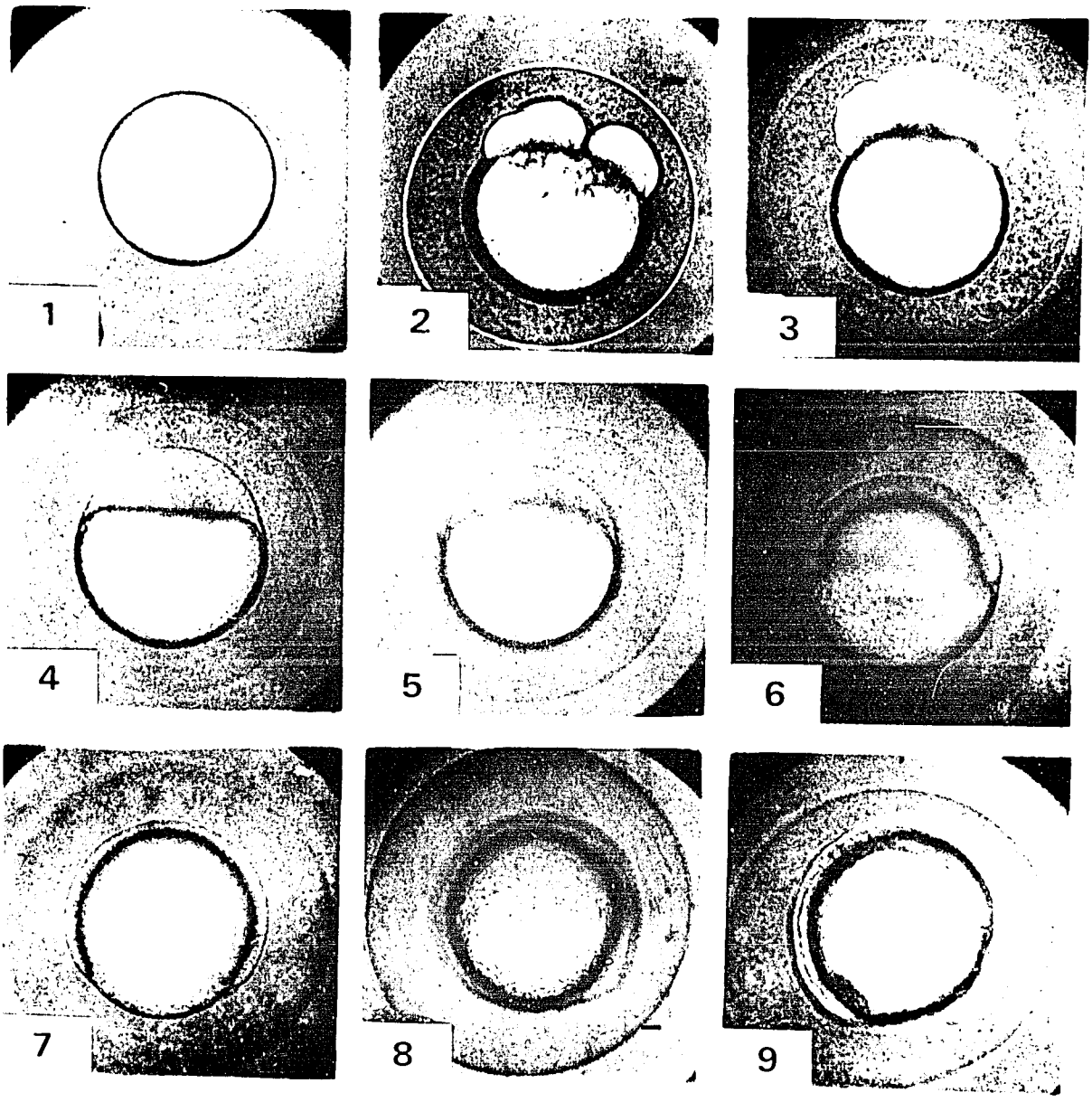


PLATE 3.2

Main developmental stages of Brachydanio rerio (continued) (reproduced from Hisaoka and Battle, 1958).

Fig. 10-13 photomicrographs, phase contrast microscope x 46.

Fig. 14 photomicrograph, light microscope x 20.

Fig. 10. Stage 19 - Auditory placode; 15 somites; tail beginning to be elevated from yolk; 20 hr.

Fig. 11. Stage 20 - Optic cup; 20 somites; characteristic constriction of posterior portion of yolk mass; muscular movements have been initiated; 24 hr.

Fig. 12. Stage 21 - Otolith formation; 30 somites; heartbeat and circulation initiated; 27 hr.

Fig. 13. Stage 22 - Retinal pigment; 32 somites; ventricles of the brain visible; 37 hr.

Fig. 14. Stage 23 - Body pigmentation; 33 somites; stellate melanophores distributed over head and yolk surface; 49 hr.

Fig. 15. Stage 24 - Xantophore development indicated superficially by diffuse yellow coloration; 34 somites; 72 hr.

Plate 3.2

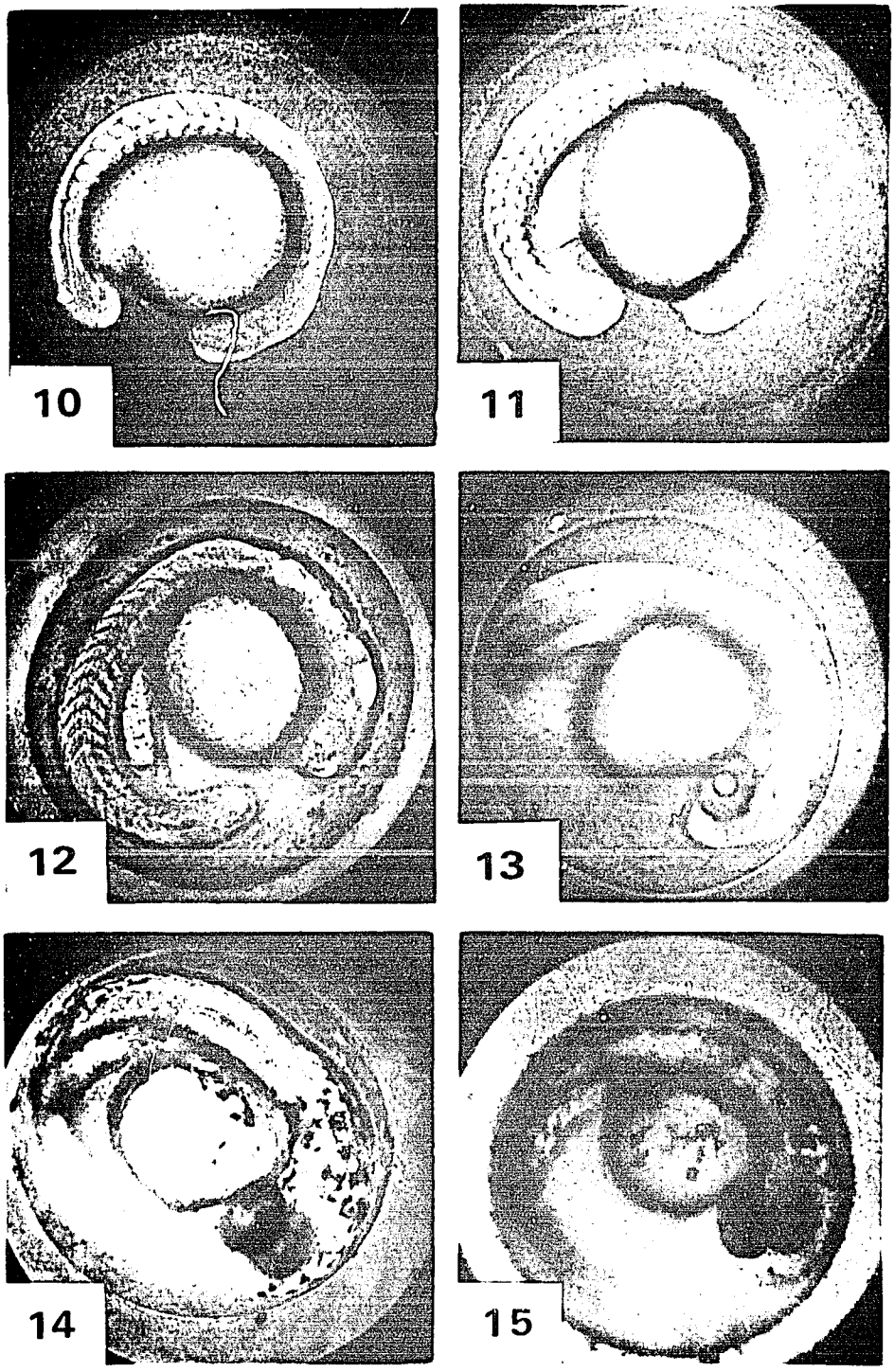


Plate 3.2

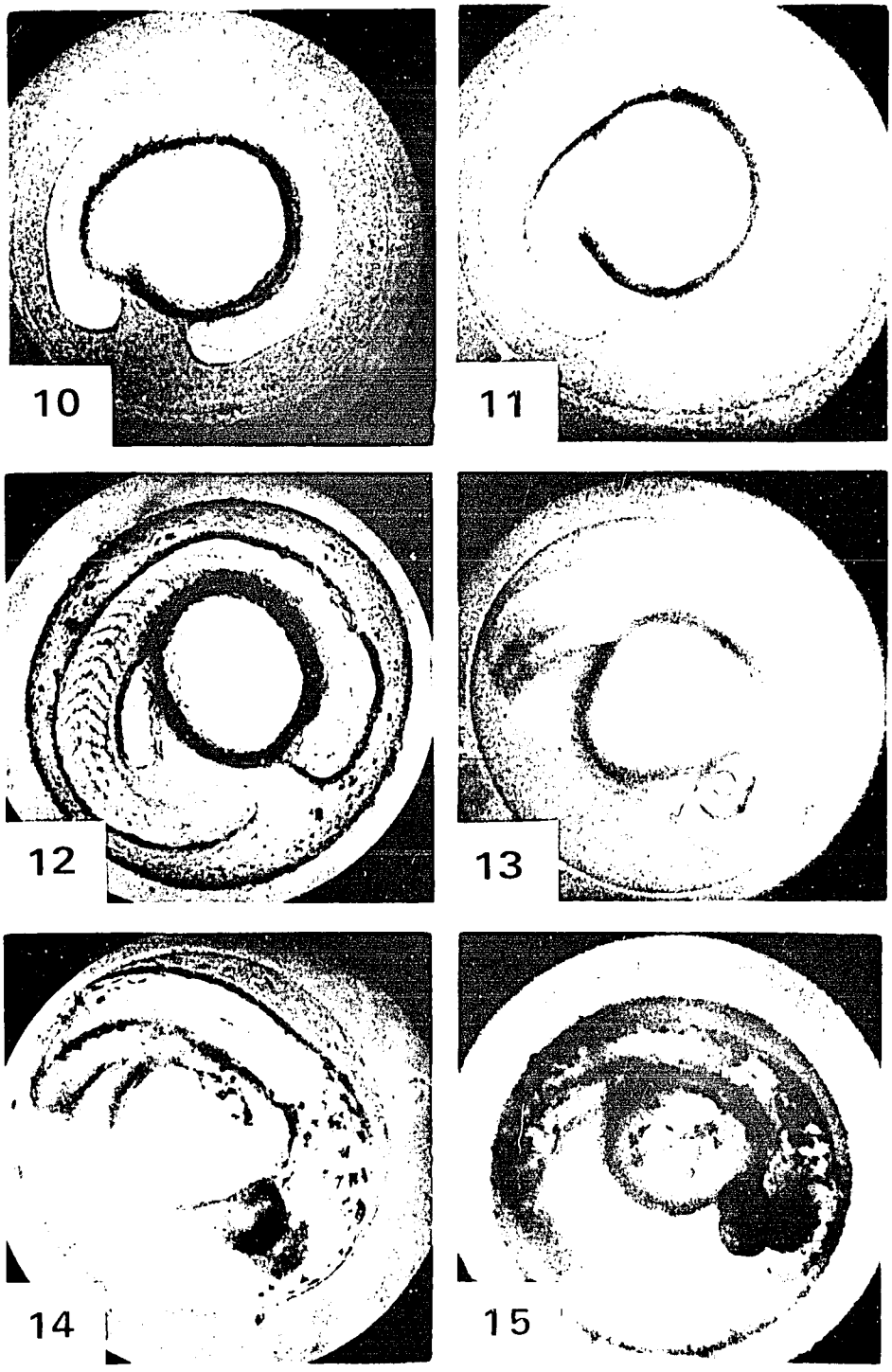
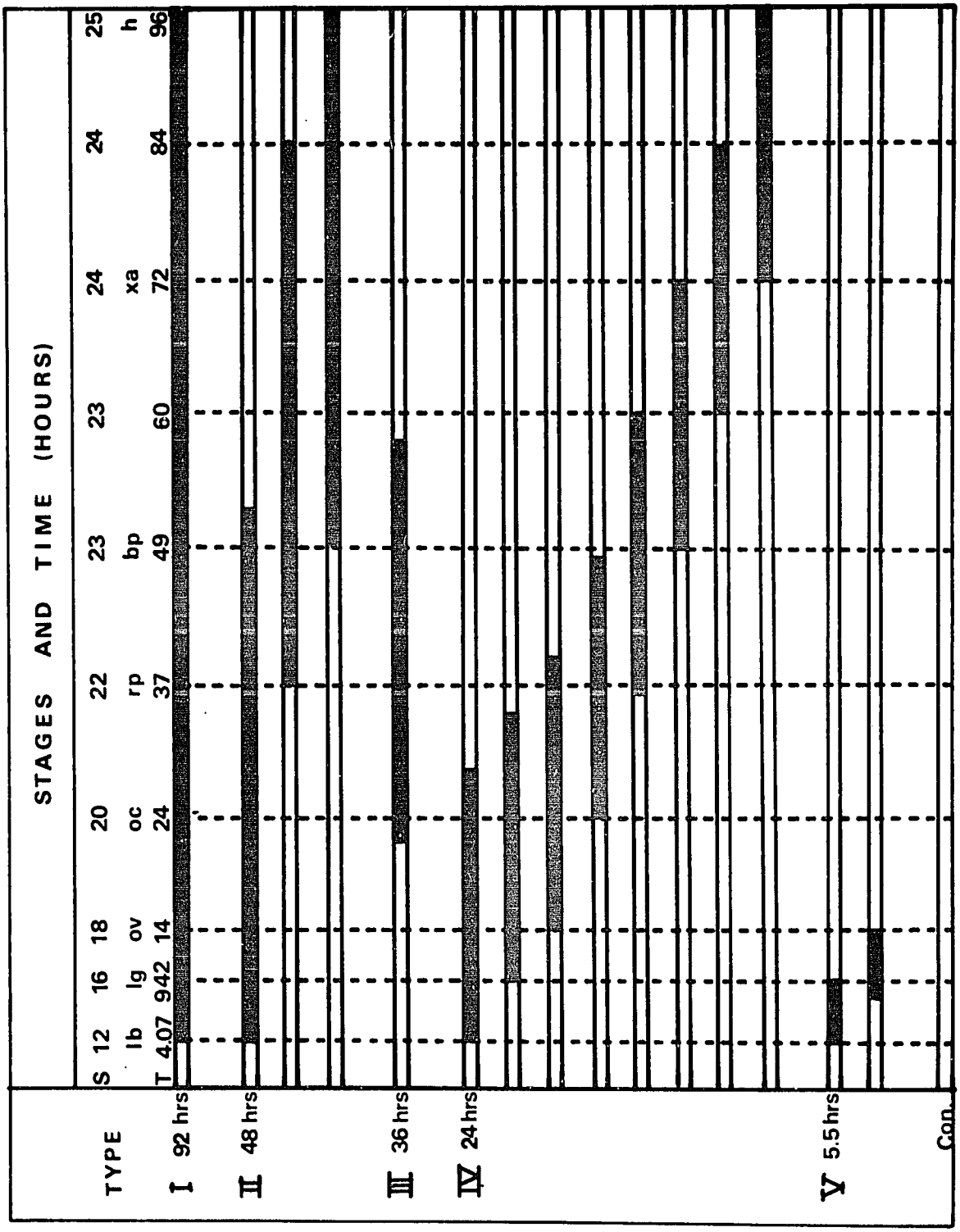


Fig. 17. Types of exposure to Phosphamidon during embryogenesis of Brachydanio rerio. Treatment periods are indicated by dark areas. S: stages; T: time; lb: late blastula; lg: late gastrula; ov: optic vesicle; oc: otic capsule; rp: retinal pigmentation; bp: body pigmentation; xa: xanthophore development; h: hatching; Con: control.


STAGES AND TIME (HOURS)
TYPE
S 12 16 18 20 22 23 24
lb lg ov oc rp bp xa
T 4.07 9 42 14 24 37 49 60 72
23 24 25
h 84 96
I 92 hrs
II 48 hrs
III 36 hrs
IV 24 hrs
V 5.5 hrs
Con.

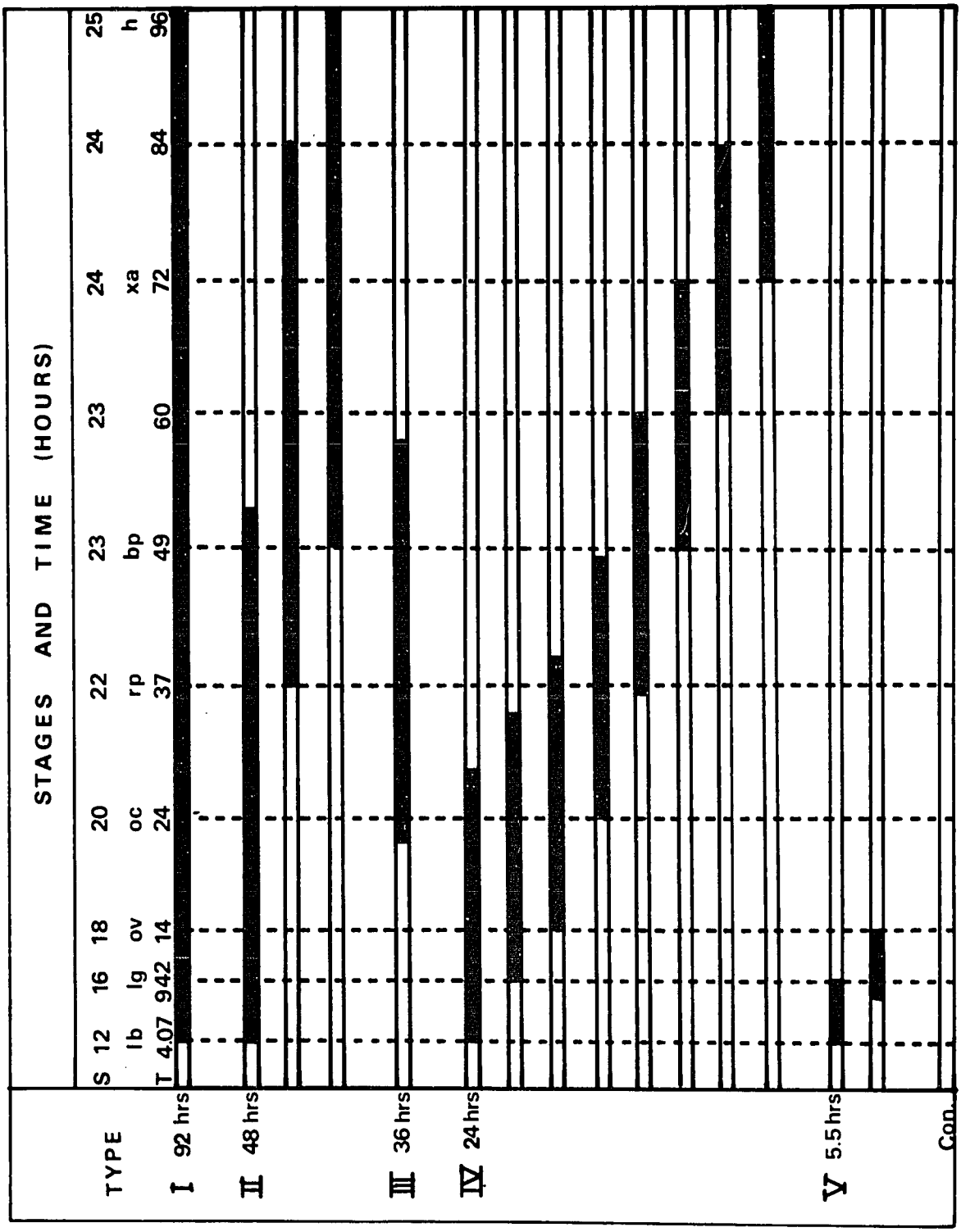

STAGES AND TIME (HOURS)
TYPE
S 12 16 18 20 22 23 24 24 25
lb lg ov oc rp bp xa h
T 4.07 942 14 24 37 49 72 84 96
I 92 hrs
II 48 hrs
III 36 hrs
IV 24 hrs
V 5.5 hrs
Con.

PLATE 3.3

Brachydanio rerio larvae at 105 hr.

Embryos were treated with Phosphamidon.

Fig. 18. Control larva. 25 x.

Fig. 19. Larva with cervical and deformed yolk sac. 600 ppm for 24 hr from Stage 16 (late gastrula). 50 x.

Fig. 20. Larva with normal body but shortened tail. 600 ppm for $5\frac{1}{2}$ hr from Stage 12 (very late blastula). 50 x.

Fig. 21. High magnification of control. n=notochord, oc=otic capsule. 50 x.

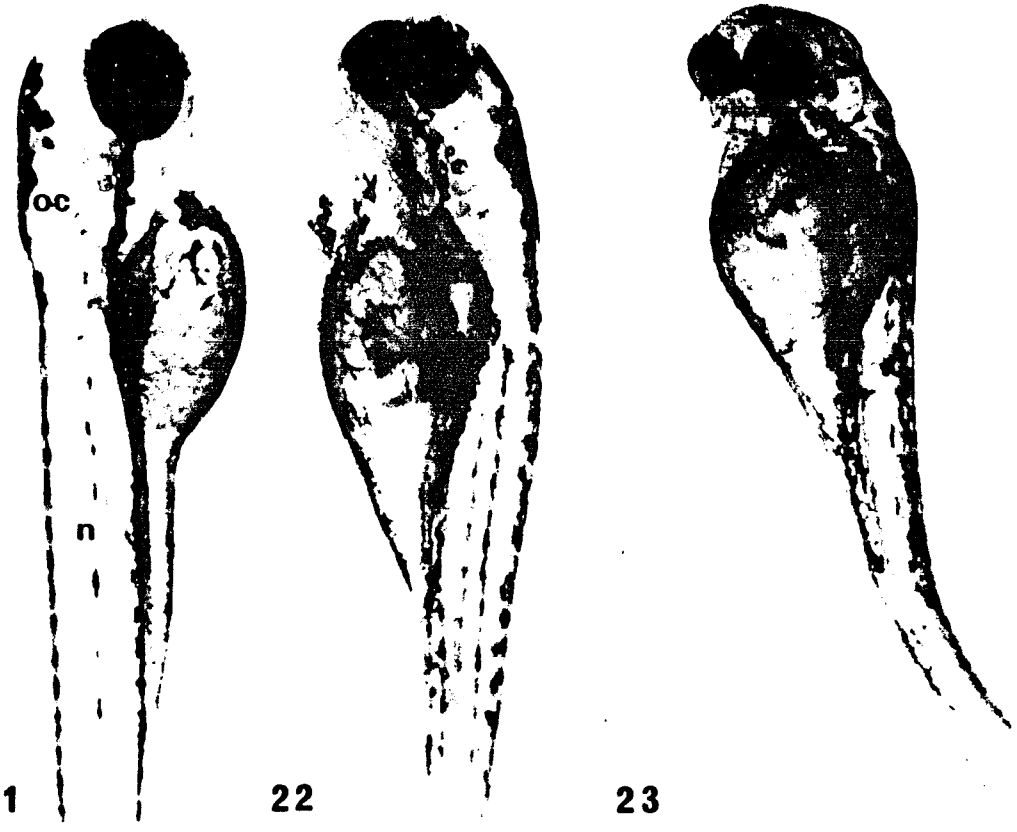
Fig. 22. Larva with normal body but deformed yolk pouch and enlarged cardiac vesicle. 400 ppm for 24 hr from Stage 12 (very late blastula). 50 x.

Fig. 23. Larva with a general reduction of the body and a large yolk pouch. 600 ppm for $5\frac{1}{2}$ hr from Stage 12 (very late blastula). 50 x.

Plate 3.3

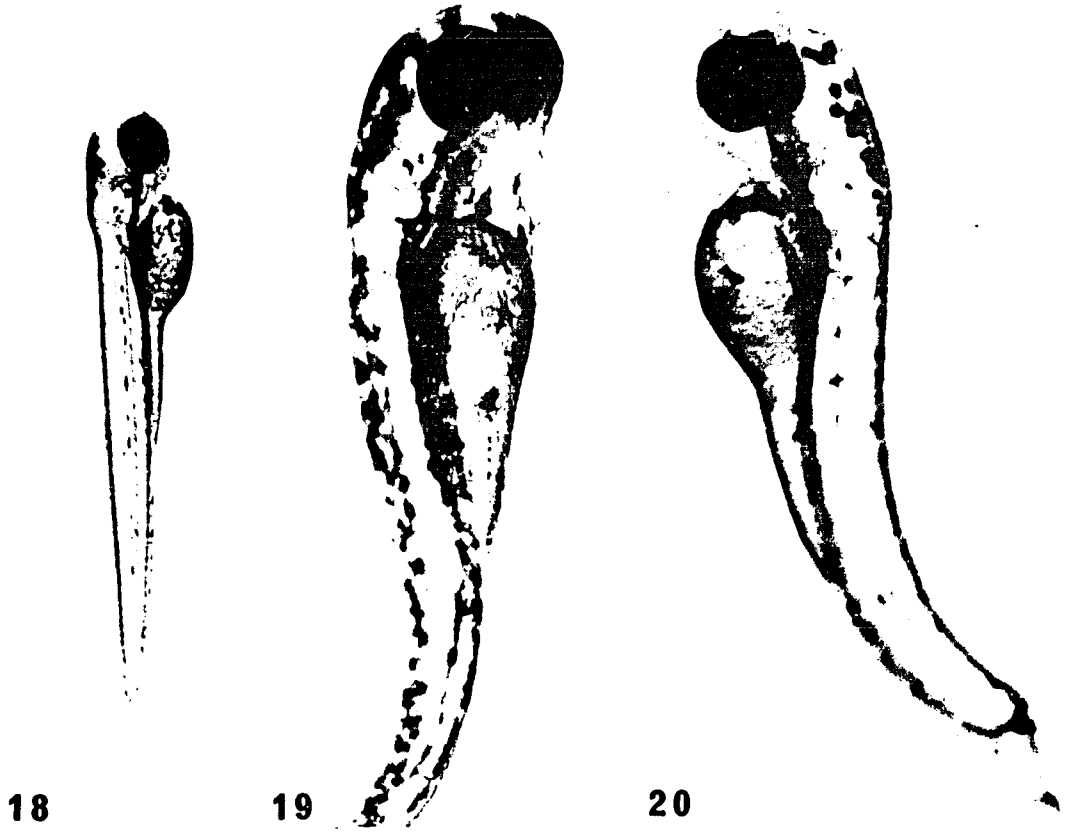


18 19 20

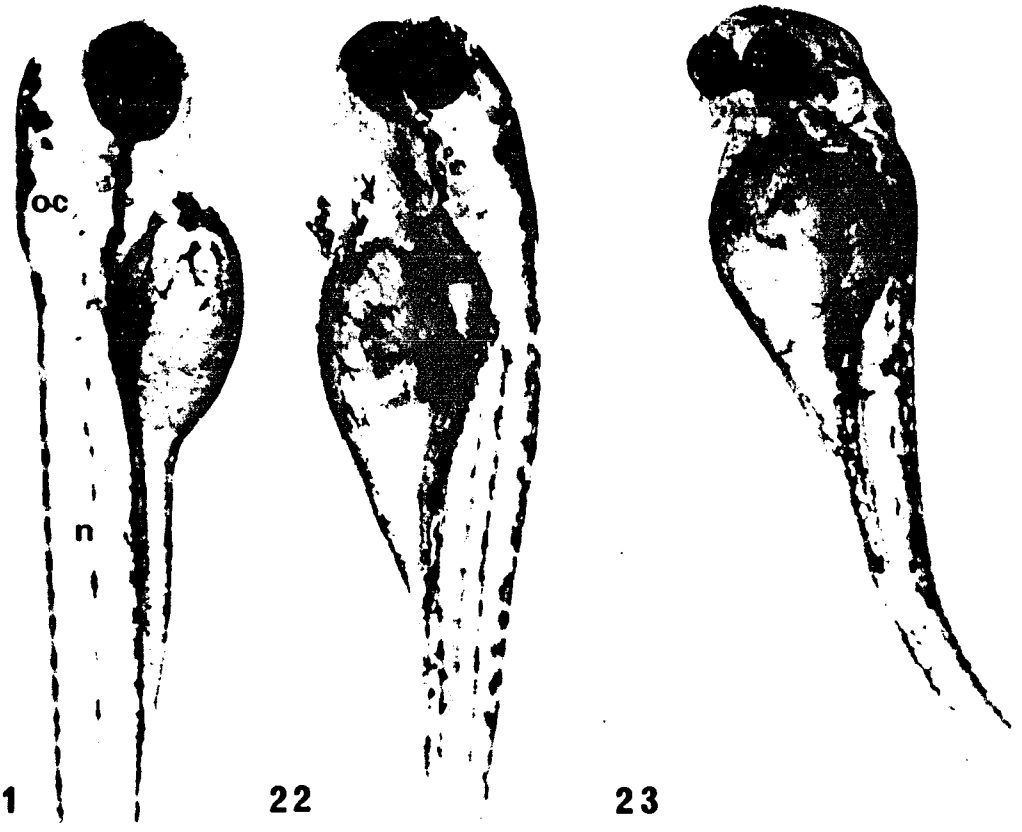


21 22 23

Plate 3.3



18 19 20



21 22 23

PLATE 3.4

Abnormal Brachydanio rerio larvae at 105 hr.

Embryos were treated with Phosphamidon.

Fig. 24 hatched larva.

Fig. 25-31 unhatched larvae, dissected out of capsule.

Photomicrograph magnification: 50 x.

Fig. 24. Hatched larva with normal body size but shortened curved tail (ventral view). 600 ppm for 24 hr from Stage 12.

Fig. 25. Larva with major abnormality: very short attached tail. 600 ppm for 24 hr from Stage 12.

Fig. 26. Larva with short curved tail. Note the unclosed choroid fissure of the right eye. 600 ppm for $5\frac{1}{2}$ hr from Stage 12.

Fig. 27. Larva with very short crooked tail. 600 ppm for $5\frac{1}{2}$ hr from Stage 12.

Fig. 28. Larva with blobby tail and large yolk pouch. 600 ppm for $5\frac{1}{2}$ hr from Stage 12.

Fig. 29. Larva (dorsal view) with no tail and dorso-ventral flattening of the body. 600 ppm for $5\frac{1}{2}$ hr from Stage 12.

Fig. 30. Larva with no tail and with eye abnormality. 600 ppm for $5\frac{1}{2}$ hr from Stage 12.

Fig. 31. Larva with no tail and no eye. Note the tubular heart (arrow). 600 ppm for $5\frac{1}{2}$ hr from Stage 12.

Plate 3.4



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Plate 3.4



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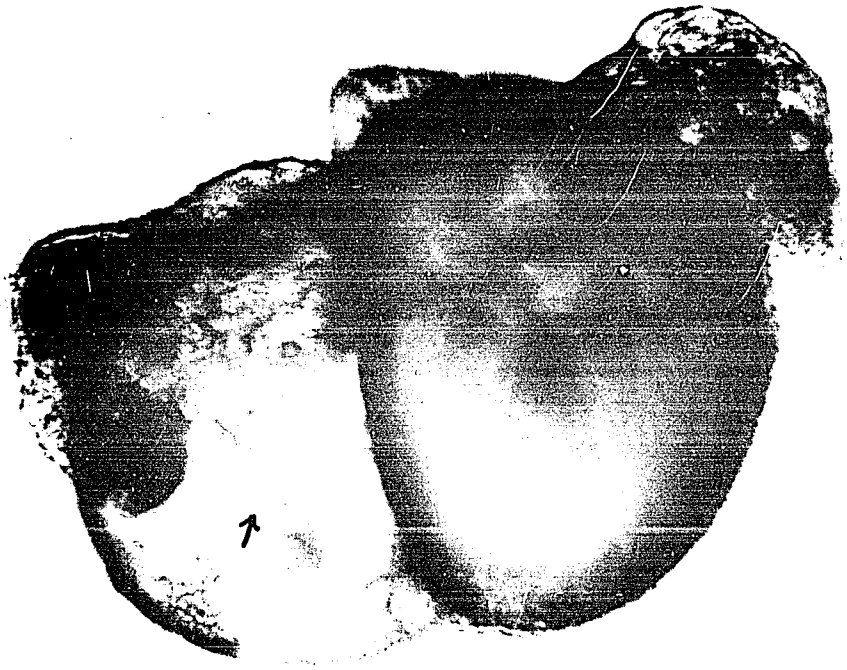


Fig. 32. Unhatched larva with major abnormality at 105 hr. The cardiac vesicle and the yolk mass are of the same size. The tubular heart (arrow) in the cardiac vesicle and the otolith in the otic capsule are evident. Phosphamidon, 600 ppm for $5\frac{1}{2}$ hr from Stage 12. 85 x.

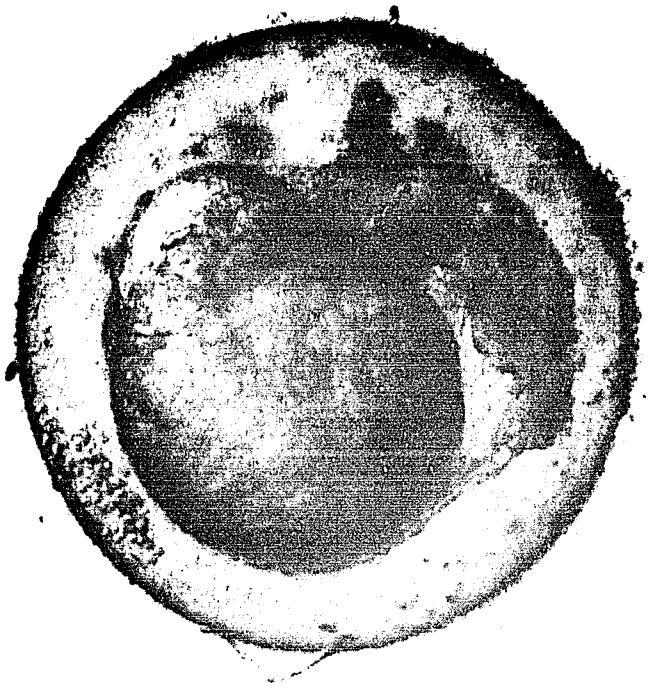


Fig. 33. Unhatched embryo at 96 hr consisting of a disorganized mass of tissue above the yolk. The epiboly around the yolk sphere has not been completed. Phosphamidon, 600 ppm for $5\frac{1}{2}$ hr from Stage

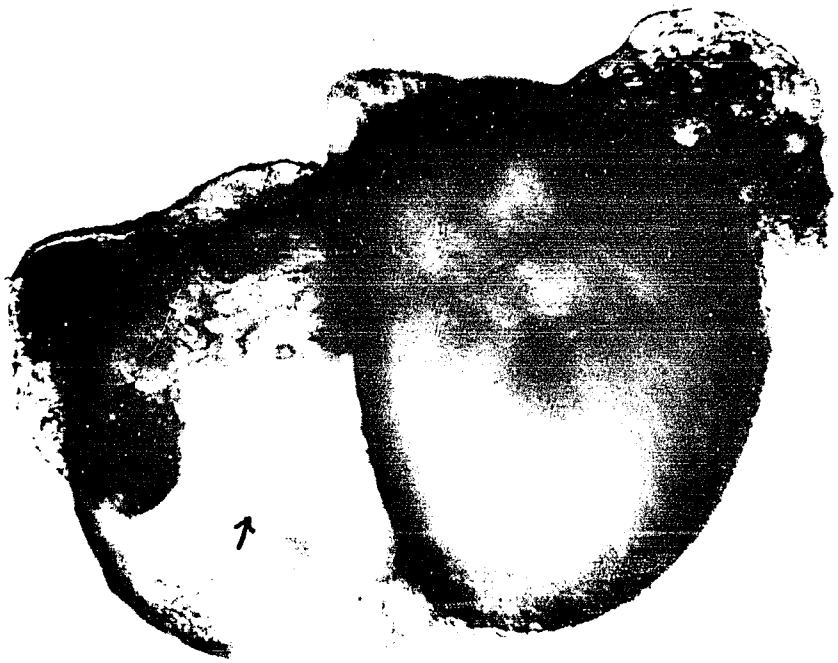


Fig. 32. Unhatched larva with major abnormality at 105 hr. The cardiac vesicle and the yolk mass are of the same size. The tubular heart (arrow) in the cardiac vesicle and the otolith in the otic capsule are evident. Phosphamidon, 600 ppm for $5\frac{1}{2}$ hr from Stage 12. 85 x.

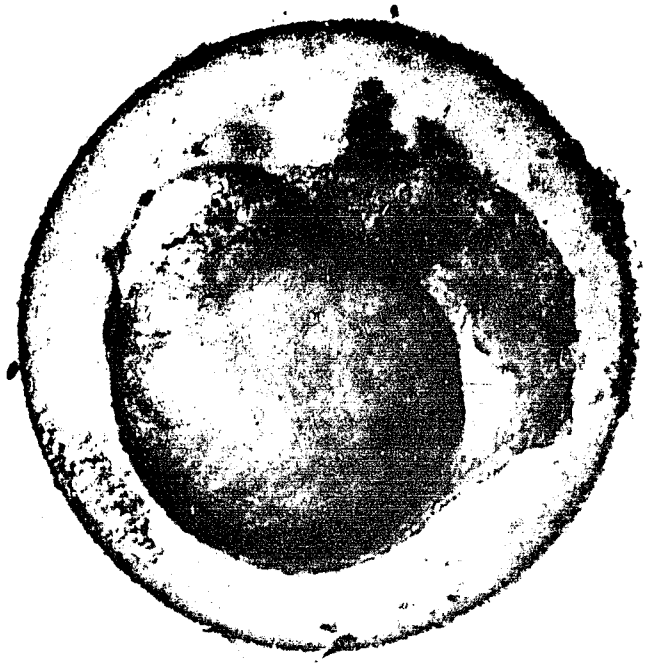


Fig. 33. Unhatched embryo at 96 hr consisting of a disorganized mass of tissue above the yolk. The epiboly around the yolk sphere has not been completed. Phosphamidon, 600 ppm for $5\frac{1}{2}$ hr from Stage

PLATE 3.5

Brachydanio rerio embryos.

All treated embryos were exposed to 600 ppm of Phosphamidon for $5\frac{1}{2}$ hr from Stage 12. Magnification: 50 x.

Fig. 34. Control embryo at 13 hr.

Fig. 35. Treated embryo at 13 hr. The embryo is elongated and the germ ring constricts part of the yolk mass.

Fig. 36. Control embryo at 27 hr.

Fig. 37. Treated embryo at 27 hr. The embryo is abnormal and the blastopore is still open.

Fig. 38. Control embryo at 37 hr. Note the divisions of the brain and the presence of the otic capsule.

Fig. 39. Treated embryo at 37 hr. Blastopore is not yet completely closed and embryonic development is delayed and abnormal.

Fig. 40. Treated embryo at 49 hr. Part of the yolk material is included in the tail.

Fig. 41. Treated embryo at 49 hr; abnormal eye and very short tail.

Fig. 42. Treated embryo at 49 hr with short crooked tail.

Plate 3.5

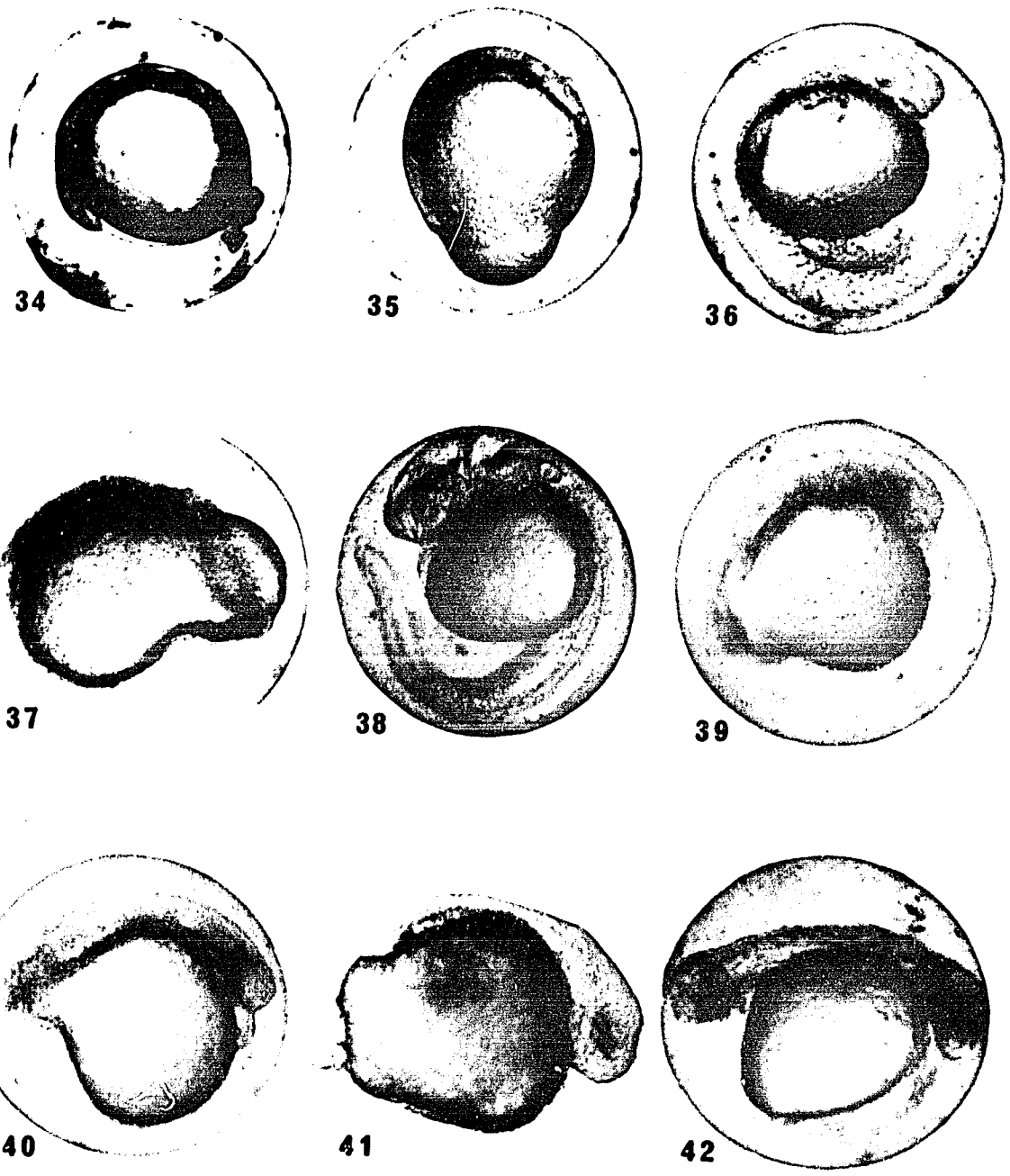


Plate 3.5

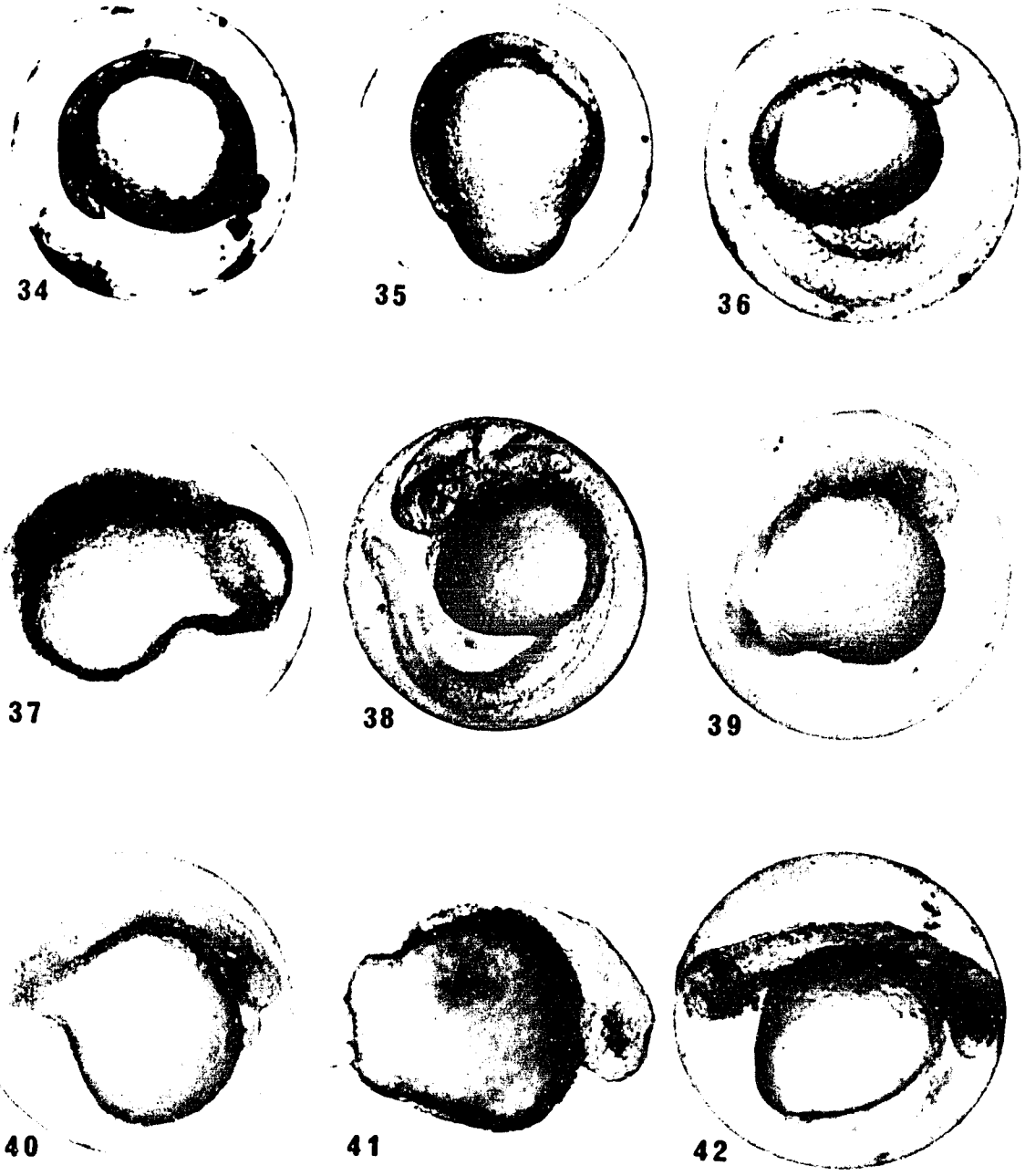


PLATE 3.6

Histology of Brachydanio rerio embryos at 32 hr.

Treated embryos were exposed to 600 ppm of Phosphamidon for $5\frac{1}{2}$ hr from Stage 12 (very late blastula).

Fig. 43. Saggital section through the head of a control embryo. bc: brain cavity; oc: otic capsule. 150 x.

Fig. 44. Saggital section through the head of an abnormal (treated) embryo. Almost no differentiation of tissue. 150 x.

Fig. 45. High magnification of the central area of the head of Figure 44 showing dense cells with pycnotic nuclei and moderate amount of cytoplasm. 600 x.

Fig. 46. Saggital section through the body of a control embryo. N: notochord; NT: neural tube. 600 x.

Fig. 47. Saggital section through the caudal portion of an abnormal (treated) embryo. A little piece of notochord is present in the mass of undifferentiated cells. The huge pale cell with several nuclei is part of the yolk syncitium. 150 x.

Fig. 48. Frontal section through an abnormal (treated) embryo. Some brain differentiation is present. The body is surrounded by a large edematous vesicle. ov: optic vesicle. 150 x.

Fig. 49. High magnification of the central lower portion of Figure 48, showing free dark necrotic cells lining the margin of the body. 600 x.

Fig. 50. Saggital section through an abnormal (treated) embryo showing the partially differentiated notochord and the undifferentiated nervous (above notochord) and muscular tissue (on the left side). 150 x.

Plate 3.6

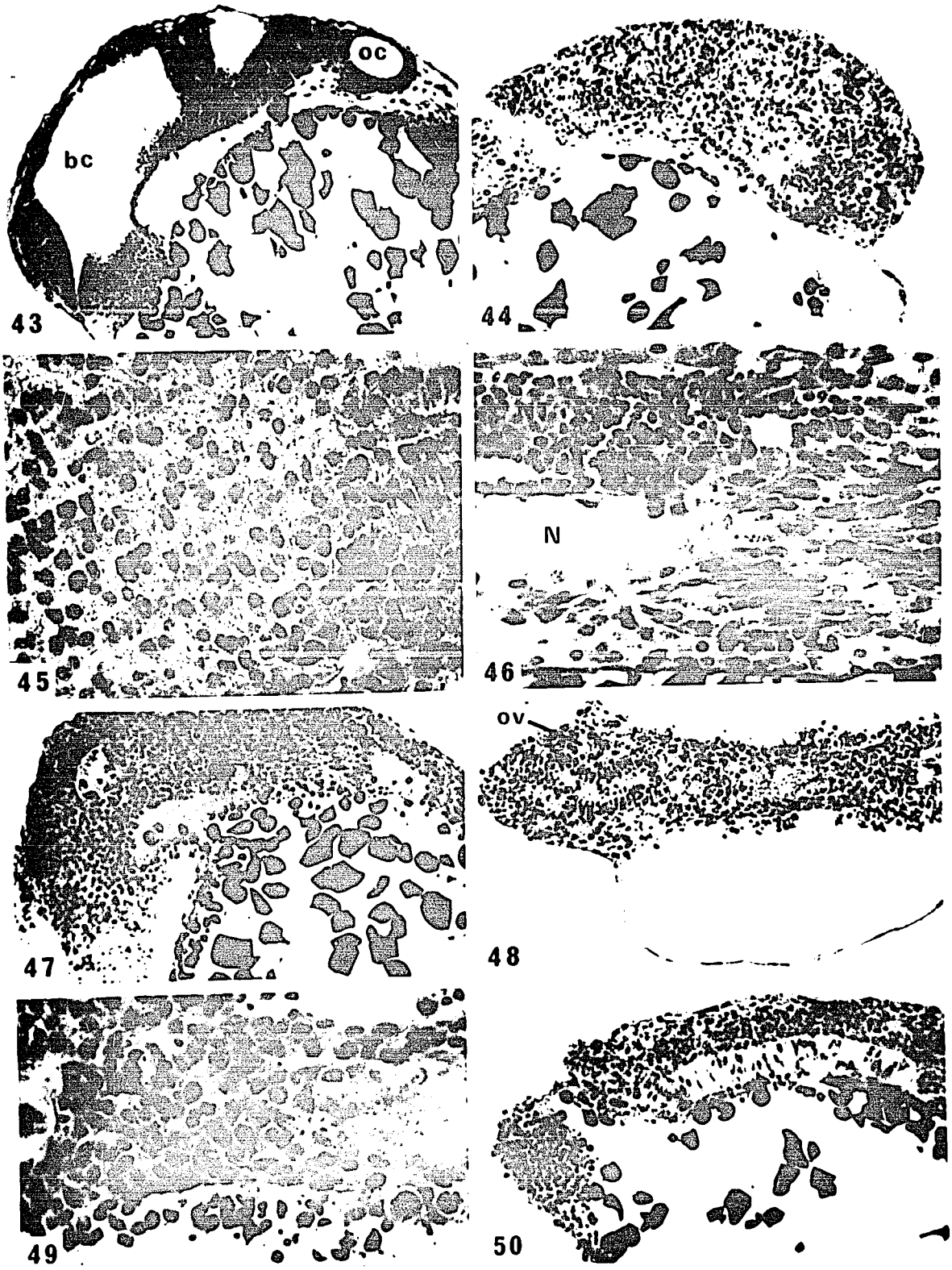


Plate 3.6

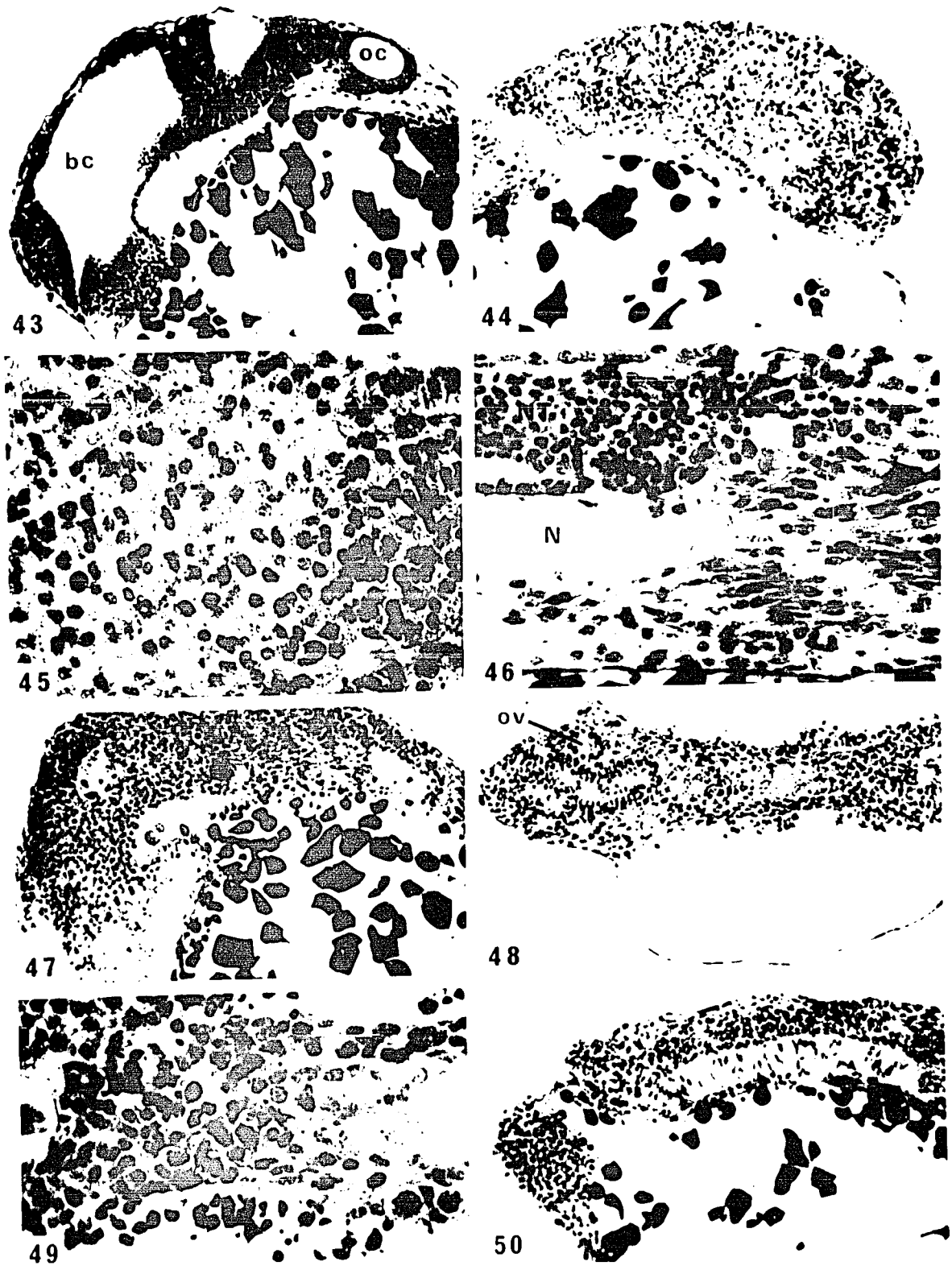


PLATE 3.7

Muscle histology of Brachydanio rerio larvae at 105 hr. Treated embryos were exposed to 600 ppm of Phosphamidon for $5\frac{1}{2}$ hr from Stage 12. 600 x.

Fig. 51. Frontal section through the body muscle of a control larva.

Fig. 52. Saggital section through the right side of the body muscle of a treated larva having a curved tail. Small groups of fibers are oriented in different directions.

Fig. 53. Frontal section through the body muscle of a treated larva with a short crooked tail. Fibers are poorly differentiated and lack proper orientation.

Fig. 54. Frontal section through the body muscle of a treated larva with blobby tail. Nerve muscle and blood cells are mixed together.

Plate 3.7

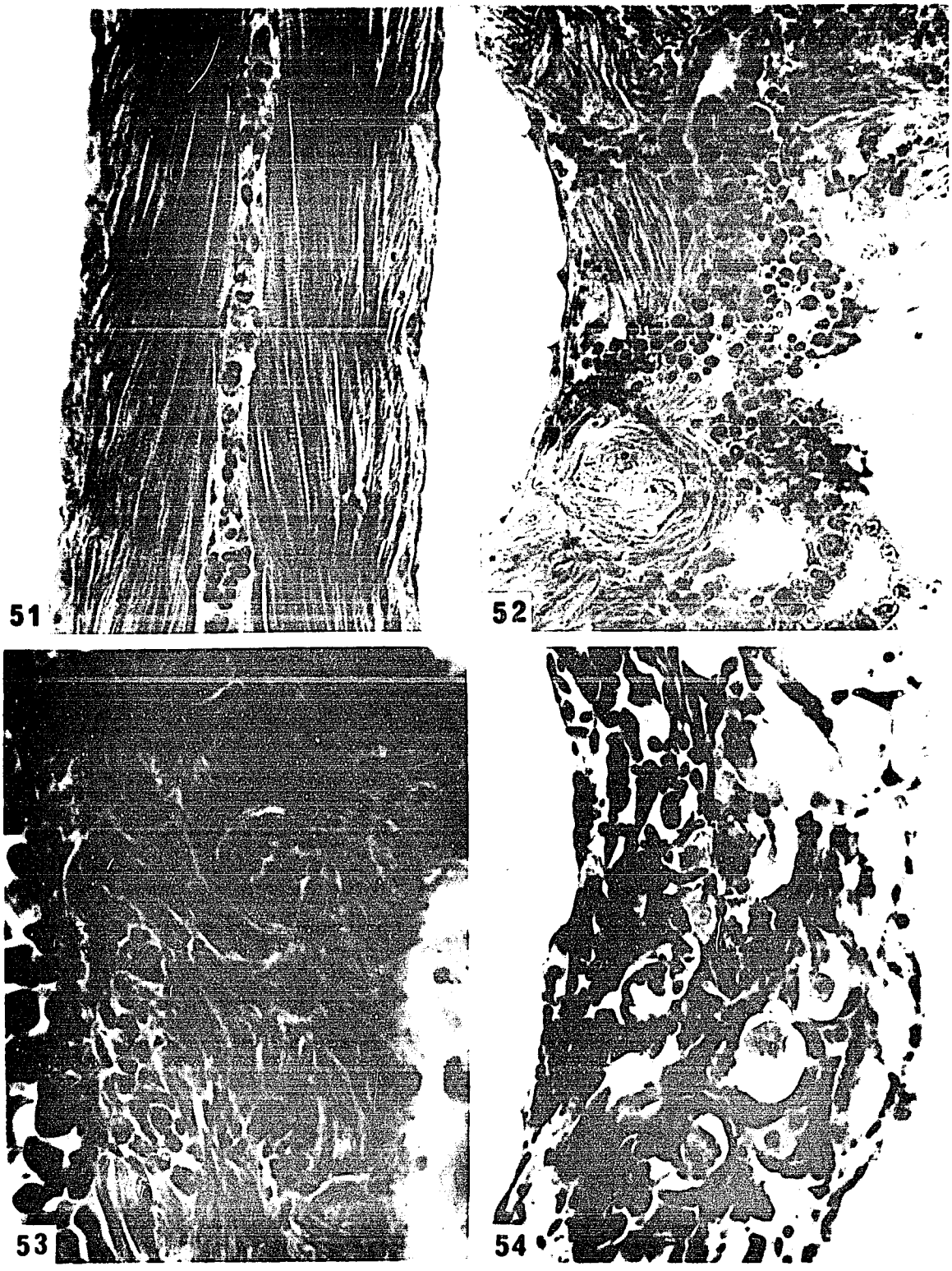
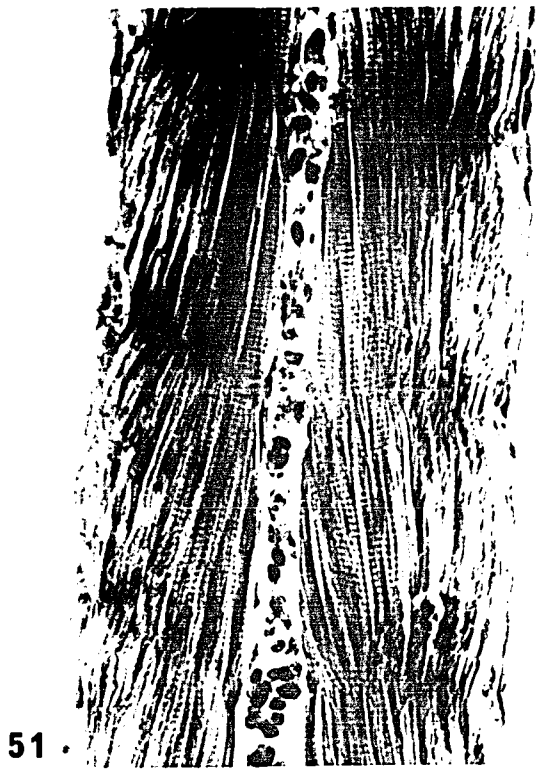


Plate 3.7


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PLATE 3.8

Histology of Brachydanio rerio larvae at 105 hr.

All treated embryos were exposed to 600 ppm of Phosphamidon for $5\frac{1}{2}$ hr from Stage 12.

Fig. 55. Saggital section through the head region of a control larva. B: brain; M: mouth; H: heart; Y: yolk. 94 x.

Fig. 56. Cross section through the body of an abnormal larva. Tissue lateral to the neural tube is replaced by a vesicle containing thin mesenchyme-like cells. Large amount of blood is present in the large vesicle surrounding the yolk. 94 x.

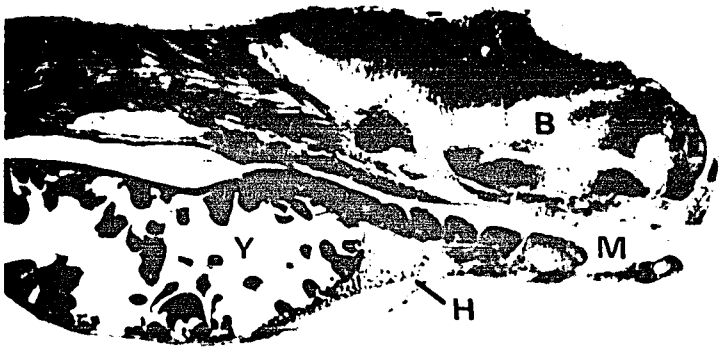
Fig. 57. Saggital section through the head and the body of an abnormal larva. The notochord (N) appears discontinuous because it is contorted. Brain and neural tube are not clearly delineated. The tail contains a blood vesicle (arrow). 94 x.

Fig. 58. Cross section through a control larva showing the heart (H) filled with blood. 150 x.

Fig. 59. Saggital section through the body of an abnormal larva. The brain has some cavities. The tail contains a large blood vesicle and several small vesicles. 94 x.

Fig. 60. Cross section through an abnormal larva showing thin tubular heart (H) within huge cardiac vesicle and "rosette cells" (rc) along the membrane. 150 x.

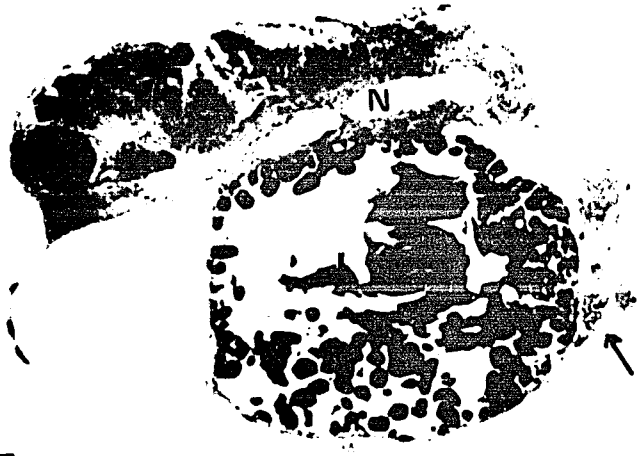
Plate 3.8



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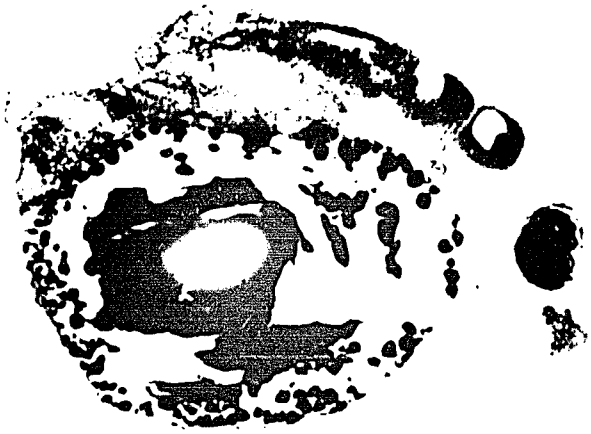
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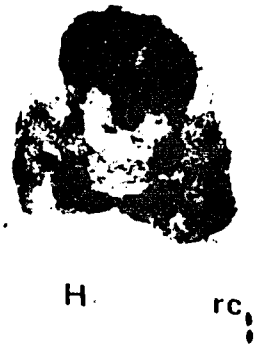
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Plate 3.8



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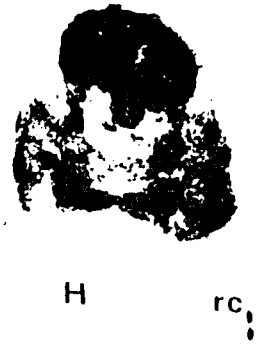
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CHAPTER FOUR

EXPERIMENTS WITH

SALVELINUS FONTINALIS

EMBRYOS

I. EXPERIMENTALS.

A. Normal development.

The normal development of the speckled trout resembles closely the development of other species of trout and salmon described by Henneguy (1888), Battle (1944) and Knight (1963). The main stages in relation to the time schedule followed in our experiments are the following:

Day 0 Recently Fertilized Egg. The germinal disc lay on the upper pole of the yolk sphere and was surrounded by several small oil droplets. During the first 48 hr it underwent repeated cleavage. The average equatorial diameter of fixed eggs was 3.4 mm.

Day 2 Spreading Blastoderm. The diameter of the round blastodisc was 1.5 mm and was composed of several layers of small cells which started to spread over the yolk surface (fig. 1).

Day 4 Embryonic Shield. The blastodisc was slightly oval (long axis: 2.2 mm; width: 2.0 mm) and was showing some regional differentiation. The tissue composing the germ ring and the primordial shield was denser and thicker than the central part of the blastoderm (fig. 2).

Day 5 Neural Keel. The spreading of the blastoderm continued while the primordial shield became narrower and thicker. The axis of the embryo was then visible and extended .85 mm long from the dorsal margin of the blastoderm (fig. 3).

Day 6 Mid Gastrula. By epiboly, the blastoderm had spread until it covered three-fifths of the yolk sphere. The embryonic axis was about 1.8 mm long. The optic anlaga appeared and six to eight somites were

present (fig. 4).

Day 7 Yolk Plug. More than three-fourths of the yolk surface was covered by the growing blastoderm and the length of the embryo averaged 2.5 mm. The optic vesicles were well-formed and 23 somites had differentiated (fig. 5).

Day 8 Closed Blastopore. The embryo was then 3.4 mm long and had 35 somites. The lens of the eye had formed and the optic vesicles had constricted from the brain. The divisions of the brain were evident (fig. 6).

Day 9 Otic Capsule. The optic lobes had started expanding and the otic capsules were clearly defined. The embryo measured 3.8 mm and had 43 somites (fig. 7).

Day 11 Optic Lobes. The head was twice the width of the body and the tail had elevated from the yolk. The cavities of the brain were well-formed and the gut was evident. The pectoral fin buds had appeared. Fifty somites had differentiated (fig. 8).

Day 12.5 Sixty Somites. The embryo covered about one-half of the yolk circumference and was 4.9 mm long. The median fin fold, present since day 9, was well-developed. All organs were present (fig. 9).

Day 14 Rolled Tail. The embryo was 5.5 mm long and lay on the yolk mass in the shape of a J (fig. 10).

Day 18.5 Eye Pigmentation. Considerable growth in width of the embryo had taken place. The head was proportionally very large and the cavities of the brain had expanded considerably. The otolith was evident within the otic capsule. Eye pigmentation, present on day 17, was

well-advanced (fig. 11).

Day 26 Body Pigmentation. The embryo encircled about three-fifths of the yolk. The brain was still high and the fourth ventricle was still clearly seen. Pigmentation in the form of fine grains appeared on the dorsal surface of the head and on the body.

Day 32 Prehatching Embryo. The embryo completely encircled the yolk. The head was dorsally flattened and tissue was more opaque. Pigmentation forming a fine irregular reticulum covered the surface of the head and body (fig. 12).

B. Types of experiments.

During the fall of 1966, 16,000 eggs were separated into 28 lots of 570 eggs each. Twenty-five lots (labelled from A to X) were treated with organophosphate for different lengths of time and at various stages of development with either 30, 100 or 200 ppm of Phosphamidon or with 100 or 200 ppm of Dylox. Results obtained from experiments on zebra fish and preliminary experiments with speckled trout embryos, indicated that these would be suitable concentrations. Three lots were used as controls and were labelled Z. Figure 13 summarizes the various kinds of treatments (p. 147).

During the course of the experiments, dead eggs (white) were counted and removed at 3 days intervals from day 2 to day 20 and also on days 26 and 32. Ten samples in each lot were taken at random each time for morphological studies. This number was reduced to five in the lots where mortality was high, and at 32 days, 28-32 samples were fixed. After careful examination, some of the representative samples were processed for histological examination.

In calculating the total mortality corrections for the samples which had been removed were made. At each day of observation the mortality rate was also computed. This expresses the percentage of embryos that have died out of the number of embryos that were still alive on the last day of counting.

The chi-square method (Juillet, 1968) was used for statistical analysis of total mortality and of mortality rate occurring on the following days: a) the day on which treatment began; b) the day on which treatment ended; c) the day of highest mortality rate (either during or immediately following treatment); d) the twenty-sixth day; e) the thirty-second day. The variables tested were: types of organophosphates, concentrations and periods of exposure.

The effects of the various treatments on hatching were also studied. The embryos alive at day 32 were recorded and considered as 100%. At day 36 the number of hatched, unhatched and dead was counted in each group. The chi-square test was used to compare the different groups when the difference was not self-evident.

The effects of the various treatments on the morphology were also studied. The samples (28-31) taken from the different groups at day 32 were examined and classified into four types: a) prehatching (normal); b) slightly delayed; c) delayed and abnormal; d) small and very abnormal. The differences between the groups which received different treatments were also tested by the chi-square.

In the fall of 1967, experiments similar to those of the fall of 1966, were repeated in order to obtain samples for histochemical studies. Acetylcholinesterase and dehydrogenase activities were tested every day from day 7 to day 17 and thereafter every third day, in embryos of Type 1 and Type 2 (see p. 98 and 99).

The effects of prehatching treatment were also studied by exposing lots of 180-200 embryos to 30, 100, 200 ppm of Phosphamidon or to 100 and 200 ppm of Dylox for six days prior to the expected hatching time of the controls. Death and hatching percentages were recorded on day 0, 3, 5, 7, 11, 16 and 20 after the end of treatment. Measurements of 10 larvae from each group were done on day 7, 11 and 20 following the end of treatment.

II. RESULTS.

A. Effects of treatment on viability.

There was no significant difference in mortality between the control and the different groups on the day on which treatment started.

Both Phosphamidon and Dylox did produce mortality if the embryos were exposed to them during their development, however, the dose level and the stage of exposure were important factors (see Plates 4.2 to 4.14).

i - Phosphamidon.

At a concentration of 30 ppm the effect of Phosphamidon was for the most part negligible. Prolonged treatment (six days) had a slight effect provided the entire process of gastrulation occurred during treatment (B). Although the mortality rate at the end of treatment was in all groups just significantly higher than in the control, it is only in the group B that the total mortality at the end of treatment, at day 26 and day 32, was significantly higher. At day 26 and day 32, the total mortality in the groups A, C, D and E was even slightly lower than in the control (see Appendix II, no. 5, p. 332).

With doses of 100 and 200 ppm, the effect was profound and dependent on both the dose level and the length of exposure if treatment was started at the onset of gastrulation (see Plates 4.4 and 4.7 and Appendix II, no. 6, p. 332). Treatment during the second half of gastrulation only (H) appeared to have a weak effect at a dose level of 100 ppm. If the dose was doubled (M), however, normal development could not occur and mortality was very high. If submitted to a prolonged exposure of 100 and 200 ppm (I, J, N, O) during a later period (from the closed blastopore stage or from the sixty somites stage), the embryos were generally more resistant than they were when treated during the gastrulation period. In the case of embryos treated for six days from the closed blastopore stage, the effect was noticeable mainly at the end of treatment and was maintained for four days following treatment.

ii - Dylox.

Dylox appeared to be less toxic than Phosphamidon especially at a concentration of 100 ppm (see Plates 4.4, 4.5 and 4.10 and Appendix II, no. 6, p. 332). In several cases, however, this difference was not as great as it appeared because the toxic action of Dylox seemed delayed and lasted for a longer period of time. Direct correlation of high mortality percentage with time of treatment was not as clearly defined as it was in the case of Phosphamidon.

The effect of the dose level was more apparent with Dylox. This was particularly noticeable when treatment coincided with gastrulation (P-U, Q-V and R-W; see Plates 4.10, 4.12 and 4.13 and Appendix II, no. 6, p. 332). Contrary to what was found with Phosphamidon, very little mortality occurred during the first three days of gastrulation.

In both the control and treated groups, a sharp rise in mortality occurred on day 32 when the embryos appeared to hatch prematurely. The capsules were broken and the young larvae immerged only half out of the capsule. Further effort by the larvae to extricate themselves resulted in rupture of their yolk sacs, quickly followed by yolk coagulation. In the control, mortality rate which had always been below 8% during previous days, rose to 25%. In most cases, however, where there was previous important mortality due to the treatment, there was also a much higher mortality due to the breakage of the yolk sac (exceptions to this rule are groups F, V, W and the groups treated between days 12.5 and 18.5, J, O, T, Y). The reasons for this sudden important mortality will be discussed later (p. 121).

B. Effects of treatment on hatching.

The percentages of hatching and of mortality were determined at day 36. The number of larvae alive on day 32 was considered as 100%.

A study of Figure 27 shows that hatching is strongly reduced in most of the treated groups. Except for treatment with 30 ppm of Phosphamidon (A, B, C, D and E) and early gastrulation treatment with 100 ppm of Dylox (P), it did not go above 36%. (Although much less important than in the other groups, the reduction in hatching was also significant in groups B and E; see Appendix II, no. 7, p. 333). With Dylox, the reduction of hatching was generally more important than with Phosphamidon, when treatment occurred after completion of gastrulation (see Appendix II, no. 8, p. 334).

Four types of minor tail abnormalities were present in treated larvae. The tail could either be curved dorsally (fig. 28) or

strongly curved sideways so that the embryo was U-shape. Both were more frequent in groups where treatment occurred during gastrulation. In a third type (fig. 30) the tail was slightly shorter, ventrally-curved and appeared to be partially attached to the yolk sphere. The tail could also be rolled on itself (fig. 31). This type which had also been observed in the control to a small extent, was particularly frequent in larvae treated with 30 ppm of Phosphamidon. In all the groups where hatching did not reach 35%, the majority of the larvae had one of these minor tail abnormalities. Normal and abnormal larvae of all treated groups, except for the groups treated during gastrulation with 30 ppm of Phosphamidon (A, B and C), were weaker and less active than the control.

C. Effects of treatment on morphology.

Observations of the samples at day 32 showed that several embryos were abnormal. Embryos have been classified into four types: 1) prehatching (normal); 2) slightly delayed; 3) delayed and abnormal; 4) small and very abnormal.

1) Prehatching (normal) (fig. 32).

The embryos completely surrounded the yolk mass and the body appeared bulky. The head, heavily pigmented, was twice the width of the body and was slightly flattened dorsally. The lower jaw lay on the yolk. The brain tissue was compact with evident thick optic lobes; the olfactory pits were deep; the eyes, darkly pigmented, were slightly oval; the otic vesicles, transparent and somewhat diamond-shaped, lay posteriorly to the optic lobes. The gill bars were thin, long and directed anteriorly and the pectoral fins were thin and leafy. Heavy

pigmentation outlined the division of the strong body musculature into myotomes. Near the tip of the tail, the notochord narrowed and curved dorsally while ventral fin rays were forming as evidenced by pigment deposition. Two canals opened at the anus: the gut and the single pronephric duct which went dorsally and disappeared into the body tissue.

2) Slightly delayed (fig. 33).

These larvae had a shorter and smaller body and had less pigmentation. The smaller head still lay partially on the side.

3) Delayed and abnormal (fig. 34).

The body axis was reduced to two-thirds of normal and was underdeveloped: the muscle mass was reduced. In general, the head and the brain though reduced in size had a normal morphology, however, vacuolar areas or denser patches of tissue in the optic lobes were observed in a few cases. The olfactory pits were of normal proportions. Gills were often distorted and the pectoral fins often absent. About 50% of the embryos had eye abnormalities: smaller size of at least one eye, irregular surface and displaced or extruded lens. The otic vesicles, sometimes distorted, were normal in size when compared to the controls but the reduction in size of other head structures gave them the impression of being abnormally large. The stomach was also disproportionally large for a small embryo and it usually lay beside the body instead of underneath. A final observation was the presence, in 75% of the cases, of a large edematous vesicle lying on the yolk near the head.

4) Small and very abnormal (fig. 35-41).

This type included a variety of abnormalities all of which had in common a very small short body (fig. 36, 37 and 38). Usually

the embryo was almost completely flat (fig. 38 and 39). The flat head contained a dense brain which was small and abnormal. Cavities were absent and only in a few cases could the divisions of the brain be seen. Olfactory pits were absent and very often one eye (fig. 40) and sometimes both (fig. 36), was missing. When both eyes were present they were of unequal size (fig. 35 and 39) and often partially cycloptic (fig. 37). Eyes seemed to be more abnormal in the Dylox treated groups. The lens could be absent or displaced. Sometimes it was seen in the unclosed choroid fissure (fig. 39).

Compared to the small head and body size, the otic vesicles though of control size, appeared to be huge. Their development did not seem to be influenced by the abnormal development of the rest of the embryo (fig. 39 and 41). Irregular invaginations of tissue replaced the gills and the mouth (fig. 40 and 41); the pectoral fins were absent. The body was flat, as wide as the head and lacked the characteristic constriction of the neck. Very little tissue covered the wide and sometimes contorted notochord. Bending of the body into a U-shape was common in the Phosphamidon treated groups (fig. 38). Vacuolization of the body was also frequent (fig. 40). The peppered pigmentation, absent over the brain, did not have the regular myotomal distribution on the trunk. The epaxial mesoderm failed to segment into the typical somite pattern.

In some embryos still alive at day 32 (fig. 36), development appeared to have been stopped at the 9 or 10-day stage.

These four types of embryos occurred in almost all groups, but the frequency was quite variable from one group to the other.

Table 4.1 gives the percentages of each type in the various groups based on a study of 28-31 samples in each group.

TABLE 4.1 Percentages of the four types of Salvelinus fontinalis embryos following treatment with Phosphamidon or Dylox, at day 32 (based on 28-31 samples for each group).

GROUP	TYPE 1 Prehatching	TYPE 2 Slightly delayed	TYPE 3 Delayed and abnormal	TYPE 4 Small and abnormal
Z[1] (control)	70[2]	22	3	5
A	36	39	11	14
B	35	42	13	10
C	44	27	10	18
D	66	24	10	0
E	47	43	7	3
F	3	23	23	50
G	5	18	32	45
H	25	50	3	22
I	37	40	17	7
J	47	30	20	3
K	35	19	29	16
L	3	13	23	60
M	0	9	48	43
N	10	23	33	33
O	3	47	36	13
P	43	37	7	13
Q	37	33	20	10
R	47	44	10	0
S	37	33	23	7
T	7	50	40	3
U	13	30	23	33
V	0	7	43	50
W	10	55	31	3
X	13	40	37	10
Y	0	33	63	3

[1]Letters refer to the different types of treatment as indicated in Figure 13.

Although normal embryos in the groups treated with 30 ppm of Phosphamidon during gastrulation (A, B and C) were significantly fewer than in the control (see Appendix II, no. 9, p. 334), the effect was not very important with this dose. Early gastrulation or total gastrulation treatment with 100 and 200 ppm had a strong deleterious effect producing important delay and severe abnormalities (F, G and K, L). Late gastrulation treatment with 100 ppm (H) slightly delayed development and produced gross teratisms in many embryos. Doubling the dose over the same developmental stages produced drastic teratisms: 90% of the embryos were either considerably delayed or grossly malformed (M). When exposed during later stages (from the closed blastopore stage or from the sixty somites stage), development was mainly delayed and gross abnormalities were not very numerous, except if treatment with 200 ppm started immediately after the closure of the blastopore (N).

On the other hand, embryos were quite resistant to a low concentration of Dylox (100 ppm) during gastrulation (P and Q). These groups were significantly different from the control groups, but did not differ significantly from the groups treated during the same period with 30 ppm of Phosphamidon (see Appendix II, no. 10, p. 335).

Large numbers of Type 3 and Type 4 embryos resulted with early gastrulation, and whole gastrulation treatment at a dose of 200 ppm (U and V). Late gastrulation treatment resulted mainly in a delayed development (W). With later treatments either with 100 or 200 ppm, severe abnormalities were not frequent, but Type 3 embryos were quite numerous. The great majority of the embryos were delayed, especially if treatment started at the sixty somites stage (T and Y).

From these results it appears that Phosphamidon manifested

its most profound effect on development during gastrulation. At developmental stages other than gastrulation, the embryos seemed to be quite resistant to Phosphamidon. On the other hand, Dylox did not approach in severity the effects of Phosphamidon during gastrulation unless the dose was very high (200 ppm) and included the entire gastrulation process. Its effect on development, when given at later stages, was somewhat greater than that observed with Phosphamidon.

D. Mode of formation of abnormalities.

At 20 days of development, forerunners of Type 3 (delayed and abnormal) could be easily identified (fig. 45). The treated embryos were smaller than the controls: they appeared as miniatures but the head was less distinctly set off from the trunk. The brain morphology was near normal except for the size of the cavities. This size differential became more exaggerated as development ensued. Gill arches and pectoral fins were usually poorly differentiated.

During the following 12 days, important growth in length took place while the tissues became thicker and more compact. This growth was parallel to the growth taking place in normal embryos (compare fig. 44 and 45 with fig. 32 and 34).

The situation was quite different in small abnormal embryos (Type 4), which could be foreseen in the early stages of development (day 11 and day 14). Two gross abnormalities were common at this time: failure of the neural tube to close (fig. 52, 54 and 56), resulting in a narrow or flat head with a small compact undifferentiated brain at later stages (fig. 36); failure of differentiation and organization of the tissues of the tail region (fig. 49, 52, 54 and 56) due to incomplete

closure of the blastopore.

Slight increase in length occurred until the 20th day, at which time the embryos had almost attained their maximal length (fig. 46, 47 and 48). However, they continued to increase in width after day 20. One gets the impression that increase in length and thickness was prevented because the embryo had been unable to elevate itself anteriorly and posteriorly from the yolk mass and as a consequence, growth processes forced expansion of the embryo laterally. Measurements of the "heck" and notochord showed that both almost doubled during the 12-day period (between day 20 and day 32). At 20 days, the stomach was proportional to the body size but abnormal disproportional growth thereafter resulted in a cavernous-appearing organ. Myotomes were not clearly defined at 20 days and no improvement occurred later on.

The changes which took place after day 20 occurred mainly between day 20 and day 26, and very little growth occurred after the 26th day. Comparison of Figure 35 and Figure 43 shows that embryos of the 26th day can hardly be distinguished from embryos at day 32. This contrasts sharply with the rapid rate of growth and differentiation taking place in normal embryos during these six days (compare fig. 42 and 32).

E. Histology of delayed and abnormal embryos.

i - Type 1 - prehatching (fig. 58, 65 and 66).

In order to facilitate comparison between normal and abnormal structures, the histology of the different tissues of prehatching embryos (normal) will be described below along with that of abnormal embryos.

ii - Type 2 - slightly delayed (fig. 34).

The tissues of these slightly delayed embryos closely resembled those of prehatching embryos (Type 1) except for the incomplete differentiation of the caudal musculature.

iii - Type 3 - delayed and abnormal (fig. 60, 61, 70, 71 and 74).

Both forebrain and midbrain were abnormal. They were flat and compact: the structures usually appeared squeezed and pushed laterally while the cavities were reduced. The optic lobes which are normally found above the diencephalon were difficult to identify (fig. 60). The ventral portion of the optic lobes are normally made out of acellular white matter (fig. 58). This white matter was almost completely absent in the abnormal embryos. The infundibulum was small and poorly differentiated. Ventral gray matter of the diencephalon and the infundibulum often contained several small necrotic cells.

The posterior portion of the midbrain and the hindbrain had a more normal size and structure. White matter was present and the cells of the gray matter were more or less placed in vertical rows (fig. 61) as in normal embryos (fig. 63). The fourth ventricle, however, was absent and necrotic cells and vacuolization either in the gray or white matter might be present.

The neural tube in some cases appeared normal but in others was squeezed and displaced. The cells lacked the normal elongation and orientation seen in the control (fig. 68). The eye, often ventrally or dorsally displaced, usually had a thinner and only partially differentiated retinal layer which failed to form a true cup. The retina was contorted and sometimes completely surrounded the lens (fig. 60). The optic nerve only developed in those embryos where differentiation of the

retinal layer approached the normal condition. The otic capsule, as previously stated, was fairly normal except for the delayed formation of the cartilage around the different chambers.

The mouth, often displaced to one side (fig. 60), was a thick slit-like opening lined with squamous epithelium. The cartilage of the gill arches was present, but the gill slits had not perforated as they did in control embryos (fig. 65). The digestive tract and liver appeared normal. In the kidney, pronephric ducts were present but the tubules and stroma were poorly differentiated. Muscle differentiation was generally delayed (fig.49-54). The white fibers were shorter (fig. 71) than normal (fig. 72) and the nuclei were still big, round and central. Myofibrils were present but not clearly defined and lacked the typical peripheral distribution (fig. 70 and 77). In the case of the small red fibers found, for instance in the carinalis and ocular muscles, differentiation was even more delayed. Cell boundaries were not evident and only a few myofibrils surrounded the round nuclei (fig. 74 and 76).

iv - Type 4 - small and very abnormal (fig. 54, 62, 64 and 67).

As expected, the tissues were much more abnormal in these embryos.

The epiphysis was always absent while the forebrain was either absent or reduced to a small compact mass of cells in which a small dorsal slit was the only indication of the normal large V-shape cavity of the cerebral lobes.

The structures of the midbrain were hardly recognizable (fig. 59). White matter was entirely absent and the tissue that did develop was in scattered patches of varying compactness. The hindbrain did not appear to be grossly affected (fig. 64 and 66). In Dylox treated

embryos (fig. 64 and 66), the dorsal vertical parallel rows of cells were present but vacuolization was frequent in both the gray and white matter. In those treated with Phosphamidon (fig. 62), this parallel arrangement was lost but vacuolization was not frequent. In embryos which were treated either with Phosphamidon or Dylox, the neural tube was much smaller than normal (fig. 67) and always displaced (fig. 75) or squeezed between the epithelium and the notochord which appeared to be normal (fig. 75 and 67). The white matter was almost always absent, and the cells of the gray matter were fewer in number and lacked the normal orientation.

The eyes were quite abnormal. Usually they consisted of a dense mass of small cells, frequently enclosing a lens. In this case, the regular refractile concentric layer of acellular material was replaced by a substance of irregular density which was sometimes vacuolar. Optic nerves were always absent and when partial cyclopia occurred, fusion involved mainly the posterior portion of the eye. The otic capsule had a normal size with a large cavity, but the cartilaginous walls (fig. 65) were replaced by a thin layer of partially differentiated cartilaginous cells (fig. 66). The infoldings dividing the capsule into three separate chambers were also absent. The epithelium varied from squamous to columnar in different regions as in the control, but the aggregates of columnar cells forming ganglions (fig. 65) were absent. The mouth was displaced and reduced to a narrow slit (fig. 62, 64 and 66), lined by squamous epithelium in which were scattered large mucous cells. The jaws, pharynx, gills and operculum failed to differentiate. Only a mass of undifferentiated small cells in which were found islands of partly differentiated cartilaginous cells formed the

ventral part of the head (fig. 66). No muscles were seen in the head.

The mouth was connected by a narrow opening with the differentiated gut, and the oesophagus, stomach and intestine could be easily identified. The liver was also normal. Cuboidal epithelium formed the two large pronephric ducts which often contained an orange amorphous material. The space between the two ducts, varied in width and could be empty or filled with blood cells. The stroma and tubules were never seen. Pools of deformed blood cells were present in the muscular tissue which was found near the intestine; these were also found near the eyes and even in the optic lobes of the midbrain. No blood was seen in the heart, which was made of two tubes side by side, having thick walls and almost no lumen.

Figure 75 shows the typical structure of muscle cells in grossly abnormal embryos. Normal cells with full differentiation were extremely rare. At best, the muscle resembled muscle of Type 3 embryos and consisted of a mass of cells lacking definite boundaries. They had large round nuclei and granular cytoplasm in which some fibrils could be seen. More frequently however, the cells were more abnormal; they were larger and their swollen nuclei were chromatin poor and had a single peripheral nucleolus. In Phosphamidon treated embryos, the cytoplasm of these cells was homogeneous and opaque. In longitudinal section, some irregular myofibrils were found at the extremities of the cells (fig. 79 and 83). In Dylox treated, the appearance was slightly different: myofibrils were more numerous (fig. 80 and 82) but were present around the nucleus only. In the most severe cases, the cells were extremely large and almost completely void of sarcoplasm or inclusions except for a granular or fibrillar central core.

F. Cholinesterase in trout embryos.

The histochemical appearance of cholinesterase in trout embryos does not appear to have been previously described. Therefore, it was necessary to trace its appearance in normal embryos prior to studying the effect of any treatment.

i - Ontogenesis of cholinesterase.

ChE was detectable for the first time in a few restricted areas of day 12 embryos. At the level of the otic capsules, the hind-brain is a thick crescent-shape structure over which a large cavity is present (fig. 84). Near the medio-ventral edge, two or three cells on each side stained lightly. As we proceed caudally the reaction was found in the more lateral portions (fig. 85). It occupied the same position but in a discontinuous pattern in the neural tube (fig. 85). The reaction was more intense indicating a greater concentration than in the brain. A medium reaction was also present in the myotomes along a vertical line which at later stages of development became the myotomal septum. The margin of oval or globoid cells along this line also gave a medium reaction. This reaction was also periodically interrupted. The third area where cholinesterase was present was the heart which consisted of a tube lined with loose cells. Some of the cells next to the lumen gave a slight reaction (fig. 84).

The intensity of the reaction increased in these areas and especially in the ventral myotomes (fig. 87) until the 15th day, at which time a weak reaction was also found in two small ventro-medial areas of the posterior part of the optic lobes (fig. 88). In the myotomes, the reaction was no longer restricted to a vertical line but had spread to involve more cells (fig. 89). Figure 91 shows

that the strong reaction in the cells of the neural tube occurred in the cytoplasm and in the cytoplasmic extensions. In the heart which then had a much wider lumen, the reaction was restricted to the thin internal lining (fig. 90). At 19 days, cellular differentiation in the midbrain was more advanced. The cells of the optic lobes and of the diencephalon (fig. 95) were compact but slightly elongated and oriented toward the lumen. In the ventro-mediad portion of the optic lobes, above the crescent shape acellular portion, there was a group of cells with no specific orientation which were positive for cholinesterase (fig. 95). Serial sections showed that they formed two longitudinal rods in the floor of the midbrain, which extended posteriorly to the level of the posterior margin of the eye.

In the hindbrain, at the level of the otic capsules, the reaction was limited to two or three separate cells on each side at the ventral margin of the cellular portion. In the region where the medulla becomes narrower and heart-shape, the reaction was then localized in several cells of the dorsal part. In the neural tube, the reaction was found at the ventro-lateral margin (fig. 93). The reaction in the myotomal septa was also strong. Many of the muscle cells near the septa had a dark margin but no true end-plates were found.

At 26 days, the two longitudinal rods of unoriented cells in the floor of the midbrain then extended to the anterior part of the midbrain, where the optic lobes above the main stem formed a mushroom-like structure (fig. 98). The reaction was then localized centro-mediad below the cavity of the midbrain. The ventral-most cells also gave a weak reaction (fig. 100). Layer differentiation had taken place in the eyes but no ChE was present yet. The ocular muscles were well

differentiated and the reaction was quite strong (fig. 99 and 101). The exterior margins of the peripheral cells were darkly stained (fig. 96). In the posterior region of the midbrain, the reaction was very strong at the base of the optic lobes (fig. 101). The two longitudinal parallel rods eventually fused and formed a U-shape area staining strongly (fig. 102). In the hindbrain, two or three groups of a few cells reacted strongly in the ventral and dorsal horns. At the junction of the medulla and the neural tube the reaction was stronger and involved more cells.

In short: ChE appeared first in the ventro-lateral part of hindbrain and neural tube, and in the future myotomal septa. It eventually developed in the midbrain in two longitudinal rods of cells while in the hindbrain and neural tube, the reacting cells are arranged in groups situated more mediad.

ii - Effect of treatment on cholinesterase.

Table 4.2 shows the cholinesterase percentage activity estimated from histochemical reactions. Phosphamidon did produce an important reduction of activity even if treatment occurred during a time at which cholinesterase could not be detected. The extent of inhibition was dependent upon the dose level and upon the period of treatment. Treatment during stages following gastrulation (from the closed blastopore stage, 8-14) produced more important inhibition.

Noticeable recovery of ChE activity took place in the embryos during the six-day period between day 26 and day 32. But the activity still remained much below the level found in the control. Dylox differed from Phosphamidon in its inhibitory effect in two ways. First, the inhibition observed at day 26 and day 32 was much less important.

Second, treatment during the second part of gastrulation (5-8) produced a stronger inhibition than treatment occurring from the closed blastopore stage (the reverse situation was true with Phosphamidon).

TABLE 4.2 Estimated percentages of cholinesterase activity at day 26 and day 32 in Salvelinus fontinalis embryos treated with Phosphamidon or Dylox.

Day of Treatment	Dose	ChE Percentage Activity			
		Phosphamidon		Dylox	
		Day 26	Day 32	Day 26	Day 32
2-5	30	50	80	-	-
	100	35	70	80	90
	200	35	50	80	90
5-8	30	40	60	-	-
	100	25	40	65	70
	200	20	40	65	70
8-14	30	35	50	-	-
	100	20	35	80	80
	200	20	25	65	60

G. Malic dehydrogenase in trout embryos.

i - Ontogenesis of the enzyme.

At 12 days, the reaction was weak and uniform except in three regions: a) the latero-ventral acellular region of the brain: in this area, the reaction appeared more diffuse because of the extremely small grains; b) the group of cells of the hindbrain which were positive for ChE (fig. 85); c) the cells along the future myotomal septa (fig. 86).

At 15 days, the distribution was similar, however, two regions of the midbrain gave a slightly stronger reaction: the dorso-lateral portion of the diencephalon and the ventral region of the floor of the optic lobes (fig. 92).

At 19 days, the latter region which was acellular, was much stronger. The cellular portion above was also stronger than the rest of the tissue (fig. 94). Figure 97 shows the MDH distribution in a section at the level of the posterior portion of the medulla. Some areas reacted much stronger than others: the acellular ventro-lateral margin of the medulla, the lateral margin of muscle (future cutaneous muscle fibers), the pronephric ducts, the epithelium of the gut, and the heart. The areas of strong ChE activity had also a strong MDH reaction.

Embryos at 26 days had somewhat the same MDH distribution. Structures such as ocular and cutaneous muscles, kidney, heart and epithelium of the gut were strongly positive (these structures were well differentiated at this stage and are among the structures which in the larval stages gave a definitely stronger reaction). The reaction was also somewhat stronger in the acellular portions of the brain and in the same large cells of the neural tube which were rich in ChE.

ii - Effect of treatment on malic dehydrogenase.

Far from being inhibited, the reaction appeared slightly stronger in Type 1 and Type 2 embryos which had been treated with high doses of organophosphate (100 and 200 ppm). No peculiarity was noted in the distribution and in the shape of the formazan deposits in these embryos.

H. Prehatching experiment.

i - Prehatching mortality.

There was no mortality of embryos, either in the control or in the treated during this period of treatment. Later on, a 2-5% mortality occurred in eggs of both the control and treated groups except for the group treated with 200 ppm of Dylox where mortality reached 8% (non-significant difference, see Appendix II, no. 11, p. 335).

ii - Hatching.

In the control hatching started on the last day of treatment. However, most of the embryos hatched between the 5th and the 11th day following treatment. As seen from Table 4.3, treatment had an effect on hatching since it occurred sooner in the treated than in the control. In the control, 50% hatching would have been obtained on the 8th day, while in the embryos treated with either Phosphamidon or Dylox the same percentage would have been generally obtained as early as the second day following the end of the treatment. High hatching percentages (90%) were obtained sooner with a high dose treatment of Phosphamidon than with Dylox.

TABLE 4.3 Hatching percentages in Salvelinus fontinalis embryos treated with Phosphamidon and Dylox during six days before hatching.

TREATMENT	DOSE (ppm)	DAYS AFTER THE END OF TREATMENT						
		0	3	5	7	11	16	20
Control		1	5	8	40	92	96	96
Phosphamidon	30	30	46	55	74	87	96	96
Phosphamidon	100	40	68	80	92	94	96	97
Phosphamidon	200	37	67	83	95	98	98	98
Dylox	100	41	69	69	75	87	95	95
Dylox	200	35	66	75	77	85	92	92

iii - Behaviour and mortality of hatched larvae.

Control.

When the capsule of unhatched eggs was artificially opened, control embryos immediately unravelled their tails and exhibited good activity. Quick contraction of the body and tail slashing were frequent. The heartbeat was 60/min.

Treated.

In the treated groups, when the capsule was opened, the tail remained partially rolled around the yolk and the embryos showed very little activity. The heartbeat was only 35-40/min. After spontaneous hatching, the embryos had a similar behaviour during the first few days. After three days of recovery, the heartbeat of treated larvae was only 42-47/min. There was, however, faster and stronger movements of the gill apparatus. It was only after five days of recovery that hatched larvae manifested a normal behaviour.

Larvae that hatched before the end of the treatment and were thus left in organophosphate for a period of 2 to 24 hr, were not killed by the organophosphate. Mortality of larvae started to occur only on the 13th day. In the control, mortality reached 13% on the 20th day; similar percentages occurred in larvae treated with Dylox. With high doses of Phosphamidon (100 and 200 ppm), mortality reached 30% ($p < .01$) and 21% ($p < .05$) (see Appendix II, no. 12, p. 335).

iv - Morphology.

Larvae had a normal appearance, except that they seemed slightly shorter. Table 4.4 gives the length measurements of 10 larvae from each lot after 7, 11 and 20 days of recovery.

TABLE 4.4 Average length (millimeters) of Salvelinus fontinalis larvae hatched from embryos treated before hatching.

TREATMENT	DOSE (ppm)	DAYS AFTER THE END OF TREATMENT		
		7	11	20
Control	0	11.8 (± .17)@	13.7 (± .57)	16.0 (± .33)
Phosphamidon	30	10.4 (± .28)*	13.5 (± .53)	15.6 (± .30)
Phosphamidon	100	10.7 (± .13)*	12.9 (± .33)	15.3 (± .49)
Phosphamidon	200	10.5 (± .24)*	12.9 (± .25)	15.1 (± .41)
Dylox	100	10.5 (± .45)*	13.4 (± .26)	15.2 (± .55)
Dylox	200	10.1 (± .24)*	13.8 (± .33)	15.2 (± .23)

@ Standard deviation.
* Significant difference from control.

Statistical analysis (t-test) showed that the difference was significant ($p < 0.05$) in all groups on the 7th day. Although treated larvae were always shorter than the control at days 11 and 20, the difference for each group was not significant. Even if at 20 days the treated larvae were still shorter than the controls, the growth that took place between day 7 and day 20 was greater in the treated groups than in the control group as seen by the increase in length (mm) between day 7 and day 20.

Control		4.2
Phosphamidon	30 ppm	5.2
Phosphamidon	100 ppm	4.6
Phosphamidon	200 ppm	4.6
Dylox	100 ppm	4.7
Dylox	200 ppm	5.1

Histological studies showed that at the end of treatment the tissues were normal, with the possible exception of the masseter muscle in which the fibers were paler and hyaline in appearance. Cross striations were also difficult to identify.

III. DISCUSSION.

A. Ontogenesis of enzymes.

i - Cholinesterase.

The ontogenesis of cholinesterase has been reviewed by Karczmar (1963). In amphibian and fish, the development of ChE has been correlated with the morphological and functional differentiations of the nervous system (Youngstrom, 1938; Sawyer, 1943a, 1943b, 1944).

In the fish Fundulus heteroclitus, for instance, ChE concentration remains low until the motility and true reflex stages are attained, at which time there is a rapid increase. Similar results were also obtained in chick. In the opinion of Burt (1968), however, the functional differentiation of the nervous system would be more closely associated with the levels of the enzyme choline acetyltransferase than with the levels of cholinesterase.

Although histochemical staining is not a precise quantitative tool, the results obtained confirm those of Uesugi and Yamazoe (1964) who found ChE to be low during the first third of development and then to increase rapidly during the second third and even more rapidly during the last third of development. The results also agree with Filogamo's and Gabella's (1967) findings that outside of the nervous system, ChE first develops in the more anterior somites and that light ChE activity associated with the subneural apparatus (end-plates) occurs only late in development. The sequential localization in spinal cord, hindbrain, midbrain and forebrain is also similar to that found in amphibians (Sawyer, 1943b; Boell and Shen, 1950).

The diffuse localization of ChE in somites during its first period of ontogeny would indicate that at this time, ChE does not originate from nervous but from presumptive muscular cells. The non-nervous origin of ChE has been observed several times in denervated presumptive muscle (Sawyer, 1943b; Durante, 1959), in tendon (Pecot-Dechavassine, 1961; Gerebtzoff, 1959) and in cultured muscular cell (Engel, 1961). The bulk of ChE, however, would come from nervous structures: Boell and Shen (1949) have shown that ChE was highly reduced in the optic lobe after extirpation of one eye and Sawyer (1943b)

has observed that after denervation the AChE content of denervated muscle was only one third that of control. Fukuda and Koelle (1959) have also shown that the de novo synthesis of ChE occurred in the Nissl bodies of the neurons.

ii - Malic dehydrogenase.

Histochemical localization of the progressive development of malic acid dehydrogenase (MDH) has shown that during the first half of development, the enzyme has a uniform distribution except in the ventral acellular part of the brain in that group of cells which are positive for ChE, in the cellular portion and in the cells lining the future myotomal septa. Biochemical differentiation proceeds rather rapidly and at day 19, the pattern of distribution found in the adult is already present.

Boell (1948), Boell and Shen (1950) and Shen (1958) have shown with biochemical methods that higher concentration of the respiratory enzymes succinic acid dehydrogenase and cytochrome oxidase appeared in the different parts of the brain in the reverse order than that found for ChE. Higher concentration of the respiratory enzymes are found first in the forebrain, then in the midbrain and finally in the hindbrain. The same authors have also shown that for whole embryos, the activity of these enzymes was parallel to the rate of respiration, and increased at a steady rate. Similar findings were obtained with the fish Oryzias latipes (Hishida and Nakano, 1954). The results obtained in speckled trout embryos partially agree with these since MDH is differentially distributed in the midbrain at a time when there is very little ChE present. The results, however, are not in perfect agreement since in the brain, areas of high ChE also show higher MDH

activity. This observation points out to the necessity of complementing biochemical measurements by histochemical staining. In larvae, large neuron cells show a strong activity for both enzymes. If one accepts the idea that biochemical differentiation is related to both morphology and function and that increased activity of ChE is a sign of function differentiation, it appears normal to find that enzymes, which show high activity in well-differentiated adult cells, also appear at about the same period during development.

B. Cause of high mortality rate at day 32.

A high rate of mortality was observed in both control and treated embryos at the beginning of hatching. Battle (1944) has observed that mortality occurred at a higher rate during the hatching period than during the preceding period, in normally developing salmon eggs. She also quoted experiments showing that Salmonidae eggs were particularly sensitive to several injurious agents at that time. The observed mortality would then be partially due to the natural sensitivity of the eggs.

The factor which is most probably more important is the effect of the hatching enzyme. Hatching is a physiological process which normally occurs when the embryo is morphologically and physiologically ready for it. Hayes (1942) has shown that the hatching enzymes appear in the egg a few hours before the breakage of the capsule. On the other hand, all eggs do not hatch at the same time. Since in the experimental design, the water was recirculating, accumulation of hatching enzyme in the water probably occurred and digested the capsules of eggs not ready to hatch. Incomplete hatching of the embryos

resulted in physical damage to the yolk sac. Furthermore, tissues of Salmonidae embryos are known to be quite sensitive to the deleterious effect of the hatching enzyme during the period preceding hatching (Hayes, 1930a).

Finally, it is possible that the presence of foam on the water, resulting from the digestion of capsules, lowered the gas exchange with the surrounding air so that the oxygen content of water decreased, leading to premature hatching. Buznikov (1964) has indeed shown that hatching glands were stimulated by reduced oxygen tension.

C. Effect of treatment on hatching.

Treatment with 100 and 200 ppm of Phosphamidon during the first half of development lowered the hatching percentages. Hatching was generally also more reduced with Dylox, although Dylox appeared less toxic when the mortality produced by treatment during the development is considered. Herbicides (Hiltibran, 1967) and artificial light (Eisler, 1958) are known to reduce hatching in fish eggs.

The reduced hatching in the treated groups is certainly related to the fact that several embryos were abnormal or delayed and were thus unable to hatch. But it also seems that the physiological process of hatching in treated embryos which were normal or slightly delayed was interfered with. Such an indication is given by the comparison of the hatching and abnormality percentages of group B with those of group A. The number of abnormal and delayed embryos is similar in both groups but hatching was reduced in group B. The comparison of percentages between groups P and S shows that there is a big difference in hatching while the difference in the number of abnormal

embryos is small. Furthermore, hatching was generally more reduced when treatment occurred at a later stage.

Was the failure to hatch due to ChE inhibition? In hen eggs, injection of Phosphamidon and Dipterex reduces the hatching (Dunachie and Fletcher, 1966). This effect is probably related to ChE inhibition since the movements of the hatching chick are very important in hatching. In speckled trout, it is unlikely that the failure to hatch was due to ChE inhibition since important recovery of the inhibited enzyme and important synthesis of the enzyme was found to occur during the last six days prior to hatching and since hatching can occur even if there is a strong ChE inhibition. Behaviour of the embryos treated just prior to hatching strongly indicates that there was important ChE inhibition, but hatching was not reduced in these embryos.

It is possible that the organophosphates have affected the hatching enzyme, but it seems unlikely that this is so. Inhibition of the enzyme is ruled out by the fact that the enzyme which appears shortly before hatching does not seem to be inhibited by treatment occurring just before the hatching period. It has been suggested that organophosphates may affect protein metabolism and synthesis (Karczmar, 1963) and it is thus possible that the synthesis of the enzyme would be interfered with. It appears dubious, however, that synthesis of hatching enzyme would be inhibited when important synthesis of proteins, associated with growth in treated embryos which are normal, does not seem to be affected.

Although no firm conclusion can be reached on the effect of organophosphate on the physiological process of hatching, the results suggest that hatching should be studied in detail in embryos which have

been treated during the first half of development but which have a normal morphology.

Treatment during the period preceding hatching induced earlier hatching. This, at first, seems rather surprising and one would expect the reverse situation since the growth process was slowed down, and the activity of the embryos was also greatly reduced. It seems that the movements of the embryos, in speckled trout, play a very minor role in the breakage of the capsule and that dissolution of the egg covering by the hatching enzyme associated with water flow is sufficient.

The earlier hatching would be due to an indirect effect of the treatment on hatching glands. Less oxygen is probably available to the embryonic tissues due to the reduced blood circulation resulting from the observed decreased heartbeat rate. Baravik (1963) has shown that in rainbow trout, hatching is associated with a low oxygen consumption and Buznikov (1964) has shown that a reduced oxygen tension stimulates the activity of the hatching glands. The premature hatching would then be due to the fact that the hatching glands are stimulated by the lack of oxygen.

It was observed that the embryos that hatched before the end of the treatment did not die. This is rather surprising since larvae (see chapters five and six) were quickly killed by concentrations lower than those used in this experiment. This would mean that either larvae are much more resistant to organophosphates immediately after hatching than later, or that prehatching exposure lowers their sensitivity. Sawyer (1944) has observed that Fundulus embryos were more resistant to the ChE inhibitor eserine at the "reflex stage" if they had been exposed to it prior to the acquisition of motility.

In trout, a possible explanation for the resistance of the young larvae to organophosphate brought about by the prehatching treatment, is the maintenance of the larvae in a kind of embryonic state of metabolism. In normal Salmonidae, the respiration rate abruptly increases immediately after hatching (Hayes, 1930). Treatment with organophosphates would prevent this change in the rate of respiration because of the reduced blood circulation, so that, although hatching had occurred, the metabolism is maintained at the same rhythm as that which prevailed before hatching. On the other hand, in older larvae, respiration rate is high and would be abruptly reduced by the treatment, thus causing a marked change in the metabolism. These results differ from those obtained in zebra fish, where there was rapid death of the larvae if they remained in the organophosphate solution after hatching (see p. 58). The explanation proposed above, would then not hold true for zebra fish embryos and larvae since no protection is observed. This difference could possibly be due to the difference in the spontaneous activity that embryos and larvae of the two species exhibit before and after hatching. Zebra fish embryos are much more active and the larvae swim freely while in trout, activity of the larvae is reduced and true swimming occurs only a few days after hatching. Because of the inhibited ChE, the higher rate of function of the nervous system normally associated with higher spontaneous activity would lead to early exhaustion of the young zebra fish larvae.

D. Effect of treatment on mortality and morphology.

i - Comparison with the effect found in Brachydanio.

In the zebra fish, major malformations of the embryo are

produced only if organophosphate treatment includes the first part of gastrulation. In trout, however, treatment occurring after the completion of gastrulation does produce lethal and teratogenic effects, even if these effects are less frequent or less severe than those resulting from treatment during gastrulation. Although this difference is most likely due to the higher sensitivity of trout embryonic tissues to deleterious agents (trout eggs develop abnormally if they are exposed to light or if they are moved or disturbed during the first half of development and spontaneous malformations in optimal external conditions are relatively frequent), the morphology of the embryo at the end of gastrulation is probably of importance too.

After completion of gastrulation and closure of the blastopore, the body axis compared to the total size of the egg is much bigger in zebra fish than in trout. In zebra fish, the body axis almost completely surrounds the yolk while in trout it is reduced to a small longitudinal mass of tissue on the surface of the yolk. So that growth taking place both in the head and body regions after closure of the blastopore is more important in trout than in zebra fish. Any factors which can interfere with growth will then have a more noticeable effect in trout than in zebra fish.

ii - Comparison with teratisms produced by other factors.

Spontaneous malformations are not infrequent in Salmonidae embryos. These include such teratisms as fission of the embryonic axis with production of partial or complete siamese twins (Garman and Denton, 1886; Gemmill, 1912; Stockard, 1921; Ross, 1934), microcephaly and reduction of body size (Gemmill, 1912), microphalmia with poor development or absence of optic nerve and optic muscle (Gemmill, 1903,

1906, 1912; Ross, 1934) and screwed or rolled tail (Ross, 1934).

Several agents have been found to be lethal or teratogenic during the development of Salmonidae. Some of these are: artificial light (Eisler, 1958; Brannon, 1965), irradiations either of the parent fishes or of the embryos (Foster et al., 1949; Neutroev and Podymakhin, 1966; Newcombe and McGregor, 1967; McGregor and Newcombe, 1968), lack of oxygen, or presence of carbon dioxide and respiratory inhibitors (Vernidub, 1941; Domurat, 1956, 1964; Devillers et al., 1957a; Alderdice and Wickett, 1958; Garside, 1959, 1966), temperature (Vernidub, 1941), lithium chloride (Zenner, 1965), neotetrazolium (Perlmutter and Antopol, 1963). In some species of fishes, specific teratisms can be produced by certain agents (Stockard, 1907b, 1921; Hisaoka and Hopper, 1957; Swarup, 1959b; Anderson and Battle, 1967). However, it is usually difficult to associate specific teratisms with a particular teratogenic agent: frequency and details of the teratisms and histological studies are often lacking in the literature.

The teratisms which are produced by Phosphamidon and Dylox are similar to the abnormalities which are spontaneously occurring and also similar to those produced by x-rays (Foster et al., 1949; McGregor and Newcombe, 1967), low oxygen levels (Garside, 1966), lithium chloride (Zenner, 1965) and neotetrazolium (Perlmutter and Antopol, 1963).

During the development of the Salmonidae eggs there are periods or stages during which they are more sensitive. These vary both with the species and the teratogen, but generally, the sensitivity is higher during cleavage and gastrulation and also during the period preceding the pigmentation of the eye and the period preceding hatching. McGregor and Newcombe (1968) irradiated embryos with x-rays and were

able to correlate the higher occurrence of certain teratisms with some specific stages. Zenner (1965) found that exposure of rainbow trout embryos to lithium chloride during cleavage resulted in reduction of the growth of mesodermal structures, while exposure during gastrulation mainly produced reduction of the nervous and head structures.

iii - Cause of gross teratisms.

The results of the experiments show that the gastrulation period, among the periods tested, is the period during which the eggs are most sensitive to Phosphamidon treatment. Sensitivity of the eggs is also higher during the first half than during the second, although comparison of groups L and M (see p. 101) seems to indicate that eggs are more sensitive to 200 ppm during the second half. It must be remembered, however, that treatment with 200 ppm lasted only 60 hr during the first half while it lasted 72 hr during the second half. Treatment was stopped at 60 hr simply because the death rate was high and it was feared that it would reach 100% before the end of treatment.

These results are not in agreement with those of Vernidub (1941) who observed that eggs of speckled trout were more sensitive to asphyxia and high temperature during the second half than during the first half. The high sensitivity observed by that author following the closure of the blastopore and prior to eye pigmentation was not observed in the present experiments.

The results also show that Phosphamidon acts rapidly since the deleterious effects (death and retardation of gastrulation) occur soon after it is added to the circulating water. It thus affects the cytoplasmic structures rather than the nuclear structures since when these are affected there is usually a delay before the effect becomes evident

as, for instance, is the case when eggs are submitted to ionizing radiations (Solberg, 1938; Neyfakh, 1959; Lasher and Rugh, 1962; McGregor and Newcombe, 1968).

The effects produced by Phosphamidon during or shortly after gastrulation cannot be attributed to its anticholinesterase action, since during these periods, ChE is either absent or present in such a small quantity that it cannot be detected. DFP, a powerful ChE inhibitor, has also been found to be lethal to premotile urodele embryos, which at this period contain very little ChE (Karczmar and Koppanyi, 1953). Both the lethal and teratogenic actions of Phosphamidon appear to result from an impairment of the embryo from undergoing the normal morphogenetic movements. In both zebra and trout embryos, the epiboly movement is delayed and the blastopore often fails to close properly. When the normal relationship between the processes of epiboly and invagination are disturbed, abnormalities are produced (Swarup, 1959), due to incomplete or faulty induction from the invaginating material (Zenner, 1965; Collini and Ranzi, 1967; Noto, 1967). Impairment of normal morphogenetic movements in fish eggs produced by organophosphates has never been reported before.

Several factors have been associated with normal morphogenetic movements of gastrulation: these are protein synthesis, synthesis and presence of RNA, presence of proteins containing -SH groups and respiration.

Several studies have shown, as reviewed by Brachet (1960), that in amphibians appreciable protein synthesis begins only at gastrulation. Inhibitors of protein synthesis such as chloramphenicol are known to affect gastrulation and to produce abnormalities (Anderson and

Battle, 1967). The similar action exerted by lithium ions has been partly attributed to the fact that it opposes the destruction of pre-existing proteins (Brachet, 1960). Although not much is known about the existence of aliesterases in embryos and their precise physiological role, it is possible that aliesterase inhibition would lead to abnormal protein metabolism, since it has been suggested that they might be involved in protein metabolism (Myers et al., 1957; Dubois et al., 1968). The effect of anticholinesterase inhibitors on amino acid metabolism has also been suggested by Winteringham and Harrison (1956). However, it remains difficult to decide whether inhibition of protein synthesis by organophosphates can be of importance.

A large amount of work has been done in amphibians to relate the RNA distribution with the morphogenetic movements (Brachet, 1960). Inhibition of RNA synthesis by chemical analogs of purines and pyrimidines lead to the arrest of development at different stages. The stage at which the development was stopped varied with the analog used, suggesting that new enzymatic mechanisms of RNA synthesis appear at definite stages. Several analogs were found to block development at gastrulation. The similar deleterious effect of some antibiotics have also been attributed to its action on RNA synthesis (Collini and Ranzi, 1967) and it has been suggested that part of the effect of lithium ions would also result from RNA synthesis inhibition (Lallier, 1954; Thomason, 1957; Zenner, 1965). In fishes, Devillers (1951) has found that the invaginating and the peripheral cells of the blastoderm were rich in RNA, while RNA inhibitor such as barbituric acid was found to alter the development of the zebra fish (Hisaoka and Hopper, 1957), showing that in fishes as well as in amphibians, RNA distribution and

synthesis would be important for normal development. Although there is no indication in the literature that there could be a relationship between the synthesis and distribution of RNA and the impaired morphogenetic movements produced by Phosphamidon, it seems logical that this aspect of the problem should be further investigated.

Brachet (1960) has emphasized the importance of the -SH proteins in the normal morphogenetic movements and Mulherkar et al. (1966) have shown that -SH groups were involved in induction. The reaction of an organophosphate with -SH groups is theoretically possible; it could result in the inactivation of the -SH proteins and thus disturb the morphogenetic movements and the induction process. Such a possibility should also be investigated.

Respiration is certainly of the utmost importance during development and especially during such critical stages as gastrulation, since it is the source of energy for such organic processes as protein and RNA synthesis. In Salmonidae, the lack of oxygen during gastrulation leads to arrest of gastrulation and abnormal development (Devillers and Rosenberg, 1953; Devillers et al., 1957a). Since it has been suggested that the abnormalities produced by ChE inhibitors (Landauer, 1954) could be related to carbohydrate metabolism, the importance of respiration will be shortly examined. There seems to be some confusion about the importance of carbohydrate metabolism during development of Salmonidae. Some authors have shown (Hayes and Hollet, 1940; Smith, 1940, 1952, 1957; Daniel, 1947) that glycogen and glucose were present in very small quantities during the first half of development, suggesting that carbohydrate metabolism has very little importance, in comparison to proteins and lipids, as a source of energy. Devillers et al.

(1957), however, have shown that carbohydrate was important for gastrulation to take place, since rainbow trout blastoderms explanted in saline would not continue their development if glucose was not added to the medium. Williams (1967) agrees that carbohydrate metabolism is the main source of energy during the gastrulation period. Vernidub (1941), on the other hand, has shown that in Salvelinus fontinalis both oxygen consumption and the amount of lactic acid produced were smaller during gastrulation than during cleavage, and that there was a slight increase of both after closure of the blastopore. Considerable increase of oxygen consumption occurred during eye pigmentation.

In examining the periods of higher sensitivity of the embryos to asphyxia in relation to respiration, the last author claimed that there is a relationship between the periods of higher sensitivity and the periods of high oxygen consumption. Close examination of his results rather suggest that aerobic respiration, even though it is lower during certain stages, would be of more importance during these stages; for instance, during closure of the blastopore, oxygen consumption is only one half of that occurring during cleavage, but the embryos are twice as sensitive during closure of the blastopore as during cleavage. Similarly, the respiration is very high during eye pigmentation but the eggs are quite resistant to asphyxia. The general conclusion that can be drawn about the effect of inhibition of respiration is then that the stage during which any change from the normal pattern is critical is of more importance than the stage during which respiration is the highest.

Changes in the amount of energy normally supplied by the catabolism of carbohydrate can result from the inhibition of the anaerobic respiration, from the inhibition of the dehydrogenase enzymes

normally involved in the citric acid cycle and from the uncoupling of the oxidative phosphorylation. According to Denis and Devlin (1968), inhibition of the direct use (without the intervention of ATP as an intermediate) of high energy intermediates normally formed during electron transport could also be of importance in the energy requirements of embryonic development.

Breakage of the glycogen into glucose units, and simultaneous phosphorylation of the glucose is the first step of glycogenolysis; this reaction is carried by the enzyme phosphorylase. Phosphorylase can exist in two forms: the inactive "b" form and the active "a" form. Transformation of the "b" form into active "a" form involved a phosphorylation of two serine hydroxyl groups of the "b" form with the aid of ATP (West et al., 1966; Fisher et al., 1967). As will be seen in chapter six, phosphorylase activity was found to be reduced in larvae treated with organophosphate. Since the site of phosphorylation (serine hydroxyl groups) of phosphorylase "b" are identical to the active site of ChE, which becomes phosphorylated by organophosphates, it is not unlikely that these sites could be phosphorylated by the organophosphates, thus interfering with the transformation of the "b" form into the "a" form and producing a lower activity. The reduced activity of phosphorylase could then explain the teratogenic action of organophosphates during gastrulation if the association of glycogen breakdown with the invaginating material, shown for Oryzias latipes (Hishida and Nakano, 1954), is also true for speckled trout.

Mitochondria isolated from the early gastrula blastoderm have been found to stimulate glycolysis, while mitochondria from cleavage or blastula stages blastoderm do not have this effect (Milman and Yurowitzky,

1966). Mitochondria with abnormal structures have been observed in larval muscle (see chapter six, p. 241) and Battle and McLeese (1954) have shown that Urethane, which does produce embryonic abnormalities in zebra fish (Battle and Hisaoka, 1952), modifies the structure of blastodermal mitochondria. Mitochondria have not been studied during early stages of Salvelinus fontinalis, but it is not impossible that a modification would occur. This could be of importance in the glycolysis since it was shown that abnormal enzymatic reactions in mitochondria are often associated with abnormal structure (Lehninger, 1965).

The major part of the energy released in carbohydrate breakdown comes from the aerobic respiration. The inhibition of respiratory enzymes involved in the citric acid cycle and the electron transport system was found to have a profound and radio-mimetic effect on the development of trout embryos (Perlmutter and Antopol, 1963). The possible inhibition of aerobic respiration by ChE inhibitors is suggested by the fact that nicotinamide and nicotinic acid act as alleviating agents for the teratologies produced by such ChE inhibitors as pilocarpine, eserine, Bidrin and other organophosphates (Landauer, 1953; Roger et al., 1964; Upshall et al., 1968). The alleviating mechanism, however still remains unclear since Roger et al. (1969) have recently shown that the metabolism of nicotinic acid was unchanged by the organophosphate Bidrin.

Although concentrations of organophosphates, necessary for the inhibition of enzymes involved in the aerobic respiration, are higher than the concentrations necessary for ChE inhibition, the inhibition of several dehydrogenases and oxidases have been reported (Greenberg and LaHam, unpublished results; Michaelis et al., 1949;

Bochavora and Ukrainets, 1958; Heath, 1961) and could be of importance in the impairment of the morphogenetic movements.

Oxidative phosphorylation and the direct use of high energy intermediates during electron transport is very important in aerobic respiration, since they are the channels by which most of the energy coming from carbohydrate catabolism is used by the cells. Such processes are important during embryonic development: uncouplers of oxidative phosphorylation are powerful teratogens (Ishida et al., 1958; Thompson, 1967; Denis and Devlin, 1968). That phosphorylating agents such as organophosphates might have an effect on oxidative phosphorylation is then a possibility.

Although no firm conclusion can be reached about the inhibiting effect of organophosphates on carbohydrate breakdown and its relationship with abnormal gastrulation, there are some indications that it could be an important factor. Detailed studies on the effect of compounds such as Phosphamidon on phosphorylase enzymes, aerobic respiration and oxidative phosphorylation should then be carried out. Finally, it must be emphasized that even if the induced modifications of these processes are small, they can have important consequences such as formation of excess intermediates leading to a shift in metabolism, or the decreased formation of some intermediates required for some synthesis, or the lack of energy available for such endergonic processes as protein and RNA synthesis. Thus, small changes in the respiratory metabolism can have important consequences during embryogenesis.

D. Cause of some abnormalities.

Lack of differentiation of the nervous and muscular structures

were typical of abnormal embryos (especially of Type 4). However, the development of the endodermal structures was normal.

Endodermal organs were found to be hypertrophied in hypomorphic trout embryos resulting from early treatment with lithium chloride (Zenner, 1965). In amphibians, isolation experiments have shown that the endoderm can develop independently of other tissues (Holtfreter and Hamburger, 1955). The results obtained indicate that this would be true also for speckled trout embryos, since the development of the gut proceeds without being greatly influenced by the general hypomorphism of the embryos.

Cholinesterase inhibitors were found to have no effect on muscle differentiation in Ciona, even though the development is partly inhibited. In vertebrates, however, abnormal muscle differentiation was associated with foot deformity in ducks injected with EPN (Khera et al., 1966).

The muscle morphology observed in speckled trout most probably represents incomplete differentiation followed by degenerative changes, since several fibers and nuclei were extremely vacuolar. The lack of differentiation could be due to a direct effect of the organophosphate. During muscle differentiation, important protein synthesis is going on for the production of myofibrils which make up the bulk of the cell. The possible effect of organophosphate on protein synthesis has already been discussed. It is true that in abnormal embryos, resulting from gastrulation treatment, muscle differentiation would normally take place long after the eggs are removed from the organophosphate solutions. However, as will be discussed later, important quantities of the organophosphate could be stored in the yolk and could

affect the cells at stages following the removal of the eggs from the organophosphate. In this regard, it would be interesting to study in tissue culture, the effect of organophosphates on muscular differentiation. Some stages during differentiation seem to be particularly sensitive to deleterious agents as shown by Klishov (1967).

Lack of muscle differentiation can also be explained by the changed relationship between nerves and muscles. In the strongly abnormal embryos the small amount of white matter in the nervous system and the lack of differentiation of neurons strongly suggest that nerve fibers were either absent or importantly reduced. On the other hand, muscle fibers are known to degenerate after denervation (Bosanquet et al., 1960) and the final stage of differentiation is not attained when the differentiating muscle cells are chemically denervated with the use of tubocurarine, indicating that the integrity of the nerve-muscle association must be preserved in order to obtain complete differentiation (Ahmed, 1966). Sullivan (1967) has also shown that tensile stress arising from both skeletal growth and contraction originating from the nervous system were necessary for normal differentiation and orientation of muscle fibers.

Absence of complete muscle differentiation could then result either from the absence of a myoneural junction or from the lack of integrity of this junction due to ChE inhibition. Comparison of muscle differentiation in grossly abnormal embryos resulting from a physical or chemical agent, and of muscle differentiation of abnormal embryos resulting from organophosphate treatment would help clarify this point.

It must be remembered that the circulatory system was poorly developed in the abnormal embryos; such a deficiency certainly resulted

in the lack of proper nutrition of the embryo. But this is certainly not the only cause of lack of muscle differentiation since other structures, such as notochord could differentiate normally.

Nervous structures were also very poorly developed in abnormal embryos. Severe abnormalities of the neural structure are very often characteristic of embryonic abnormalities produced by physical or chemical agents (Zenner, 1965; Mulherkar et al., 1967; Suzuki, 1967). It has been known for quite a long time that ectoderm does not differentiate in the absence of proper induction from the chordamesoderm (Holtfreter and Hamburger, 1955). The observed malformations could be explained by a faulty induction: abnormal morphogenetic movement during gastrulation changes the spatial relationship between the ectoderm and the chordamesoderm.

It was observed that in grossly abnormal embryos there was, during the second half of development, very little growth in length but important growth in width. Is the absence of growth in length related to the poor differentiation of the somites at the time when the normal number of sixty is attained and to the absence of elongation of myoblasts normally occurring during differentiation? It is quite possible, but then the question can be asked: why does the notochord which has a normal structure does not grow? Detailed study of growth taking place in abnormal embryos between day 20 and day 26 should then be made in order to find what are the factors restricting growth in length while permitting growth in width.

E. Cause of delayed development.

Embryos which were treated just before hatching had a slower

growth rate than controls, and several embryos, treated either during or after gastrulation were delayed or underdeveloped. Abnormalities such as micromelia and dwarfism are frequent in chick embryos treated with ChE inhibitors (Landauer, 1954; Khera and Lyon, 1969; Greenberg and LaHam, 1969) and eserine was found to reduce development in Fundulus heteroclitus (Sawyer, 1944). Upshall et al. (1968) have shown that there is no correlation between the teratogenesis and the inhibition of ChE activity since nicotinamide can alleviate the teratisms without having an effect on ChE activity.

Reduced development could be due to several factors: inhibition of cellular respiration, nutritional deficiency, inhibition of cell growth, reduction of blood circulation. The first factor has already been discussed and will not be commented on here. Nutritional deficiency would result from the inability of the embryo to obtain the normal ester hydrolysis products from the yolk (Upshall et al., 1968) (yolk contains large amounts of phosphoproteins and phospholipids), which are used as a source of energy and for synthesis. The inhibition of cell growth by organophosphates is probably of importance. Dipterex (Dylox) for instance, has been found not only to be a strong in vitro growth inhibitor of both human and mouse cells, but also to kill cells. These effects were obtained with concentrations which normally inhibit aliesterase and ChE (Gablicks and Friedman, 1965; Gablicks et al., 1967). Mendel et al. (1953) had previously observed that concentrations of Paraoxon inhibiting aliesterase could inhibit the growth of malignant cells of mouse lymphosarcoma, but were without effect on normal fibroflasts. This observation is interesting since McKinnell (1962) has shown that embryonic and malignant cells have some similarities.

Reduction of the blood circulation resulting from the brachycardia associated with organophosphate poisoning is certainly of importance, especially during the last stages of development. The reduced blood circulation results in lowered oxygen supply and increased carbon dioxide. Both rate of development and size of the embryo are affected by low oxygen supply in fishes (Alderdice and Wickett, 1958; Alderdice et al., 1958; Silver et al., 1963; Garside, 1966) especially after the onset of blood circulation (Winnicki, 1968).

Larvae hatched from embryos treated immediately before hatching were reduced in size. However, during the following days, growth was faster so that the reduction in size observed immediately after hatching was compensated for during the recovery period. This mechanism of homeostasis could be related to the possible accumulation of substrates which should have been used during normal growth and which had a stimulating effect on the metabolism after the inhibiting factor had been removed.

F. Comparison of the effect of Dylox with the effect of Phosphamidon.

Both Phosphamidon and Dylox proved to be lethal and teratogenic to Salvelinus fontinalis embryos, but their effects were not perfectly parallel.

When the treatment was applied during the gastrulation period, Dylox was less lethal and less teratogenic than Phosphamidon. When treatment was applied after completion of gastrulation or closure of the blastopore, Dylox was also less lethal, but generally retarded the development of the embryos more than did Phosphamidon.

Whether the action exerted by Dylox on the embryo is due to

the intact compound or to its derivative DDVP can hardly be decided. Most authors believed that the anticholinesterase action of Dylox would be due to the derivative DDVP which is formed by dehydrochlorination. However, as already discussed (see p. 129), it is most likely that the effect observed on embryos by both Dylox and Phosphamidon is not due to their anticholinesterase action. It is then possible that the intact Dylox would be the effective molecule. Metcalf et al. (1959) have shown that at 37 C and pH 7.0, 50% of Dipterex is transformed into DDVP within 10 hr. The rate of transformation increases with the pH but decreases with the temperature. From the rate constant, it can be calculated that at 10 C and pH 7.4, about 20-30% of Dylox is transformed into DDVP, after 24 hr. Since solutions were renewed every day, the effective concentrations of DDVP were then rather low. On the other hand, if the toxic action is due to DDVP, it is quite possible that Dylox was taken up into the egg and transformed there into DDVP, since the effect of Dylox usually appeared delayed. In such a case, the effective concentration of DDVP in the embryo would be less affected by the daily renewal of the solutions.

G. Effect of treatment on cholinesterase and malic dehydrogenase.

No decrease in the activity of MDH was observed; on the contrary, a slight increase was observed in some cases. With biochemical methods, Greenberg (personal communication) has observed a 30% inhibition after injection of Malathion in hen eggs. This inhibition, however, was not universal and occurred only with injections given on the 7th day of incubation. It is possible that a slight inhibition in some cases has passed unnoticed since histochemical methods are not

recognized as a very precise quantitative tool. Quantitative measurements with biochemical methods would be necessary to evaluate the effect of treatment on the enzyme activity.

Strong ChE inhibition was observed at day 26 even in the groups where treatment occurred at times when no ChE was present. The inhibition was stronger with Phosphamidon; with both compounds the degree of inhibition was dependent upon the duration and the stage of exposure: exposure during earlier stages produced a slightly less important inhibition.

The important inhibition observed at day 26 could be explained by the inhibition of ChE synthesis or by the phosphorylation of some precursors of ChE. The inhibition, however, is most likely due to the accumulation of the compounds in the embryonic tissues and in the yolk: Phosphamidon is soluble in oil (Anonymous, 1967) while DDVP is known to bind reversibly with proteins (Van Asperen and Dekhuijzen, 1958). As the organophosphate, stored in the egg, comes in contact with newly synthetized enzyme, inhibition would take place. Little detoxification of the organophosphate is expected to occur in the embryos since it normally takes place by liver enzymes. According to Murphy (1966a) and Terriere (1968) adult fish contains very little detoxifying enzymes; moreover, important development of the liver takes place mainly during the last stages before hatching, so that it is unlikely that embryonic liver could effect extensive detoxification.

During the last six days of development before hatching, a good recovery of the enzyme occurred. This recovery of enzyme activity is partly due to the reactivation of the enzyme but the major part of it can be attributed to the de novo synthesis since important synthesis

takes place during this period: the bulk of the ChE present at hatching is synthesized during these six days.

H. An hypothesis about teratisms.

Interactions between different parts or different tissues during embryonic development are very complex and it is not the purpose here to review all these interactions. In general, it can be said that there are three kinds of tissues: some tissues like the endoderm can differentiate independently of other tissues; other tissues like the neural ectoderm require the stimulus of other tissues in order to undergo differentiation; finally, some tissues like the chordamesoderm can give the induction necessary for differentiation of other tissues. When isolated, the chordamesoderm can undergo differentiation without receiving induction, but the differentiation is then slightly different from what it is in a normal embryo: myoblasts and notochordal tissues are produced while normally the chordamesoderm produces only notochordal tissues. Spatial relationships are thus important in normal development. Another type of relationship is also important: the "time" relationship. Competence or ability of a tissue to react to an induction and the inducing capacity of a tissue are properties which are limited in time; each tissue has a certain period during which it can either react to an induction or give an induction. Furthermore, all tissues do not grow and differentiate at the same time.

Let us suppose that a teratogenic agent is applied to a developing embryo during a certain period. The rate of development of tissue A could be stopped or delayed during this stage, while tissue B will not be affected during this stage. Tissue B, however, could be

affected later on because it will not receive the proper induction from tissue A, because tissue A has been delayed in its development and has not attained the developmental stage during which it can give proper induction.

The hypothesis is the following. If tissue B cannot develop or differentiate properly, it might not be only because it does not receive a proper induction, but also because it is inhibited from undergoing partial auto-differentiation or auto-development by the nearby presence of tissue A which has not attained the proper stage. The presence of a tissue which is delayed in its development could become inhibitory for the development of another tissue.

Although we have no personal experimental data to support this hypothesis, it was suggested by the presence of extremely abnormal and poorly differentiated muscular tissue in abnormal embryos. And it is known, on the other hand, that muscle can undergo differentiation in tissue culture.

Some recent work would support this idea of the inhibitory effect of a "young" tissue upon another tissue. Shimada (1968) has found that in monolayer culture, myogenesis of 12-day embryos was either delayed, reduced or suppressed by the presence of heterotypic cells of younger embryos probably due to the mechanical interference of the heterotypic cells with the fusion of the myoblast necessary for the myogenesis. Suzuki (1967) has shown with frog embryonic tissue that inhibition of the self-differentiating ability of a tissue can be inhibited by the presence of an abnormal tissue. When chordamesoderm is irradiated with neutrons and wrapped in non-irradiated neural ectoderm, the chordamesoderm retains its inducing and self-differentiating ability.

However, when neural ectoderm is irradiated and wrapped around non-irradiated chordamesoderm, the chordamesoderm does not undergo self-differentiation. And Strittmatter (1968) has shown the presence in frog embryos and larvae of a soluble, heat labile component which could inhibit the incorporation of amino acids into proteins in a cell free system obtained from adult frog liver.

It is thus quite possible that abnormalities of some tissues during development would result from the inhibition produced by adjacent delayed or abnormal tissues.

PLATE 4.1

Main developmental stages of Salvelinus fontinalis.

Embryos reared at 10 C ± 0.5 C. Average egg diameter: 3.4 mm. Magnification: 15 x.

Fig. 1. 48 hours; spreading blastoderm; diameter of blastoderm: 1.5 mm.

Fig. 2. 4 days; embryonic shield; diameter of blastoderm: 2.6 mm.

Fig. 3. 5 days; neural keel; length of body axis: 0.8 mm.

Fig. 4. 6 days; mid-gastrula; 6-8 somites; length of body axis: 1.8 mm.

Fig. 5. 7 days; large yolk plug; 23 somites; length of body axis: 2.5 mm.

Fig. 6. 8 days; closed blastopore; 35 somites; 3.4 mm.

Fig. 7. 9 days; otic capsule; 43 somites; 3.8 mm.

Fig. 8. 11 days; optic lobes; 50 somites; 4.3 mm.

Fig. 9. 12.5 days; 60 somites; 4.9 mm.

Fig. 10. 14 days; rolled tail; 5.5 mm.

Fig. 11. 18.5 days; eye pigment; 6.1 mm.

Fig. 12. 32 days; prehatching; 10 mm.

Plate 4.1

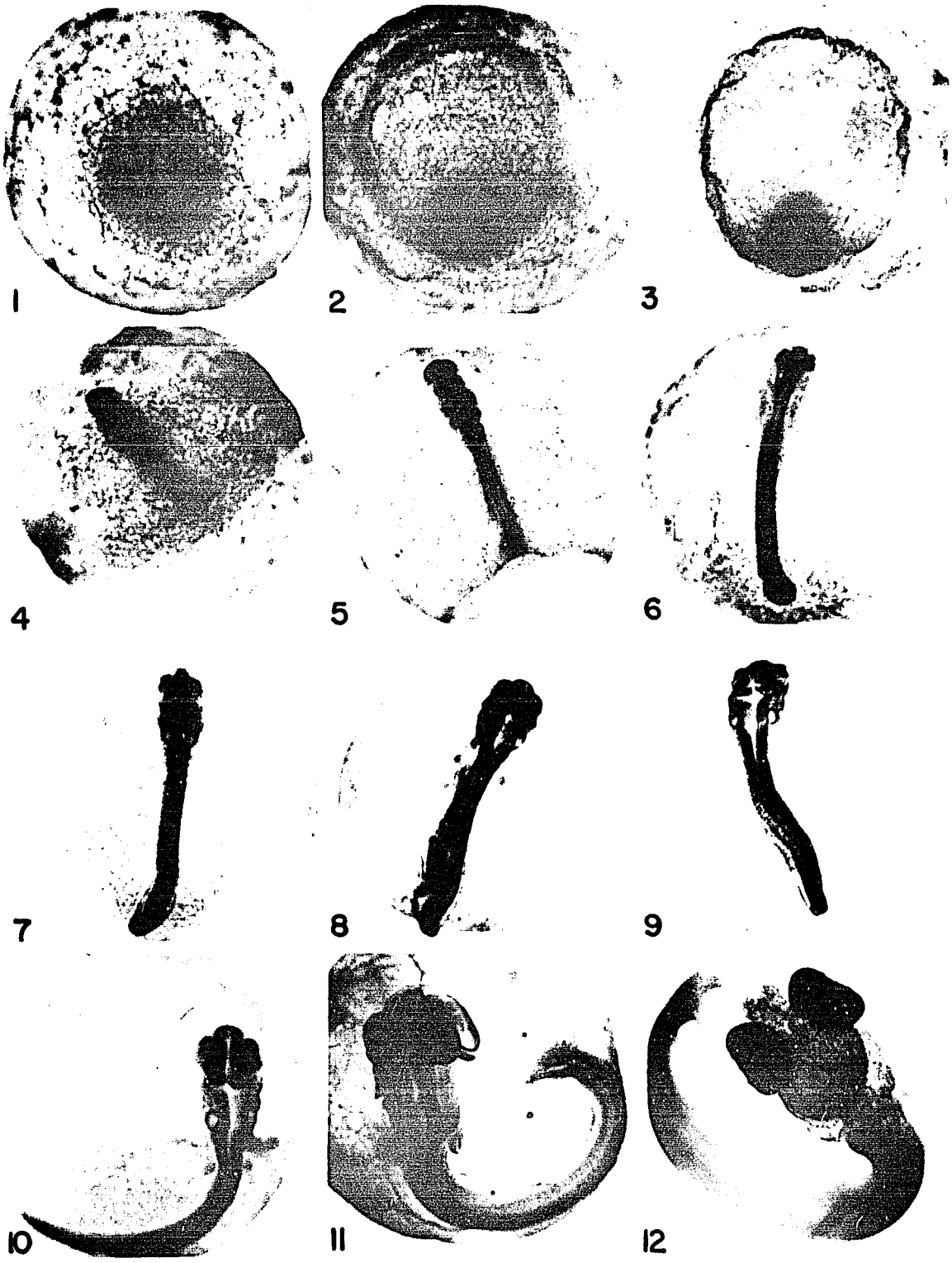
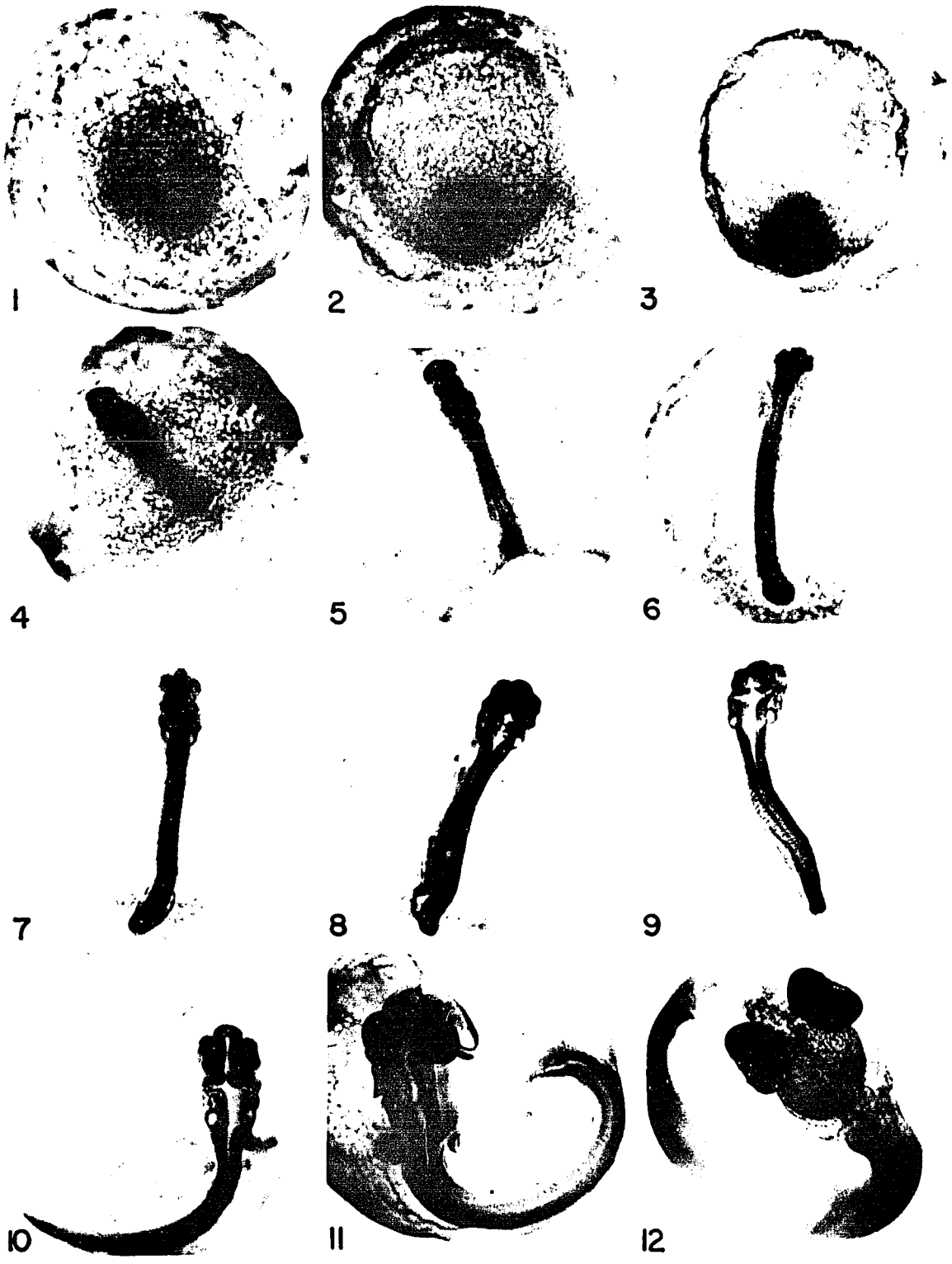


Plate 4.1



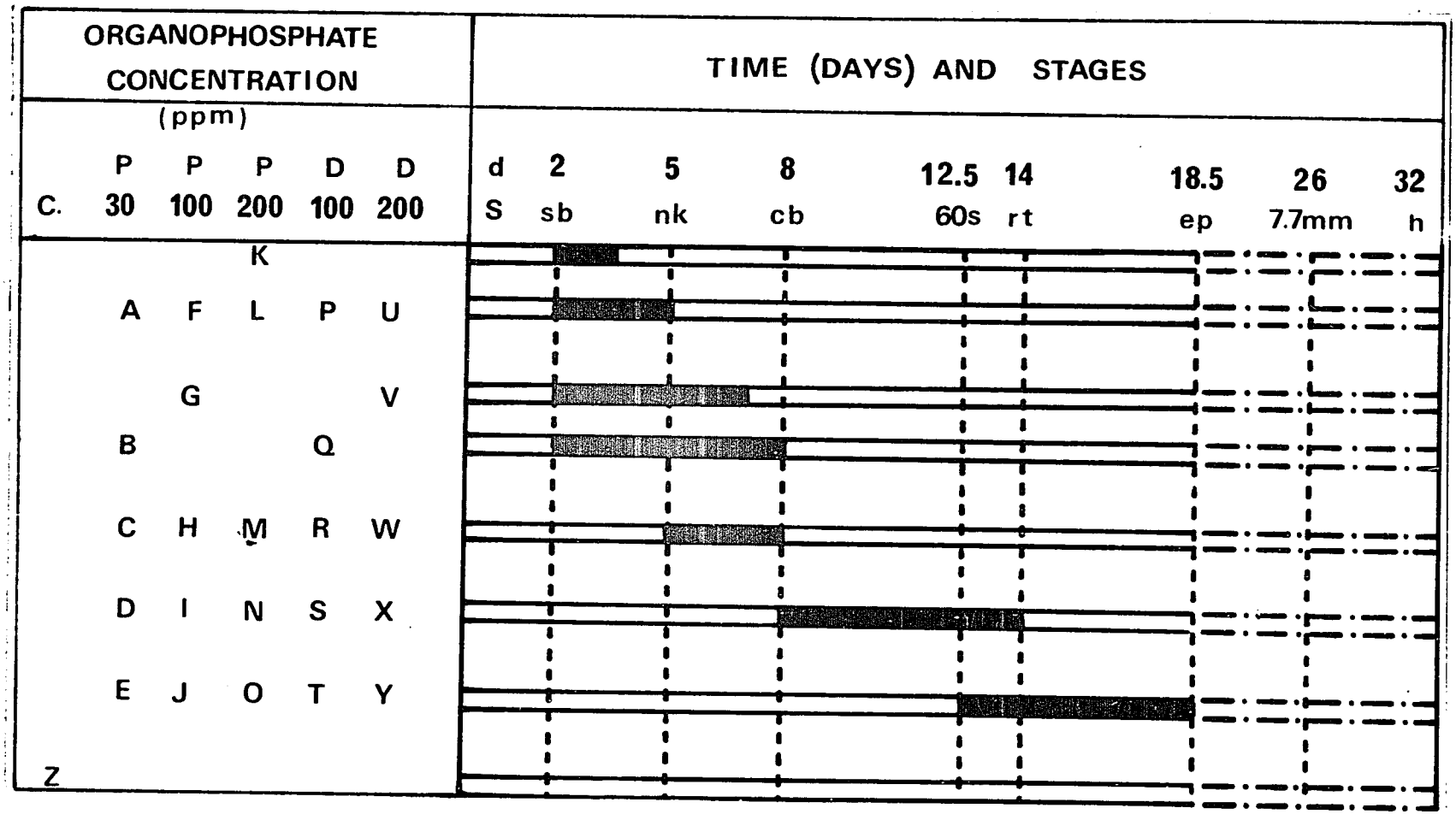


Fig. 13. Types of exposure to Phosphamidon and Dylox during embryogenesis of Salvelinus fontinalis.

The dark areas indicate periods of exposure.

C: control; P: Phosphamidon; D: Dylox; d: days; S: stages; sb: spreading blastoderm; nk: neural keel; cb: closed blastopore; 60s: sixty somites; rt: rolled tail; ep: eye pigmentation; 7.7 mm: 7.7 mm long; h: prehatching.

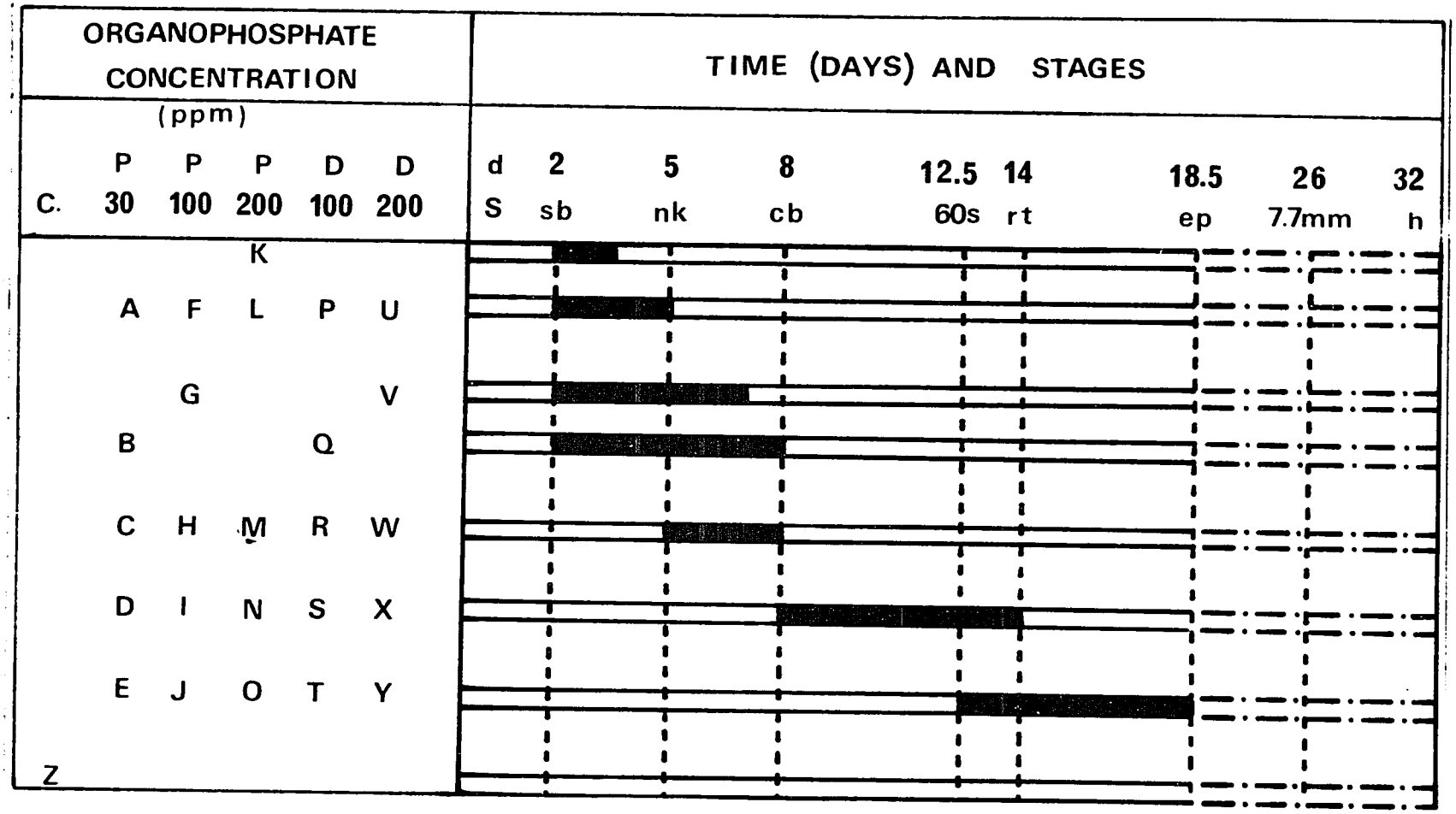


Fig. 13. Types of exposure to Phosphamidon and Dylox during embryogenesis of Salvelinus fontinalis.

The dark areas indicate periods of exposure.

C: control; P: Phosphamidon; D: Dylox; d: days; S: stages; sb: spreading blastoderm; nk: neural keel; cb: closed blastopore; 60s: sixty somites; rt: rolled tail; ep: eye pigmentation; 7.7 mm: 7.7 mm long; h: prehatching.

PLATES 4.2 to 4.14.

Figures 14 to 26.

Mortality percentages of Salvelinus fontinalis embryos exposed to 30, 100, 200 ppm of Phosphamidon and to 100, 200 ppm of Dylox during various periods of development.

Letters refer to the type of exposure as shown in Figure 13. Arrows indicate the beginning and the end of treatment. Dotted line: cumulative mortality percentages. Full line: mortality rate for each interval of three or six days (see p. 94).

Plate 4.2

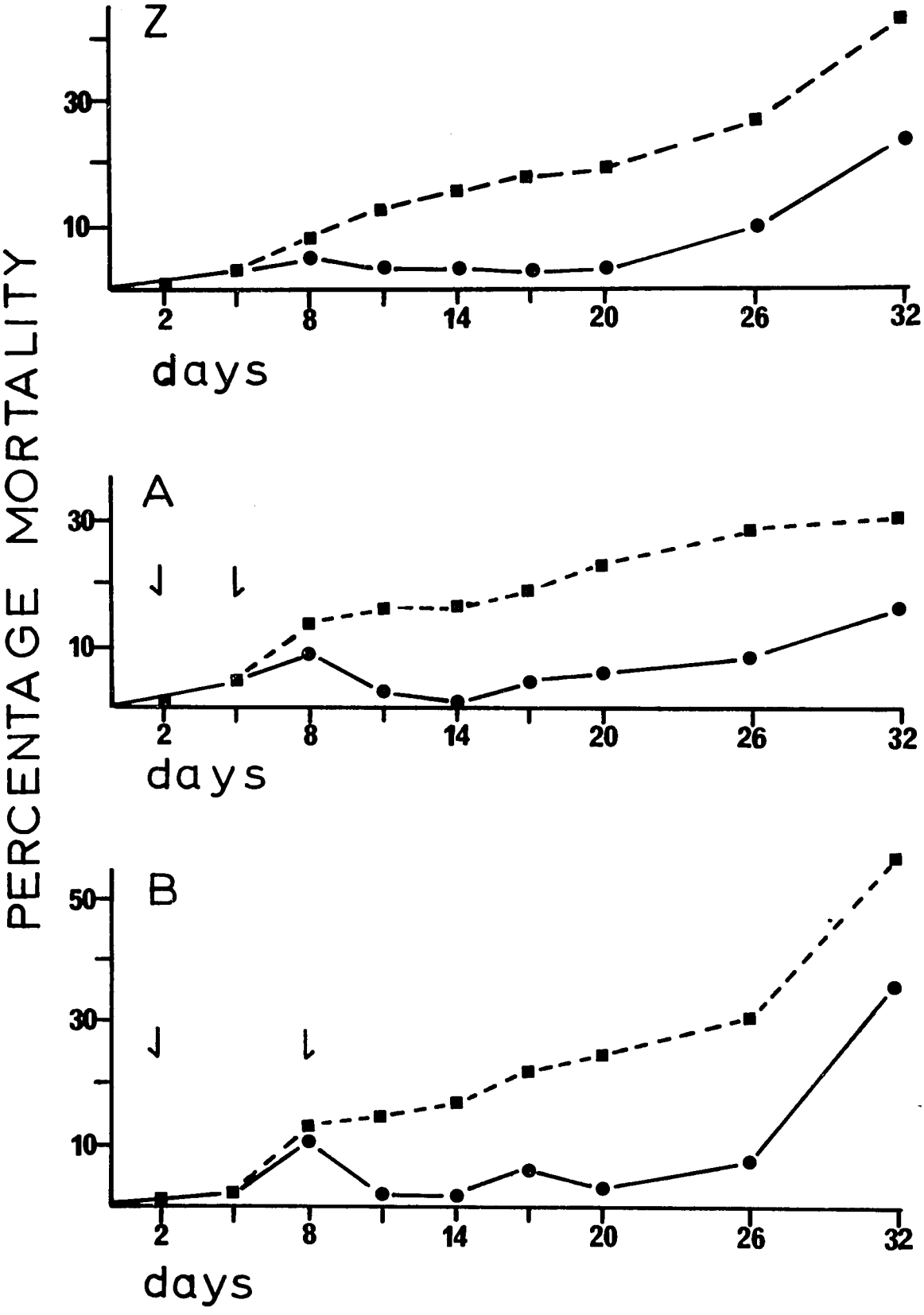

Z
30
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2
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14
20
26
32
days
PERCENTAGE MORTALITY
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days
B
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14
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26
32
days

Plate 4.3

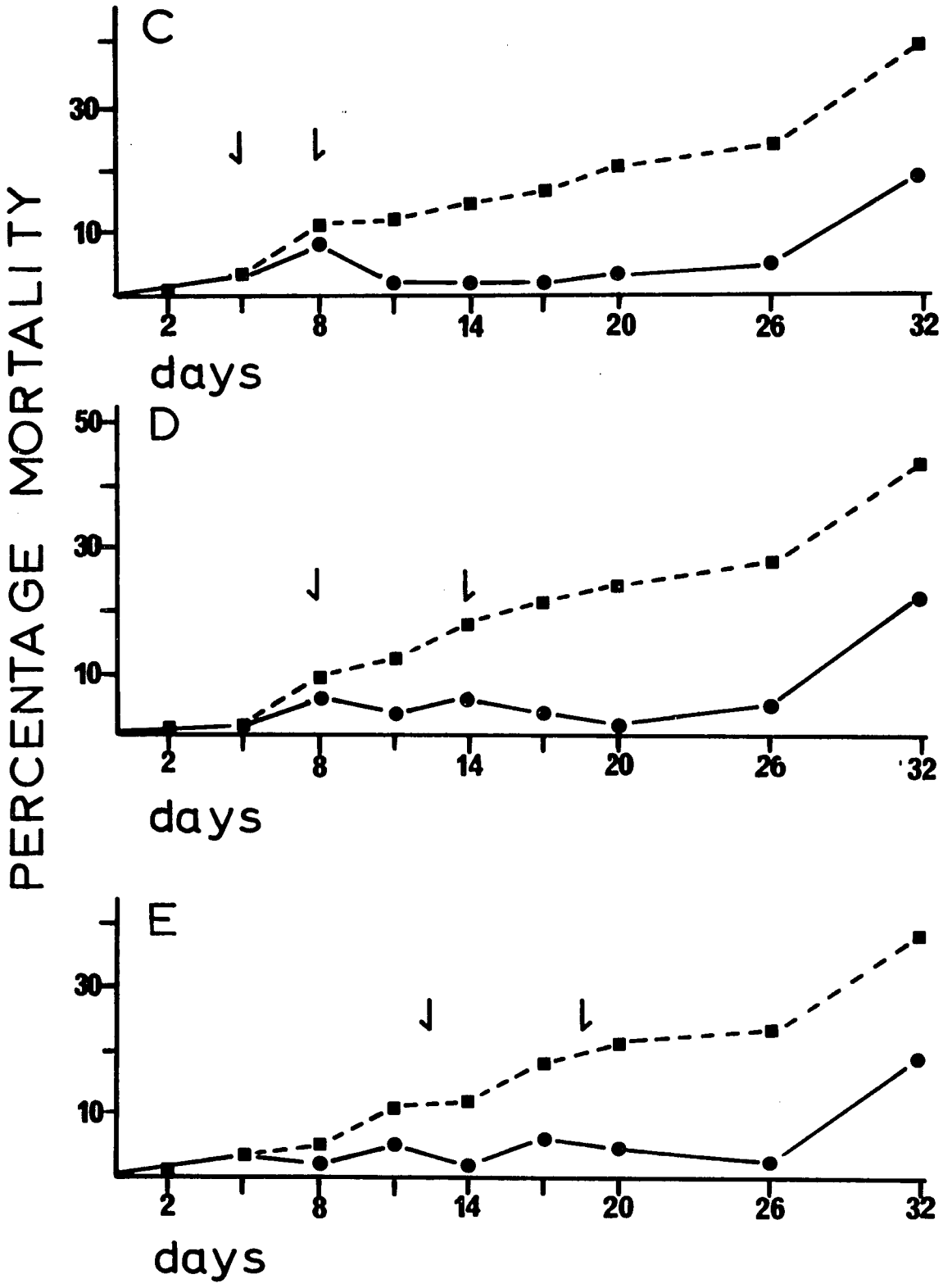


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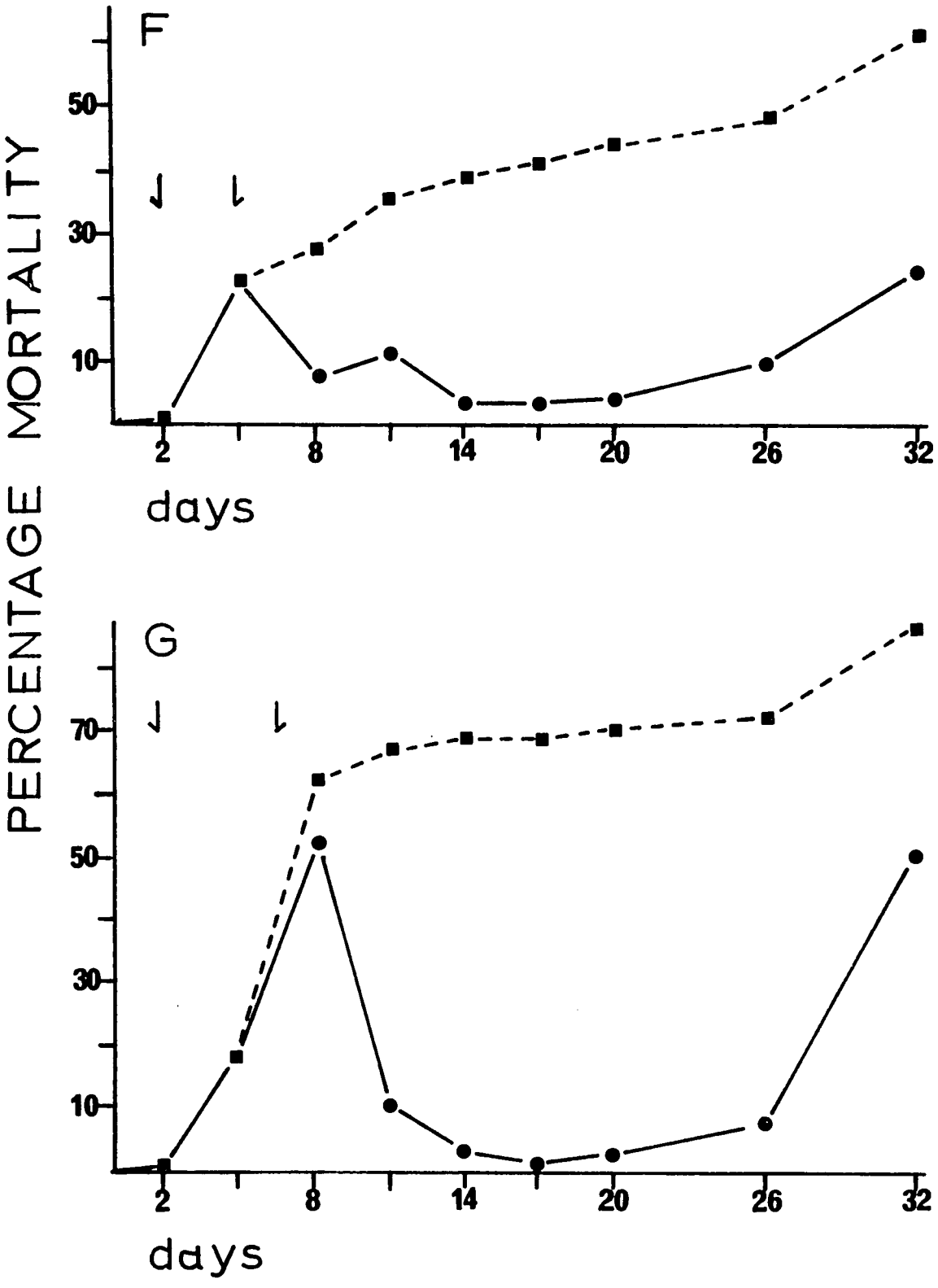

F
G
PERCENTAGE MORTALITY
50
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26
32
days
days

Plate 4.5

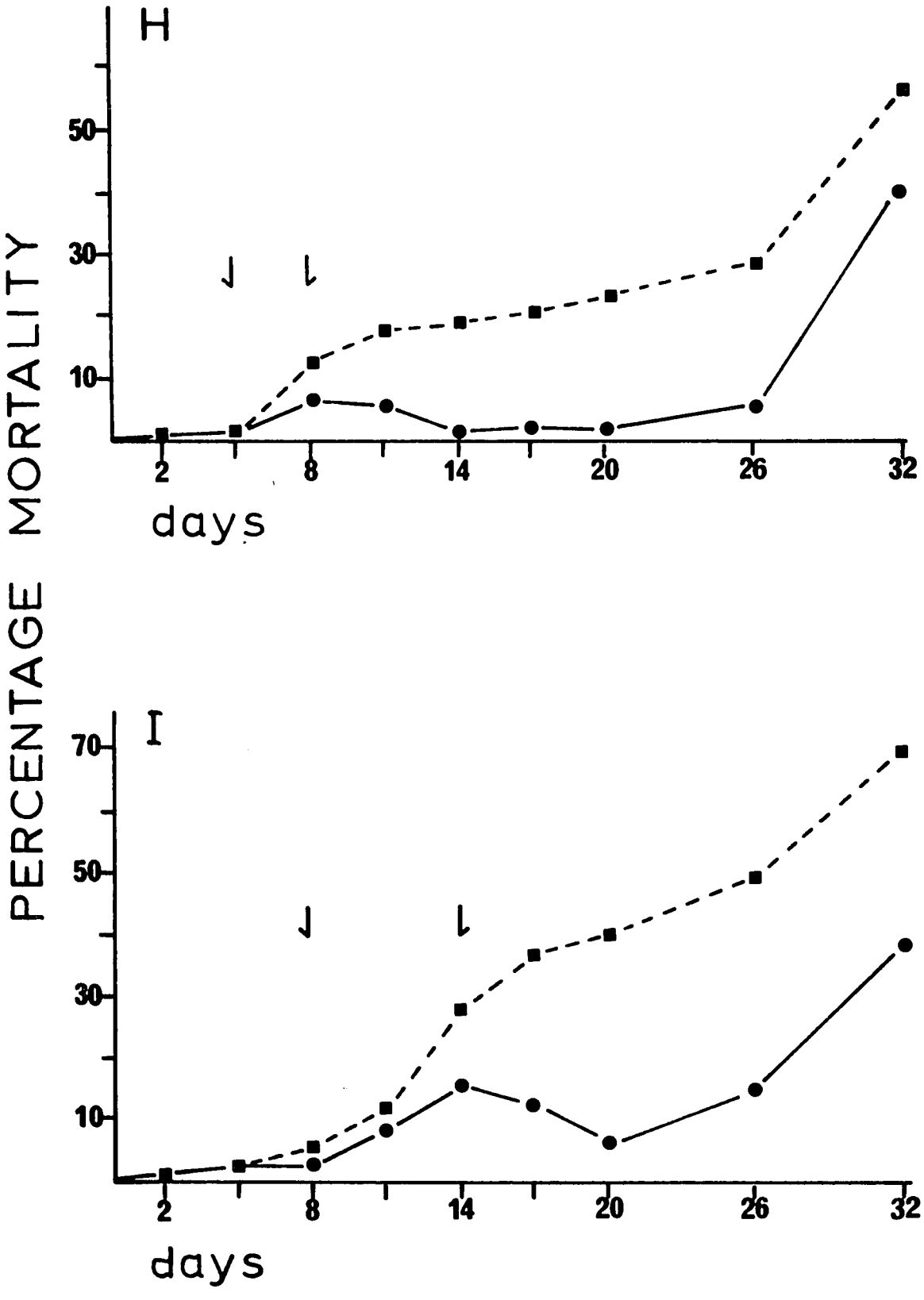


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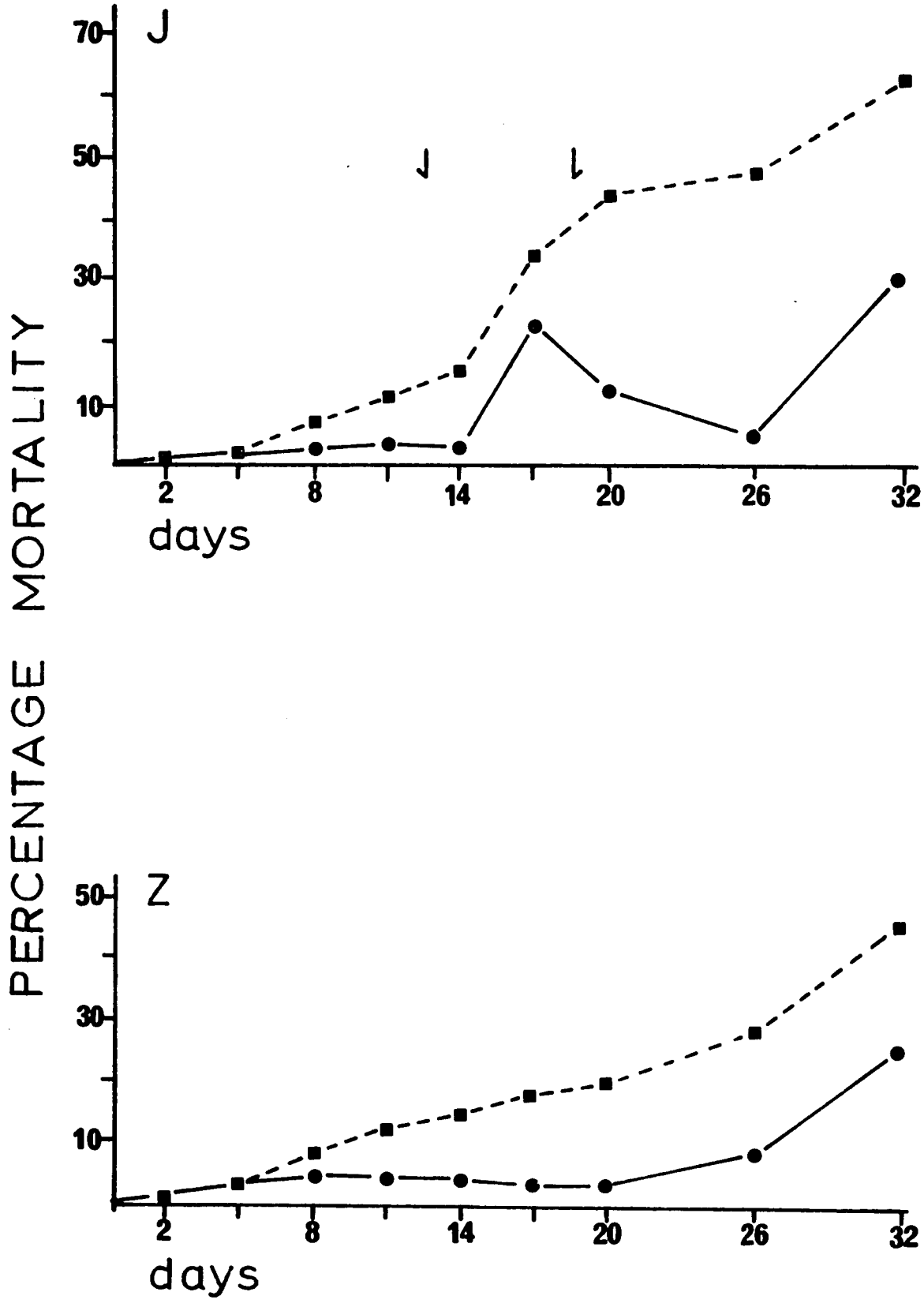

J
70
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days
PERCENTAGE MORTALITY
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8
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20
26
32
days

Plate 4.7

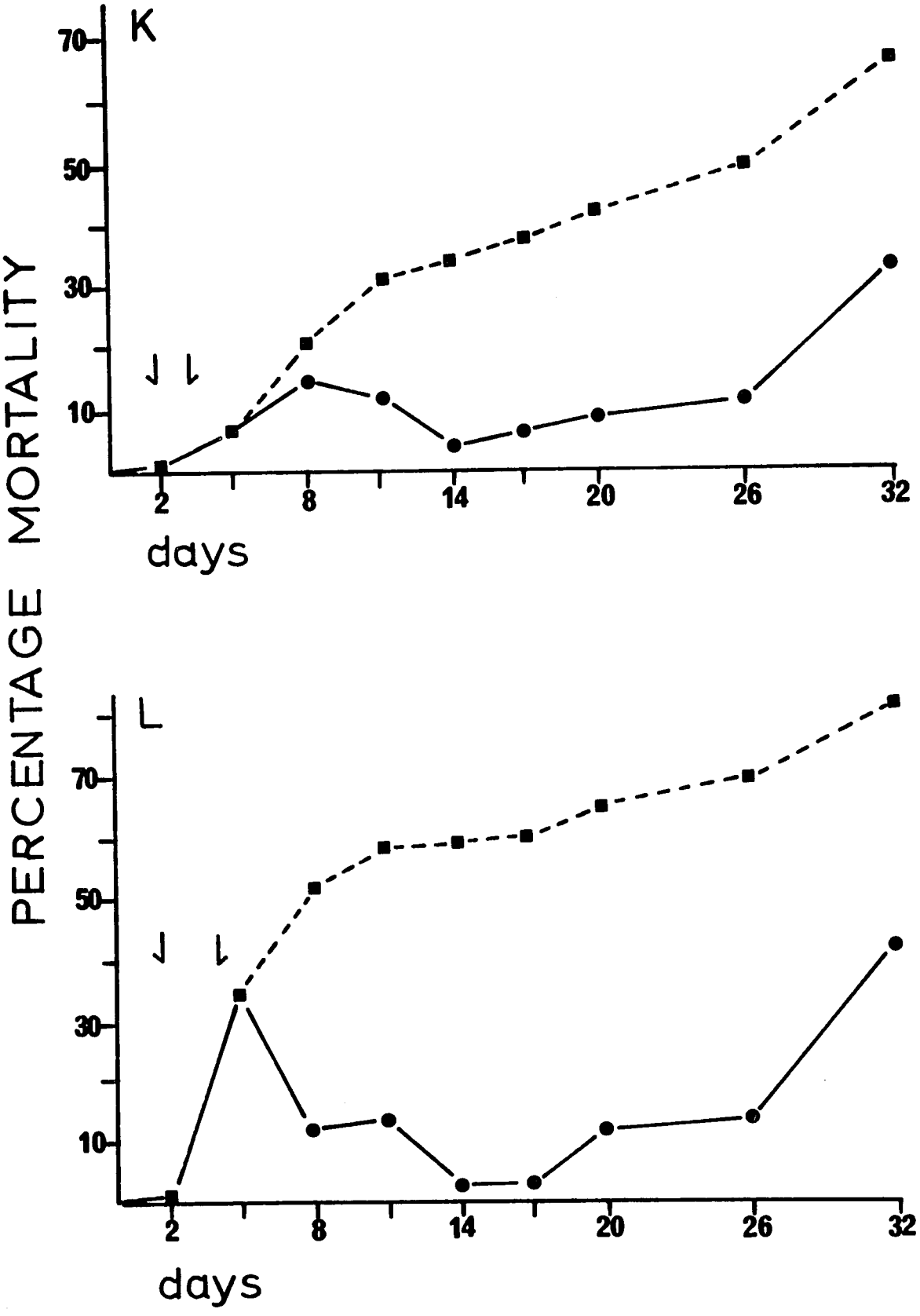

K
70
50
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26
32
days
PERCENTAGE MORTALITY
L
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days

Plate 4.8

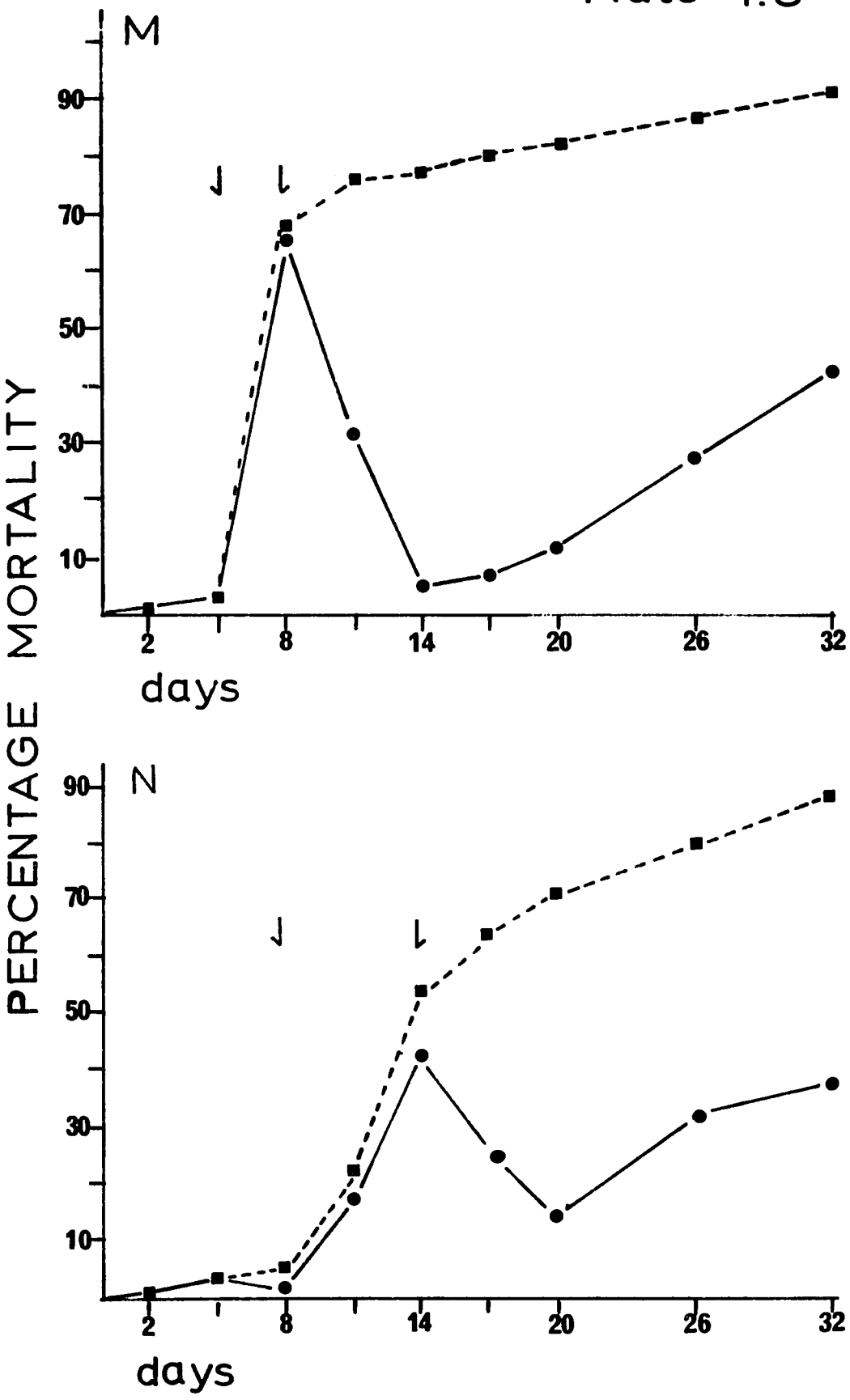

M
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days
PERCENTAGE MORTALITY
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32
days

Plate 4.9

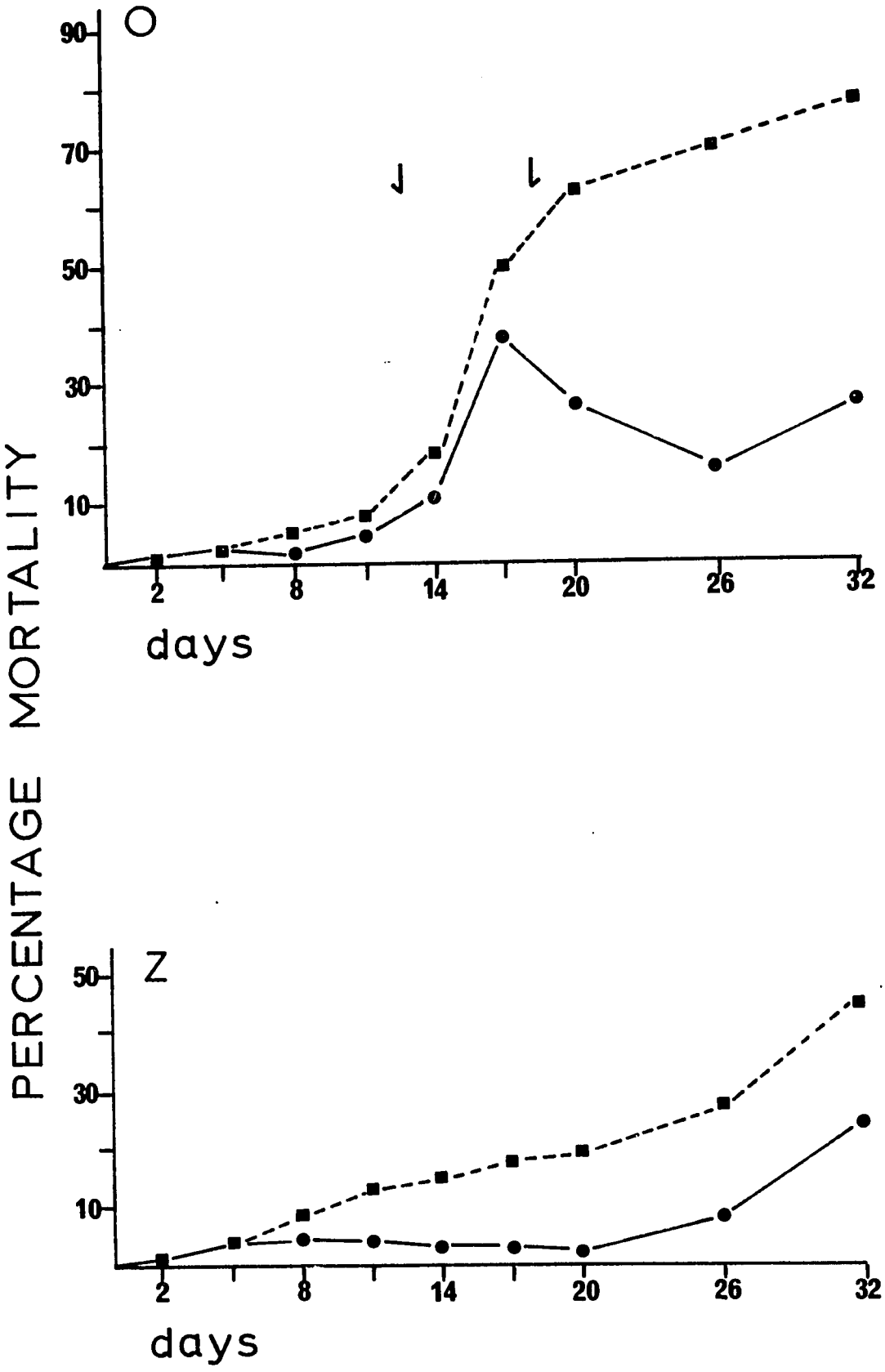

O
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70
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PERCENTAGE MORTALITY
2
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26
32
days
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32
days

Plate 4.10

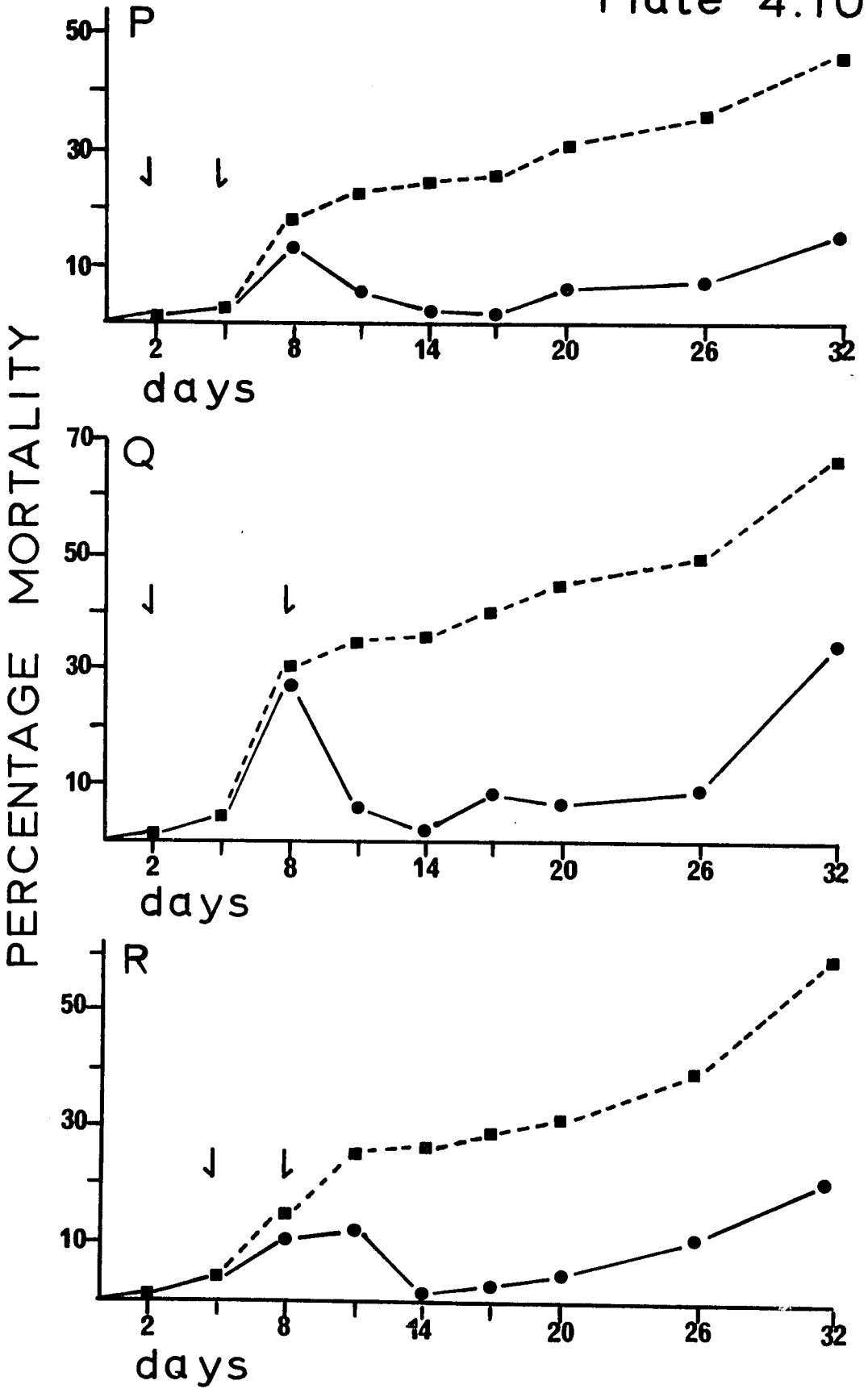


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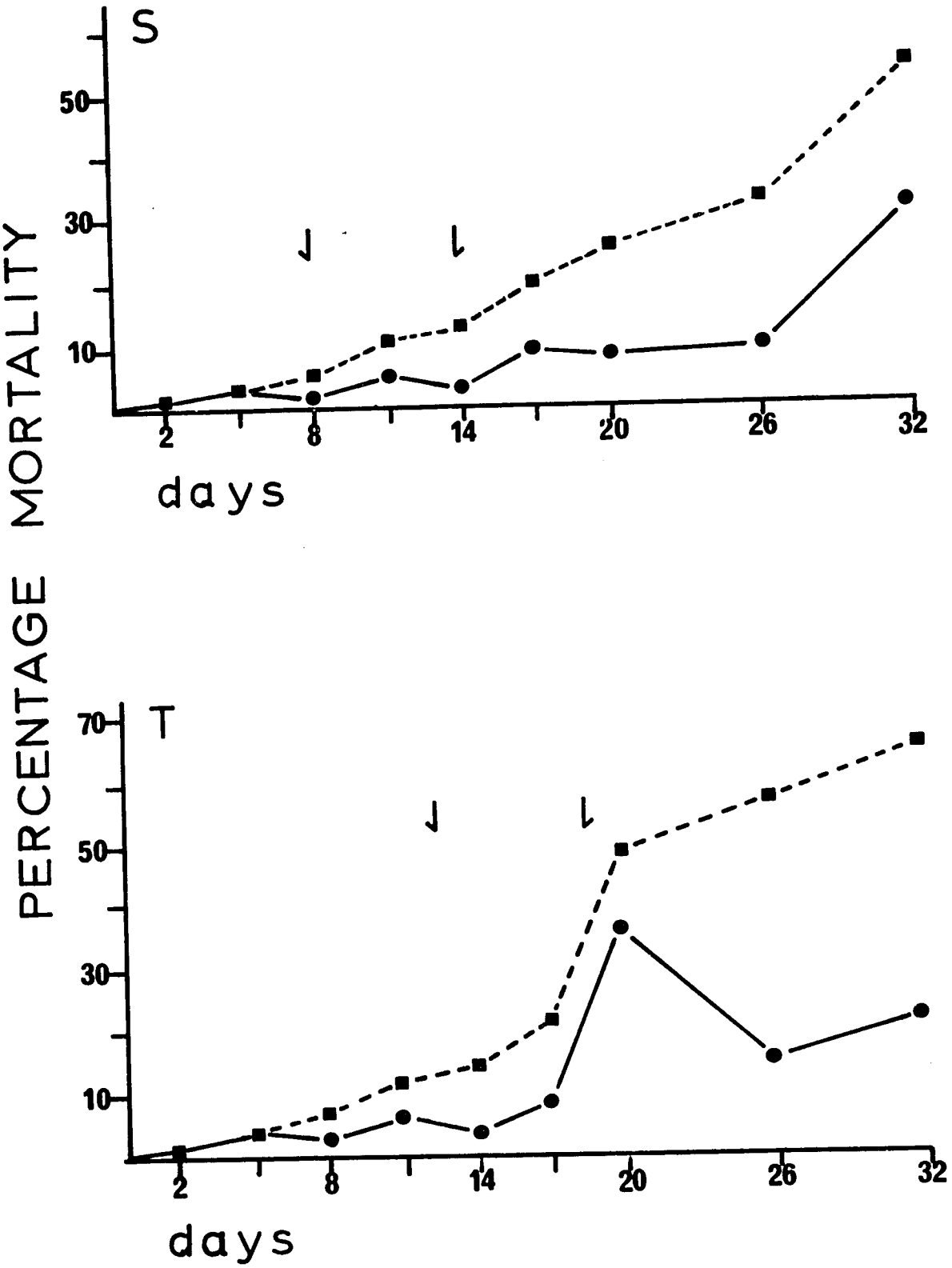

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PERCENTAGE MORTALITY
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32
days

Plate 4.12

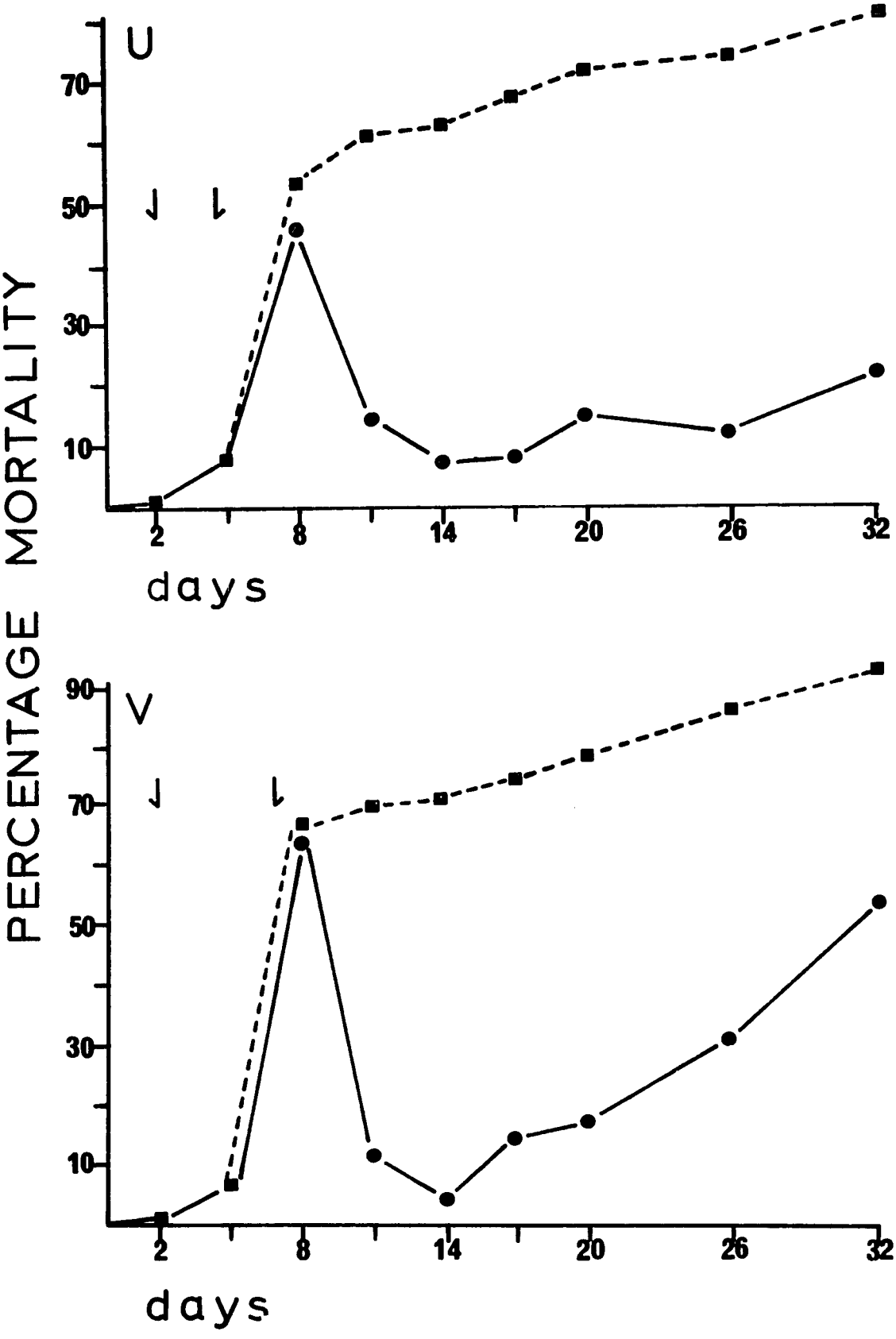


Plate 4.13

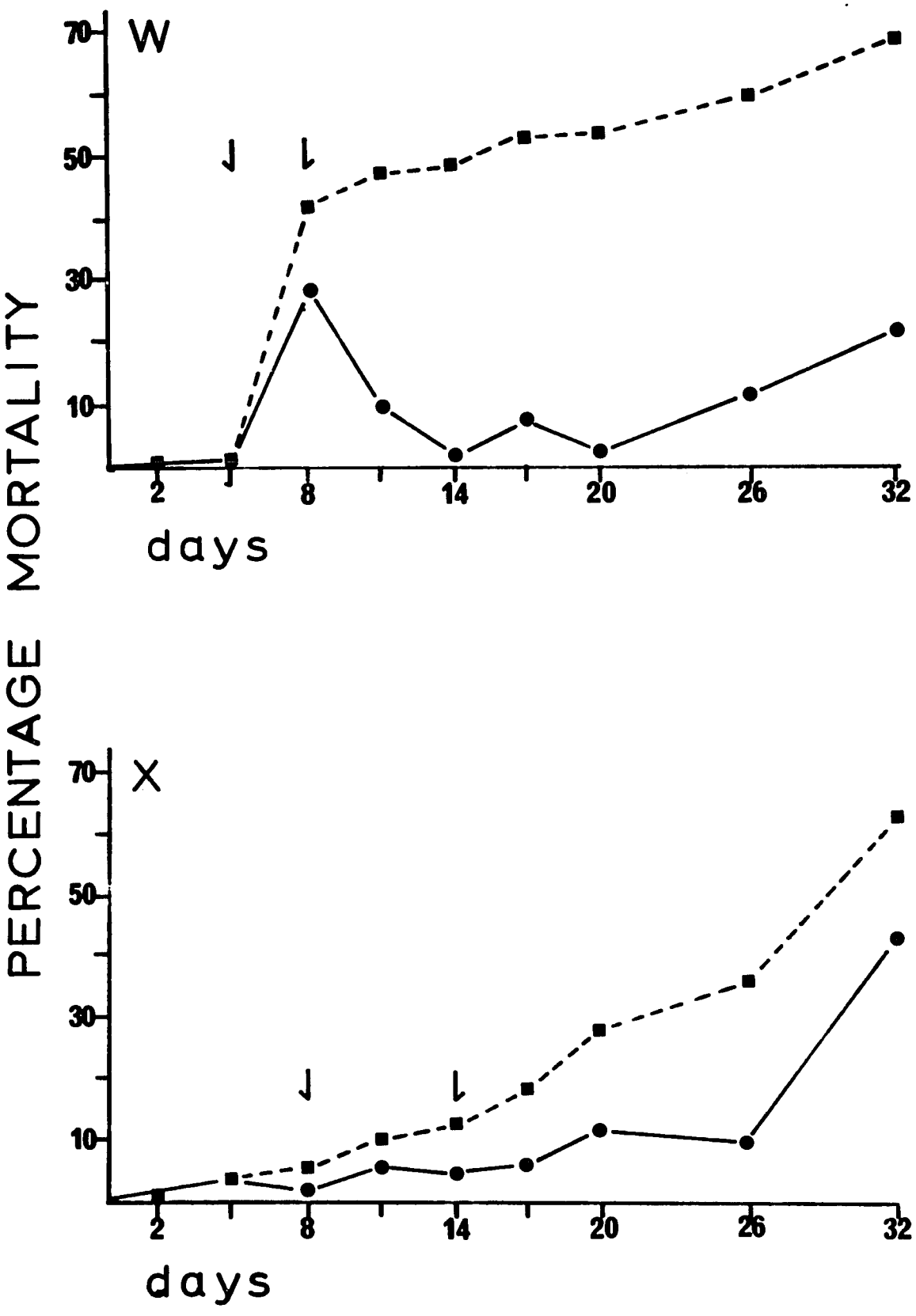


Plate 4.14

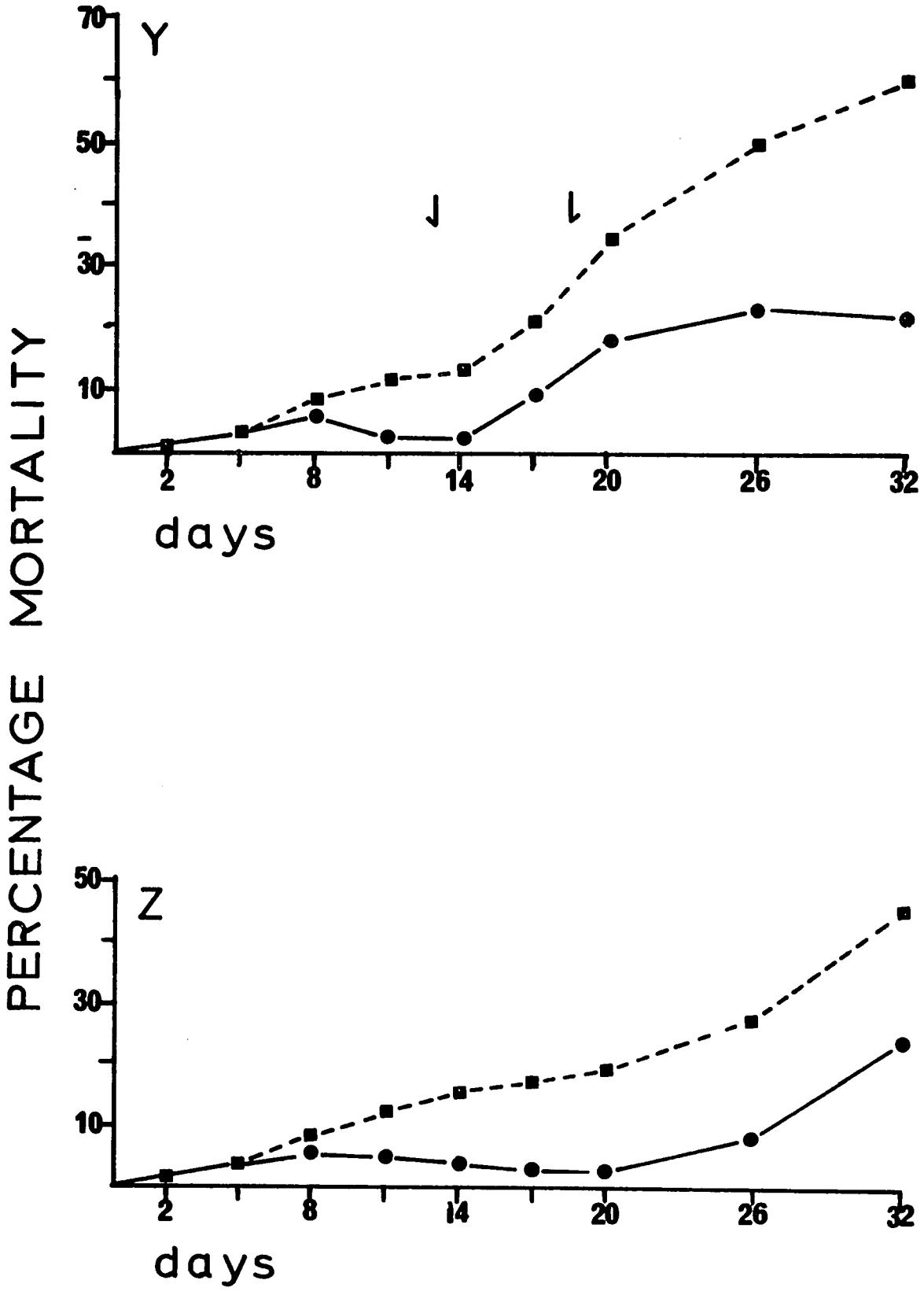

PERCENTAGE MORTALITY
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20
26
32
days
Z
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30
10
2
8
14
20
26
32
days

PLATE 4.15

Fig. 27. Percentages of mortality, hatching and unhatching in treated Salvelinus fontinalis embryos at day 36. Letters refer to the different types of treatment (see fig. 13). Hatched areas: hatching; dark areas: mortality; white areas: unhatching. The total number of live embryos in each group at day 32 was considered as 100%.

Plate 4.15

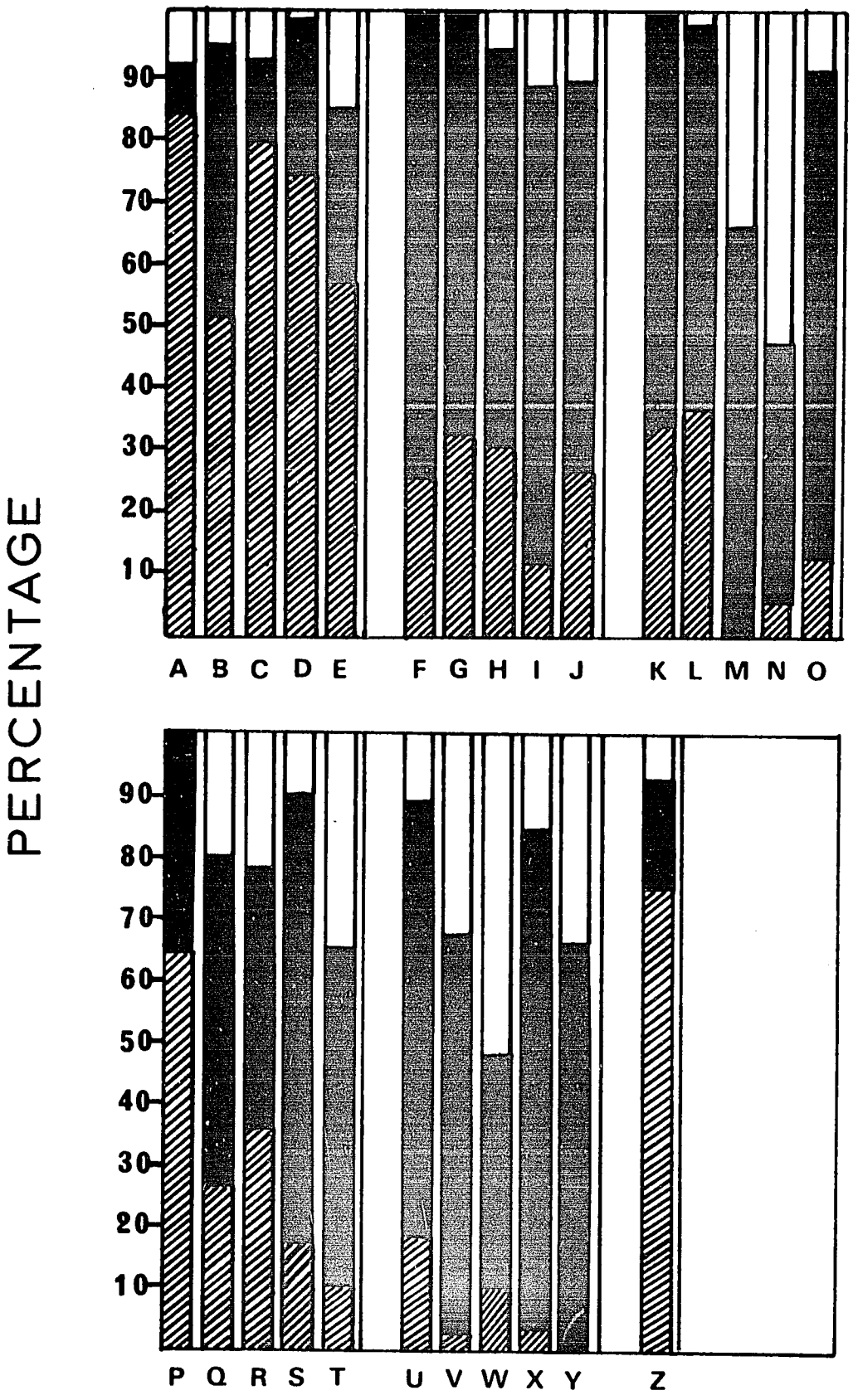

PERCENTAGE
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A B C D E F G H I J K L M N O
P Q R S T U V W X Y Z

Plate 4.15

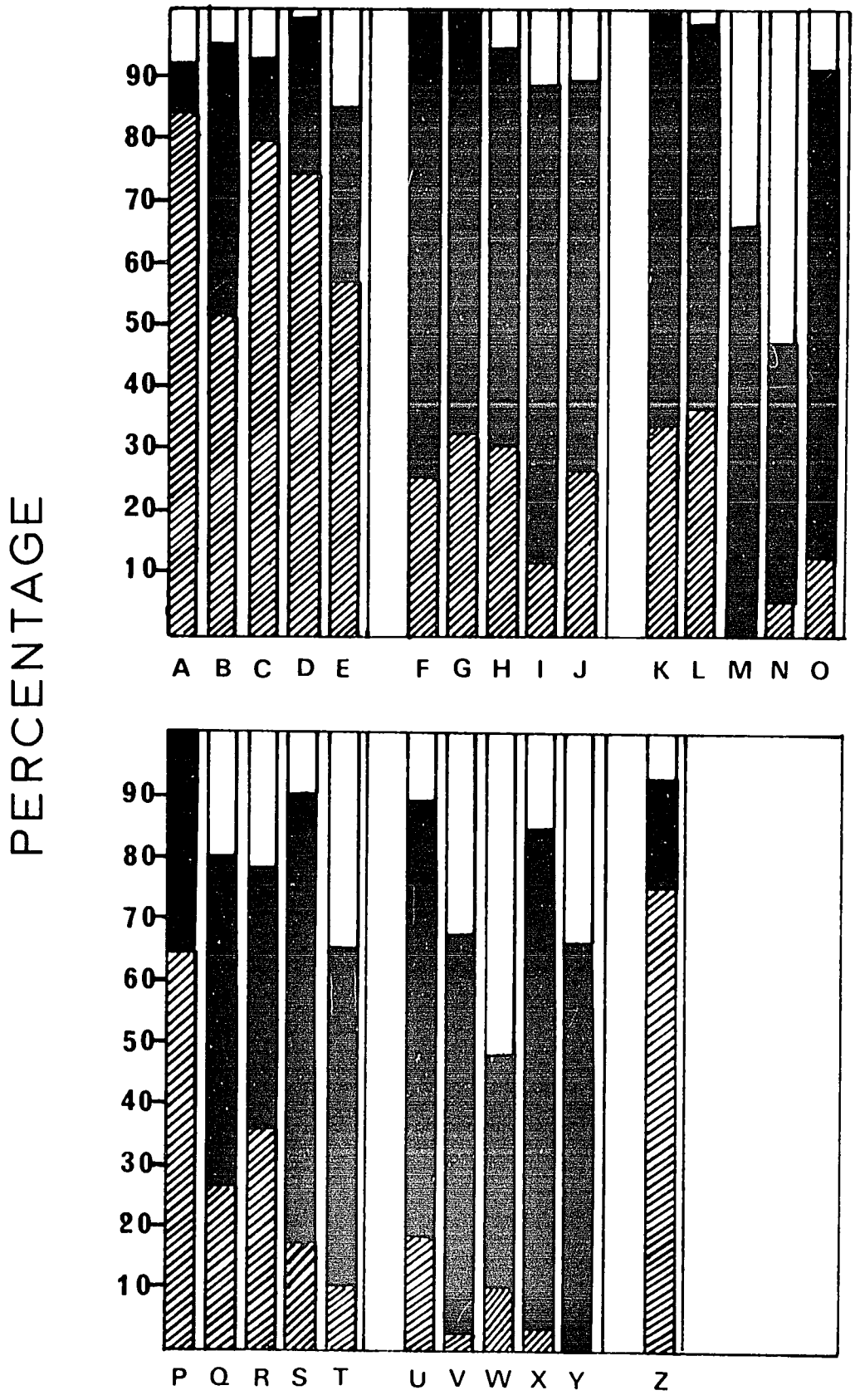

PERCENTAGE
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A B C D E F G H I J K L M N O
P Q R S T U V W X Y Z

PLATE 4.16

Tail abnormalities at day 36 in Salvelinus fontinalis larvae hatched from embryos treated with organophosphates. Magnification: 10 x.

Fig. 28. Larva with dorsally curved tail. Phosphamidon, 100 ppm from day 12.5 to 18.5; J.

Fig. 29. Normal larva (control); Z.

Fig. 30. Larva with the tail ventrally flexed and attached to the yolk. Dylox, 100 ppm from day 5 to day 8 (late gastrulation); R.

Fig. 31. Larva with rolled tail. Phosphamidon, 30 ppm from day 2 to day 5 (early gastrulation); A.

Plate 4.16

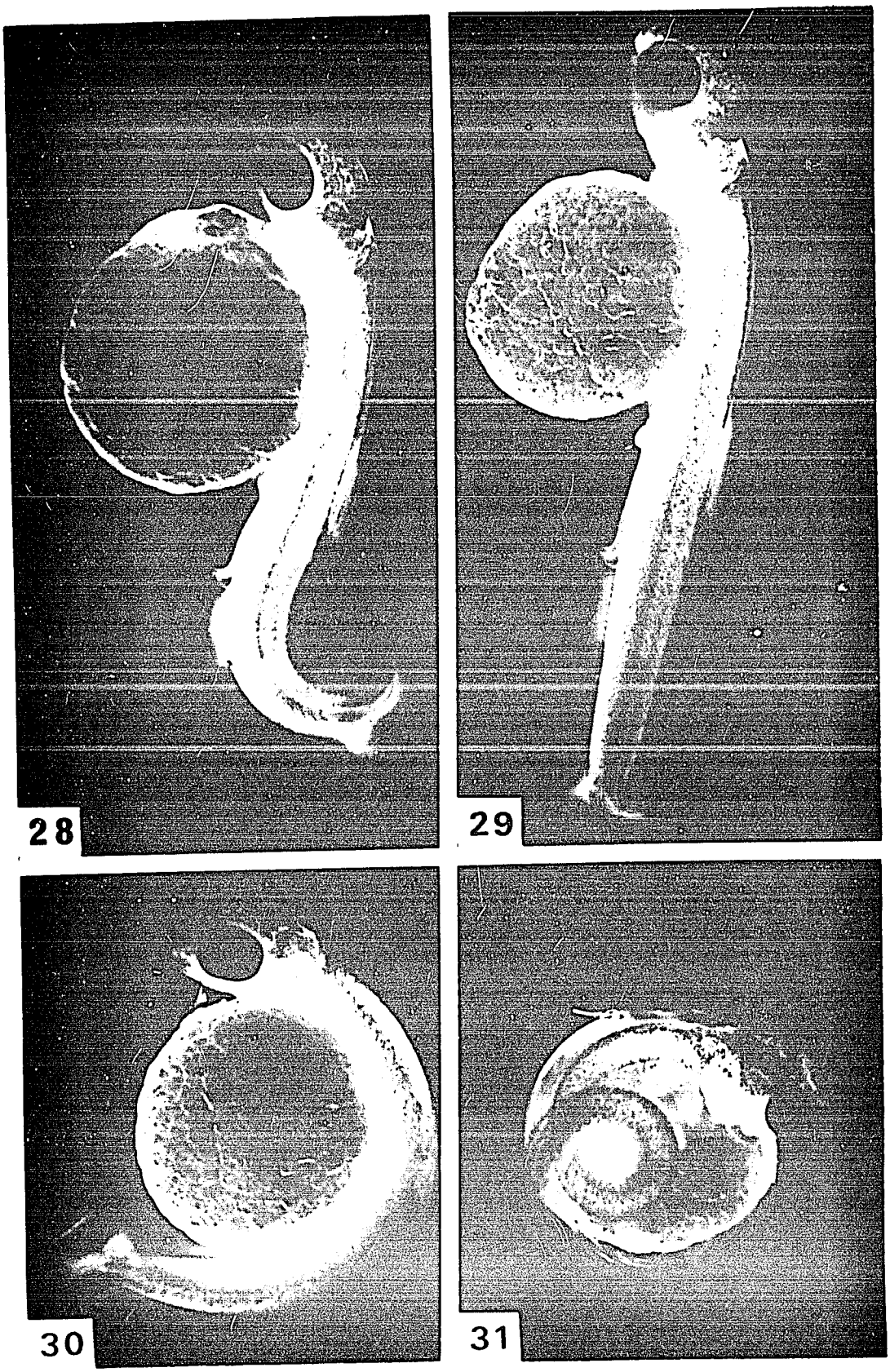


Plate 4.16

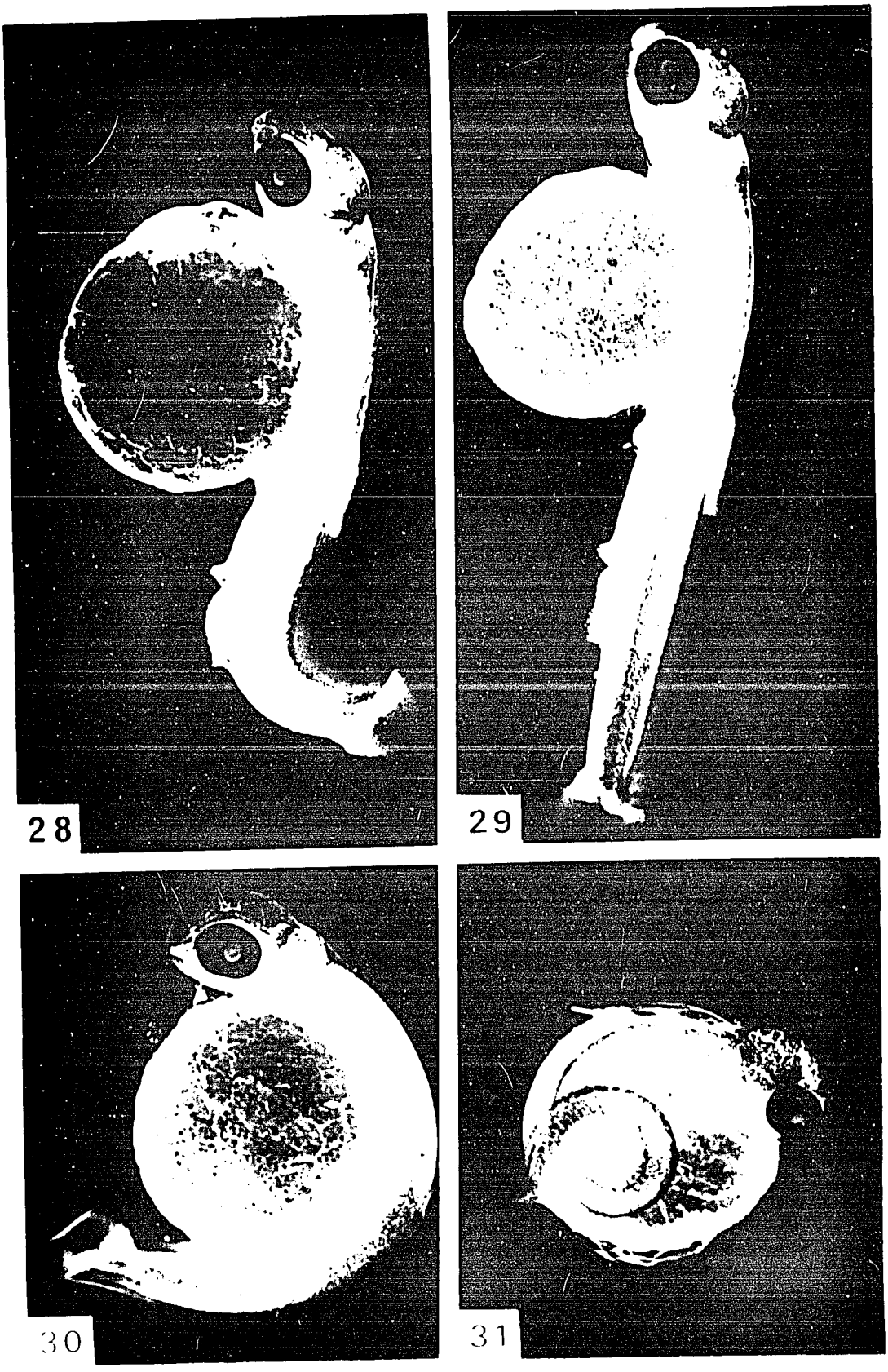


PLATE 4.17

Salvelinus fontinalis embryos at day 32. Magnification: 19 x.

Fig. 32. Control embryo with prehatching morphology (Type 1): Z.

Fig. 33. Delayed embryo (Type 2). Dylox, 100 ppm from day 5 to day 8 (late gastrulation): R.

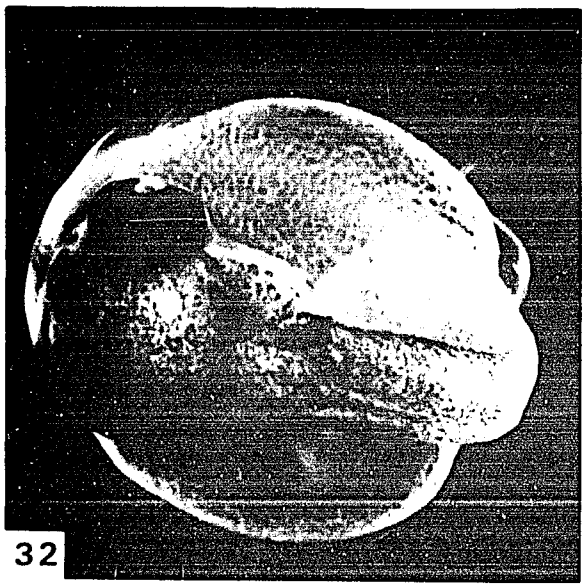
Fig. 34. Delayed and abnormal embryo (Type 3). Phosphamidon, 100 ppm from day 2 to day 5 (early gastrulation): F.

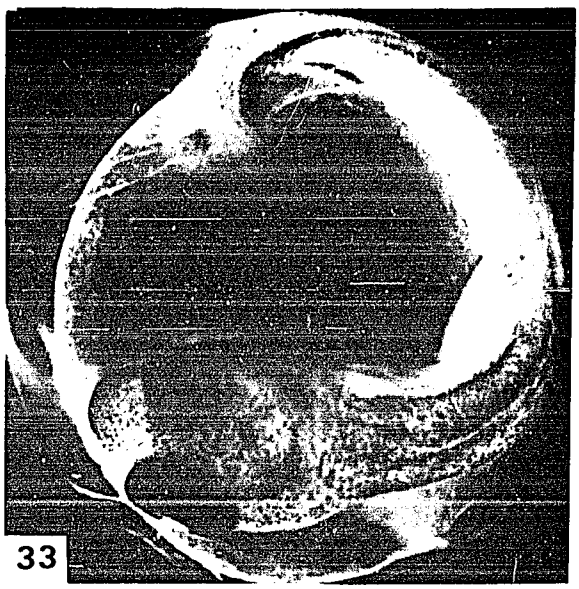
Fig. 35. Small and abnormal embryo (Type 4). Phosphamidon, 100 ppm from day 2 to day 7 (complete gastrulation): G.

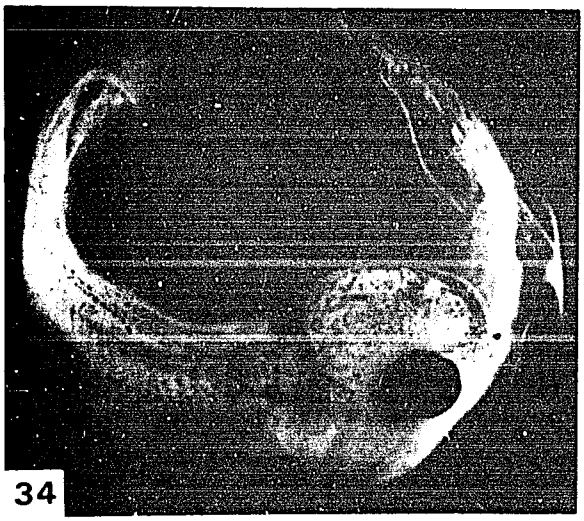
Fig. 36. Small and abnormal embryo (Type 4). The embryo is similar to embryos of day 9 or 10. Dylox, 200 ppm from day 2 to day 7 (complete gastrulation): V.

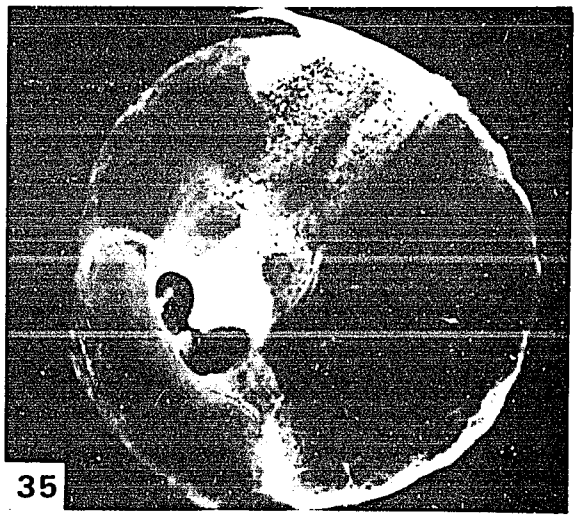
Fig. 37. Small and abnormal embryo (Type 4). Phosphamidon, 200 ppm from day 2 to day 3.5 (very early gastrulation): K.

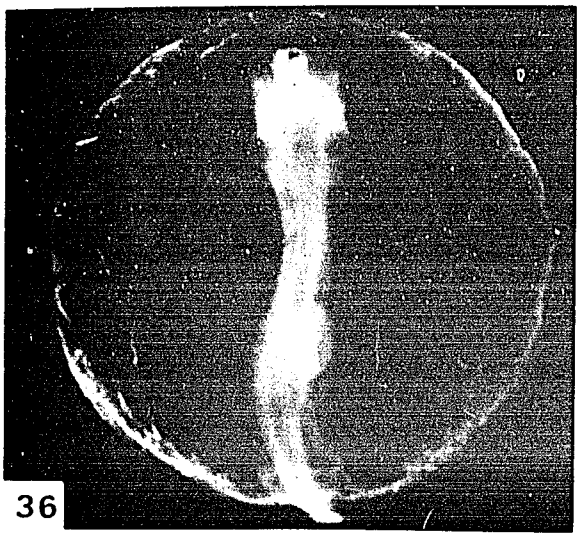
Plate 4.17


32


33


34


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36

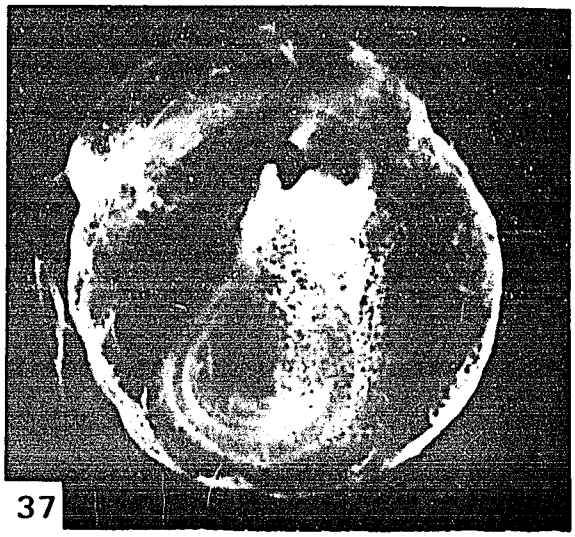
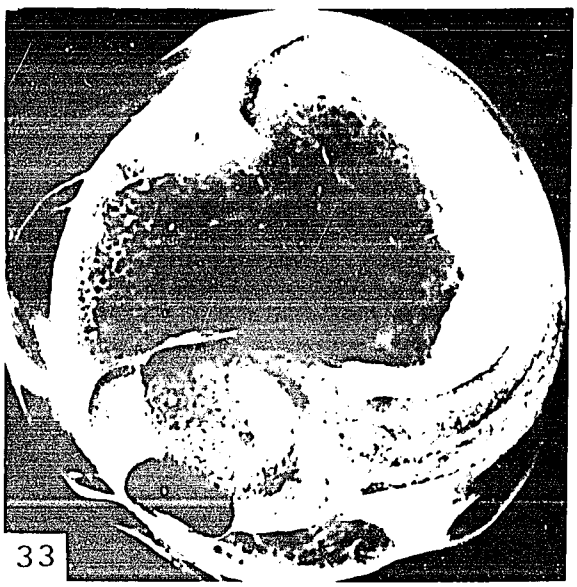
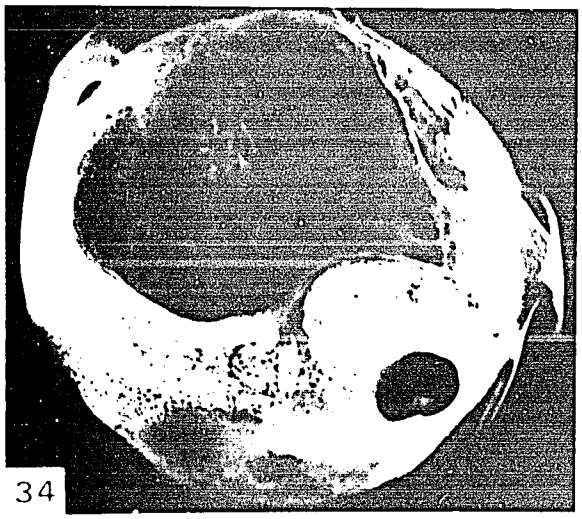
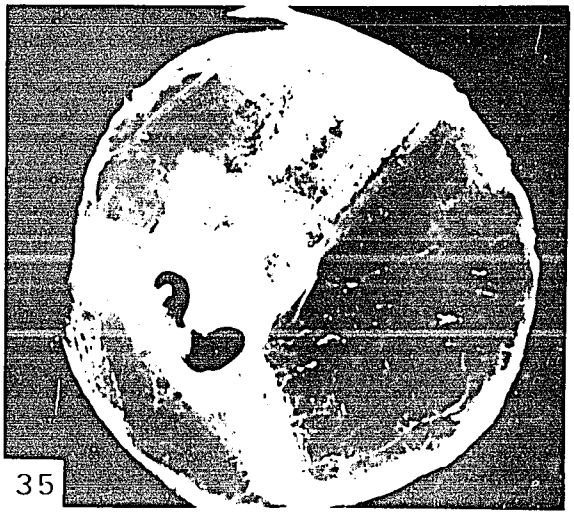

37

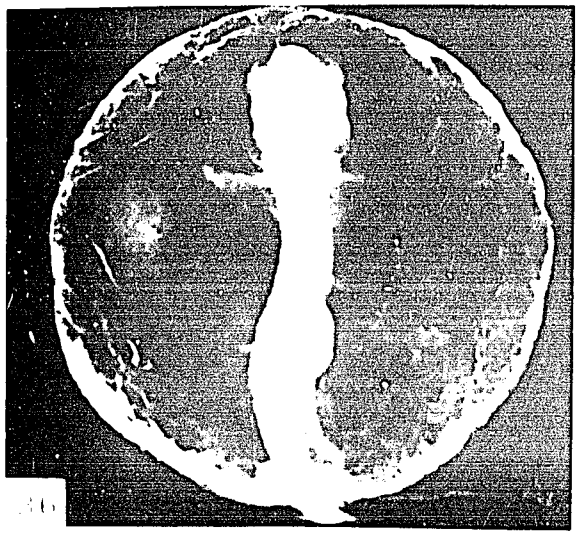
Plate 117


32


33


34


35


36

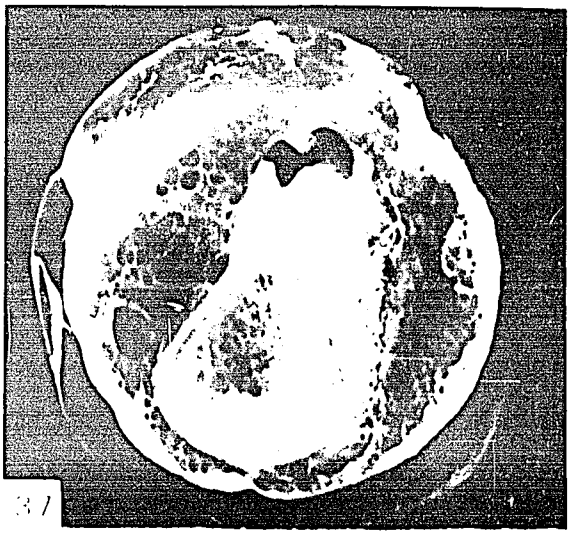

37

PLATE 4.18

Small and abnormal Salvelinus fontinalis embryos (Type 4) at day 32. Magnification: 25 x.

Fig. 38. Embryo with flat head, prominent otic capsule and unsegmented somites. Phosphamidon, 200 ppm from day 8 to day 14 (closed blastopore stage to rolled tail stage); N.

Fig. 39. Embryo with flat head, unequal eyes and large otic capsules. Phosphamidon, 100 ppm from day 5 to day 8 (late gastrulation); H.

Fig. 40. Cyclopean embryo showing vacuolization of the body. Phosphamidon, 200 ppm from day 5 to day 8 (late gastrulation); M.

Fig. 41. Cyclopean embryo with lateral mouth and large otic capsules. Dylox, 200 ppm from day 2 to day 7 (complete gastrulation); V.

Plate 4.18



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Plate 4.18



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PLATE 4.19

Salvelinus fontinalis embryos at day 26 and day 20. Magnification: 19 x.

Fig. 42. Normal embryo (control) at day 26: Z.

Fig. 43. Strongly abnormal embryo at day 26. Phosphamidon, 200 ppm from day 5 to day 8 (late gastrulation): M.

Fig. 44. Normal embryo (control) at day 20: Z.

Fig. 45. Delayed and slightly abnormal embryo (Type 3) at day 20. Phosphamidon, 100 ppm from day 12.5 to day 18.5: J.

Fig. 46. Small and abnormal embryo (Type 4) at day 20. The eyes are proeminent and the forehead is narrow. Phosphamidon, 100 ppm from day 2 to day 5 (early gastrulation): F.

Fig. 47. Small and abnormal embryo (Type 4) at day 20. The eyes are absent. Dylox, 200 ppm from day 2 to day 5 (early gastrulation): U.

Fig. 48. Small and abnormal embryo (Type 4) at day 20. The blastopore is still open and the contorted notochord is displaced. Dylox, 100 ppm from day 2 to day 5 (early gastrulation): P.

Plate 4.19

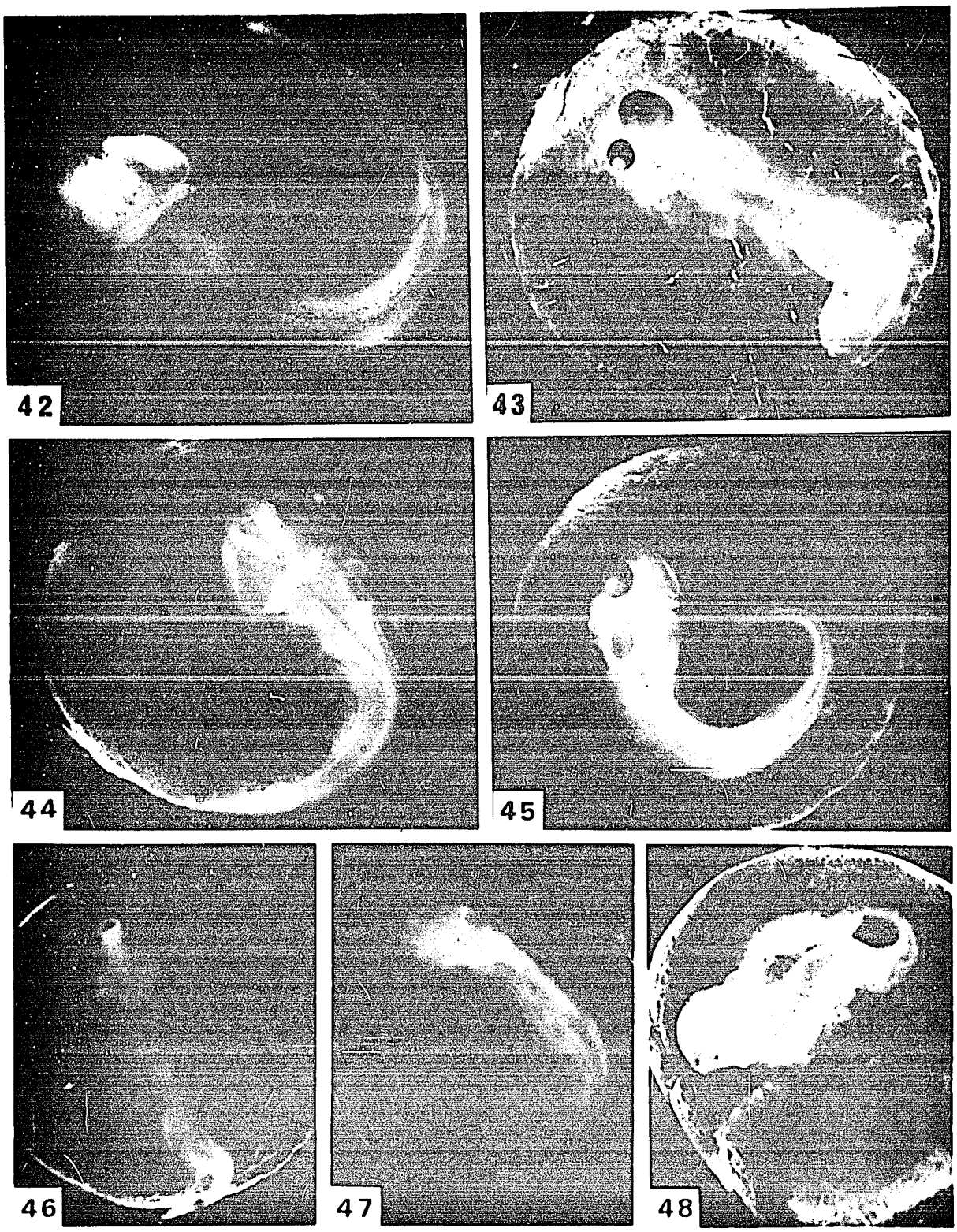


Plate 41

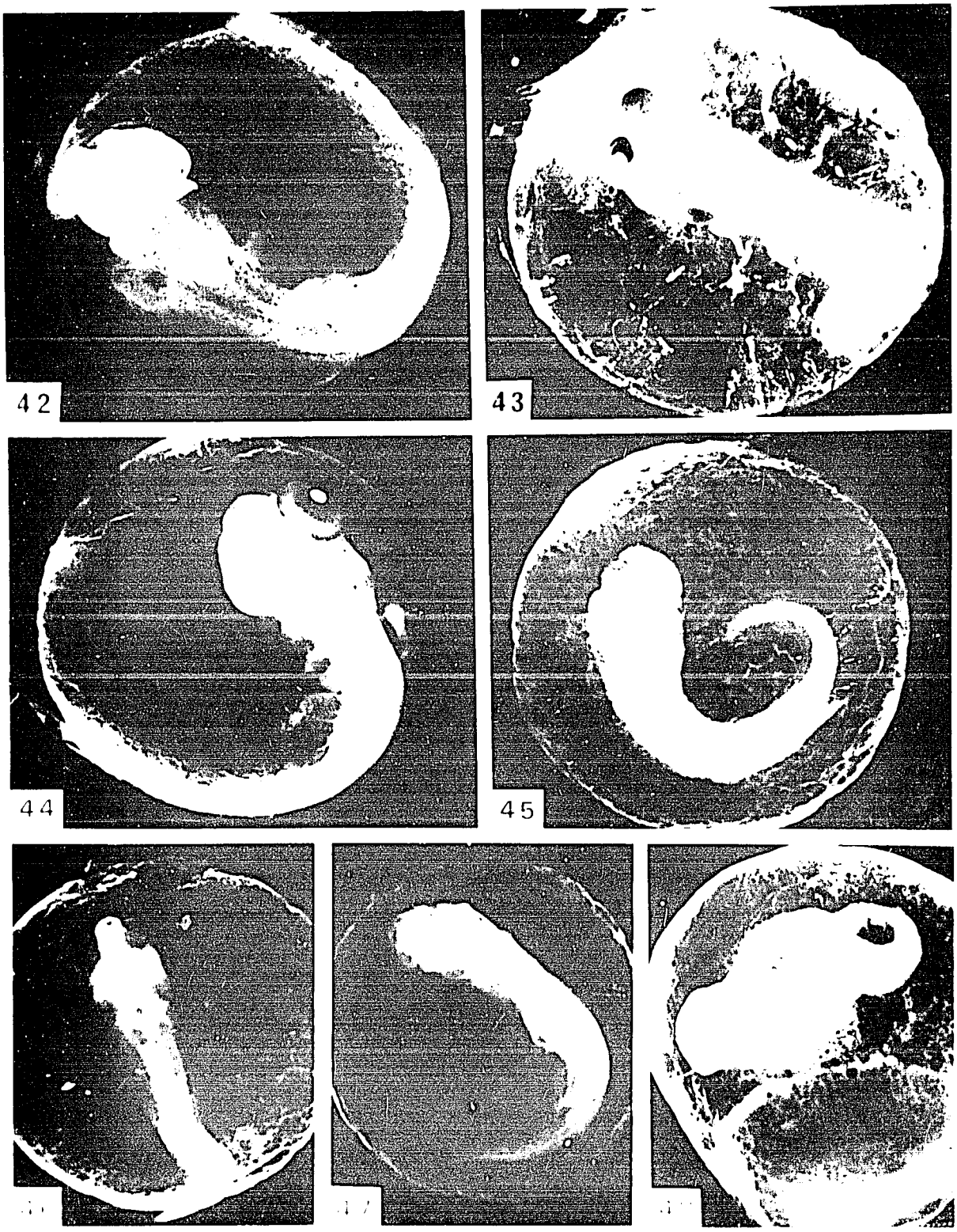


PLATE 4.20

Salvelinus fontinalis embryos at day 14 and day 11. Magnification: 19 x.

Fig. 49. Abnormal embryo at day 14. The eyes are absent and the head is wide. Phosphamidon, 200 ppm from day 8 to day 14: N.

Fig. 50. Strongly abnormal embryo at day 14. The body is almost reduced to the notochord. Dylox, 200 ppm from day 2 to day 7: V.

Fig. 51. Normal embryo (control) at day 14.

Fig. 52. Very abnormal embryo at day 14. The neural tube is unclosed and the body tissue is disorganized. Phosphamidon, 100 ppm from day 2 to day 5: F.

Fig. 53. Very abnormal embryo at day 14. One eye is absent and the head is poorly differentiated. Dylox, 200 ppm from day 2 to day 5: U.

Fig. 54. Abnormal embryo at day 11. The blastopore is unclosed. Dylox, 200 ppm from day 2 to day 5: U.

Fig. 55. Normal embryo (control) at day 11.

Fig. 56. Abnormal embryo at day 11 showing extreme underdevelopment. Dylox, 100 ppm from day 2 to day 5: P.

Fig. 57. Abnormal embryo at day 11. Poor differentiation of the head. Dylox, 200 ppm from day 2 to day 5: U.

Plate 4.20

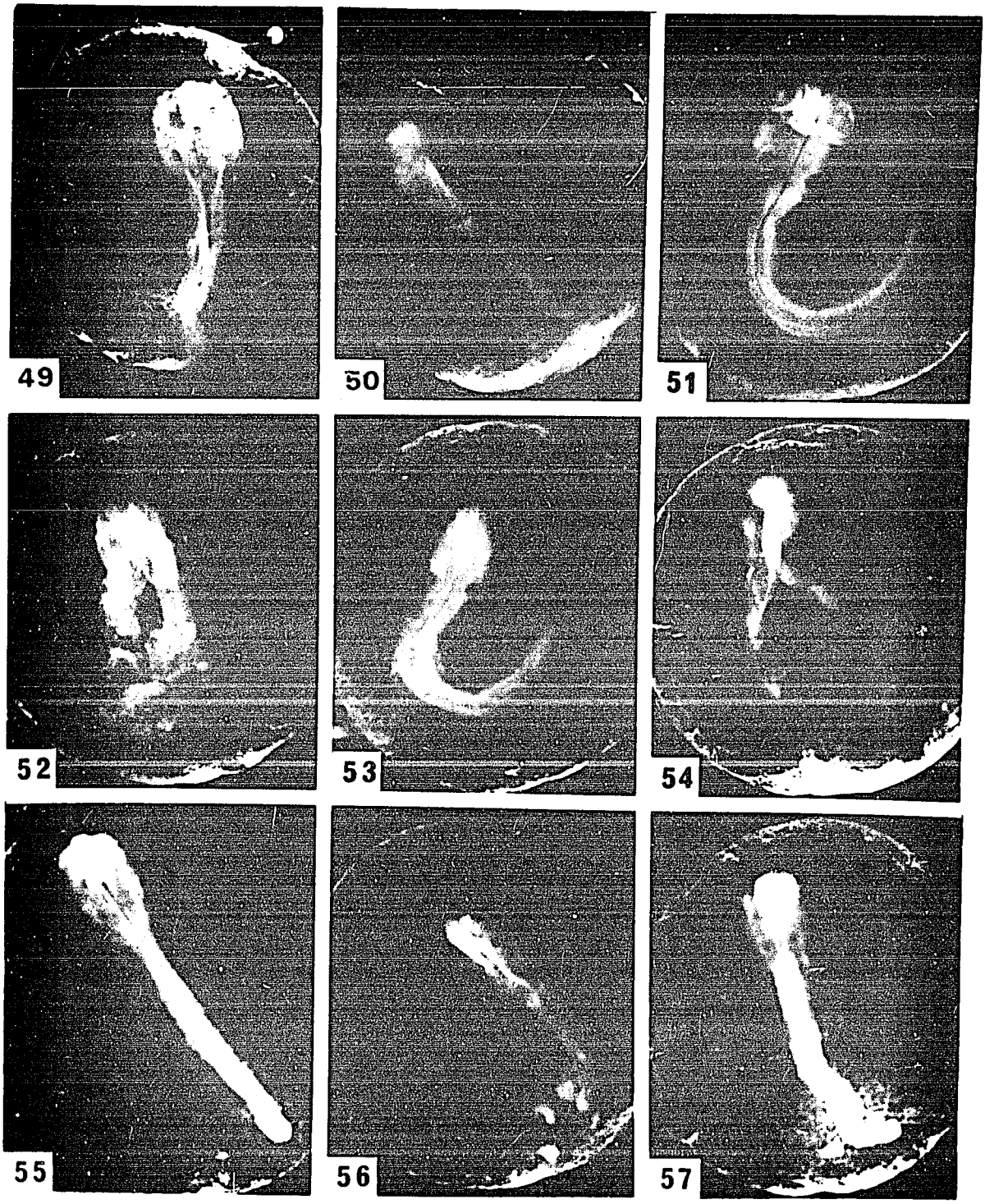


Plate 42

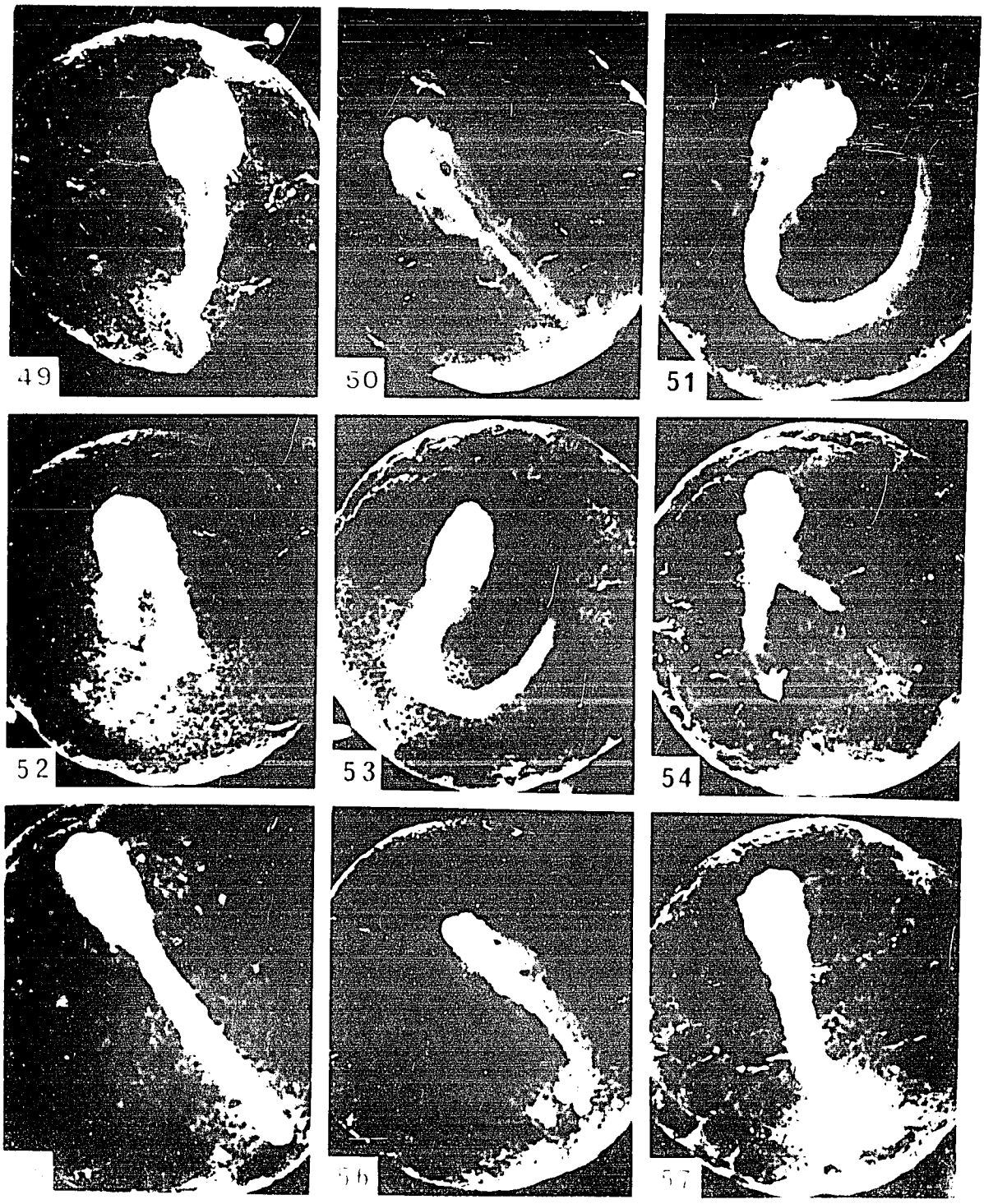


PLATE 4.21

Histology of Salvelinus fontinalis embryos at day 32. Magnification: 56 x.

Fig. 58. Cross section through midbrain of control (Z) showing the optic lobes (ol), the brain cavity, the retina of the eye differentiated into four layers and the lens.

Fig. 59. Cross section through the midbrain of Type 4 embryo (P). The dorsal expansion of the brain would be equivalent to the optic lobes. No cavities are present and there is no cellular differentiation. There is one distorted eye (e) with no lens. The mouth (m) is incomplete and is a vertical slit having some young cartilage trabeculae.

Fig. 60. Section (slightly oblique) through midbrain of Type 3 embryo (U). The brain has optic lobes (ol) and stem but the morphology is abnormal. One eye is greatly reduced, the retina is undifferentiated and the lens is enclosed. The mouth is displaced on the side and the otic capsule (oc) is proeminent.

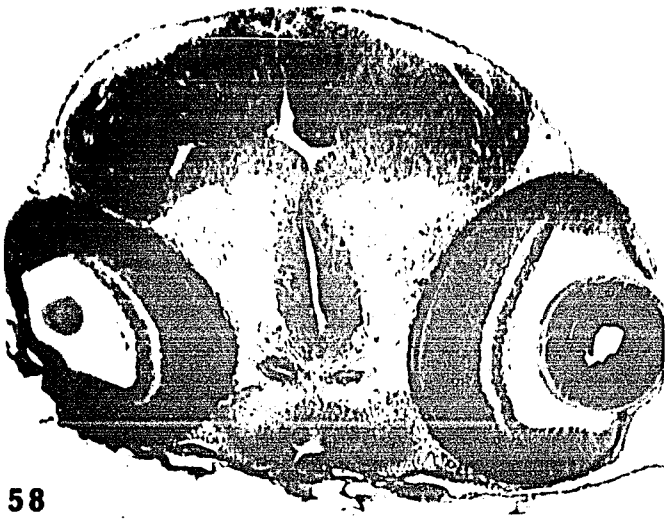
Fig. 61. Cross section through the hindbrain of Type 3 embryo (U). There is cellular differentiation (dorsal cellular and ventral acellular regions) but no cavities. The mouth (m) is an horizontal slit. There are no true gill slits between the cartilage trabeculae.

Fig. 62. Cross section through the hindbrain of Type 4 embryo (F). The hindbrain and the notochord (N) are differentiated but there is no brain cavity. All other structures are absent.

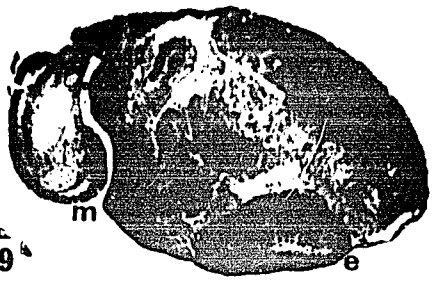
Fig. 63. Cross section through the hindbrain of a control showing the large brain cavity, the large polymorphic otic capsule (OC), the anterior end of the notochord, the horizontal opening of the mouth (m) and the gill arches with cartilage trabeculae.

Fig. 64. Cross section through the hindbrain of Type 4 embryo (M). There is partial differentiation of the brain but the tissue is vesicular. The otic capsules (oc) are large but do not have the normal infolding of

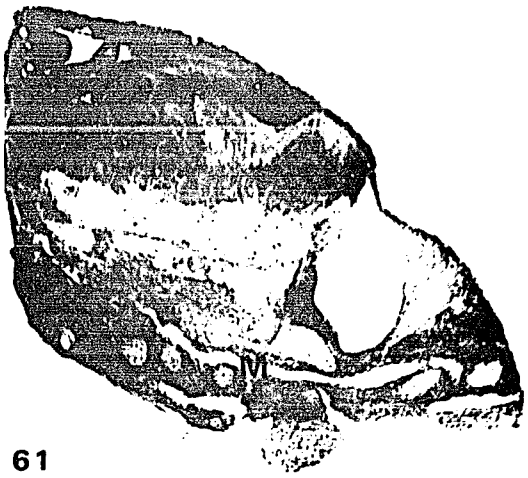
Plate 4.21



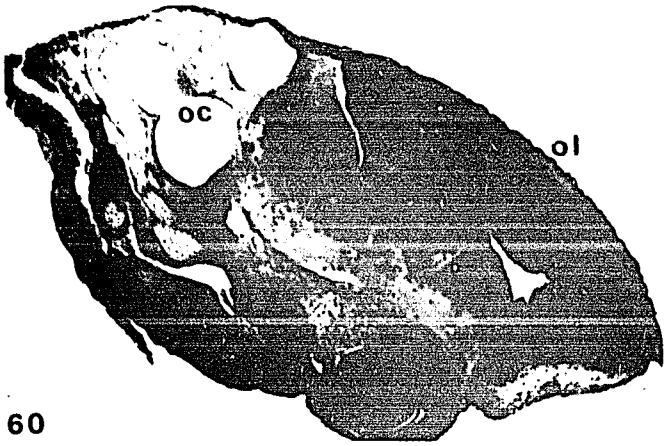
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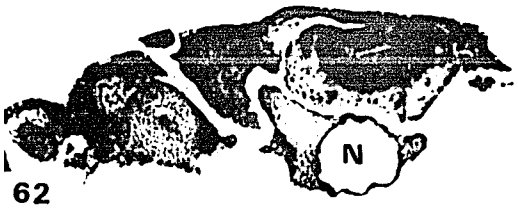
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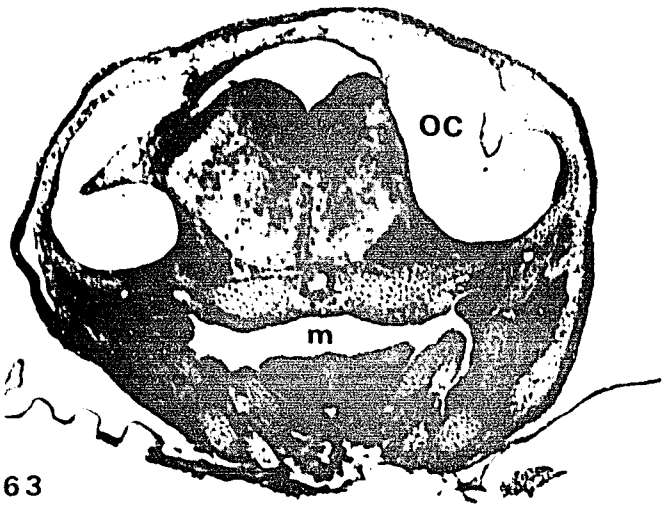
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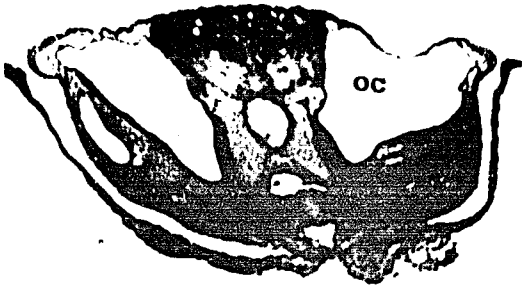
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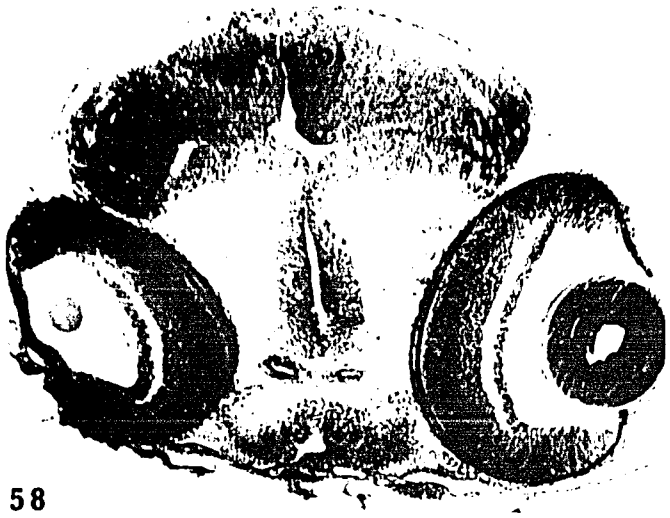


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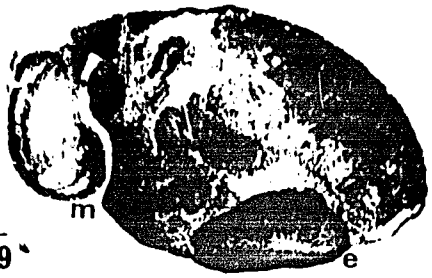


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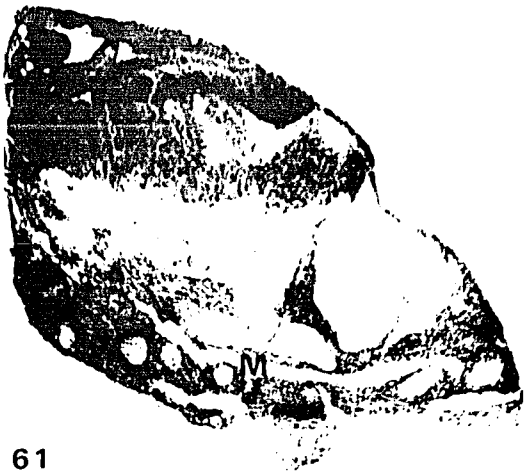
Plate 4.21



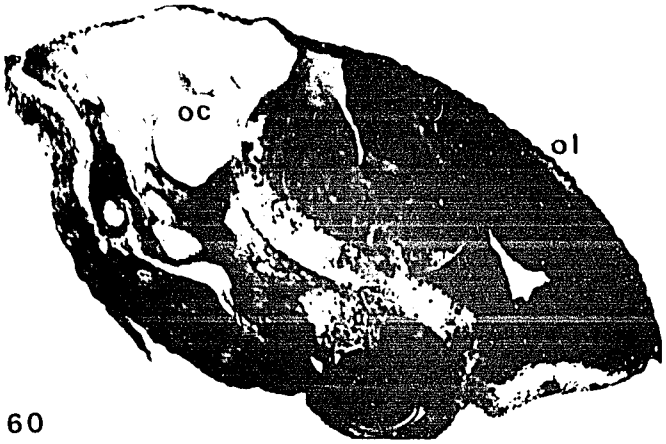
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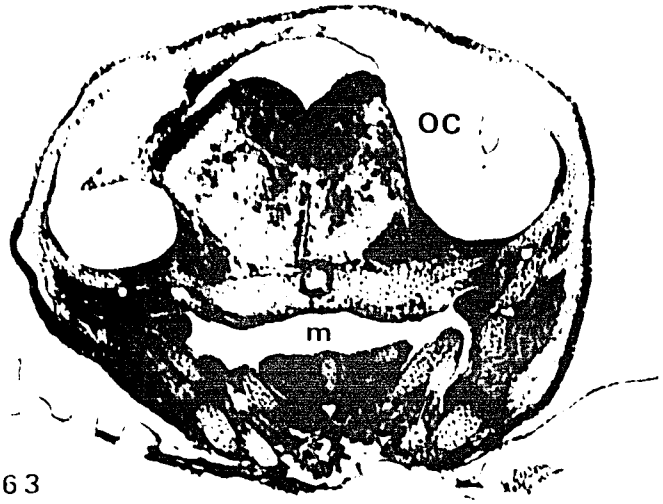
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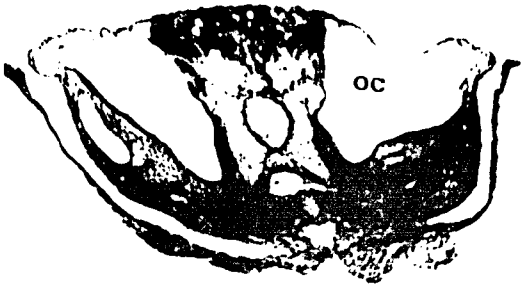
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PLATE 4.22

Histology of Salvelinus fontinalis embryos at day 32.

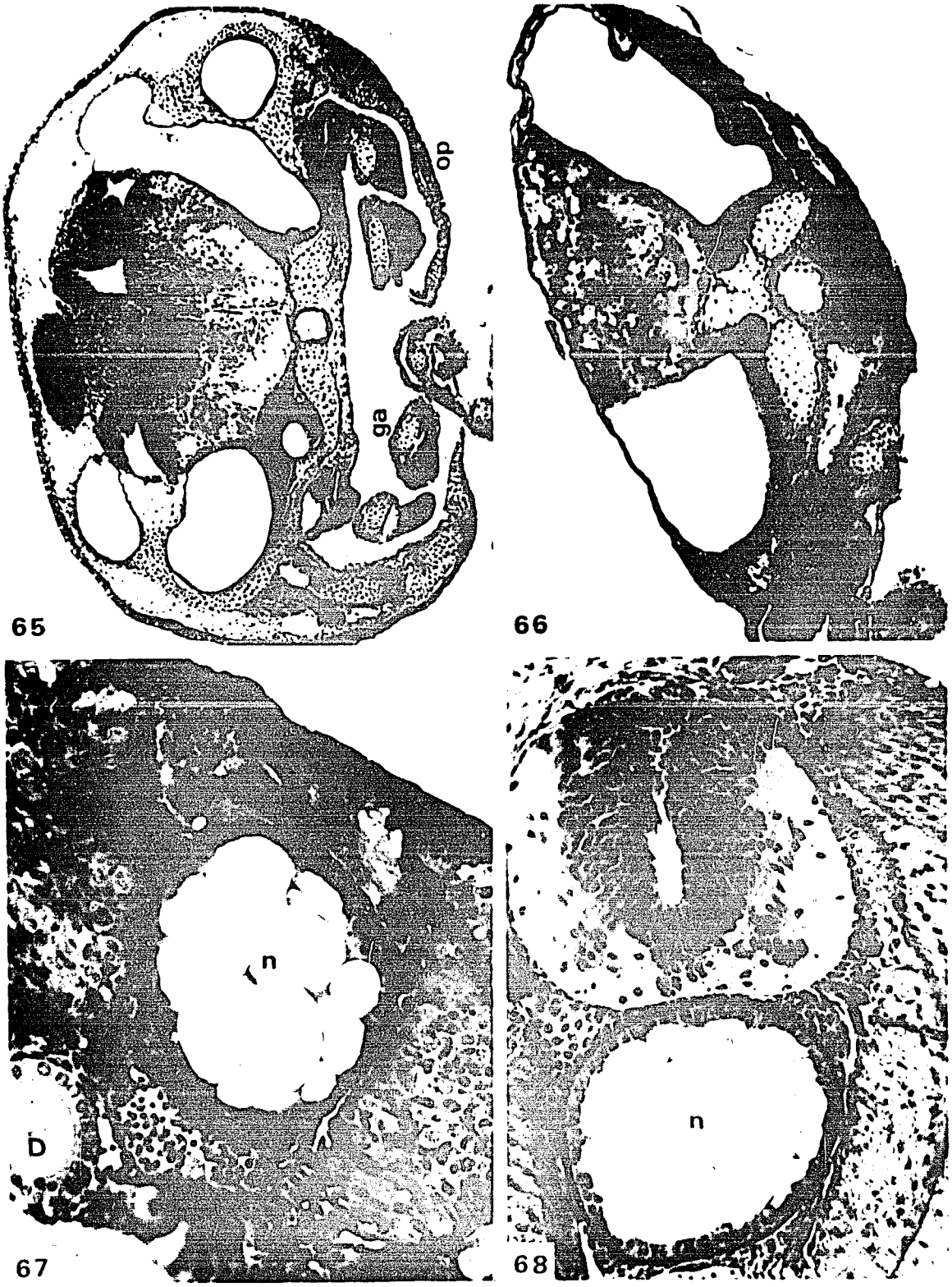
Fig. 65. Cross section through control (Z) at the level of the hindbrain showing normal morphology of the otic capsules (3 chambers), the cartilage structure of the head, the gill arches (ga), the mouth and operculum (op) and the pseudogills (in the dorso-lateral corner of the mouth). 75 x.

Fig. 66. Cross section through the hindbrain of Type 4 embryo (F). The hindbrain is vesicular and there is little differentiation of tissues in the rest of the head. Very little cartilage surrounds the otic capsule and the mouth is very small. No gill arches and operculum are present. 125 x.

Fig. 67. Cross section through the body of Type 4 embryo (U). The neural tube is small, squeezed on the side and the acellular portion is barely present. The notochord is of normal size while the embryo is much smaller. Muscle is abnormal, made of large round cells. The pronephric duct (D) is seen at the lower left corner. 185 x.

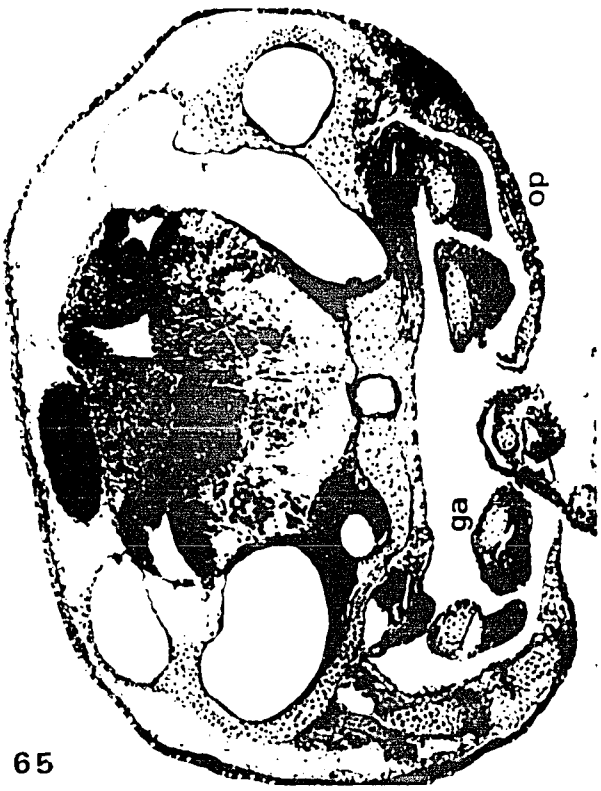
Fig. 68. Cross section through the body of a control embryo (Z) showing the neural tube with the typical cellular arrangement around the neurocoele, the notochord and the myotomes with differentiated muscle cells. 185 x.

Plate 4.22

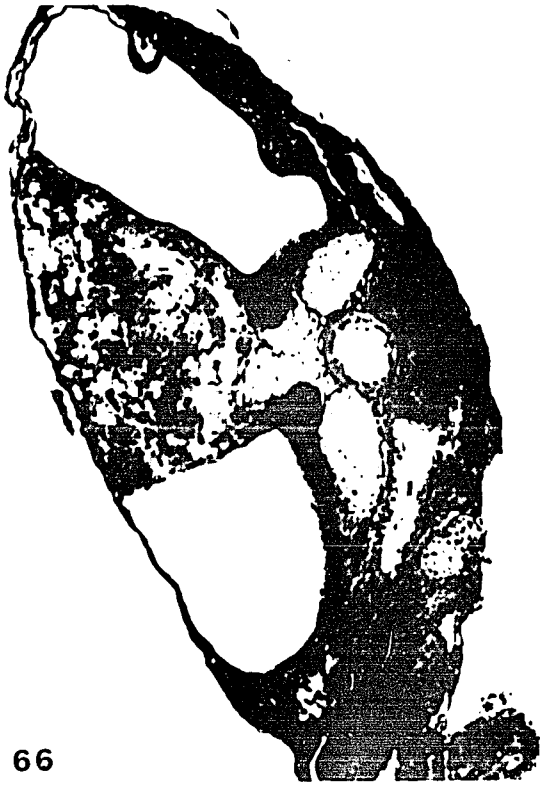


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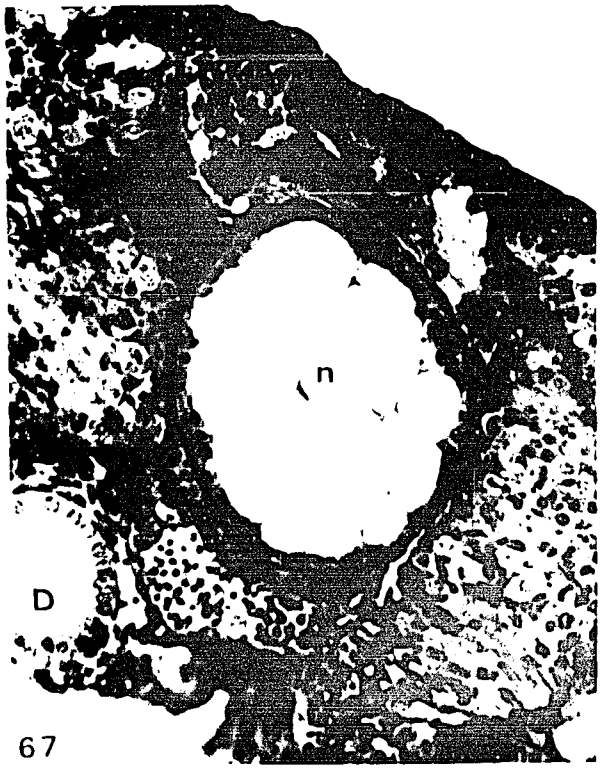
Plate 4.22



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PLATE 4.23

Histology of muscle in Salvelinus fontinalis embryos at day 32. Magnification: 750 x.

Fig. 69. Cross section through the white fibers of the body muscle of a control embryo (Z). The myofibrils are found in small bundles. Nuclei are peripheral and crescent shape.

Fig. 70. Cross section through the white fibers of the body muscle of Type 3 embryo (F). Incomplete differentiation: myofibrils are very small, more evenly distributed than in control and several cells contain central round nuclei.

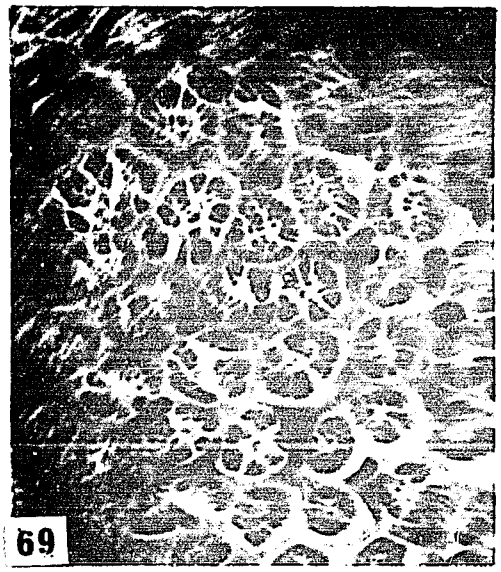
Fig. 71. Longitudinal section through the white fibers of the body muscle of Type 3 embryo (M). Fibers are partially differentiated: cells are much shorter and nuclei are round and central; fibrils are not discrete.

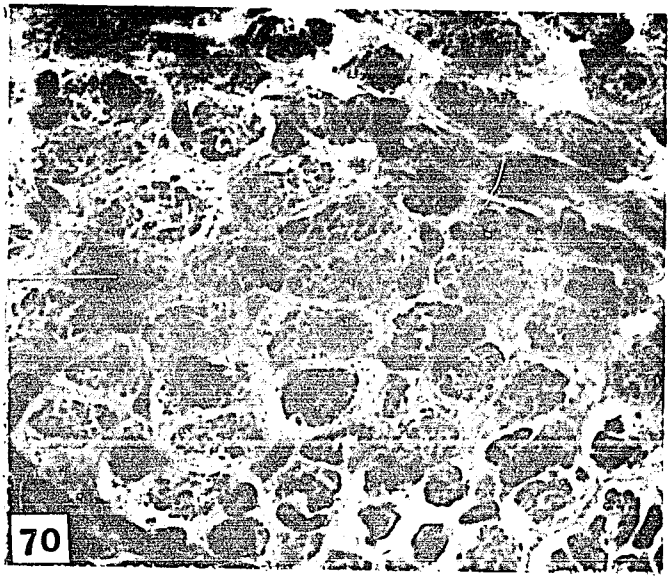
Fig. 72. Longitudinal section through the white fibers of the body muscle of a control embryo (Z). The cells are long and run from one septa to the other. The nuclei are peripheral and most of them are spindle shape. Only a few of the cross striations of the myofibrils are apparent in this figure.

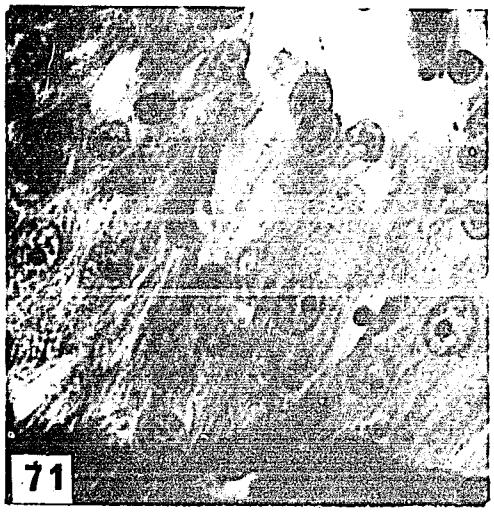
Fig. 73. Cross section through the small red fibers of the dorsal carinalis muscle of a control embryo (Z). The cells are very small, contain discrete small myofibrils and have peripheral nuclei.

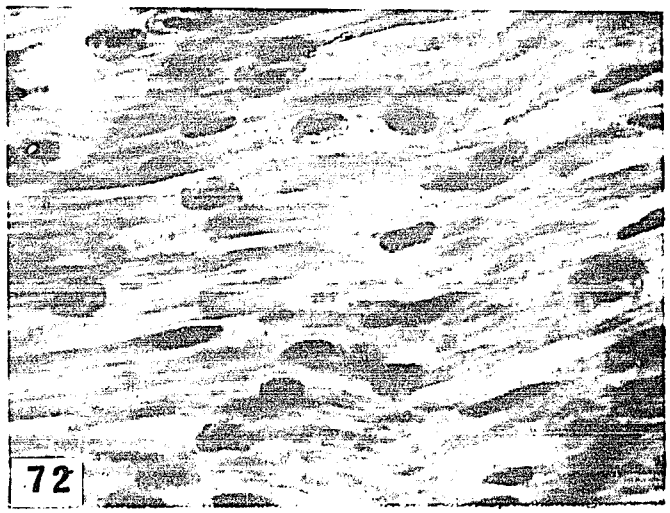
Fig. 74. Cross section through the carinalis muscle of Type 3 embryo (F). The cells are not as compact as in the control and nuclei are central, round and surrounded by myofibrils; cell boundaries are not clearly defined.

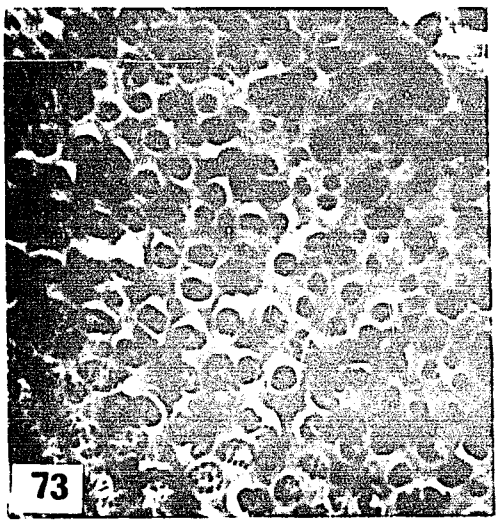
PLATE 4.23











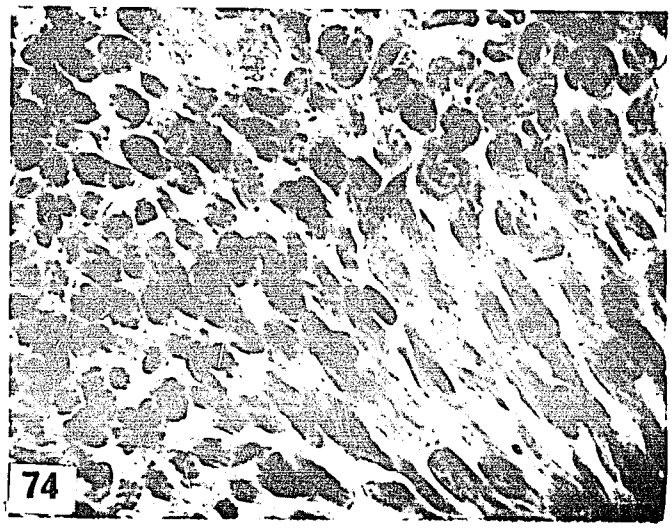
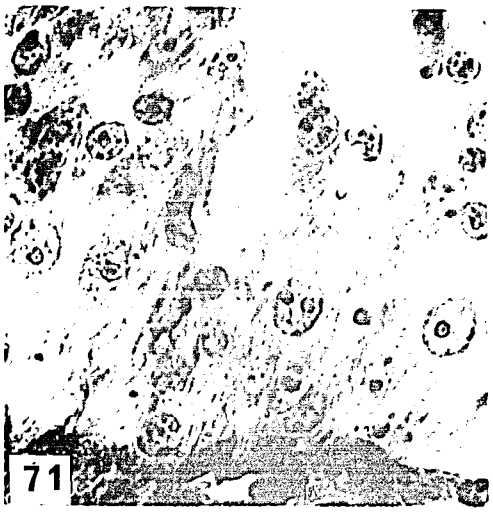


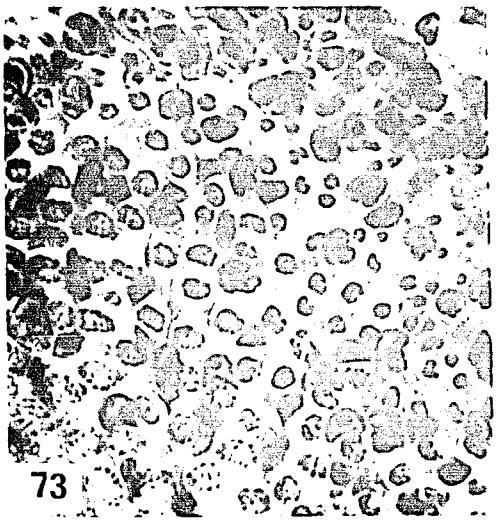
PLATE 4.23











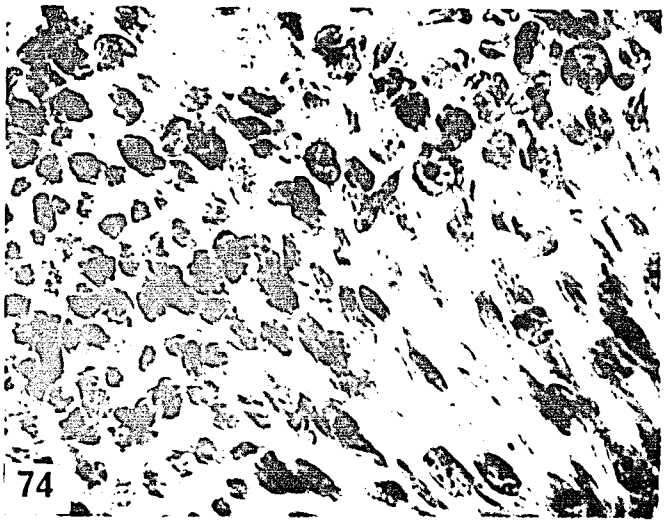


PLATE 4.24

Histology of muscle in Salvelinus fontinalis embryos at day 32.

Fig. 75. Cross section through the body of Type 4 embryo (M) showing the structure of the muscle cells. These are either small, densely packed, and undifferentiated or quite big and vacuolar. 185 x.

Fig. 76. Cross section through the region of the carinalis muscle[1] of Type 3 embryo (M). The cells are small and have dense cytoplasm and big round central nuclei; undifferentiated cells are also seen. 750 x.

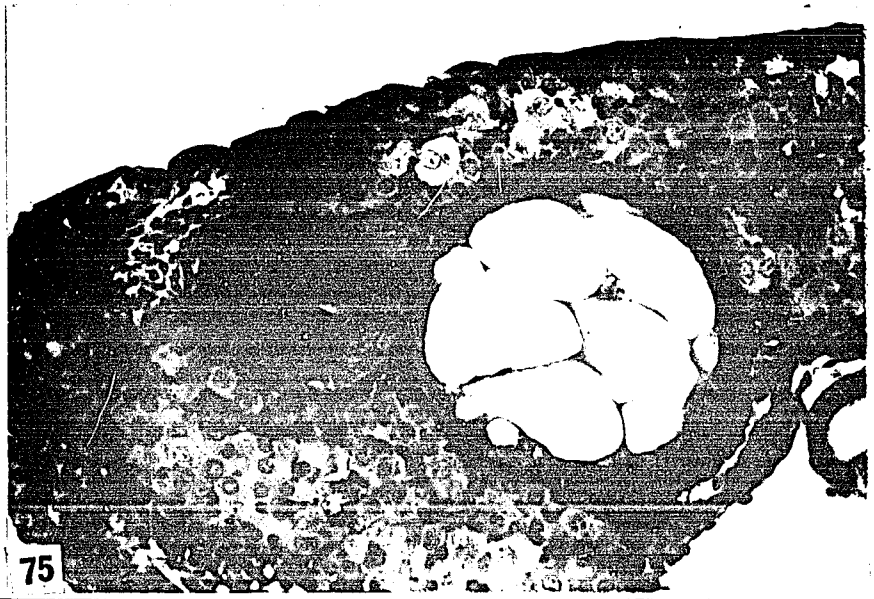
Fig. 77. Cross section through the masseter muscle of Type 3 embryo (O, Dylox treated). The cells are poorly differentiated. True myofibrils are absent but some fibrillar material is present. The nuclei are round and dense and the fibers appear short. 750 x.

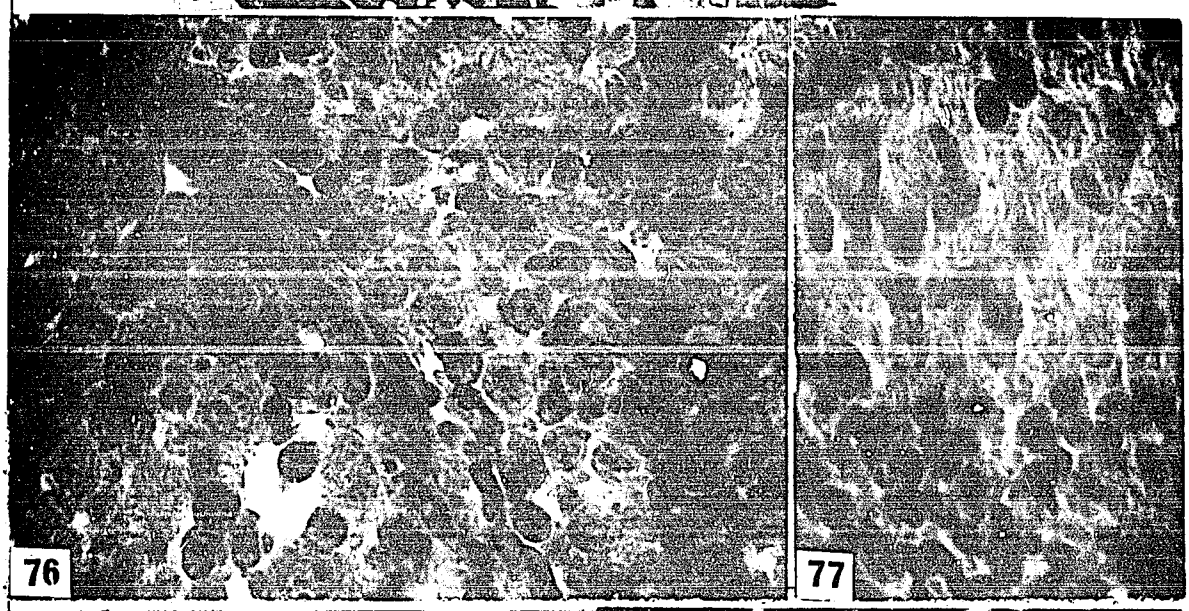
Fig. 78. Cross section through the white fibers of the body muscle of Type 4 embryo (M, Phosphamidon treated). The cells are quite large with large central nuclei. The cytoplasm is homogeneous and pale, and some fibrils are absent. This morphology could result from degeneration process. 750 x.

Fig. 79. Longitudinal section of cells similar to those of Figure 78. Some fibrils are seen at the ends of the cells. 750 x.

1. The carinalis muscle is the muscle located dorsally between the two main masses of myotomes.

PLATE 4.24





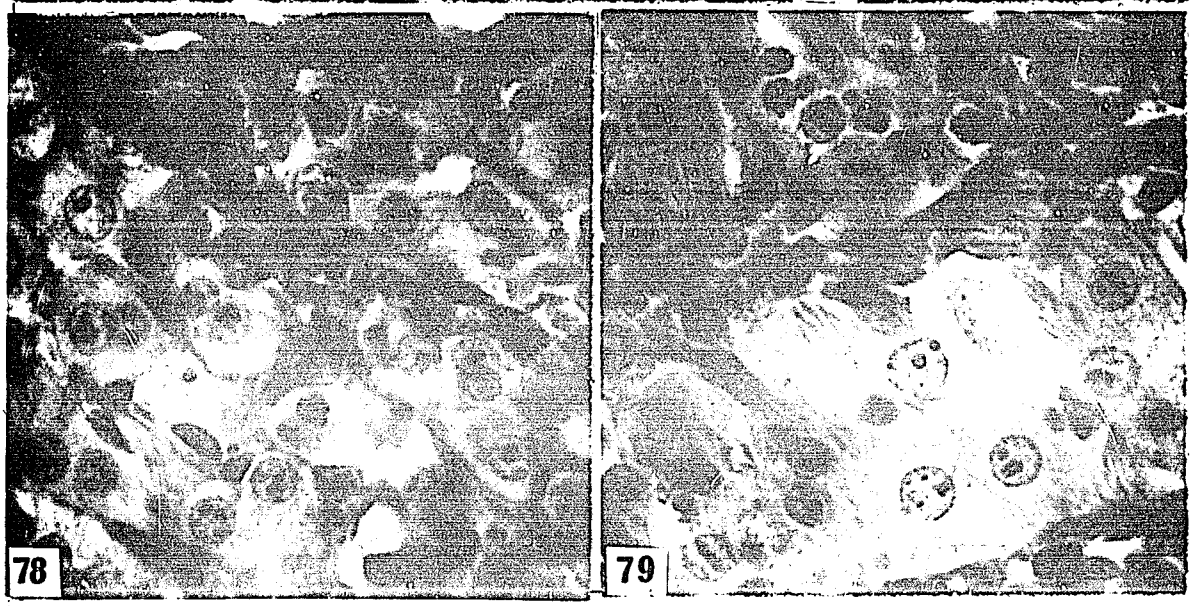
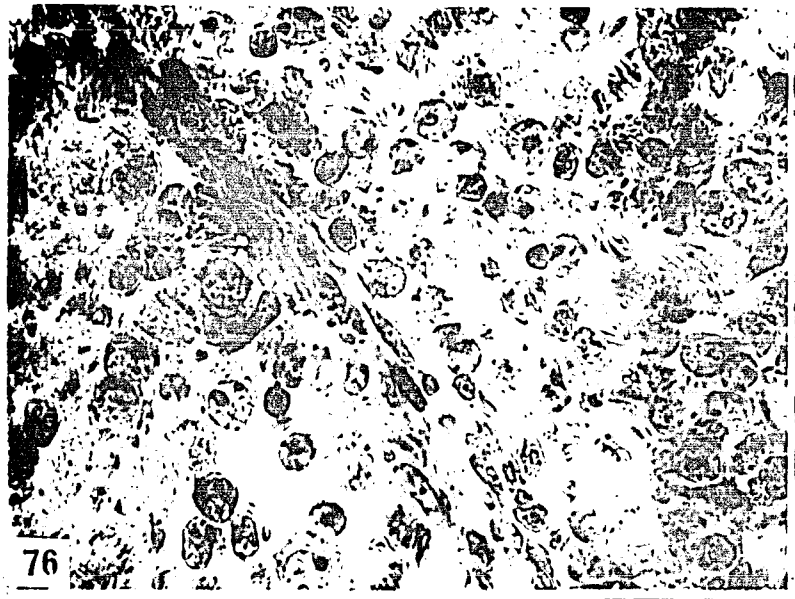


PLATE 4.24



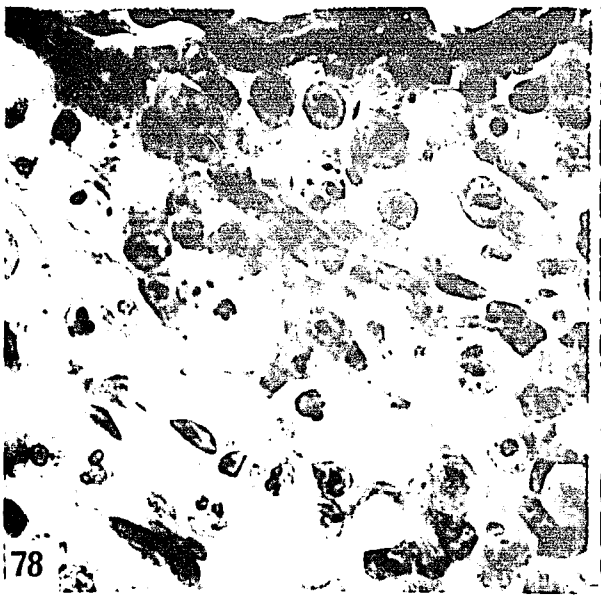
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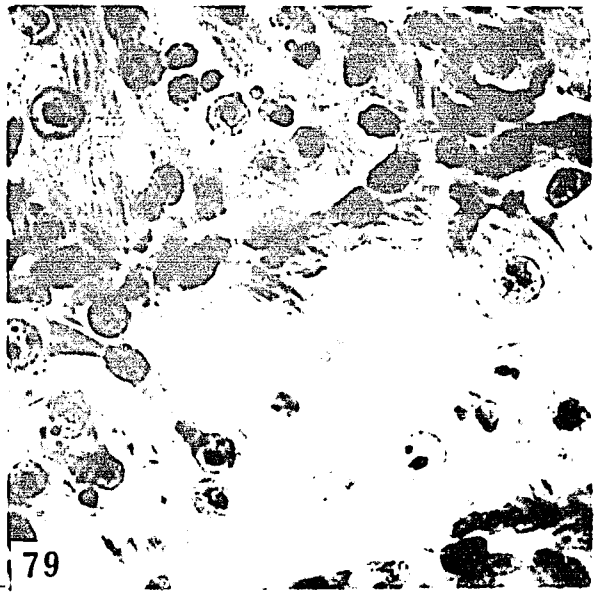
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78



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PLATE 4.25

Histology of muscle in Salvelinus fontinalis embryos at day 32. Magnification: 750 x.

Fig. 80. Cross section through the white fibers of Type 4 embryo treated with Dylox (P). The cells are large and nuclei are centrally located surrounded by fibrillar material (swelling of the cell or lack of differentiation).

Fig. 81. Longitudinal section through white fibers of Type 4 embryo treated with Dylox (P). Some cross striations are seen and the nuclei are found at the ends of the cells. Differentiation is incomplete.

Fig. 82. Cross section of the white fibers of Type 3 embryo treated with Dylox (P). The cells are big with granular material surrounding the nucleus. Some cells are extremely large, devoid of cytoplasm and contain only necrotic nuclei.

Fig. 83. Longitudinal section of white fibers of Type 4 embryo treated with Phosphamidon (G). The cells have almost no cytoplasm except for some desintegrating fibrillar material. The nuclei are central and round and nucleolus is next to the membrane giving the appearance of a ring.

Plate 4.25

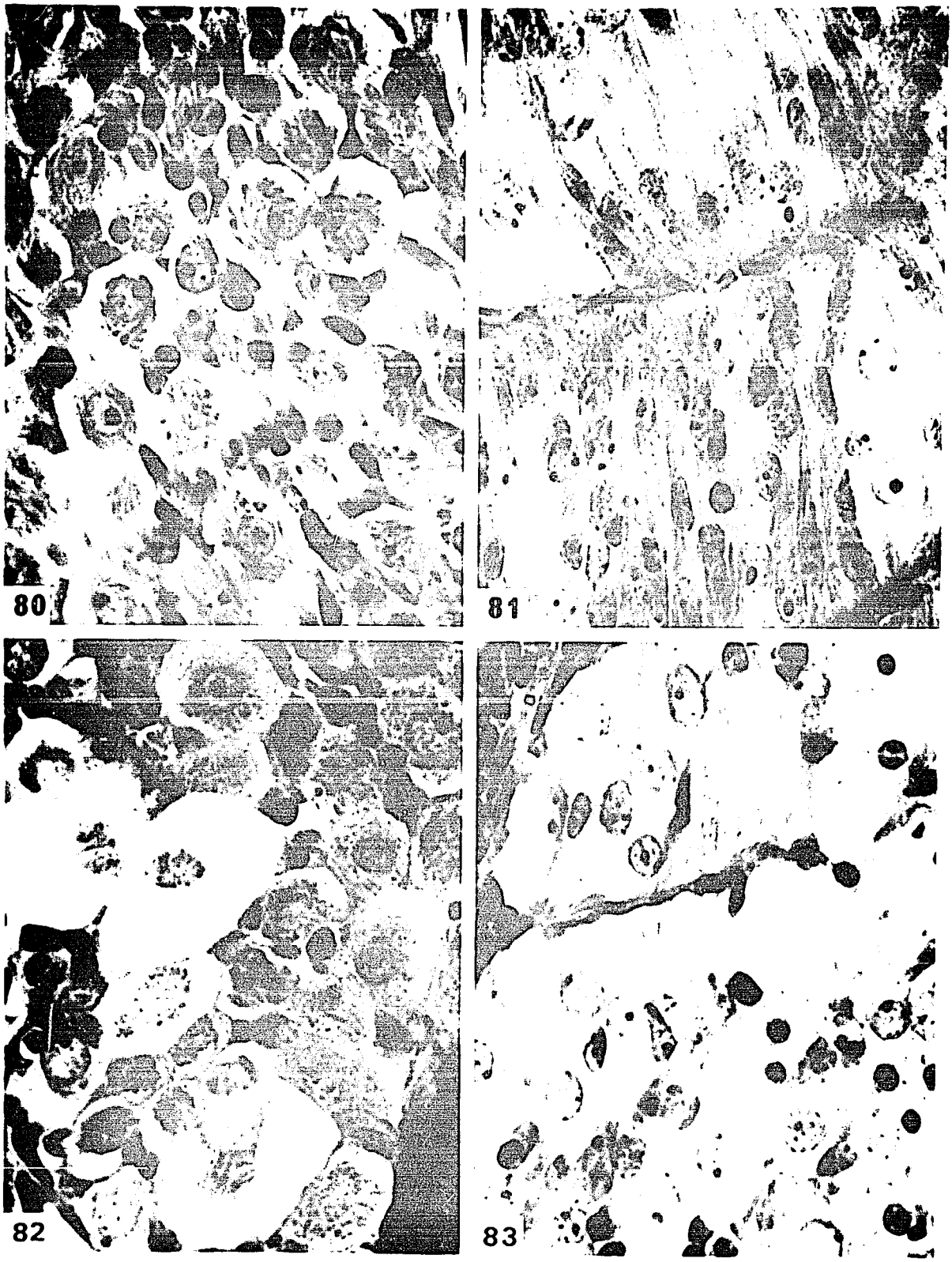


Plate 4.25

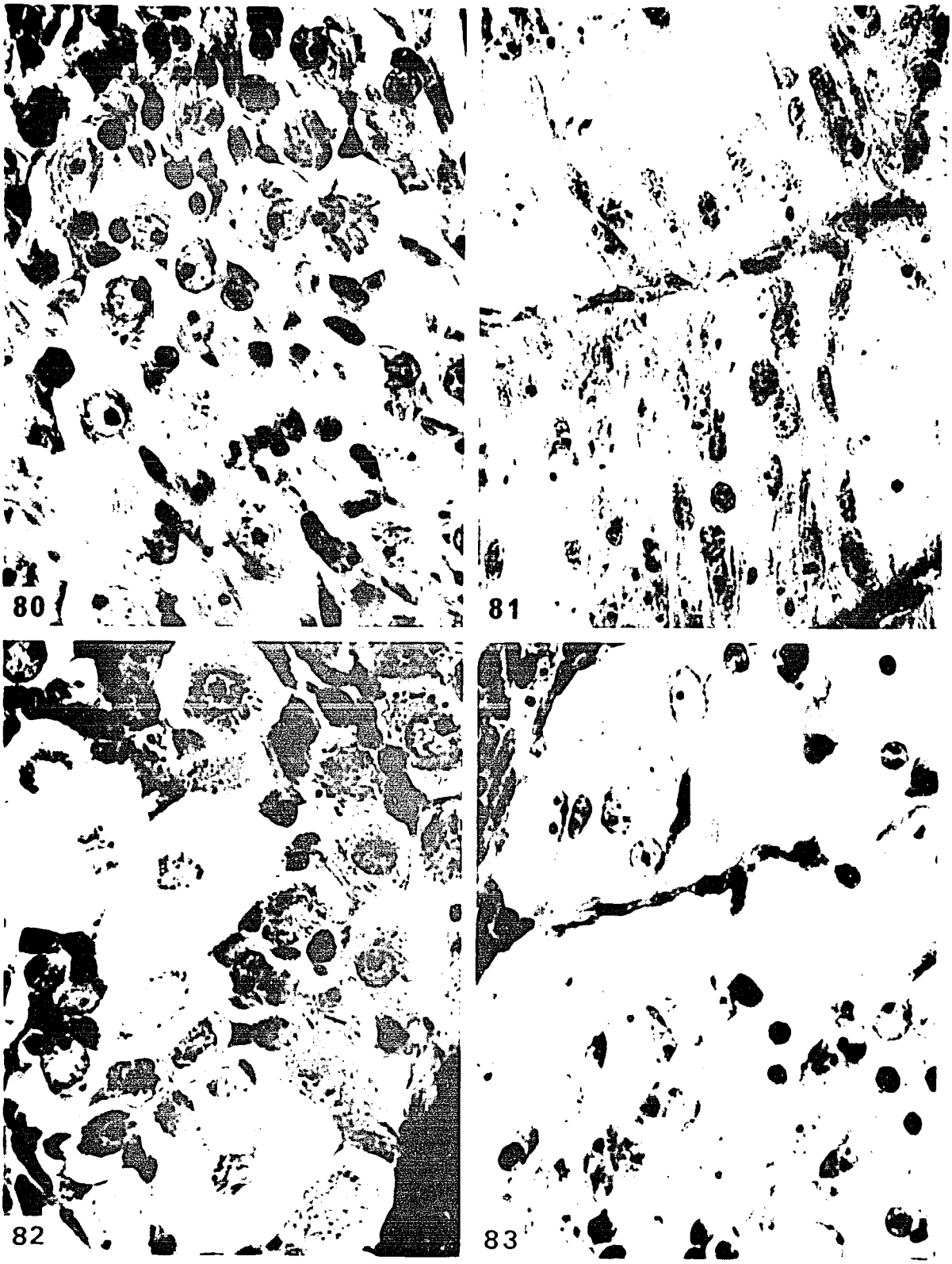


PLATE 4.26

Cholinesterase and Malic Dehydrogenase in Salvelinus fontinalis embryos.

Fig. 84. Cross section through a 12-day embryo at the level of the hindbrain (HB) showing the ChE reaction in the heart (H). 180 x.

Fig. 85. Cross section through the posterior portion of the hindbrain of a 12-day embryo. ChE is present in the ventro-lateral part of the brain and along a vertical line in the myotomes. 180 x.

Fig. 86. High magnification of the region marked on the previous figure showing the distribution of MDH in the cells lining the future myotomal septa. 720 x.

Fig. 87. Cross section through the posterior part of the hindbrain (HB) of a 14-day embryo. ChE is strong in the ventro-lateral margin of the hindbrain and in the myotomes. 180 x.

Fig. 88. Cross section through the midbrain of a 15-day embryo. ChE is present only in the meso-ventral part of the floor of the optic lobes (arrow). 72 x.

Fig. 89. Cross section through the body of a 15-day embryo showing ChE distribution in the neural tube (NT) and the somites. (N.B. Figure is rotated of 90°). 180 x.

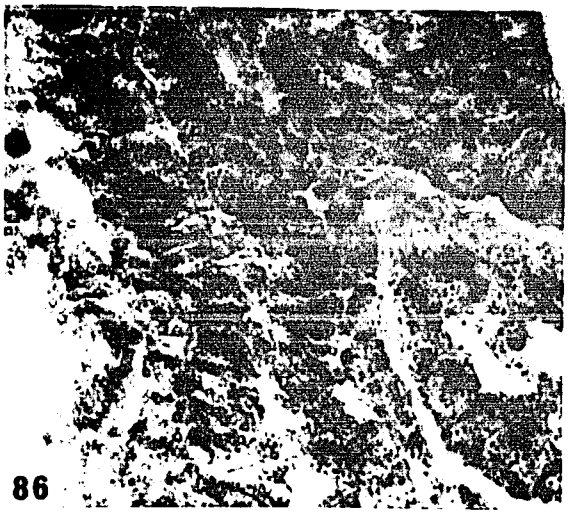
Plate 4.26



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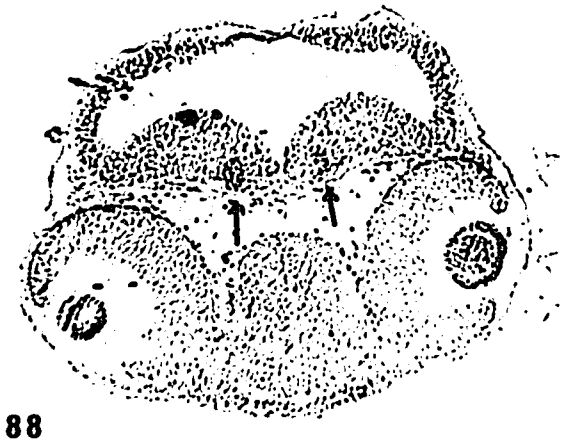
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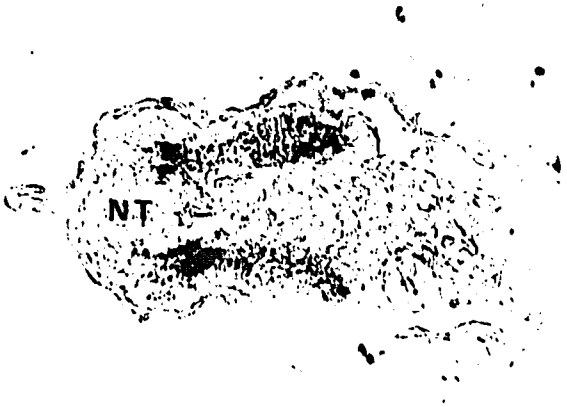
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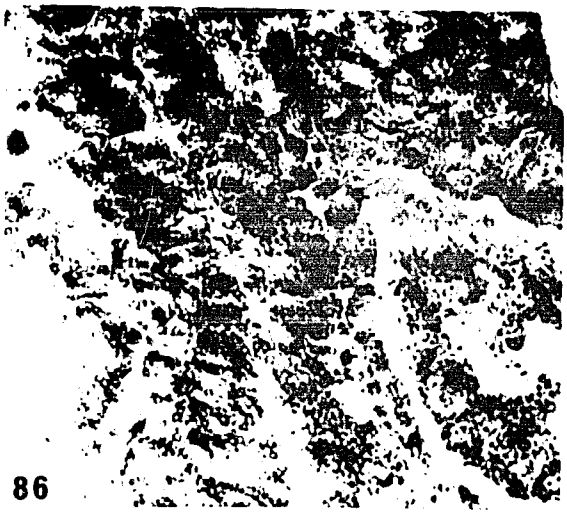
Plate 4.26



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PLATE 4.27

Cholinesterase and Malic Dehydrogenase in Salvelinus fontinalis embryos.

Fig. 90. Cross section through the heart region (H) of a 15-day embryo showing the ChE reaction in the thin internal lining. 180 x.

Fig. 91. High magnification of the ChE reaction in the cells of the neural tube of a 15-day embryo. 1,800 x.

Fig. 92. Cross section through the midbrain of 15-day embryo showing the stronger MDH reaction in the ventro-medial part of the floor of the optic lobes (OL, at the top) and in the dorso-lateral part of the diencephalon (Di). 180 x.

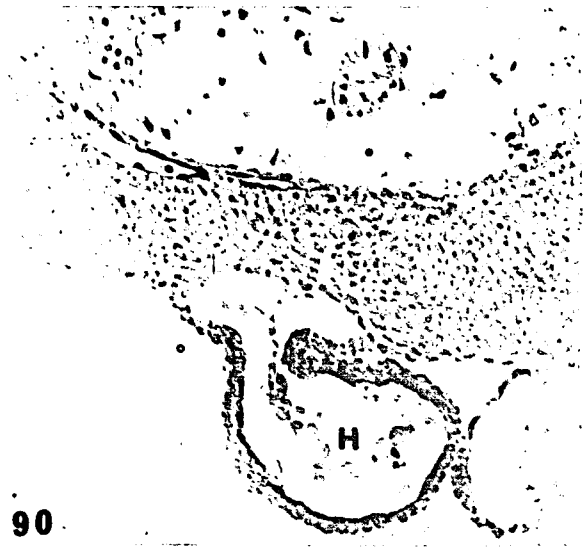
Fig. 93. Cross section through the neural tube and the somites of a 19-day embryo showing the ChE present in the ventro-lateral cells and in the myotomal septa. 720 x.

Fig. 94. Cross section through a 19-day embryo at the level of the midbrain showing the MDH distribution. Darker areas in the ventral part of the section are artefacts due to folding of tissue. 72 x.

Fig. 95. Cross section through the midbrain of a 19-day embryo. ChE is present in two medio-ventral areas made of unoriented cells. 72 x.

Fig. 96. Cross section through the ocular muscle of a 26-day embryo showing the ChE distribution. 720 x.

Plate 4.27



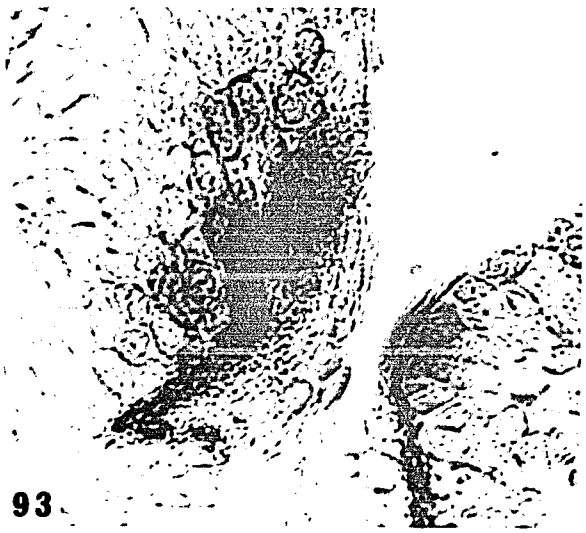
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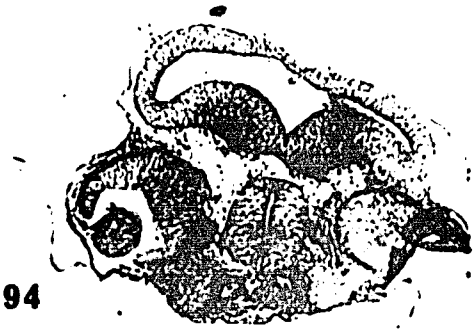
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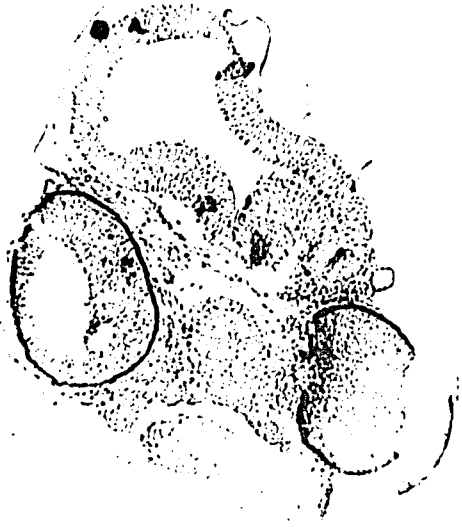
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Plate 4.27

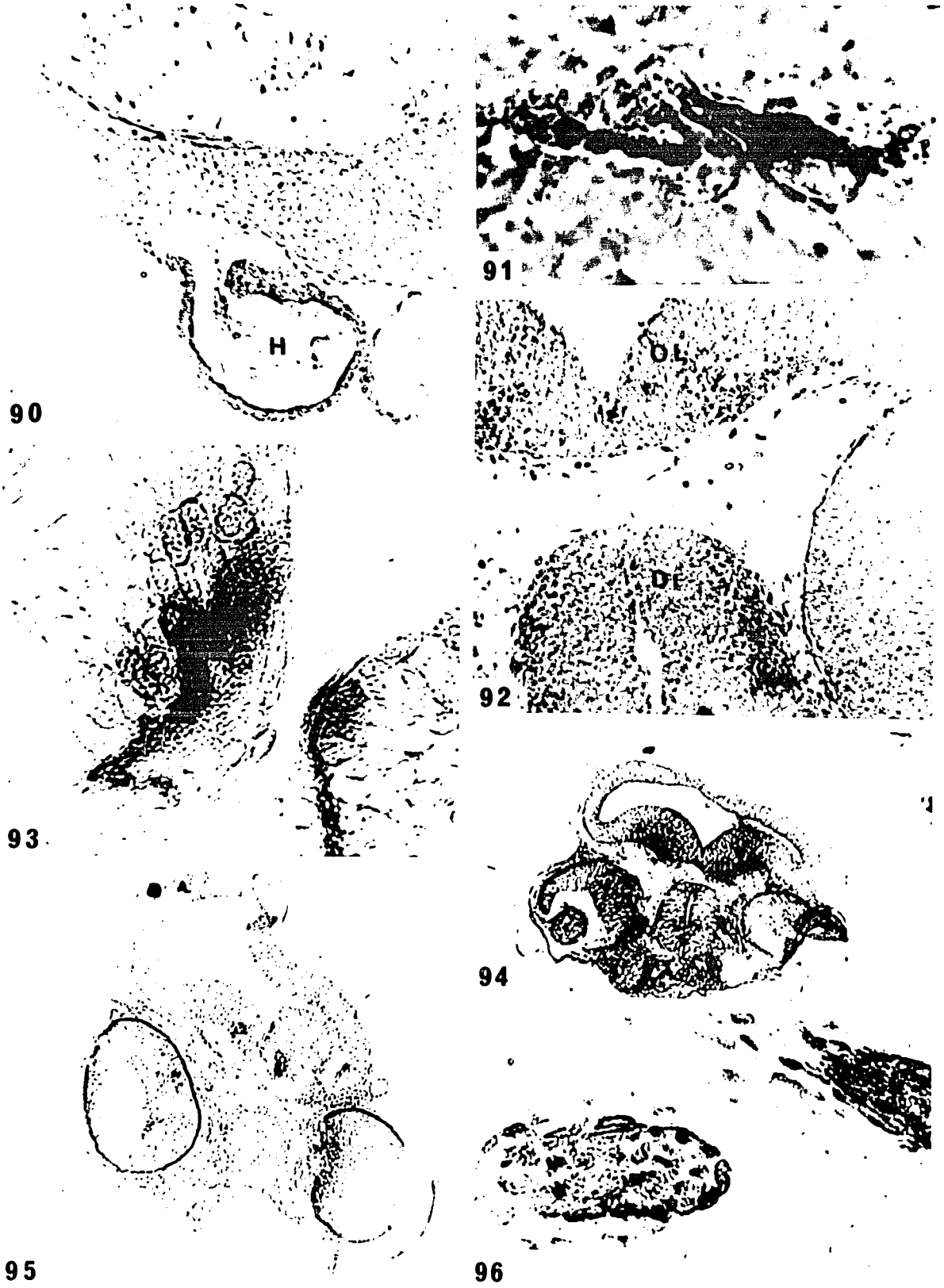


PLATE 4.28

Cholinesterase and Malic Dehydrogenase in Salvelinus fontinalis embryos.

Fig. 97. Cross section at the level of the posterior hindbrain of a 19-day embryo showing the MDH distribution: it is strong in the brain (HB), in the future cutaneous muscle (arrow), the pronephric duct (D), the gut (g) and the heart (H). (N.B. The figure is rotated of 90°). 180 x.

Fig. 98. Cross section through the anterior midbrain of a 26-day embryo treated with 100 ppm of Phosphamidon from day 5 to day 8 (H) (late gastrulation). No ChE activity is seen. In the control, the reaction would be present in the areas indicated by arrows. 72 x.

Fig. 99. Cross section through the anterior part of the midbrain of a 26-day normal embryo. ChE is present at the base of the midbrain (arrow) and in the small ocular muscle (OM). (N.B. The figure is rotated of 90°). 72 x.

Fig. 100. High magnification of the base of the diencephalon of a 26-day embryo treated with 200 ppm of Dylox from day 5 to day 8 (W). The ChE reaction is weak in the cells along the ventral margin. 720 x.

Fig. 101. Cross section through the posterior portion of the midbrain of a 26-day normal embryo. The strong ChE is situated only in the islands at the base of the diencephalon. The infundibulum (I) gives no reaction. OM: ocular muscle. 72 x.

Fig. 102. Cross section through the most posterior portion of the midbrain of a 26-day embryo showing the strong ChE reaction in the ventral part. The dark folded line at the lower right is an artefact (folding of the pigmented layer of the eye). 180 x. (control embryo).

Plate 4.28

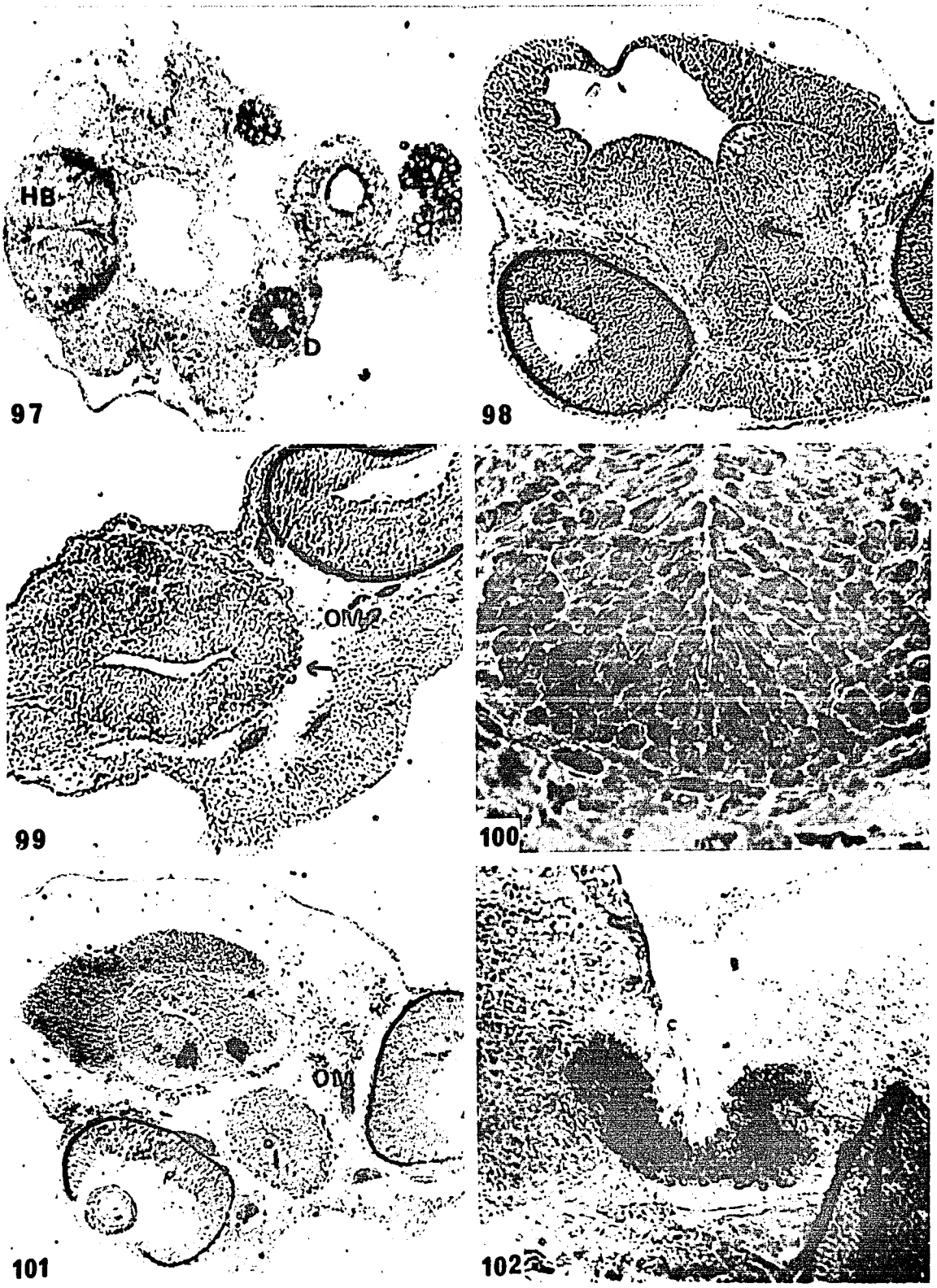
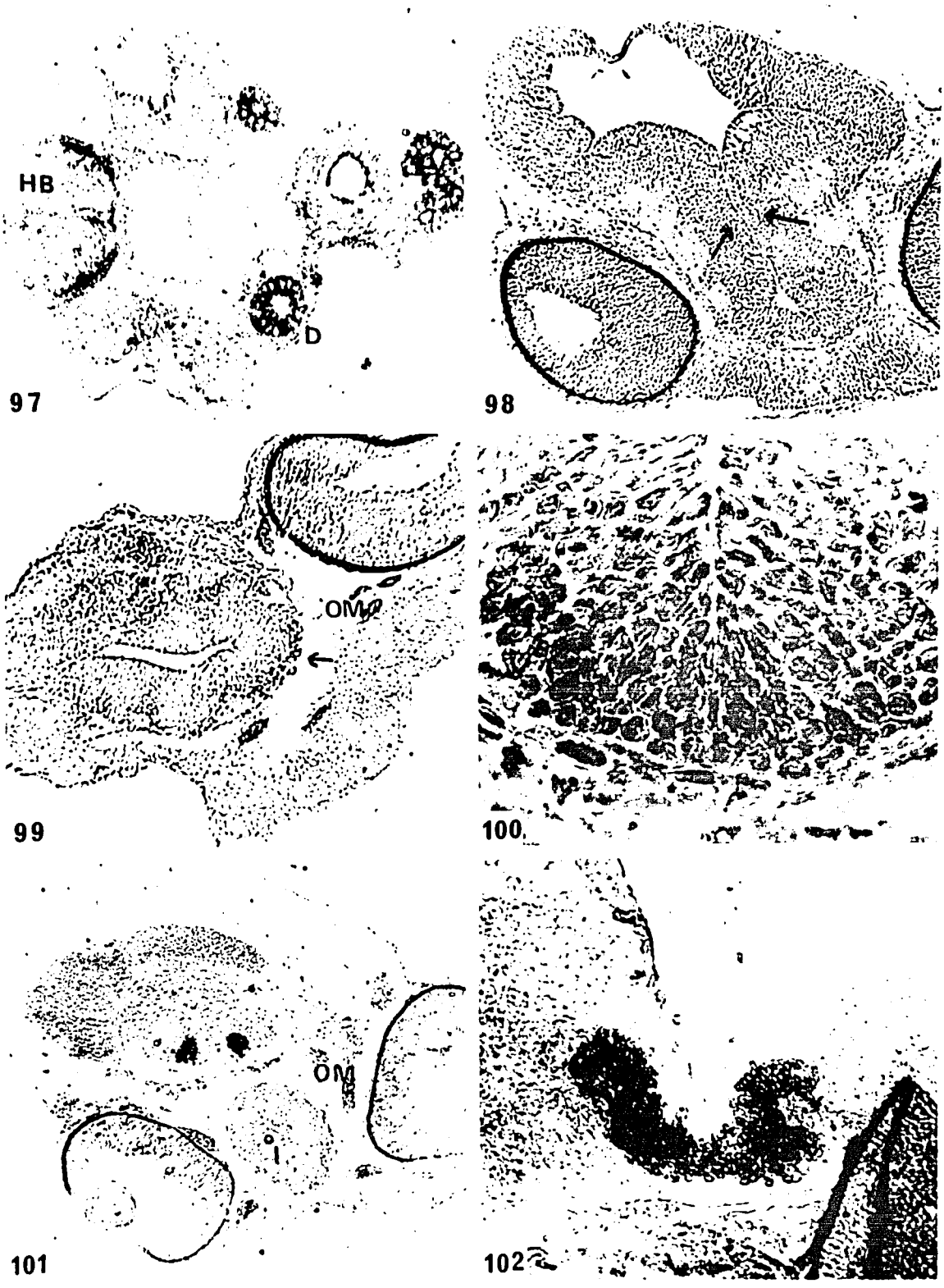


Plate 4.28



CHAPTER FIVE

EXPERIMENTS WITH

SALMO GAIRDNERI IRIDEUS

LARVAE

I. EXPERIMENTALS.

Rainbow trout larvae of two different ages were used in these experiments: a) young larvae (14 days old); the yolk sac was partially resorbed and the larvae measured 21 mm (fig. 1); b) old larvae (40 days old); the yolk sac was resorbed and the larvae, 26 mm long, had started feeding (fig. 2).

The larvae were divided into groups of 150 specimens and placed in trout hatching trays provided with a closed circuit of running water. Each group was treated with 0 (control), 10, 30, 50 and 100 ppm of Dylox for 16 hours. An additional group was treated with 5 ppm for 40 hours.

Behaviour of the larvae, histochemical and histological studies were undertaken.

The behaviour of the larvae during the experiment was observed at 6, 10, 16, 32, 40, 72 hr and 12 days, using the following parameters: 1) spontaneous activity; 2) reaction to light; 3) reaction to a touch stimulus; 4) heartbeat rate; 5) mortality.

Samples of larvae of both ages were taken for serial histological sections at 10 and 32 hr while samples of young larvae were taken for histochemical study of acetylcholinesterase, malic and lactic dehydrogenases and NADH diaphorase at 6, 16 and 32 hr. An estimate of the acetylcholinesterase activity of each specimen was obtained by comparing the intensity of the reaction of larvae treated at different concentrations of organophosphate with the intensity of the reaction of control samples, and by varying the incubation time of sections of treated larvae so as to obtain color intensities equal to the color intensities of

the controls.

II. RESULTS.

A. Behaviour of larvae.

Before treatment, the larvae were in a normal position, that is, with the yolk pouch touching the bottom of the tray, usually in groups in the shadowed corners. From time to time, they would swim for a distance of 6 to 10 inches before resting again. After exposure to the organophosphate the larvae first became hyperactive, swam very fast and erratically for short distances and often changed direction. This activity was followed by a progressive lowering of activity until eventually they came to rest on their sides. At this time they no longer avoided light and their response to the touch stimulus gradually became weaker, more delayed and less efficient. A jump produced by a single long-lasting contraction of the body followed a jerky forward swimming action. Later, this contraction produced only a curling of the body on itself and finally there was no grossly observable response. When observed under the stereoscope, however, the body showed an almost continuous fascicular twitching.

The typical sequence of changes in behaviour of the larvae was generally the same at different concentrations but occurred more rapidly with increasing concentrations; e.g., after 6 hr the young larvae treated with 10 ppm were hyperactive and responded strongly to a stimulus while larvae treated with 50 ppm were already lying on their sides and no longer responded to a touch stimulus.

During recovery, the symptoms disappeared in the reverse order

of their appearance. In the young larvae, treated with a dose of 50 ppm, surviving larvae had a near normal behaviour after 24 hr of recovery, but later on, their behaviour became more abnormal: lower spontaneous activity and overexcitability. With lower doses, recovery was slower at the beginning but was completely normal on the 12th day. In the larvae exposed at a low dose (5 ppm) for 40 hr, the first signs of recovery appeared later than in the others: 36 hr after the end of treatment they had no spontaneous activity and failed to respond to a touch stimulus. After six days, there was noticeable improvement in their behaviour but it was still far from normal. On the 12th day, however, recovery was complete. In the old larvae, the return to a normal behaviour was similar to that found in the young larvae but it occurred faster.

B. Heartbeat rate and mortality.

The heartbeat rate and mortality are given in Tables 5.1 and 5.2. Decrease in the rate and the strength of contraction of the heart usually came when the larvae were lying on their sides.

Comparison of Tables 5.1 and 5.2 shows that older larvae are much less resistant than the younger larvae. In young larvae, mortality occurs only at concentrations above 10 ppm while it occurs at all concentrations in older larvae.

TABLE 5.1 Heartbeat[1] (H.B.) rate per minute and total percentage mortality (Mort.) in young Salmo gairdneri irideus larvae during and after treatment with Dylox[2].

DOSE (ppm)		TIME					
		6 hr	10 hr	16 hr	32 hr	40 hr	76 hr
0	H.B.	s 120	s 120	s 120	s 120	s 120	s 120
	Mort.	0	0	0	0	1	1
5	H.B.	s 120	s 120	s 120	s 80	s 80	m 80
	Mort.	0	0	0	1	3.5	4
10	H.B.	s 120	s 120	m 90	s 106	s 120	s 106
	Mort.	0	0	0	0	0	1.5
30	H.B.	m 106	m 40-95	m 80	s 110	s 70	s 95
	Mort.	0	1	27	45	50	51
50	H.B.	m 110	w 50	w 60	s 120	s 80-120	s 120
	Mort.	0	0	61	78	79	79
100	H.B.	w 54	-				
	Mort.	71	100				

[1]The strength of the heartbeat was classified as strong (s), medium (m), or weak (w).

[2]Treatment with 5 ppm lasted for 40 hr whereas treatment with 10, 30, 50 and 100 ppm lasted for 16 hr. After treatment the trout larvae were allowed to recover in pre-cooled aerated water.

TABLE 5.2 Heartbeat[1] (H.B.) rate per minute and total percentage mortality (Mort.) in old Salmo gairdneri irideus larvae during and after treatment with Dylox[2].

DOSE (ppm)		TIME					
		6 hr	10 hr	16 hr	32 hr	40 hr	76 hr
0	H.B.	s 120	s 120	s 120	s 120	s 120	s 120
	Mort.	0	0	2	3	4	4
5	H.B.	s 120	s 120	m 100	w 90	w 90	s 90
	Mort.	0	0	0	90	98	98
10	H.B.	s 110	m 100	w 90	s 100	s 100	s 90-110
	Mort.	0	59	85	90	90	91
30	H.B.	m 90	w 90	-			
	Mort.	0	94	100			
50	H.B.	w 80	-				
	Mort.	90	100				
100	H.B.	-					
	Mort.	100					

[1]The strength of the heartbeat was classified as strong (s), medium (m), or weak (w).

[2]Treatment with 5 ppm lasted for 40 hr whereas treatment with 10, 30, 50 and 100 ppm lasted for 16 hr. After treatment the trout larvae were allowed to recover in pre-cooled aerated water.

C. Histological observations.

i - Young larvae.

Histological damage was observed after 10 hours of treatment.

The pseudogills, found in the posterior dorso-lateral part of the mouth, are normally arranged in parallel rows, each row being made of three layers. There are two layers of elongated narrow cells, placed end to end, sandwiching a thin space in which are found a single row of blood cells. In the treated animals, there was a disorganization of this structure: the cell rows were no longer parallel and the elongated cells were swollen and irregular with swollen nuclei; distorted blood cells were present in the central zone (fig. 3 and 4). Some changes were also present in the cervical ganglionic cells: they were slightly smaller than normal and the basophilic cytoplasm changed to acidophilic; the nuclei became vacuolar and stained less intensely.

Extravasated blood was sometimes observed indicating capillary and arteriole fragility but more often, the walls of the blood vessels appeared distorted rather than ruptured. Blood cells of the treated larvae were irregular in shape and shrunken. In some treated specimens, the liver cells appeared vacuolar but this change was not universal. The cytoplasm of some cardiac muscle cells was vacuolated or granular whereas other fibers were streaky or glossy.

Striated muscle fibers showed extensive histological damage. Some of the large white fibers which constitute the main mass of the body musculature appeared stretched with a clear cytoplasm, while other areas of the same fibers were swollen and glossy (fig. 8). Those fibers which were normal in appearance were strongly contracted. In cross section the myofibrils were grouped in the center of the cell and

surrounded by a granular material containing large oval distended nuclei (fig. 15). In some fibers the myofibrils appeared fused (fig. 14). At high concentrations, the fibers were sometimes completely broken down with an amorphous granular material with free nuclei replacing the normal tissue. The sarcolemma seemed to have disintegrated (fig. 10). The small red fibers found in the ocular (fig. 7), masseter and carinalis muscles (fig. 6) and in the muscles of the pharynx, oesophagus and anterior part of the stomach, showed extensive damage. The fibers were either almost completely disrupted, extremely reduced in diameter or greatly swollen (fig. 7). No striations were evident. Damage was usually found in localized areas, with adjacent muscles presenting a normal or near normal appearance. The ocular muscles were the most extensively damaged of all the muscle groups.

Table 5.3 shows the relationship between the extent of the pathology and the concentration of Dylox used in the treatment.

TABLE 5.3 Extent of histological damage* in young Salmo gairdneri irideus larvae observed after 10 hr of treatment.

TISSUE	DOSE (ppm)			
	5	10	30	50
Blood	-	-	+	+
Ganglion			+	+
Heart	-	-	+	+
Pseudogills	+	+	++	++
Red fibers	+	++	+++	++++
White fibers	-	+	++	+++

In surviving larvae, foci of muscle degeneration became engorged with leucocytes, during recovery (fig. 8).

ii - Old larvae (advanced fry).

Tissue damage, similar to that found in young larvae, was found in old larvae, however, red muscle fibers were more disrupted and other tissues and organs than those found in young larvae were also affected. The blood was irregularly distributed: some areas of the heart and some vessels being almost devoid of cells while in other areas, the blood cells formed very dense masses. The blood cells were also distorted. In cross section, the normal cells were typically dumb-bell shaped with elongated nuclei (fig. 20) whereas in the treated groups they were shrunken, distorted and had pointed tips, indicative of hemolysis (fig. 23).

Extensive damage was found in the gills. The epithelium was swollen, vacuolar and partially torn (fig. 17). Sections through gill lamellae showed edema and separation of the epithelium from the capillary (fig. 19). Liver from larvae treated with a high dose (100 ppm) had an irregular appearance. Some regions were near normal while in other regions, blood sinuses were less clearly defined due to a swelling of the parenchymal cells. In these areas, the parenchymal cells appeared to be paler and foamy (fig. 21). Similarly, the mucosal glands of the stomach were also abnormal; the cells were either vacuolar or shrunken. Some were adjacent to the basement membrane while others were completely separated from it. In some areas, the mucosa was torn away and blood cells found either in the mucosa or in the lumen. Kidney tubules appeared normal except that some mucus-like material formed a dense mass in the center of the lumen. In some larvae, the

brain showed very extensive necrosis of the cells.

D. Histochemical observations.

i - Localization of dehydrogenases and diaphorase.

Serial sections of young larvae showed that the three enzymes: malic dehydrogenase, lactic dehydrogenase and NADH diaphorase have the same type of distribution. Under the same incubation conditions, MDH was just slightly stronger than LDH, and NADH was somewhat weaker than LDH and MDH. Figures 24 to 43 show the distribution of these enzymes in the various organs of the body. The strength of the reaction in the various organs have been given values from 0.5 to 5.0. Table 5.4 shows a comparison of the strength in several tissues and organs.

TABLE 5.4 Comparison of histochemical dehydrogenase activity in several tissues and organs of young Salmo gairdneri irideus larvae.

ORGAN	INTENSITY	FIG.
Olfactory placode	1.0, 3.0*	24
Forebrain	2.0	26
Epiphysis	4.0	26
Midbrain	1.0, 2.0, 3.0	28, 30
Infundibulum	1.0, 2.0	28
Hindbrain	3.0	36, 37
Neural tube	2.0	40

*Two or three values are given when there was a variation of activity in different regions of the same tissue or organ.

TABLE 5.4 (continued)

ORGAN	INTENSITY	FIG.
Otic capsule epithelium	5.0	29, 32, 34
Ganglion	4.0	32
Eye	2.0	25, 27
Epithelium	0.5	24, 43
Gill epithelium	0.5, 3.0	29, 36, 39
Pseudogills	5.0	29, 33
Lymph gland	0.5	35
Ocular muscle	5.0	25, 30
Masseter	2.0, 4.0	28
Oblique	4.0	28
Pectoralis	2.0	36
Opercular	4.0	36
Carinalis muscle	4.0	40
Pectoral fin muscle	4.0	38
Body muscle	0.5, 2.0, 5.0	40, 43
Heart ventricle	4.0	36, 37
Heart auricle	2.0	37
Notochord	0	40
Chondrocytes	2.0	31
Kidney tubule and duct	2.0, 4.0	42
Urinary bladder	4.0	40
Oesophagus mucosa	2.0	38
Stomach mucosa	3.0	-
Intestine mucosa	4.0	40
Dig. tract muscle	1.5	38, 40
Liver	4.0	41

In the brain, the intensity of the reaction varied according to the kind of tissue. The columnar ependymal cells (fig. 30, e) lining the cavities, especially on the ventral side of the optic lobes, reacted strongly and thus formed a thin dark band. In the gray matter of the optic lobes, the cells were small and small clusters of grains were found next to the nucleus in the thin crescent-shape cytoplasm (fig. 30, region 1). Widely spaced small cells also formed two lines parallel to the surface of the optic lobes and the reaction was stronger along these lines (fig. 30, arrow). In other regions of the brain (fig. 30, region 2, and fig. 38), however, the gray matter was composed of large pyramidal and globoid cells widely separated from each other, the cytoplasm of which contained numerous small grains. The acellular matrix embedding these cells gave a medium reaction. In the white matter (fig. 30, region 3), a few small cells were present in an acellular matrix which contained fine grains.

The epithelium lining the cavity of the otic capsule gave an irregular reaction: the columnar luminar cells in the cushion reacted very strongly, while the basal cells gave a weak reaction (fig. 34). The adjacent cuboidal epithelium was also weak, but other regions of the epithelium stained intensely (fig. 32).

Four main types of muscles could be identified according to their morphology and their dehydrogenase reaction.

In the large fibers, making up the bulk of the body musculature (fig. 40 and 43) the peripheral myofibrils appeared rectangular, their long axis at right angle to the surface of the fiber. The central core contained several loose small myofibrils. The reaction was weak, limited to the subsarcolemmar region and to a few big grains in the

center. The smaller intermediate red fibers found in the masseter muscles and in the lateral area of the myotomes had a similar but stronger reaction (fig. 43). The small red fibers found in the carinalis and ocular muscles were compactly filled with fine myofibrils. The reaction was strong and evenly distributed in these fibers. A single layer of very large red fibers enveloped the body musculature and formed the cutaneous muscle (fig. 40 and 43). The reaction was extremely strong and evenly distributed.

ii - Effect of Dylox on dehydrogenases and diaphorase.

The activity of the enzyme was not affected by treatment except in a few tissues. The distribution however, within certain tissues could be severely disrupted.

In the neural tube of larvae treated with high concentrations (50 and 100 ppm), the grains were agglomerated into large clumps, both in the cells and in the acellular matrix (fig. 44). Disruption of the pseudogills, apparent from histological sections, was very evident with the histochemical reaction: the reaction was generally weaker and very uneven (fig. 48) compared to that of the control (fig. 33). The reaction in the otic ganglion was also weaker and more diffuse (fig. 46 and 47).

In longitudinal sections of red muscle fibers of control larvae, the reaction appeared as small grains distributed along the myofibrils (fig. 49, 51 and 53). When the section was perfectly horizontal, the reaction revealed both longitudinal and cross striations (fig. 49). In some fibers of the treated larvae, the grains were aggregated into larger dots and irregularly distributed, with consequent loss of the longitudinal pattern (fig. 56). In other fibers, the

distribution was more irregular. Long black streaks of apparently homogeneous material replaced the individual grains, while the adjacent portions of the fibers were almost negative (fig. 50). In a more extreme situation, fibers could not be recognized and the reaction appeared as irregular masses of black deposits (fig. 52).

Histological observations had shown that some fibers were greatly swollen while other segments of the same fibers were stretched. Very intense clumping was associated with the swollen areas: all the deposit was either aggregated in the swollen area or at its periphery (fig. 54 and 56). Thick dark transverse bands were also seen in these fibers (fig. 54).

iii - Distribution of cholinesterase.

A study of serial sections showed that acetylcholinesterase was present only in certain structures.

In the forebrain, weak diffuse activity was present in the medial and dorsal areas which contained scattered cells, while in the pineal body, only two dorso-lateral spots gave a medium reaction. Both gray and white matter of the lateral wings of the optic lobes reacted to a small extent but a medium reaction was found along a line of widely spaced cells parallel to the surface of the brain (fig. 57, arrow). The medial dorsal septa and some cellular areas bordering the third ventricle gave a similar reaction (fig. 57). In the hindbrain and in the neural tube, the densely cellular zone surrounding the cavity gave no reaction; weak enzyme activity was present throughout the acellular matrix in which are found multipolar neurons, while a strong reaction was present in the cytoplasm and in the cytoplasmic extension of these neurons (fig. 58, 61 and 67).

Strong enzyme activity was also seen in the dorsal root ganglion (fig. 67) while the reaction was medium and equally distributed in the cytoplasm of the large cells of the otic ganglion (fig. 65). Similar intensity occurred in the inner plexiform layer of the retina (fig. 57).

In muscle, the reaction was variable depending on the type of muscle. Muscle composed of small red fibers, such as the ocular muscle, contained so many end plates which stained strongly that at low magnification, the whole muscle seemed to be stained (fig. 57). End-plates were much less numerous in the body muscle which is composed of white fibers (fig. 63) but the myotomal septa showed a medium reaction throughout their length (fig. 66). Muscle insertions also showed medium activity (fig. 62). A fine reticulum stained weakly in the muscularis of the digestive tract where striated muscle was present. The reaction was weak and diffuse in the ventricle of the heart.

iv - Effect of Dylox treatment on cholinesterase.

In treated larvae, the acetylcholinesterase activity was similarly located but weaker throughout. Table 5.5 gives an estimate of the values of ChE activity at 6, 16 and 32 hr. Acetylcholinesterase present in the nervous system appeared to be inhibited at the same rate as the acetylcholinesterase of the septa and myoneural junctions.

TABLE 5.5 Estimate of AChE activity (%) in young Salmo gairdneri irideus larvae during and after treatment with Dylox*.

DOSE (ppm)	TIME (hr)		
	6	16	32
Control	100	100	100
5	75	50	20
10	50	30	40
30	40	20	40
50	25	15	25

*Treatment with 5 ppm lasted for 32 hr whereas treatment with 10, 30 and 50 ppm lasted for 16 hr.

III. DISCUSSION.

A. Enzyme distribution.

Distribution of enzymes in lower vertebrates and especially during the early stages have received little attention. The study of the distribution of some oxidative enzymes (LDH, MDH and NADH) was undertaken to verify if the activity of enzymes would be more affected in some organs than in others, and to test for a possible relationship between the enzyme content and the sensitivity of the tissues to organophosphate treatment. Although the significance of the enzyme content of some tissues might not be evident and cannot be discussed, the results have been given since these results can serve as a basis for further work in this field.

The epithelial cells of the olfactory pits, of the mucosa of the auditory vesicles and the visual cells of the retina have high activity. The ganglion cells of the retina and the neurons of the cervical ganglia also give a strong reaction which is quite definite in the latter. These cells would then be expected to have a high level of respiration. In the forebrain and the neural tube, strong activity is mainly associated with large neurons; in the midbrain higher activity is mainly found in the white matter, while in the hindbrain, regional differentiation is less marked. A strong reaction was present in the ventricle of the heart. A high level of SDH and cytochrome oxidase was also found in the heart of the toadfish (Lazarow and Cooperstein, 1951). High levels of respiratory enzymes are also found in higher vertebrates and these would be related to the continuous and heavy work of the heart (Moog, 1965).

In the gills, the epithelium is generally poor in MDH, LDH and NADH, except for some cells which probably represent the so-called "chloride cells". The strong enzymatic activity of these cells is thus parallel to their abundant content of mitochondria (Philpott and Copeland, 1963; Threadgold and Houston, 1964). High respiratory needs of these cells would be related to their work in salt excretions and osmoregulation. The high activity found in the mucosal cells of the stomach and intestine is probably related to their functions of secretion and absorption, while the mucosal cells of the mouth and oesophagus which do not have this function are much weaker. It is to be noted, however, that in the stomach and intestine, the biochemical differentiation precedes considerably the functional activity since these larvae had not started feeding and would not start for at least another three weeks.

In the kidney, the reaction is strong in the ducts and medium in the tubules. High oxidative enzyme activity has also been related to function (Moog, 1965). Although cartilage gives the impression of a weak reaction, individual chondrocytes give a strong reaction. This is somewhat surprising because of the absence of vascularization of this tissue and also because the cells of other connective tissue give a weak reaction. Although weaker than in some other tissues, the enzymatic activity of the liver was intense. These results are similar to those found in the liver of several neonatal and adult vertebrates. The significance of the results obtained in pseudogills and in muscle will be discussed in the next chapter.

The distribution of cholinesterase in the brain of rainbow trout has several similarities with that of the frog brain which has

been described by Shen et al. (1955). Very little activity was found in the forebrain while in the midbrain most of the activity was found in the outer layer of the optic lobes. The rainbow trout has weak but definite enzyme activity in the richly cellular deep layer, whereas none is present in the frog whose activity in the deeper region is restricted to regions of numerous synapses. In the hindbrain, strong activity was found in the perikaryon and cellular extensions of the large neurons. The perikaryon of neurons of ganglia also show a strong activity. In the retina, the distribution of the enzyme, exclusively located in the inner plexiform layer, differs from that found in the chick retina where the ganglion cells are also positive (Gerebtzoff, 1959).

In muscle, the number of end-plates varies with the muscle type. They are much more numerous and closely packed in the ocular muscle than in the body muscle, thus resembling the situation found in avian and mammalian ocular muscles (Gerebtzoff et al., 1954). In the body muscle, the end-plates are straight extended structures similar to that seen in amphibians (Koelle, 1963), thus differing from the endomysial localization found in goldfish and guppy (Lundin, 1958). Cholinesterase is also present at the musculotendinous junctions, as in other vertebrates (Koelle, 1963) and in the myotomal septa. This last site has been observed in several species of fishes (Lundin, 1958, 1962; Pecot-Dechavassine, 1961) but the function of ChE in the myotomal septa still remains unknown.

In the digestive tract, ChE appeared as a fine reticulum in striated muscle but could not be detected in smooth muscle; in mammals smooth muscle gives a positive reaction (Koelle, 1963). ChE was observed in the gills of several species of fishes (Fleming et al., 1962); it

could not be detected in rainbow trout larvae.

B. Toxicity of Dylox.

The LC_{50} (for 16 hr) is between 30 and 50 ppm for young larvae and between 5 and 10 ppm for old larvae. When the figure given by Wilford (1967) is corrected for the amount of non active ingredients, the LC_{50} (for 24 hr) for $1\frac{1}{2}$ inch long rainbow trout larvae is 26 ppm. Although slightly lower, the LC_{50} observed in the present experiments is in the same range as that given by Wilford. The discrepancy can be explained by such factors as origin and age of larvae, water flow and temperature.

The results clearly show that older larvae are more sensitive than young larvae. These results are somewhat surprising since larger fishes of one species are usually more resistant to poisons than small ones of the same species (Kershwill and Edwards, 1967; Hoff and Westman, 1965). The results of Lukyanenko and Flerov (1966a, 1966b), however, were contrary to these and Marchetti (1965) has observed in rainbow trout larvae a change in resistance to nonyl phenolethoxylate similar to that observed with Dylox. It thus seems that the detoxification action of the liver which would be expected to increase (Hogan and Knowles, 1968) since important development takes place during the resorbtion of the yolk (Battle, 1942) has very little importance.

It could be argued that the decreased resistance could be due to the fact that the gastrointestinal tract represents a new pathway of entry for the poison since the old larvae had just started feeding; however, according to Ferguson's and Goodyear's (1967) findings, this is unlikely. The increased sensitivity of older larvae would be more

likely related to the increase in the gill surface which, according to Byczowska-Smyk (1961), undergoes important development during the stage of yolk resorption. At the time of starvation which is associated with the termination of yolk resorption and the beginning of feeding, there is a change in the type of metabolism: carbohydrate metabolism, as a source of energy, replaces the phospholipoprotein and lipoprotein metabolism which was prevailing in the earlier stages. The higher sensitivity of older larvae could also be related to this change.

C. Anticholinesterase action.

The behaviour of the larvae, similar to that observed in Fundulus and Amblystoma poisoned with eserine and DFP (Youngstrom, 1938; Sawyer, 1944, 1955), together with the cholinesterase studies, leaves no doubt about the anticholinesterase activity of Dylox. This anticholinesterase activity, however, is not thought to be due to Dylox (0,0-Dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate) itself but to its transformation into DDVP (0,0-Dimethyl 2,2-dichlorovinyl phosphate) by dehydrochlorination occurring in alkaline aqueous solution (Lorenz et al., 1955; Van Die, 1957). The very low mammalian toxicity of Dylox is indeed due to the fact that it is rapidly metabolized before being transformed into DDVP (Robbins et al., 1956). In this experiment, the formation of anticholinesterase DDVP from Dylox, either in the bathing solution or in the tissues, would explain the typical organophosphate poisoning.

The acetylcholinesterase inhibition, however, does not appear sufficient to explain all the results obtained. Comparison of Tables 5.1 and 5.5 shows no correlation between the percentage mortality and

AChE level. After 32 hr of treatment with 5 ppm, percentage mortality is very low (1%) and the AChE activity is barely higher than the AChE of larvae treated with 50 ppm for 16 hr after which time the percentage mortality has reached 61%. Furthermore, at 32 hr the AChE activity of the larvae treated with 5 ppm is somewhat lower than that found at 16 hr in larvae treated with 30 ppm but the mortality is much lower. If death was directly correlated with the AChE inhibition one would expect the reverse situation.

D. Histopathological changes.

Histological damage following organophosphate poisoning was seldom found. Demyelination, which appears specifically after poisoning with certain compounds in some species (O'Brien, 1967), still remains unexplained. Multiplication of the Schwann cells and vacuolization of their nuclei has been associated with ChE inhibition in rats (Zabusov, 1967). Abnormalities have been reported that are not unique to organophosphate exposure but that could be due to disturbance in the acetylcholinesterase metabolism or to hypoxia: dystrophic and necrobiotic changes in liver, kidneys, myocardium and endocrine glands after lethal doses of mercaptophos and disistone in rats and cats (Kagan and Makovskaya, 1960); vacuolization of the salivary glands (Denz, 1951). Changes in the internal organs following chronic poisoning by Dipterex in rats (Kirichek et al., 1964) would result from disturbed acetylcholine metabolism.

Decrease in the number of red and white blood cells, as well as degeneration of lymphocytes in spleen and thymus has also been observed (Kirichek et al., 1964; Kaplan and Glazenski, 1965; Jones and Landing, 1948).

In this experiment, the changes observed in glands, blood vessels and intestine probably result from excess acetylcholine. Although nerve cells are very sensitive to hypoxia (Van Liere and Stickney, 1963), it is possible that the necrotic changes observed in the brain result from excess acetylcholine: Ivanova (1968) has shown that abnormal quantities of acetylcholine modify the RNA content of neurons. Abnormalities observed in the heart and liver are probably due to hypoxia since the amount of oxygen available to the tissues is certainly decreased by the lowered rate and strength of the heartbeat and the lowered activity of the opercular muscle. The deformation of red blood cells and the disruption of the pseudogills suggest that the organophosphate has some effect on the electrolytic balance, altering the permeability of the cell membrane.

Pathological lesions of the gills, ranging from edema of the epithelium to complete destruction of the structure, were also observed. Although never reported before for organophosphate, such a finding is not too surprising since gill tissue appears to be quite sensitive: various toxic substances produced necrobiotic changes in gills (Collins et al., 1966; Lowe, 1964; Vasantha, 1963; Schmid and Mann, 1961; Cairns et al., 1964; Christie and Battle, 1962).

Quite surprising was the finding of important changes in muscle fibers. Except for the degeneration induced by Plasmocid (Hicks, 1950), Vincristine (Slotwiner et al., 1966) and phenols (Hicks, 1950; Waluga, 1966), striated muscles are known to be quite resistant to chemical action (Field, 1960; Adams et al., 1963). Khera et al. (1966) have observed dystrophic changes in muscles of hatched ducks that had been injected with EPN on the 13th day of incubation. Characterized by

fragmentation of some fibers, proliferation and central displacement of sarcolemmal nuclei, these changes could be due to a lack of proper differentiation rather than a positive degeneration. Degeneration of muscle induced by DDT, Pyrethrum and Parathion was observed in the insect (Ashraf, 1964) but was never reported before for vertebrates. The muscular dystrophy observed in our material bears some resemblance to the degeneration seen with Plasmocid: formation of hyaline material, swelling of segments and loss of cross and longitudinal striations.

The muscle degeneration in rainbow trout larvae may be a result of muscle fatigue due to continuous contractions. The muscle ultimately becomes exhausted, dies and degenerates. Although the cause remains obscure, muscle exertion or strain is known to produce degeneration of the fibers in cattle (Hadlow, 1959; Runnells et al., 1960; Adel, 1966). In this regard, the observations of Kumudavalli and Swami (1967) are particularly interesting. These authors have shown that there was, in fatigued frog gastrognemius muscle, a decrease in the amount of soluble and insoluble proteins accompanied by an increase of non-protein nitrogen, thus suggesting the activation of proteolytic enzymes during the state of muscular fatigue. Schmitt et al. (1966) have also shown that cod and trout tissues contain high amount of dipeptidases which would be responsible for the rapid degradation of protein in fish muscles after death.

The fact that important disruption is found in muscles which are richly innervated and which contain numerous end-plates (as seen by histochemical reaction for AChE) such as the ocular muscle, could be an indication that muscle degeneration results from exhaustion due to excess stimulation. The degeneration of the muscle, however, could

also result from the organophosphate acting directly on certain structural components or metabolic pathways. That the organophosphate could have a direct effect on the fiber is supported by the work of Loomis and Konker (1967) who have shown that Soman probably affects the muscle fiber since in denervated muscle it prolongs the contraction induced by d-tubocurarine.

The histochemical reaction for the respiratory enzymes have shown that in some tissues of treated larvae the reaction appeared weaker. No conclusion, however, can be drawn about the inhibition of these enzymes until quantitative biochemical tests are made since the difference in intensity of the reaction in control and treated larvae was small and therefore the histochemical reaction is not quantitatively reliable. These histochemical reactions, however, give some precisions about the damage occurring in muscle fibers and brain and indicate that important mitochondrial clumping takes place in treated larvae.

Acetylcholinesterase inhibition producing paralysis of muscles involved in maintenance of respiration is probably an important factor. However, Durham et al. (1957) have noted that animals given DDVP could still live with cholinesterase activity less than 20% of normal. Since in these experiments, high mortality is associated with important tissue damage while the AChE activity is weak in both cases of low and high mortality, it is suggested that causes other than AChE inhibition are involved in the poisoning of rainbow trout larvae by the organophosphate Dylox.



Fig. 1. Young Salmo gairdneri irideus larva (14 days old, 21 mm). 3.4 x.



Fig. 2. Old Salmo gairdneri irideus larva (40 days old, 26 mm). 3.4 x.

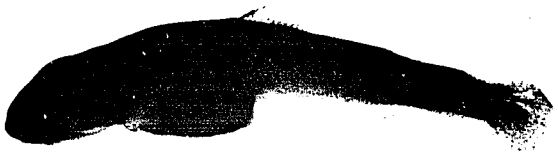


Fig. 1. Young Salmo gairdneri irideus larva (14 days old, 21 mm). 3.4 x.



Fig. 2. Old Salmo gairdneri irideus larva (40 days old, 26 mm). 3.4 x.

PLATE 5.1.

Histological damage in young Salmo gairdneri irideus larvae treated with Dylox. 600 x.

Fig. 3 and 5, control.

Fig. 4 and 6, after 10 hr of treatment with 50 ppm of Dylox.

Fig. 3. Section through the pseudogills of a control. Parallel rows of epithelial-like cells sandwiching a row of blood cells form the pseudogills.

Fig. 4. Section through the abnormal pseudogills of a treated: disruption of the parallel arrangement and desintegration of epithelial-like cells.

Fig. 5. Cross section through the dorsal carinalis muscle of a control larva. Small red fibers form this muscle.

Fig. 6. Cross section through the abnormal dorsal carinalis muscle of a treated larva. Fibers are swollen and myofibrils appear fused.

Plate 5.1

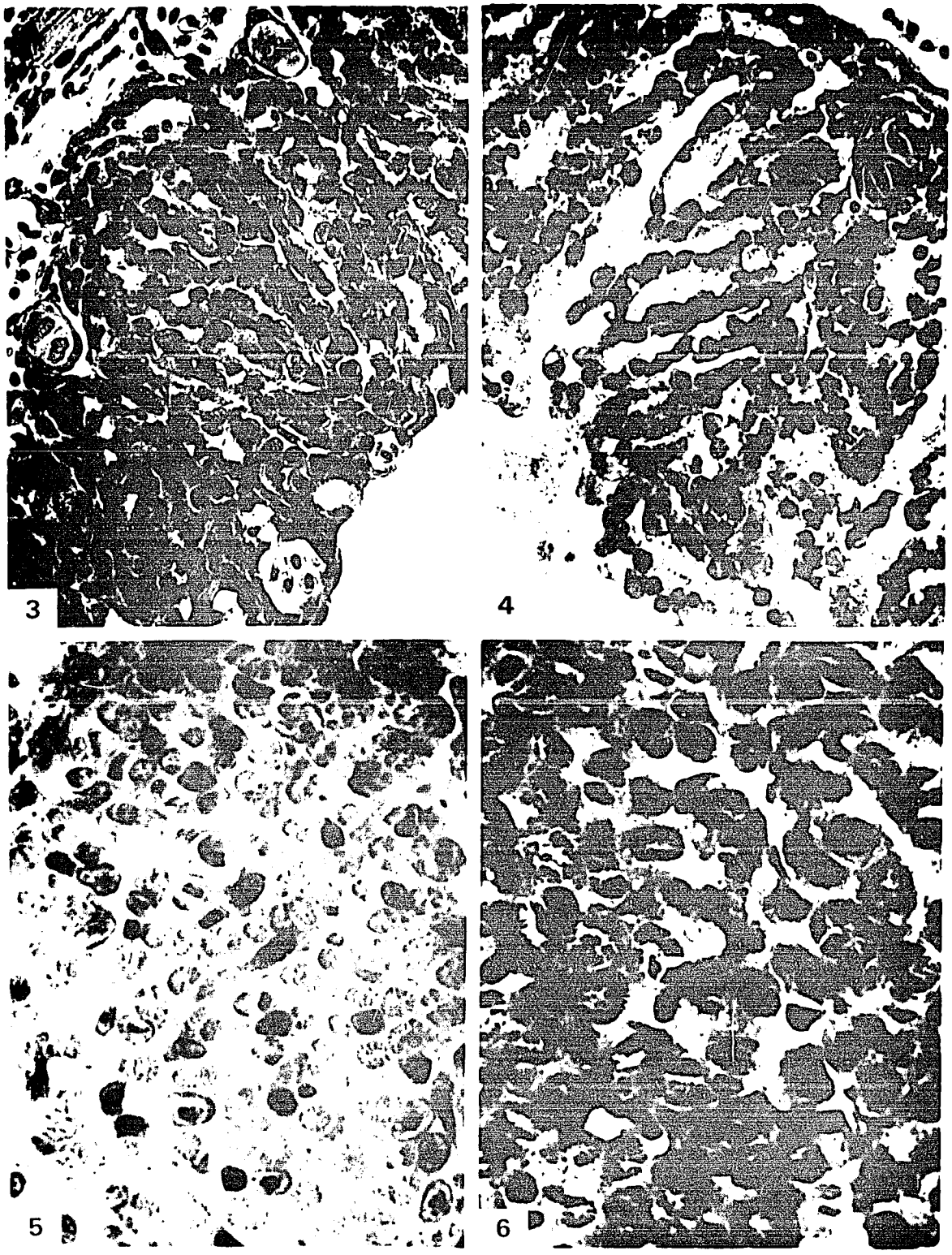


Plate 5.1

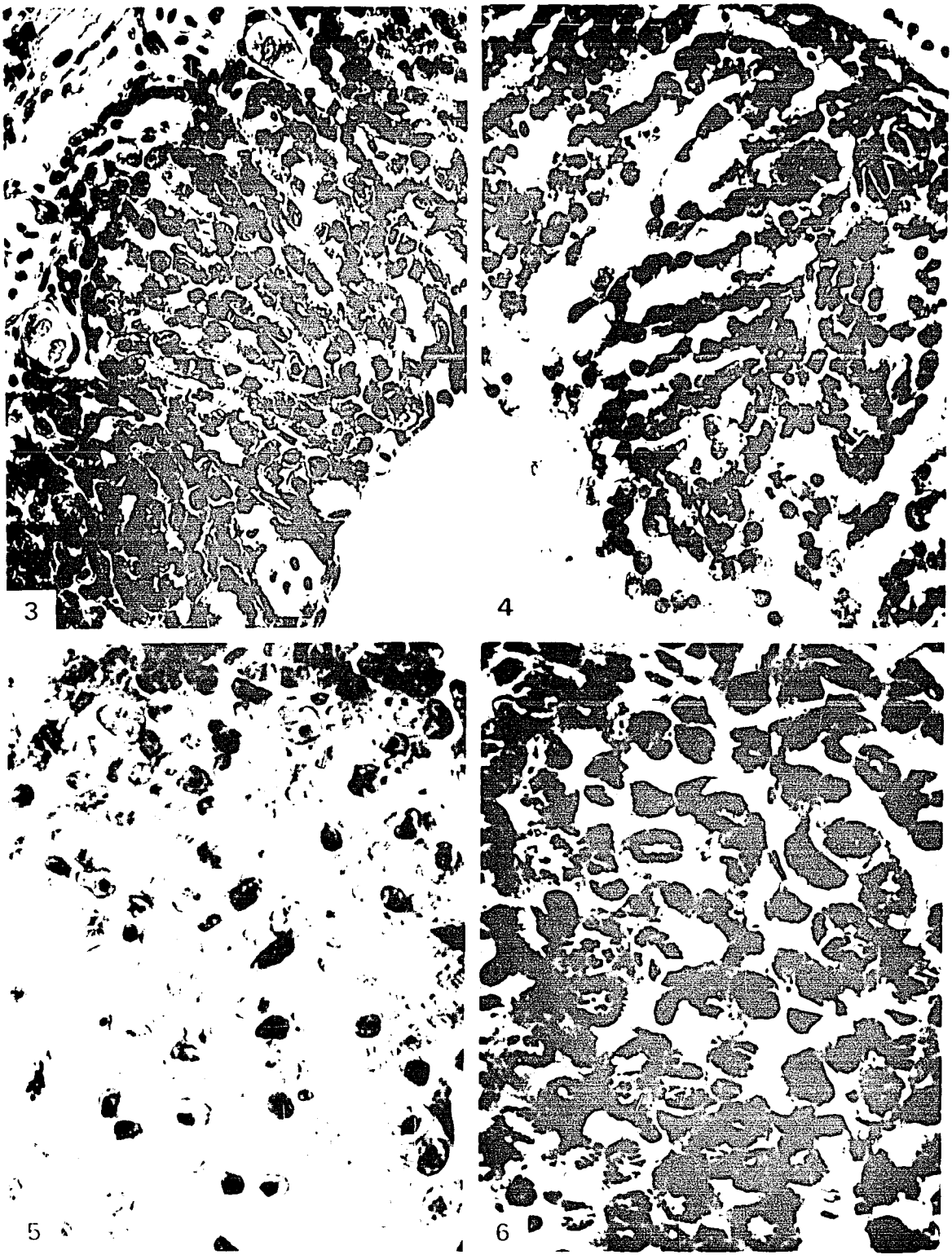


PLATE 5.2

Histological damage in young Salmo gairdneri irideus larvae treated with Dylox.

Fig. 7. Longitudinal section through the ocular muscle of a treated larva (50 ppm for 10 hr) showing swollen opaque regions similar to contraction bands (arrow) and stretched clear regions. 600 x.

Fig. 8. Longitudinal section of the tail of a treated larva (16 hr of recovery following 16 hr of treatment with 30 ppm). Two foci of muscle degeneration infiltrated by leucocytes. 125 x.

Fig. 9. Longitudinal section through the body muscle (white fibers) of a control. 600 x.

Fig. 10. Longitudinal section through the body muscle (white fibers) of a treated larva (50 ppm for 10 hr). Fibers are broken, swollen and desintegrating. Cross striations are absent. 600 x.

Plate 5.2

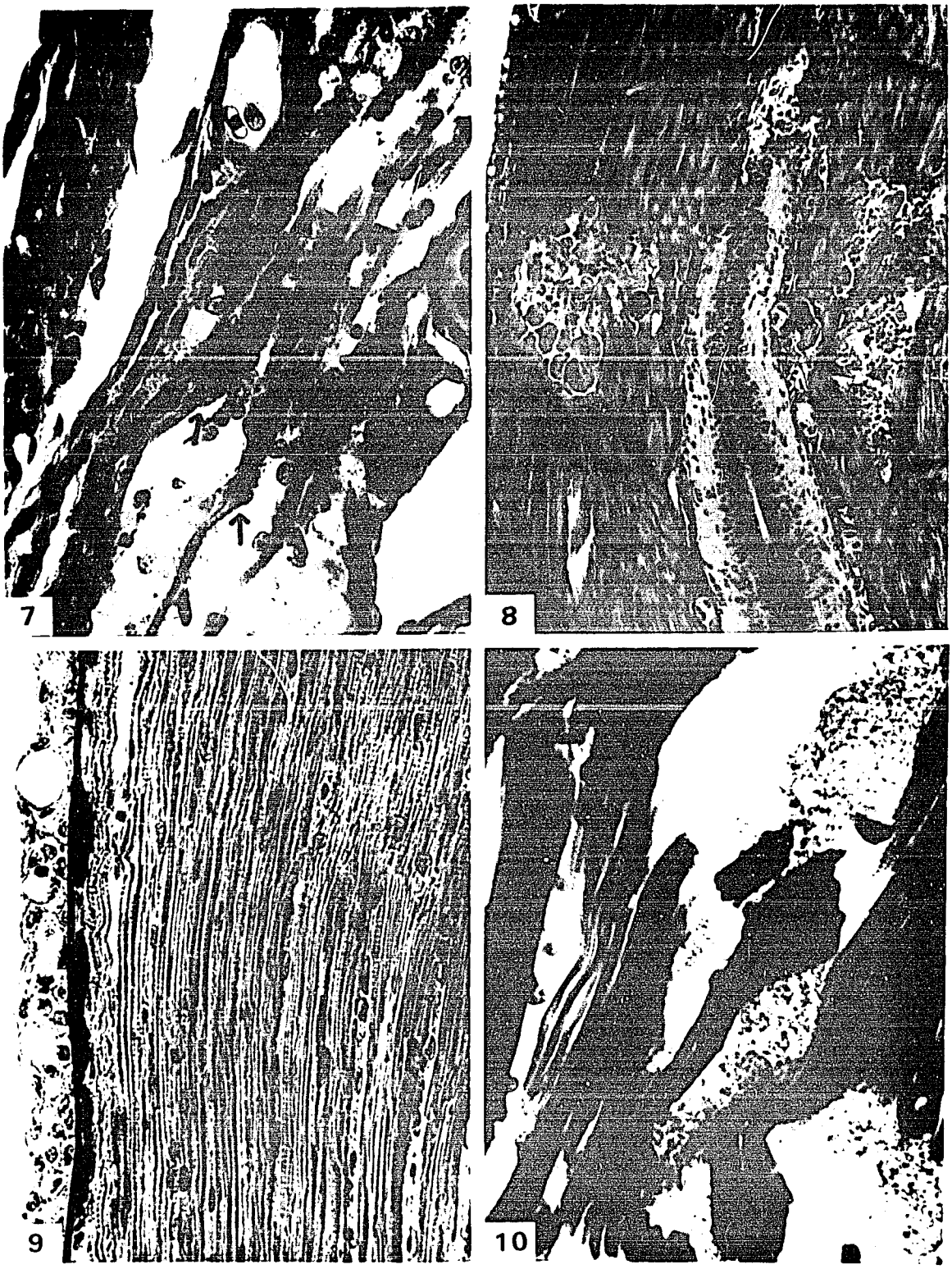


Plate 5.2









PLATE 5.3

Histological damage in young Salmo gairdneri irideus larvae treated with Dylox for 10 hr.

Fig. 11. Cross section through the cervical ganglion: cells have dense basophilic cytoplasm. 600 x.

Fig. 12. Cross section through the ganglion of a treated larva (50 ppm): cells are not as closely packed, nucleus and cytoplasm are less basophilic and nuclei are foamy. 600 x.

Fig. 13. Cross section through the white fibers of the body muscle of a control. Most of the myofibrils are regularly distributed against the sarcolemma while a few are found in the central cytoplasm. 1250 x.

Fig. 14. Cross section through white fibers of the body muscle of a larva treated with 50 ppm: myofibrils of some fibers are fused. 1250 x.

Fig. 15. Cross section through the white fibers of the body muscle of a larva treated with 30 ppm: cells are swollen and nuclei are round. Myofibrils shrink away from membrane and roll toward the center of the cell leaving a crescent-shape peripheral cytoplasm. 1250 x.

Plate 5.3

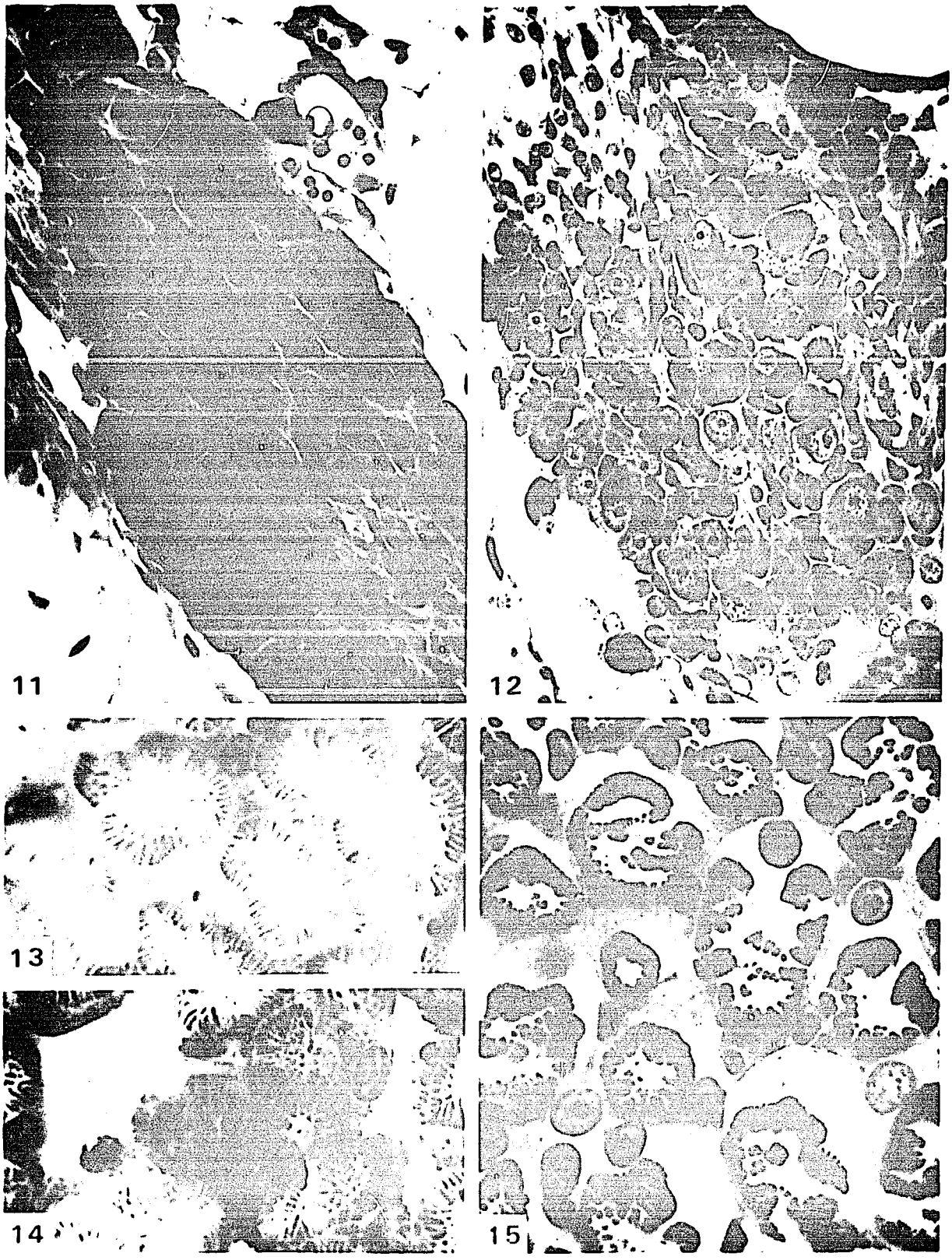


Plate 5.3

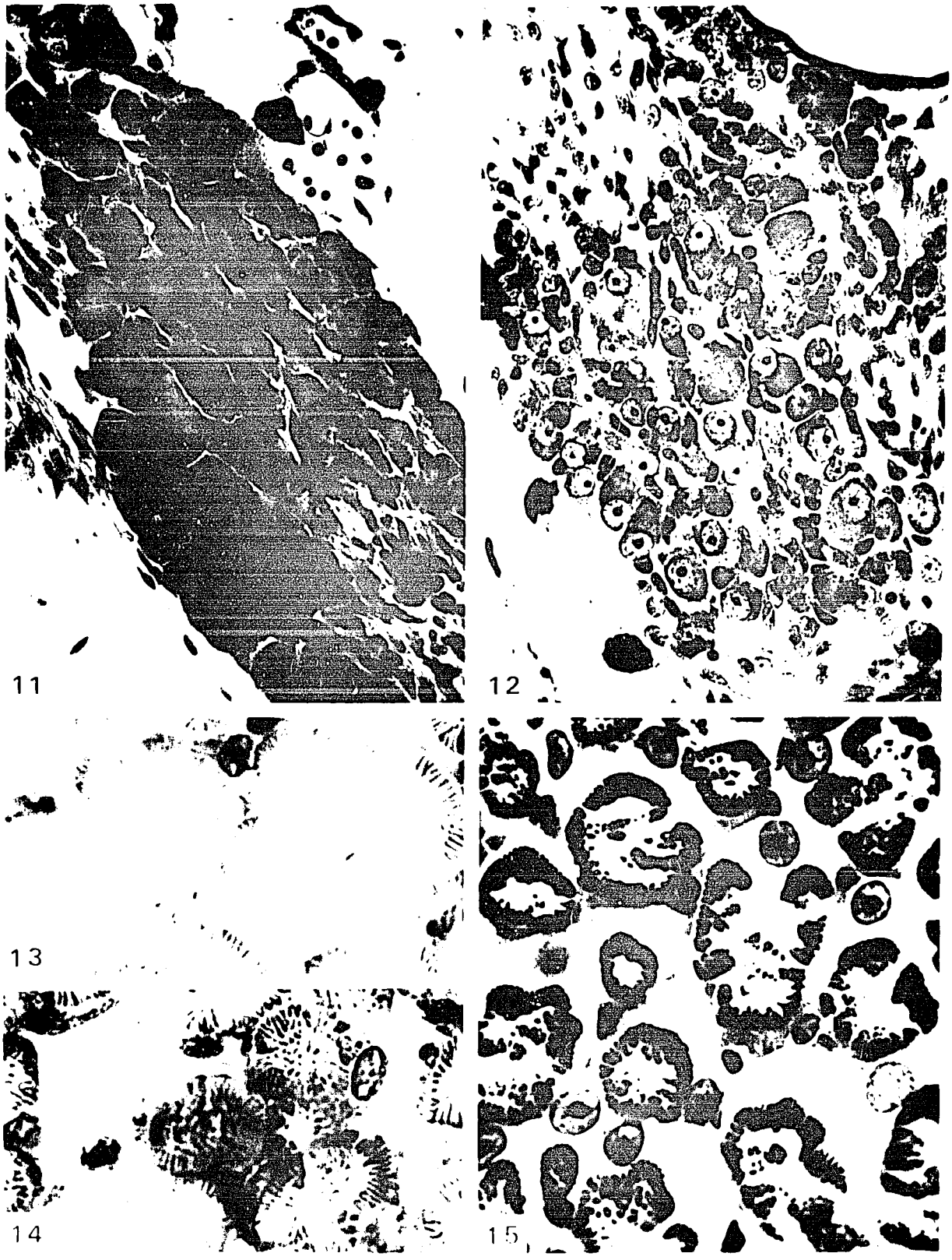


PLATE 5.4

Histological damage in old Salmo gairdneri irideus larvae treated with Dylox for 10 hr.

Fig. 16. Longitudinal section through the gill filaments of a control. 125 x.

Fig. 17. Cross section through the gill filaments of a treated larva (50 ppm). The epithelium is swollen and the tissue is necrotic. 125 x.

Fig. 18. Cross section through the gill lamellae of a control. 600 x.

Fig. 19. Cross section through the gill lamellae of a treated larva (50 ppm). The whole structure is edematous and the epithelium is separated from the capillary. 600 x.

Fig. 20. Red blood cells of a control. Cells have central nuclei and appear oval in surface view and dumb-bell shapedin lateral view. 1250 x.

Fig. 21. Cross section through the liver of a treated larva (30 ppm). Parenchyma cells are vacuolar. 600 x.

Fig. 22. Cross section through the liver of a control showing parenchyma cells with blood sinusoids. 600 x.

Fig. 23. Red blood cells of a treated larva (50 ppm). Cells are distorted and shrunken as if crenated. 1250 x.

Plate 5.4

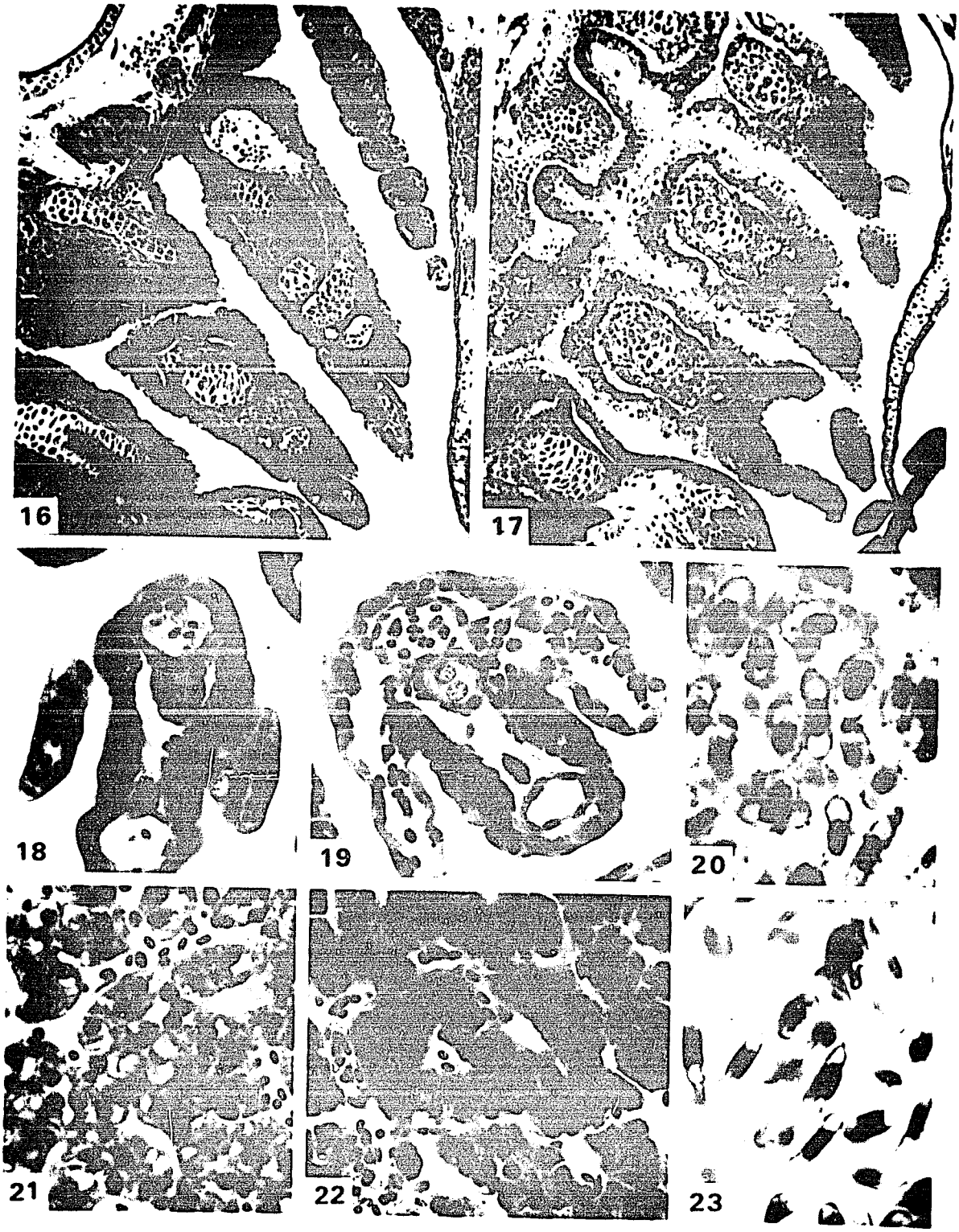


Plate 5.4



PLATE 5.5

Distribution of lactic dehydrogenase in young Salmo gairdneri irideus larvae (normal).

Fig. 24. Cross section through the anterior part of the head showing the olfactory placode (olf). The epithelium of the medial part of the luminar border gives a strong reaction which gradually decreases passing mediad. The head epithelium gives a weak reaction. 43 x.

Fig. 25. Cross section through the head at the level of the forebrain. The reaction is medium compared to the strong reaction in the ocular muscle (om). 23 x.

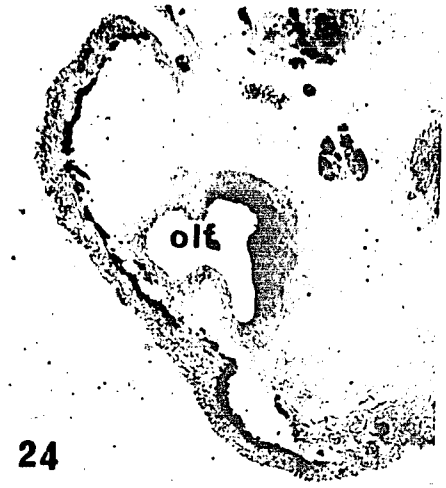
Fig. 26. Cross section through the head at the level of the anterior part of the forebrain showing a strong reaction in the epiphysis (ep). 43 x.

Fig. 27. Cross section through the eye showing the different layers. The reaction is strong in the inner and outer plexiform layer. It is medium in the layer of ganglion cells and weak in the inner nuclear layer. 450 x.

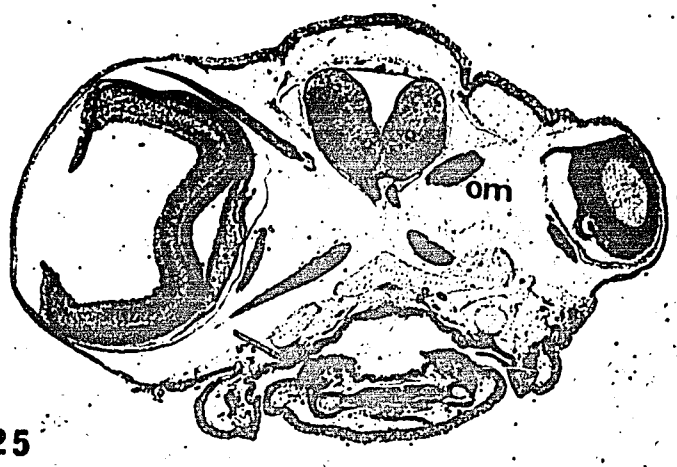
Fig. 28. Cross section through the head at the level of the midbrain. The densely cellular inner layer of the optic lobes gives a weak reaction while the outer portion is medium. The lower portion is weaker, especially the densely cellular area surrounding the cavity. Ocular muscle (oc) and oblique muscle give a strong reaction. The dorsal and medial portions of the masseter (ma) also react strongly. 23 x.

Fig. 29. Cross section through the head at the level of the posterior portion of the midbrain. The reaction is medium except in the densely cellular posterior part of the optic lobes (ol). The epithelium lining the otic cavity (oc) gives a strong reaction except in some segments. The pseudogills (pg) give a strong reaction while the reaction is generally weak in the

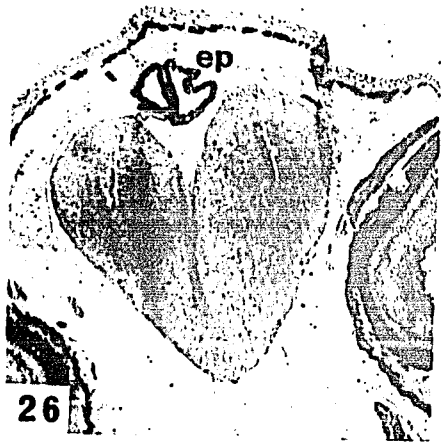
Plate 5.5



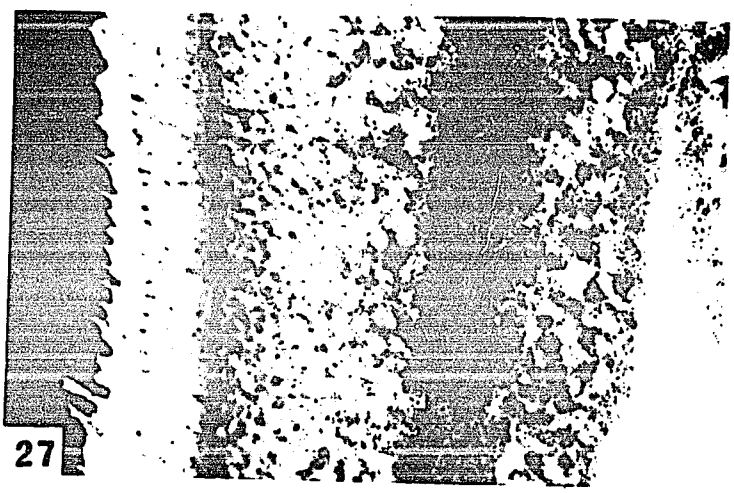
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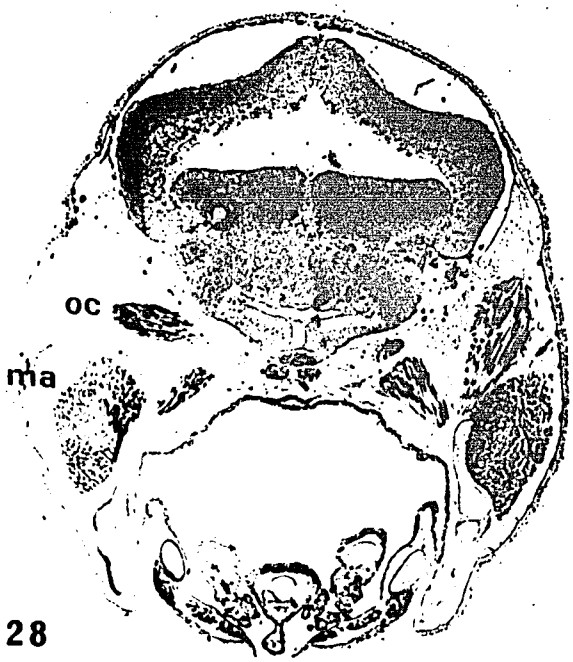
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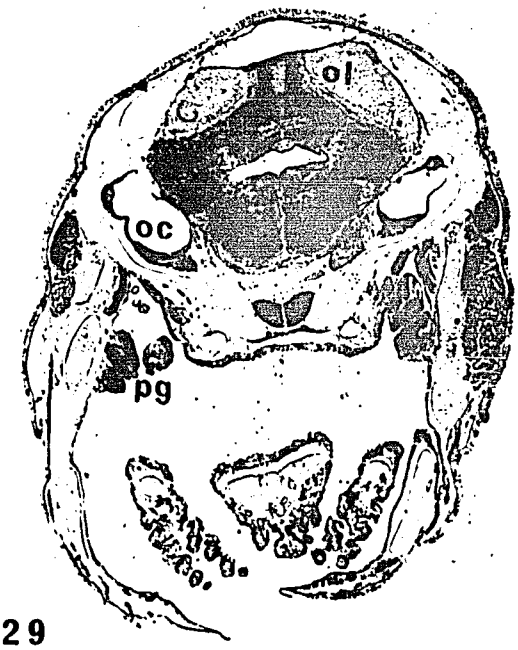
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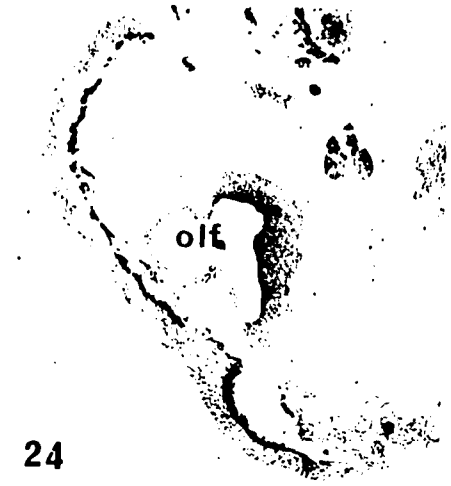


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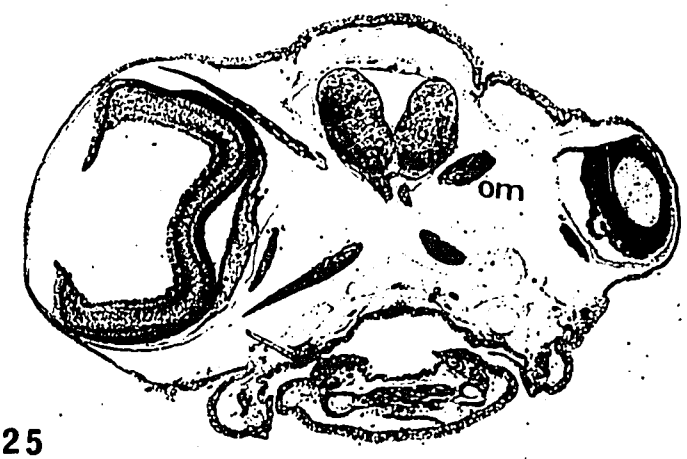


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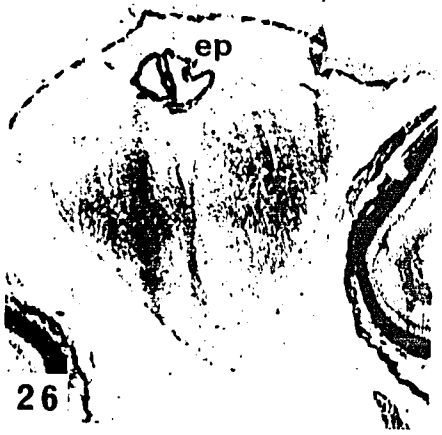
Plate 5.5



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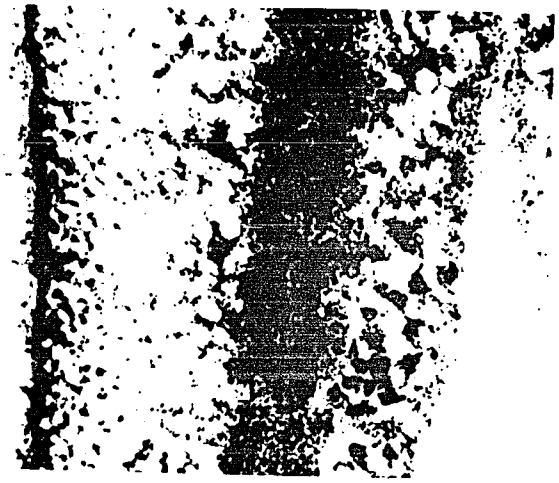
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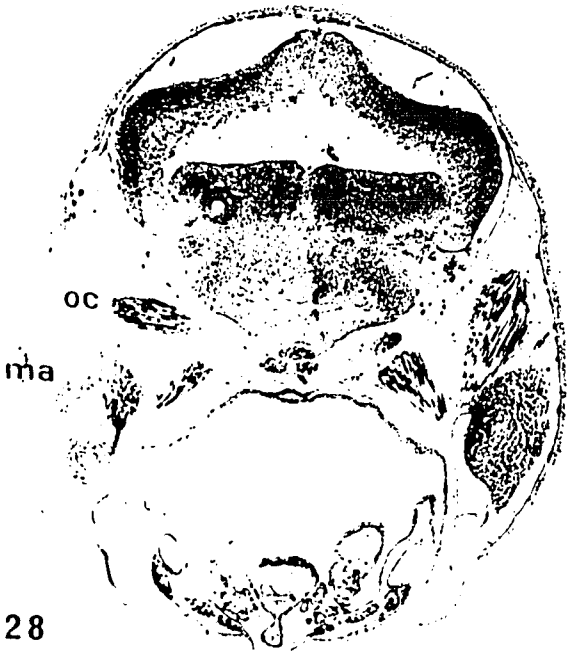


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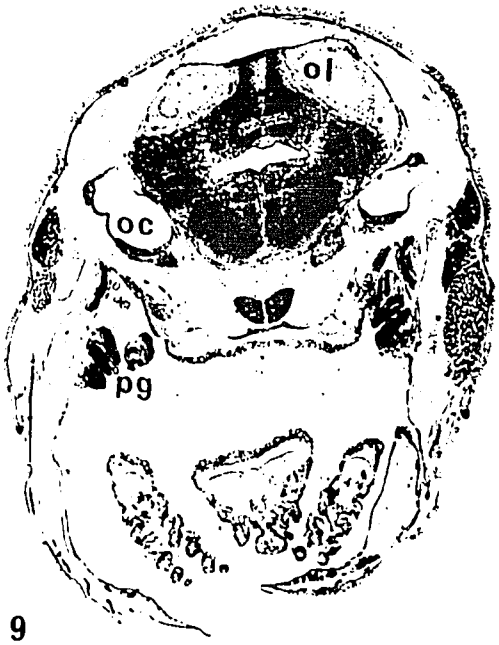


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PLATE 5.6

Distribution of lactic dehydrogenase in young Salmo gairdneri irideus larvae (normal).

Fig. 30. Cross section through the midbrain showing details of the reaction. The deep cellular layer of the optic lobes (1) gives a weak reaction, while the gray matter in the basal part of the brain (2) gives a medium-to-strong reaction. The reaction in the acellular layer of the optic lobes (3), especially along the second row of cells parallel to the surface of the optic lobes (arrow) is strong. The ependymal cells (e) lining the vertical slit (third ventricle) gives a weak reaction except at the luminar end. 43 x.

Fig. 31. Section through the cartilage of the head. The overall reaction appears weak because the cells are widely spaced. In the narrow band of cytoplasm surrounding the nucleus of the chondrocytes, the reaction is strong. 500 x.

Fig. 32. Section through the otic epithelium and the ganglion below the otic capsule. In the ganglion, the cells are irregular in shape and have an eccentric nucleus; these cells give a strong reaction. Some segments of the cuboidal cells of the otic epithelium also give a strong reaction (upper right). 500 x.

Fig. 33. Section through the pseudogills. The epithelial-like cells give an extremely strong reaction while the blood cells between these rows give no reaction. 500 x.

Fig. 34. Section through the otic epithelium. The columnar cells of the cushion-like structure give a strong reaction but the cells of the lower half give a weak reaction as do the adjacent cuboidal cells. 500 x.

Fig. 35. Section through a densely cellular area which occurs as a swelling of the epithelium in the medial upper most region of the gills. This structure, when stained with HFO, appears as lymphatic tissue. The cells have very little cytoplasm and the reaction is weak. 500 x.

Plate 5.6

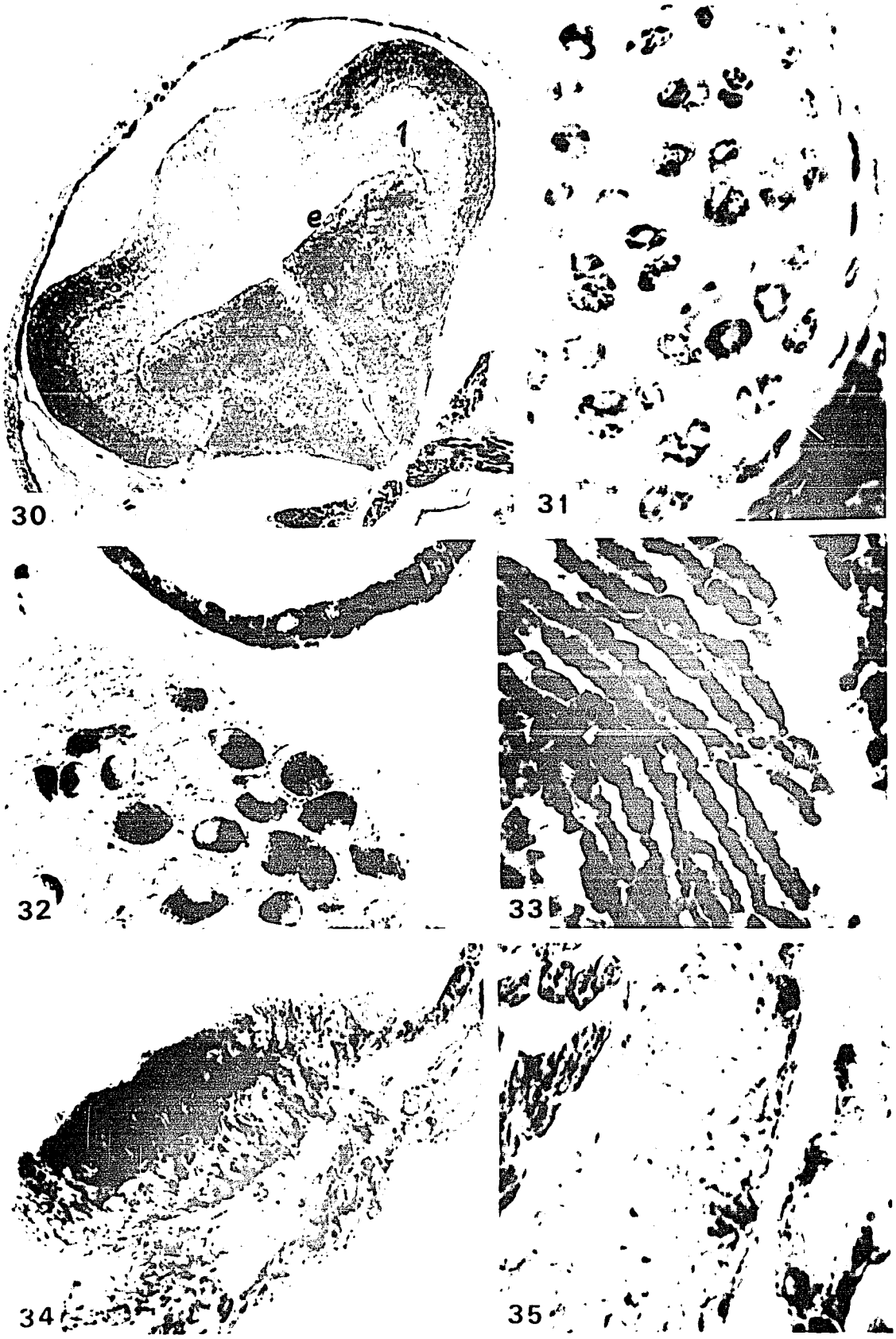


Plate 5.6



PLATE 5.7

Distribution of lactic dehydrogenase in young Salmo gairdneri irideus larvae (normal).

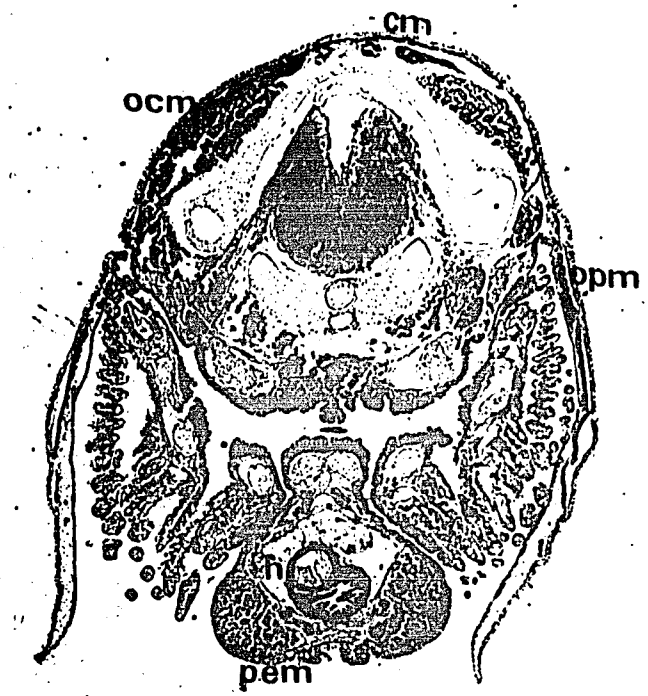
Fig. 36. Cross section through the neck at the level of the hindbrain showing the ventral aorta and part of the ventricle of the heart (h) and several muscles of the head: carinalis (cm), occipital (ocm), opercular (opm), palatine, pectoral (pem). 23 x.

Fig. 37. Cross section through the neck at the level of medulla oblongata. The single ventricle (V) of the heart and its trabeculae give a medium reaction while the wide thin-walled auricle (Au) is weaker. 23 x.

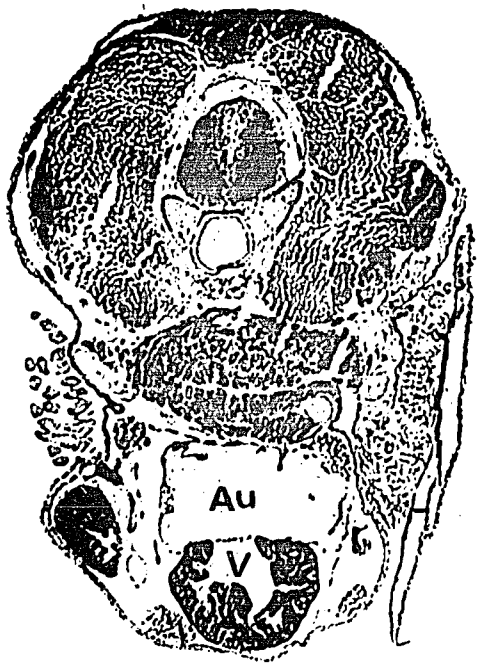
Fig. 38. Cross section through the body at the level of the anterior part of the yolk sac (ys). The notochord (n) does not stain. The reaction in the kidney tubules (T) is medium while it is weak in the oesophagus. The pectoral fin muscle (pfm) stains strongly. The dark areas in the yolk are artefacts due to a folding of the yolk. 23 x.

Fig. 39. Cross section through the gills. The reaction is very weak except in the more or less regularly spaced cells (chloride cells) in the epithelium.

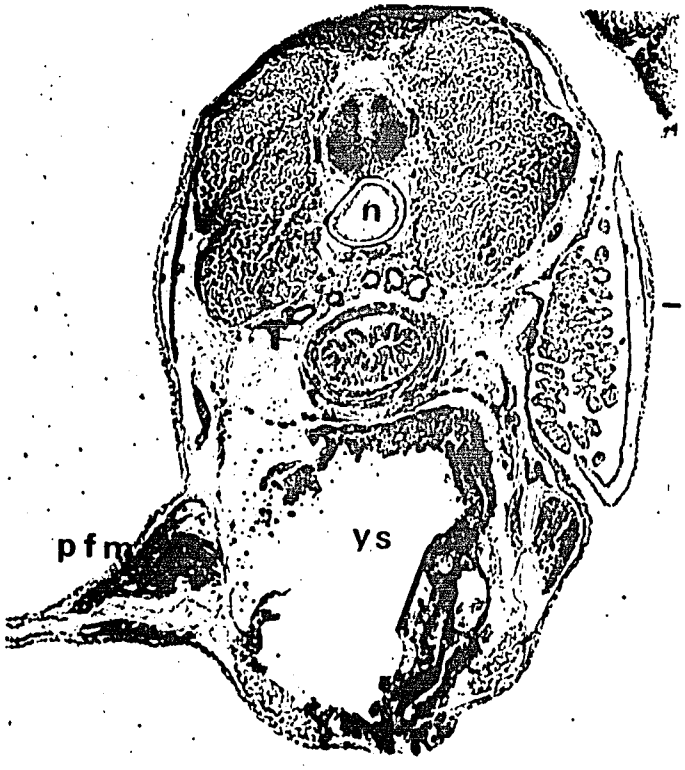
Plate 5.7



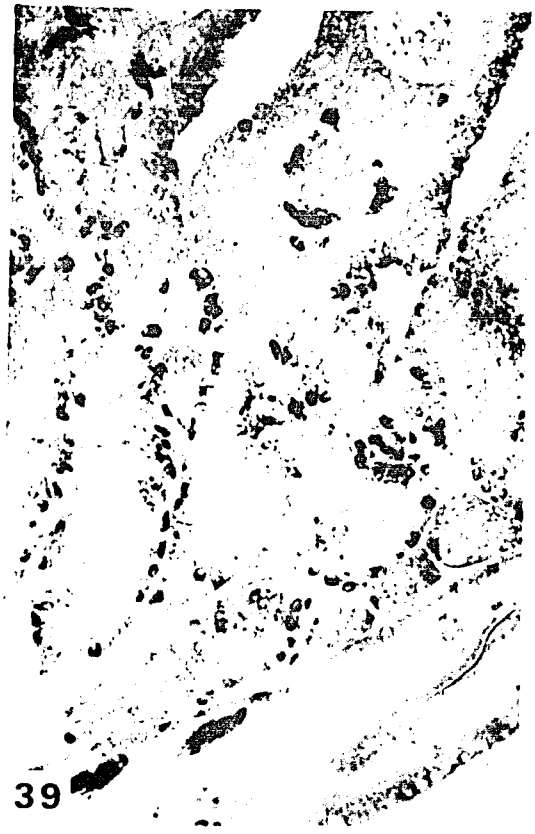
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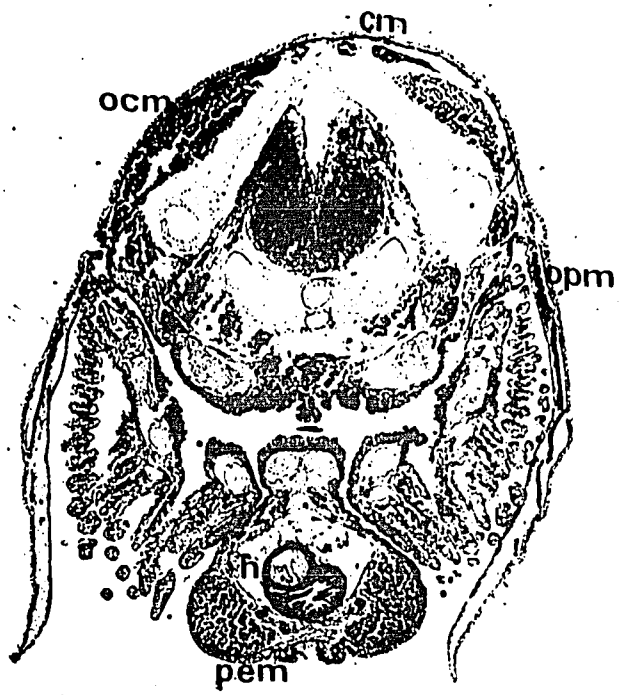


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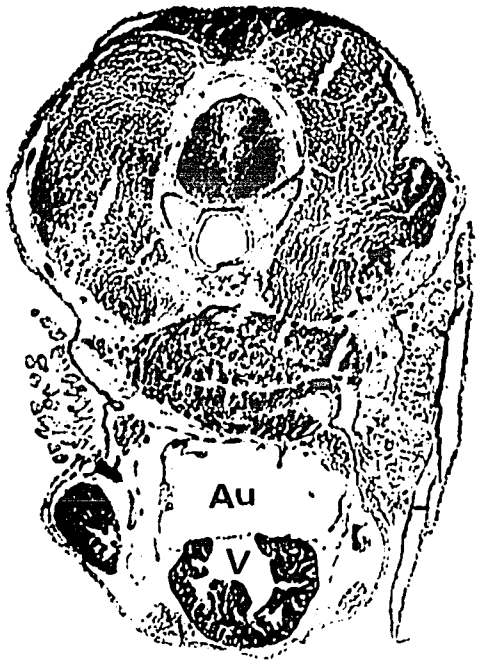


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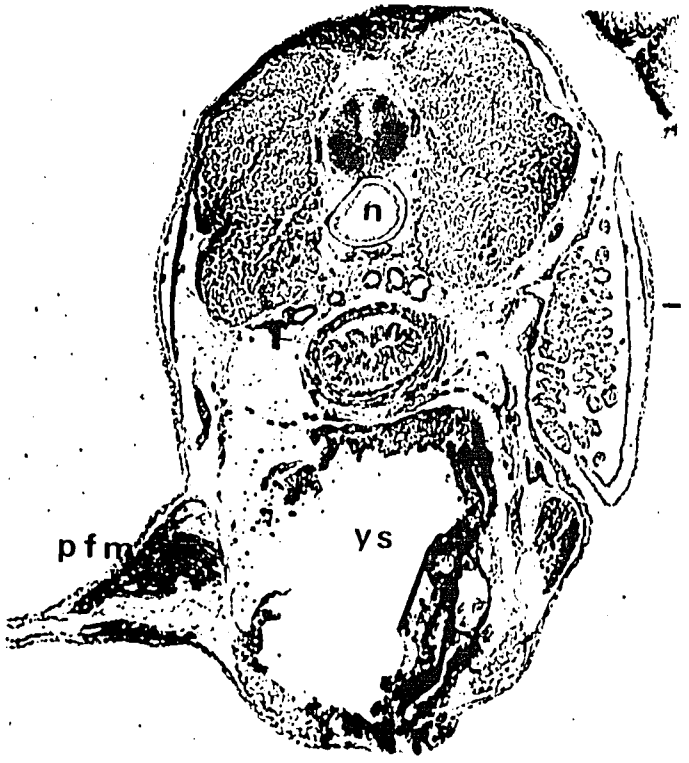
Plate 5.7



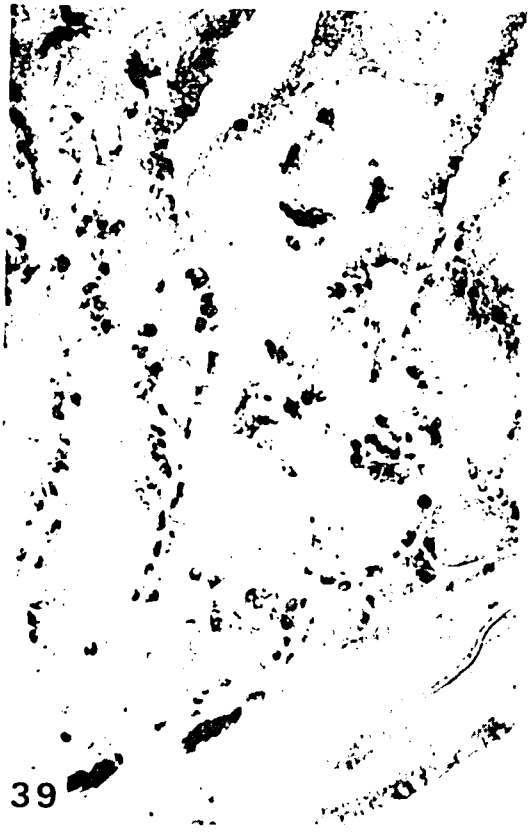
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PLATE 5.8

Distribution of lactic dehydrogenase in young Salmo gairdneri irideus larvae (normal).

Fig. 40. Cross section through the posterior part of the body showing the general distribution of various types of fibers. The bulk of body muscle stains weakly. The dorsal half is separated from the ventral by an invagination of the peripheral tissue forming a bow-like structure. The epithelium of the single mesonephric duct (D) stains very intensely.

Fig. 41. Section through the liver. The reaction is medium in the cytoplasm of the parenchyma cells. 500 x.

Fig. 42. Cross section through the kidney showing the abundant stroma (weak reaction), the mesonephric tubules (T) (medium reaction) and the mesonephric duct (D) (strong reaction). The kidney is surrounded by a thin layer of connective tissue containing pigment. 125 x.

Fig. 43. High magnification of a section through the body musculature. The epithelium (which rests on a layer of connective tissue containing pigment) stains weakly. The cutaneous muscle is made of a single layer of large cells which stain very strongly. The few outermost cells of the muscle are intermediate red fibers giving a medium reaction while the large white fibers stain weakly. Most of the stain is localized at the periphery of the muscle fibers. 500 x.

Plate 5.8

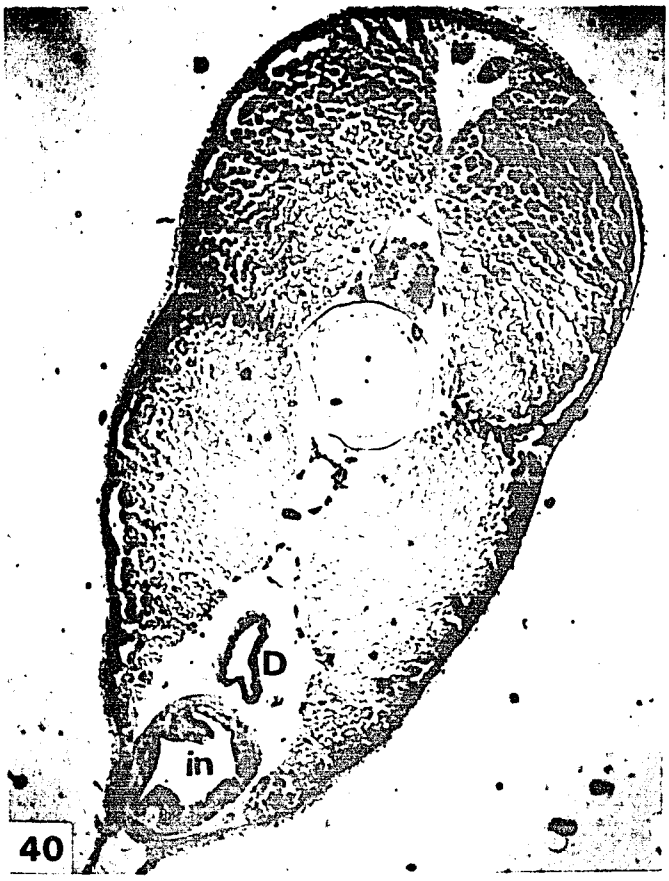
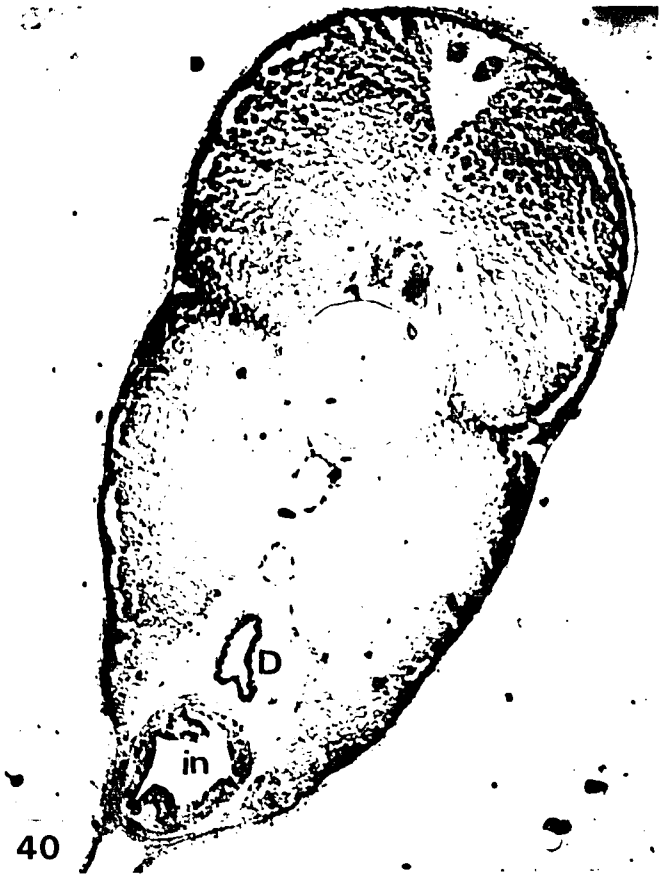








Plate 5.8



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PLATE 5.9

Distribution of lactic dehydrogenase in young Salmo gairdneri irideus larvae treated with Dylox for 6 hr.

Fig. 44. Cross section through the neural tube of a treated larva (100 ppm). The reaction is irregular: fine grains are grouped into small spots; the ependymal cells are torn away. 500 x.

Fig. 45. Cross section through the neural tube of a control larva. The reaction is medium in the cytoplasm of the large nerve cells and in the luminar portion of the ependymal cells. 500 x.

Fig. 46. Section through the otic ganglion of a control larva. The reaction is strong in the cytoplasm. 500 x.

Fig. 47. Section through the otic ganglion of a treated larva (30 ppm). The reaction is weaker and found all over the cells. 500 x.

Fig. 48. Section through the pseudogills of a treated larva (50 ppm). The general structure is disorganized and the strength of the reaction is irregular in the various cells. 500 x.

Plate 5.9

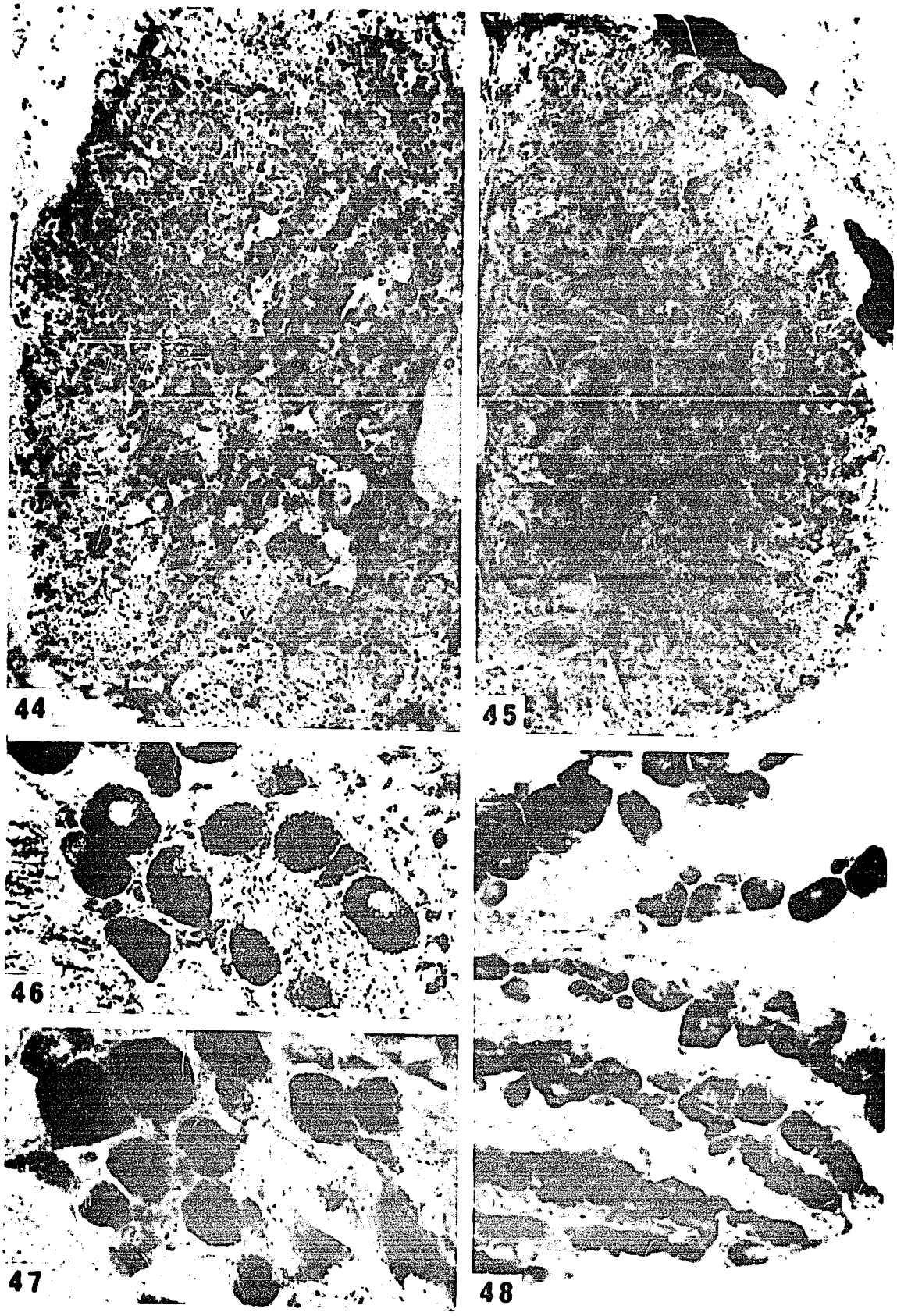


Plate 5.9

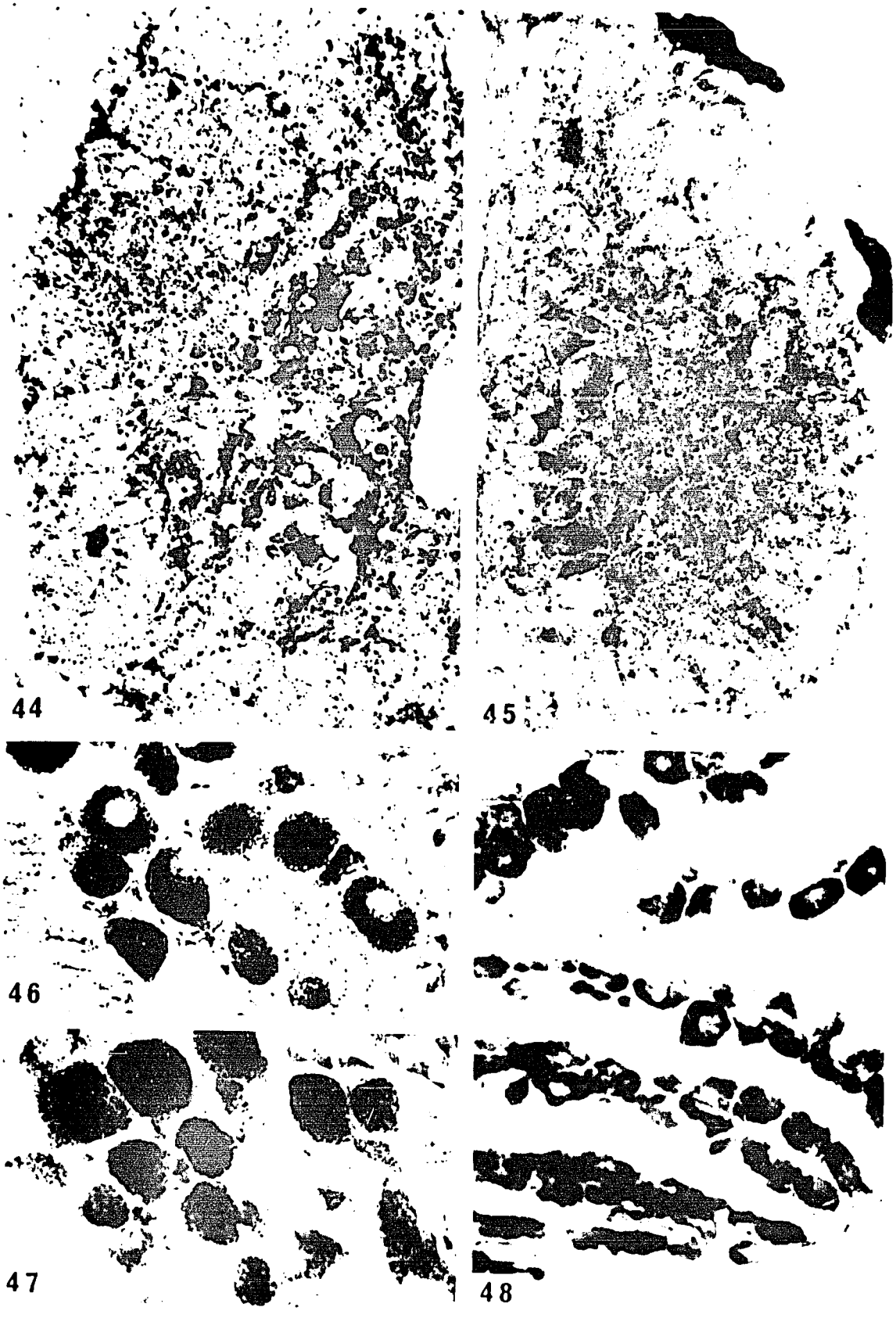


PLATE 5.10

Distribution of lactic dehydrogenase in young Salmo gairdneri irideus larvae treated with Dylox for 6 hr.

Fig. 49. Longitudinal section through pectoral fin muscle of a control. Small red fibers have a strong reaction. Note the outline of the cross striations. 500 x.

Fig. 50. Longitudinal section through the pectoral fin muscle of a treated larva (30 ppm). Most of the cross striations have disappeared. The reaction in parts of some fibers is weak while in others strong; grains are not discrete but fused into a long "trainée". Parts of some fibers are vesicular. 500 x.

Fig. 51. Oblique section through the ocular muscle (red fibers) of the control. The reaction appears as long rows of fine grains. 500 x.

Fig. 52. Oblique section through the ocular muscle of a treated larva (100 ppm). Fibers cannot be distinguished. Reaction is extremely clumpy. A fiber (upper right) is largely swollen into a node with a dark margin and a clear center crossed by thick bands. 500 x.

PLATE 5.10

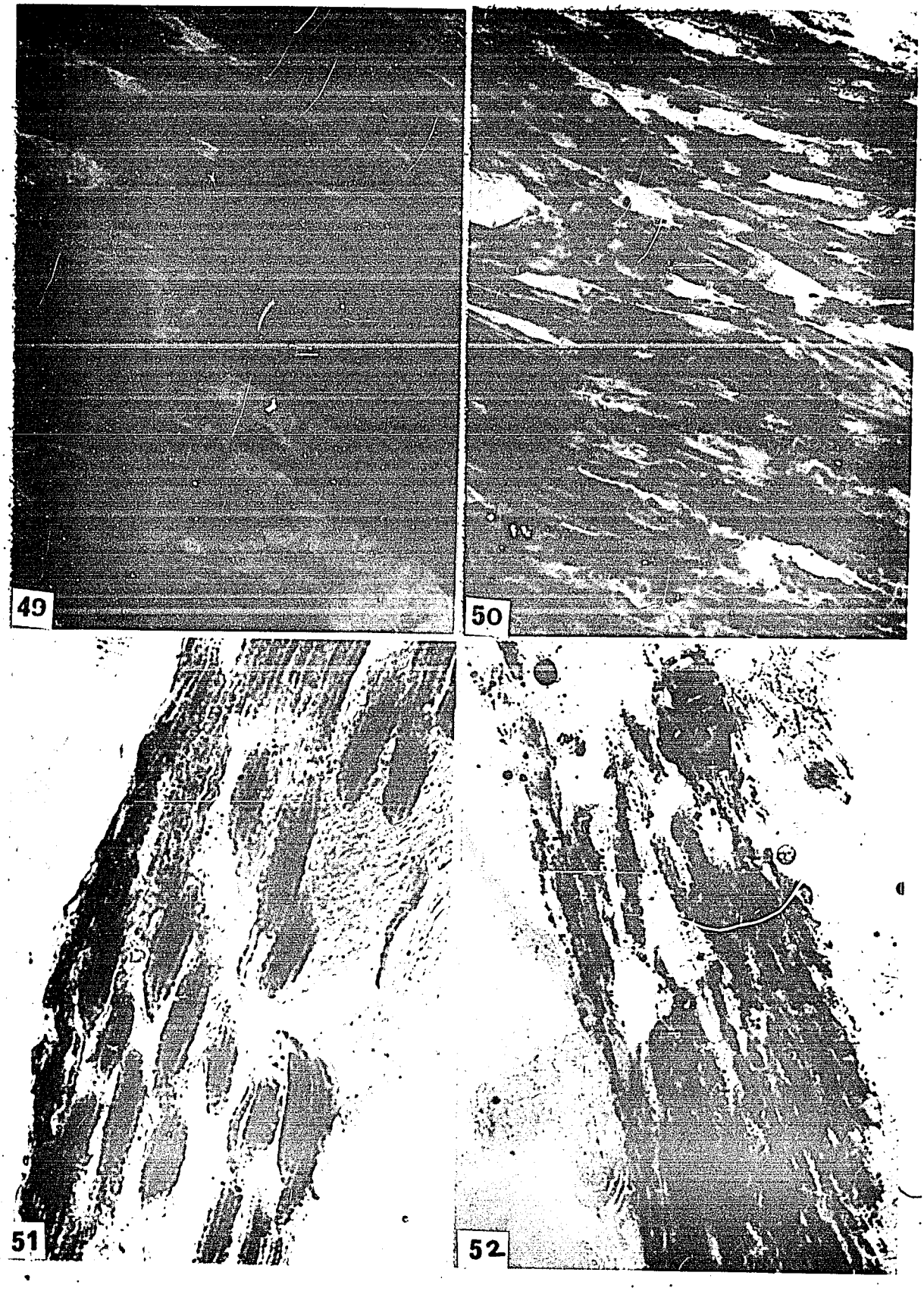


PLATE 5.10



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PLATE 5.11

Distribution of lactic dehydrogenase in young Salmo gairdneri irideus larvae treated with Dylox for 6 hr.

Fig. 53. Oblique section through the ocular muscle (red fibers) of a control. 500 x.

Fig. 54. Oblique section through the ocular muscle of a larva treated with 30 ppm. Muscle fibers are disrupted. Reaction is clumped into big dark areas. 500 x.

Fig. 55. Section through the subcapsular[1] muscle of a control. 500 x.

Fig. 56. Section through the subcapsular muscle of a larva treated with 100 ppm. The reaction is spotty. Portions of extremely swollen fibers contain large amounts of enzymes while adjacent portions are almost completely devoid of grains. 500 x.

1. The subcapsular muscle is the muscle attached to the otic capsule.

Plate 5.11



Plate 5.11



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PLATE 5.12

Distribution of acetylcholinesterase in young Salmo gairdneri irideus larvae (Karnovsky's and Roots' stain).

Fig. 57. Cross section through the head at the level of the eye and midbrain. The reaction is strong in the sensory layer of the eye and in the lateral wings of the midbrain. Note the fine dense layer parallel to the surface of the optic lobes. In the basal part of the brain, it is mainly present in the cells lining the cavity. The ocular muscles (om) give a very strong reaction. (The dark line "p" running from the top of the eye to the base of the brain is the pigmented layer of the skin which is folded). 43 x.

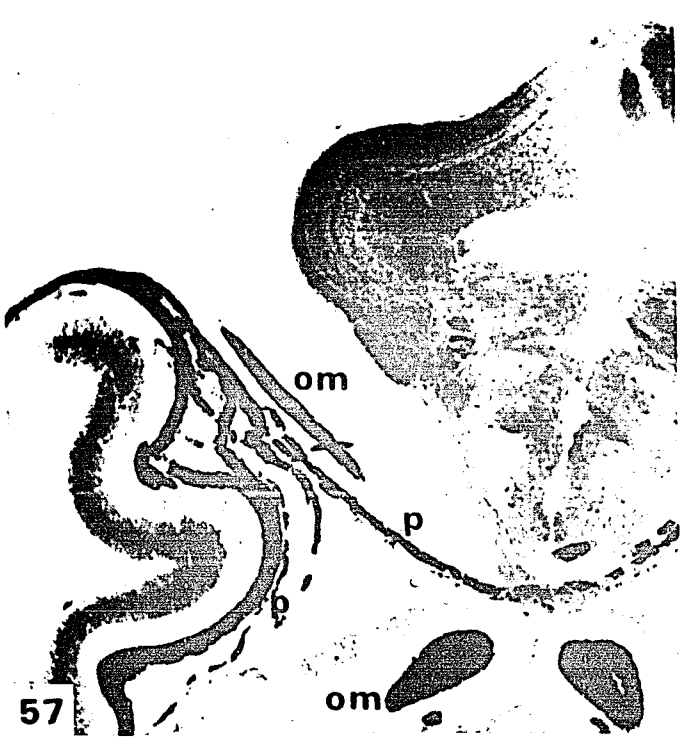
Fig. 58. Cross section through the medulla oblongata. The overall reaction is medium but strong in the large medial nerve cells. 125 x.

Fig. 59. Cross section through the head at the level of the midbrain (mb) of a larva treated with 30 ppm for 6 hr. No observable reaction in the eye (e), brain and ocular muscle (om). 43 x.

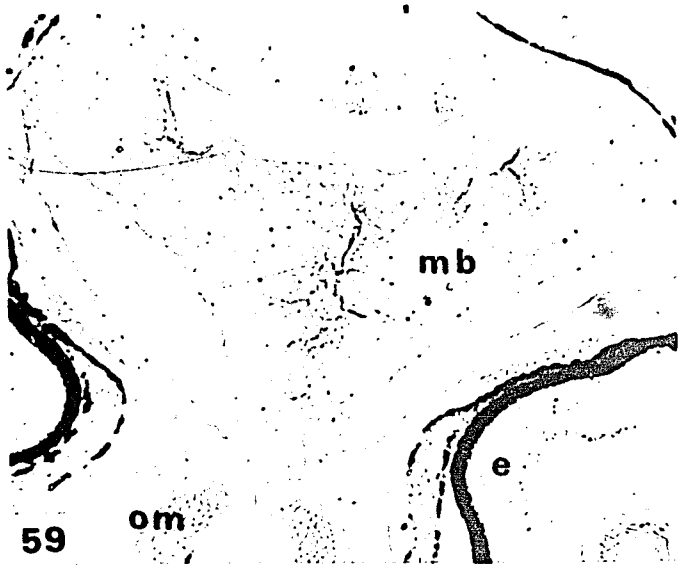
Fig. 60. Cross section through the stomach of a control larva. AChE is present in fine delicate structures within the muscle layer. 125 x.

Fig. 61. Cross section through the neural tube showing the presence of AChE in the cytoplasm of the large and small cells and in the cellular extensions. 500 x. (control larva).

Plate 5.12







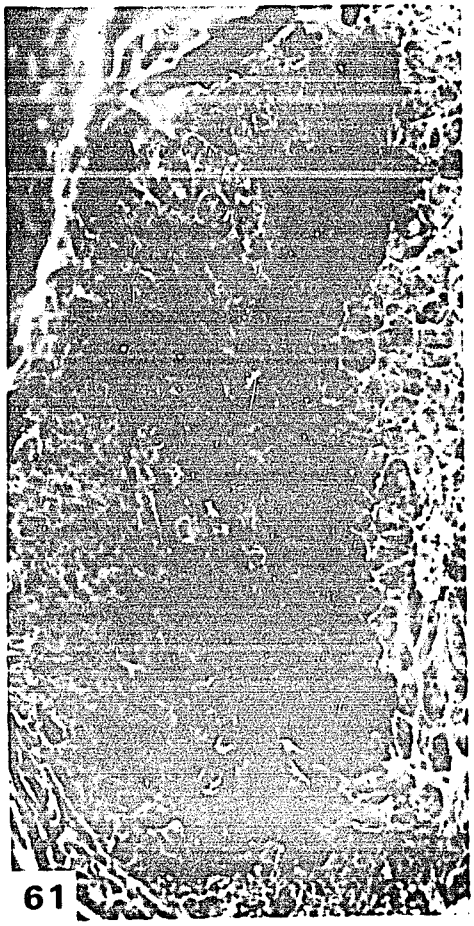




Plate 5.12

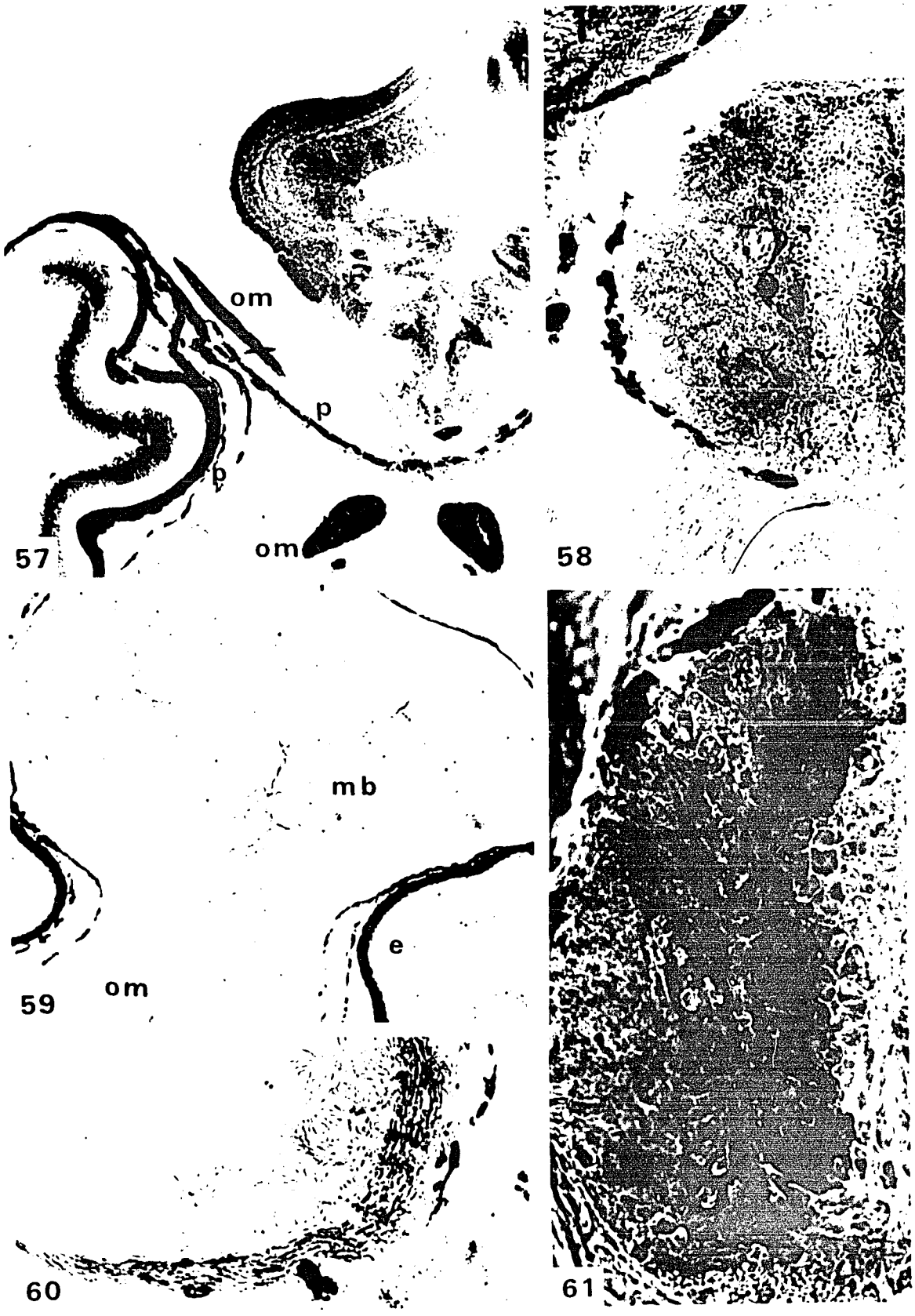


PLATE 5.13

Distribution of acetylcholinesterase in young Salmo gairdneri irideus larvae.

Fig. 62. Longitudinal section through the oblique muscle. AChE is present in the end-plates along the fibers and mainly at the point of insertion of the muscle fibers. Karnovsky's and Roots' stain. 500 x.

Fig. 63. Cross section through the large white fibers of the body muscle showing the end-plates. AChE is also present on the surface of the cells forming the medial margin of the muscle. Koelle's stain. 500 x.

Fig. 64. Cross section through the otic ganglion of a larva treated for 6 hr with 30 ppm of Dylox. No AChE is observable. Karnovsky's and Roots' stain. 500 x.

Fig. 65. Details of AChE in the cells of the otic ganglion. Note the stronger reaction next to the nucleus. Karnovsky's and Roots' stain. 500 x.

Fig. 66. Cross section through the body. AChE is present in the end-plates and along the myotomal septa (mys). Karnovsky's and Roots' stain. 125 x.

Fig. 67. Cross section through the neural tube. AChE is present in the large ventral area; it is strong in the cytoplasm of the large cells. It is also present in a few cells found in the dorsal part. The ganglionic cells situated outside the neural tube stain strongly. Karnovsky's and Roots' stain. 125 x..

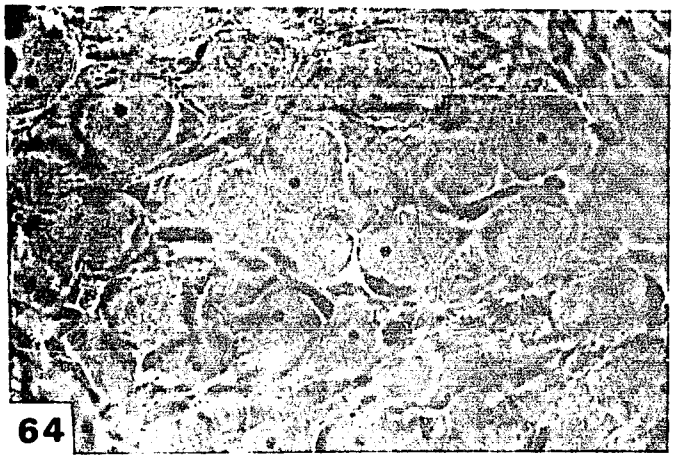
Plate 5.13



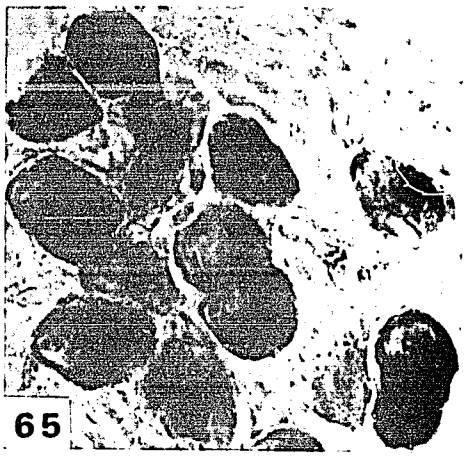
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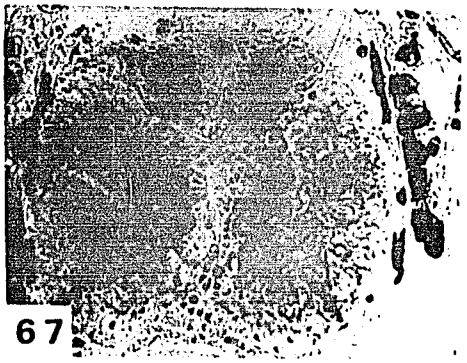
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Plate 5.13



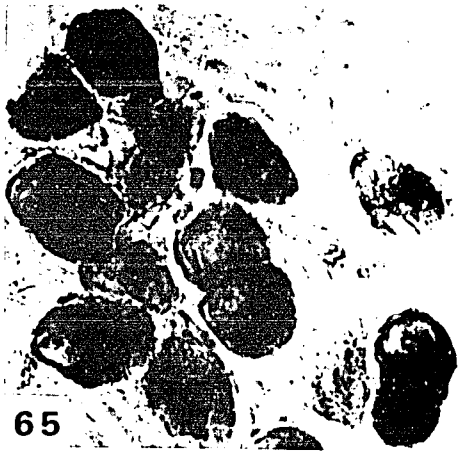
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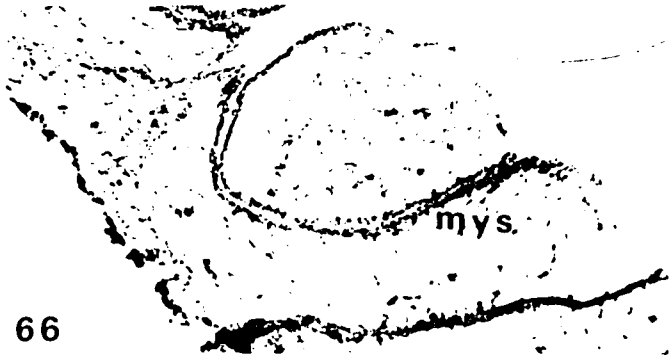
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CHAPTER SIX

EXPERIMENTS WITH

SALVELINUS FONTINALIS

LARVAE

The results obtained with rainbow trout larvae suggested that a similar but more extensive study should be made. Thus, a fairly complete picture of the effect of organophosphates on the embryonic and larval stages would be obtained.

I. EXPERIMENTALS.

An experimental design slightly modified from the previous experiment was followed. The effect of both Dylox and Phosphamidon at concentrations of 5, 10, 30, 50 and 100 ppm was separately investigated on larvae of two ages: a) young larvae, 15 days old and measuring 17 mm long (fig. 1); b) old larvae, 35 days old and measuring 21 mm long (fig. 2). In these, the yolk sac was almost completely resorbed. Trout hatching trays were replaced by the system described on page 39. Since only six units were available, treatment with Phosphamidon took place on the day following treatment with Dylox. The following parameters were studied: a) mortality; b) behaviour; c) histological sections; d) histo-enzymology and biochemical analysis of ChE; and e) electron microscopy of muscle.

Mortality and behaviour was observed at 8, 16, 24, 48 hr and 5 and 12 days after the beginning of treatment. At these periods, several samples were fixed with various fixatives for further study. Each sample was carefully examined and only samples in which the heart was still beating were taken.

Several longitudinal or cross sections of at least four samples for each group were prepared after Bouin's fixation. Histochemical reactions for the following enzymes were performed on control and

on old larvae after 24 hr of treatment with 30 ppm of Dylox and 100 ppm of Phosphamidon: phosphorilase, cytochrome oxidase, lactic and malic dehydrogenases, NADH diaphorase, acid and alkaline phosphatases, ATPase, 5'nucleotidase and acetylcholinesterase. Phosphorilase and cytochrome oxidase were studied in a few sections of the tail and of the head only (four samples). For all other enzymes, serial sections of one sample and representative sections of three other samples were used.

Samples for electron microscopy were taken after 24 hr of treatment. The larvae were placed in cold 5% gluteraldehyde and a few minutes later, the masseter muscle was dissected. This muscle was chosen because previous studies with rainbow trout had shown it to be very susceptible to treatment and because it could easily be removed even when the head muscles of the larvae were very small. In each experiment, four samples chosen at random from surviving larvae in the highest concentrations of each insecticide were studied:

a) Young larvae: Dylox 100 ppm, Phosphamidon 100 ppm;

b) Old larvae: Dylox 30 ppm, Phosphamidon 100 ppm.

Samples for biochemical analysis of AChE activity were taken at 8, 16, 24, 48 hr and 5 and 12 days (times of behaviour and mortality observations) and were stored at -20 C until needed for measurements. With the Hestrin's method, the following procedure was followed. One milliliter of head homogenate containing 60 mg/ml was incubated for 45 min at room temperature with 1 ml of 4 mM acetylcholine iodide. The reaction was stopped by the addition of 4 ml of the NaOH-Hydroxylamine mixture. The further steps of the technique are described on page 47. The composition of the incubation media and conditions of measurements with the Abou-Donia's and Menzel's method were described on page 48.

The ChE activity of head homogenate, in terms of quantity of acetylthiocholine hydrolyzed/gram of tissue/minute was determined as follows. One milliliter of head homogenate containing 6 mg/ml was incubated with 1 ml of DTNB solutions containing respectively 0.01, 0.025, 0.05, 0.075, 0.1, 0.15, 0.2, 0.25 and 0.3 µM/ml. Optical density was read after 30, 60 and 90 min of incubation at room temperature (table 6.1).

TABLE 6.1 Optical densities after 30, 60 and 90 min of incubation when 6 mg of head homogenate are incubated with 4 mM of acetylthiocholine in presence of various quantities of DTNB.

µM of DTNB	TIME OF INCUBATION		
	30	60	90
0.01	0.062	0.060	0.061
0.025	0.127	0.131	0.128
0.05	0.279	0.286	0.283
0.075	0.384	0.385	0.386
0.1	0.547	0.552	0.551
0.15	0.656	0.735	0.737
0.2	0.654	0.872	0.869
0.25	0.656	1.03	1.08
0.3	0.655	1.05	1.17

When the quantity of DTNB is smaller than 0.15 µM, the OD (optical density) does not change as the incubation time is increased,

showing that the limiting factor is the amount of DTNB, and that all the DTNB available has reacted with the product of the hydrolysis of acetylthiocholine. With a quantity of 0.15 µM and larger, the OD after 30 min of incubation does not vary even if the amount of DTNB is increased, indicating that the limiting factor is the amount of enzyme. If the values obtained after 30 min of incubation are plotted, the graph shown in figure 3 is obtained. The point of intersection of the two lines would indicate the quantity of DTNB reacting with the products of hydrolysis since in the sloping line the DTNB is the limiting factor and in the horizontal line the amount of enzyme is the limiting factor. The point of intersection corresponds to 0.12 µM of DTNB. Since this value is for 6 mg of head homogenate, the ChE activity is thus 6.6×10^{-4} µM/min.

In measuring the ChE percentage activity, all the samples were incubated and measured in groups of six. Samples of untreated larvae of corresponding ages were run each time with samples of five treated groups. Optical density obtained with the untreated was considered as representing 100% activity.

II. RESULTS.

A. Mortality and behaviour.

i - Young larvae.

The reaction of speckled trout larvae to exposure to Dylox and Phosphamidon was similar to the reaction observed in rainbow trout larvae treated with Dylox. After a period of hyperactivity during which the larvae swam fast, nervously and often changed direction, they

became less active than normal. Eventually, they lay on their sides and moved only when stimulated.

The reaction to a touch stimulus was also similar to the reaction in rainbow trout. The muscular contraction gradually became more extensive, longer lasting and less efficient.

Tables 6.2 and 6.3 give the mortality percentage and heartbeat rate of young larvae treated with various concentrations of the organophosphates.

TABLE 6.2 Heartbeat[1] (H.B.) rate per minute and total percentage mortality (Mort.) in young Salvelinus fontinalis larvae treated for 24 hr with various concentrations of Phosphamidon.

DOSE (ppm)		TIME					
		8 hr	16 hr	24 hr	48 hr	120 hr	288 hr
0	H.B.	s 130	s 130	s 130	s 127	s 130	s 125
	Mort.	0	0	0	0	7	15
5	H.B.	s 130	s 95	s 95	s 85	s 100	s 120
	Mort.	0	0	0	6	12	14
10	H.B.	s 110	s 94	m 85	m 70	s 110	s 115
	Mort.	0	0	0	3	11	14
30	H.B.	s 110	s 80	m 70	m 65	s 108	s 120
	Mort.	0	0	3	5	13	20
50	H.B.	s 90	m 45-75	m 55	m 45-75	s 100	s 105
	Mort.	0	0	3	7	14	19
100	H.B.	m 90	m 35-65	w 45	m 65	s 110	s 110
	Mort.	0	0	0	5	23	24

[1]The strength of the heartbeat was classified as strong (s), medium (m), or weak (w).

TABLE 6.3 Heartbeat[1] (H.B.) rate per minute and total percentage mortality (Mort.) in young Salvelinus fontinalis larvae treated for 24 hr with various concentrations of Dylox.

DOSE (ppm)		TIME					
		8 hr	16 hr	24 hr	48 hr	120 hr	288 hr
0	H.B.	s 130	s 130	s 130	s 125	s 125	s 127
	Mort.	0	0	0	0	5	9
5	H.B.	s 130	s 110	s 110	s 115	s 120	s 125
	Mort.	0	0	0	3	14	17
10	H.B.	s 130	s 95	s 100	s 120	s 115	s 120
	Mort.	0	0	0	1	10	17
30	H.B.	s 125	m 95	w 50	m 95	s 120	s 120
	Mort.	0	0	4	6	11	16
50	H.B.	s 115	m 60	w 30	m 110	s 110	s 110
	Mort.	0	0	2	6	11	15
100	H.B.	s 115	w 30-70	w 30-40	m 110	s 110	s 115
	Mort.	0	0	4	21	31	33

[1]The strength of the heartbeat was classified as strong (s), medium (m), or weak (w).

With both compounds, mortality was rather low becoming appreciably higher only after the end of treatment when dose levels of 100 ppm were used. Complete loss of spontaneous activity occurred only with concentrations of 50 ppm or higher and coincided with very slow and weak heartbeat and difficult "breathing" (gasping).

After 24 hr of treatment with 10 ppm of Phosphamidon, the larvae responded to a touch stimulus with an inefficient but strong long-lasting contraction of the body. With Dylox this symptom occurred only with 30 ppm. In general, symptoms occurred sooner and at lower concentrations with Phosphamidon, but at high concentrations, the symptoms were more pronounced with Dylox.

The symptoms disappeared more rapidly with Dylox: the behaviour was near normal after a 24-hr recovery period and normal by 96 hr after the end of treatment.

ii - Old larvae.

As was observed in rainbow trout larvae, old speckled trout larvae were much more sensitive to both Dylox and Phosphamidon than were early larvae (Tables 6.4 and 6.5). Dylox was also much more toxic: mortality and reduction of heartbeat were significantly higher with low concentrations of 10 and 30 ppm. Even with a concentration of 5ppm, symptoms of poisoning were evident after only 8 hr of treatment. The spontaneous activity was low and the normal swimming activity in response to a touch stimulus was replaced by a jump. In all treated larvae the symptoms were still very evident 24 hr after treatment concluded. After four days, behaviour appeared to be normal except for a slightly lower heart rate.

TABLE 6.4 Heartbeat[1] (H.B.) rate per minute and total percentage mortality (Mort.) in old Salvelinus fontinalis larvae treated for 24 hr with various concentrations of Phosphamidon.

DOSE (ppm)		TIME					
		8 hr	16 hr	24 hr	48 hr	120 hr	288 hr
0	H.B.	s 125	s 125	s 125	s 120	s 127	s 120
	Mort.	0	0	0	0	4	12
5	H.B.	s 125	s 110	s 90	m 100	s 110	s 120
	Mort.	0	0	0	2	15	19
10	H.B.	s 120	m 75	m 60	w 40	s 110	s 110
	Mort.	0	0	4	30	78	90
30	H.B.	s 110	m 43	w 27	w 40	m 90	s 125
	Mort.	0	0	45	56	95	98
50	H.B.	s 40	m 40	w 24	m 50	m 70	-
	Mort.	0	10	27	87	96	100
100	H.B.	s 24	w 33	w 30	m 50	-	
	Mort.	0	0	26	95	100	

[1]The strength of the heartbeat was classified as strong (s), medium (m), or weak (w).

TABLE 6.5 Heartbeat[1] (H.B.) rate per minute and total percentage mortality (Mort.) in old Salvelinus fontinalis larvae treated for 24 hr with various concentrations of Dylox.

DOSE (ppm)		TIME					
		8 hr	16 hr	24 hr	48 hr	120 hr	288 hr
0	H.B.	s 125	s 125	s 125	s 125	s 120	s 125
	Mort.	0	0	0	1	3	14
5	H.B.	s 110	m 45	w 30	m 100	s 100	s 120
	Mort.	0	0	0	62	68	69
10	H.B.	s 110	m 30	w 20	m 70	-	
	Mort.	0	0	45	86	100	
30	H.B.	m 30	w 20	w 20	-		
	Mort.	0	0	0	100		
50	H.B.	m 45	w 45	-			
	Mort.	0	20	100			
100	H.B.	w 36	w 10	-			
	Mort.	45	75	100			

[1]The strength of the heartbeat was classified as strong (s), medium (m), or weak (w).

iii - Effect of water flow on mortality.

Water flow during or immediately following treatment proved to have an influence on the resistance of the larvae to the toxic action of organophosphates.

TABLE 6.6 · Effect of water flow during or after treatment with Phosphamidon or Dylox (100 ppm) on the mortality (percentage) of young Salvelinus fontinalis larvae.

TREATMENT	PERCENTAGE MORTALITY		
	24 hr	24-hr recovery	
		Flowing water	Standing water
Dylox flowing water	6	18	100
Dylox standing water	72	90 (63)*	100 (100)*
Phosphamidon flowing water	3	7	-
Phosphamidon standing water	100	0	0

* 63 and 100 represent percentage mortality, when the larvae still alive at the end of the treatment (28%) are considered as 100%.

B. Effect of treatment on tissues.

Extensive histological damage was found in treated larvae. Old larvae were more affected than young larvae and the damage produced by Dylox was more extensive than that caused by Phosphamidon. Thus for instance, in old larvae, 24-hr treatment with only 5 ppm produced some tissue damage. Table 6.7 gives an estimate of damage in relation to the age of the larvae, the time of treatment and the dose.

TABLE 6.7 Estimate of tissue damage in *Salvelinus fontinalis* larvae treated with Phosphamidon or Dylox.

AGE AND TREATMENT	DOSE (ppm)	TIME (hr)		
		8	24	48
Early larvae				
Phosphamidon	100	1	2	3
Dylox	100	1	2	5
Old larvae				
Phosphamidon	30	.5	2	5
Phosphamidon	100	1	3	5
Dylox	10	1	2	5
Dylox	30	1	3	-
Dylox	100	2	-	-

With the exception of the gills, which were fairly resistant to the action of Phosphamidon, both compounds affected tissue in a similar manner. Damage was usually first observed in striated muscles, especially in the muscles of the head. This was followed by damage to the gills and pseudogills. The liver, the nervous system and the skin showed damage only at the end of treatment with a high dose. In this case, necrosis of the mucosa of the oral cavity and of the digestive tract was evident. Damage to the kidney was also observed.

Muscle damage appeared to be related to muscle type. In longitudinal section, some large white fibers of the body muscle were split into branching bundles of myofibrils. In other fibers, the myofibrils were bundled in the center or to one side of the fiber, leaving large spaces containing free nuclei (fig. 5). In more extreme situations, the nuclei were swollen indicating necrosis, and the empty spaces were filled with a granular material. In this situation, cross striations were not evident. In cross section, the fibers were smaller than normal and widely separated (fig. 6). Only a few groups of enlarged glossy orange fibers, commonly found in Dylox treated rainbow trout larvae, were seen (fig. 6). In the pharynx, oesophagus and stomach, longitudinal splitting of the fibers did not occur; rather, the cross striations were lost and the fibers, of uneven diameter, appeared homogeneous, dense and glossy in appearance. Head muscles, such as the ocular, the masseter and the oblique, had a third type of pathology characterized by extreme variation in the size and the density of the fibers (fig. 8) and by the occurrence of swollen and stretched segments. The large number of clear areas surrounding the fibers sharply contrasted with the normal dense arrangement.

Damaged gills, pseudogills and distorted blood cells resembled those observed in rainbow trout. Liver cells first became vacuolar and eventually the chromatin disappeared from the nuclei and the cells became foamy. Necrosis of the nervous system involved the brain stem, the infundibulum and the anterior half of the neural tube. The nuclei became pycnotic, while the surrounding tissue became pink or white. The optic lobes were near normal in appearance. When tissue damage was extensive, the mucosa of the mouth and pharynx completely disintegrated. Hyperplasia of the skin in the lateral mid-body region has also been observed (fig. 9). Within the stroma of the kidney the glomeruli and lymphoid tissue were normal but the cells of the tubules and of the mesonephric duct were sometimes necrotic. In this condition, a mucous-like substance was usually present in the lumen.

It should be emphasized that larvae showing such extensive damage were still living when selected for study.

C. Enzyme distribution and the effect of organophosphates on enzymatic activity.

i - Dehydrogenases and diaphorase.

The distribution of lactic and malic dehydrogenases and NADH diaphorase in old speckled trout larvae was very similar to the distribution found in young rainbow trout larvae. There were, however, some structures which were morphologically different and others which showed a different distribution pattern from that observed in rainbow trout larvae. Only these will be described here.

The pineal body is a more elaborate structure than that found in rainbow trout. The overall reaction for the three enzymes was

medium but the epithelium lining the internal invaginations gave a strong reaction (fig. 11). The morphology of the midbrain was similar to the one of rainbow trout; however, in the posterior part of the midbrain, two slight dorso-lateral expansions were lined by a few rows of cells resembling partly epithelial cells and partly ganglional cells. These gave a very strong reaction (fig. 12).

The epithelium of the head was composed of a few layers of flat and cuboidal cells throughout which were scattered goblet cells. Below the eye, the epithelium was interrupted by a structure composed of tightly packed columnar cells similar to the sensory corpuscules in the otic cavities. They were identified as the "free sensory hillock" of the lateral line system (fig. 10). The columnar cells gave a medium reaction while the rest of the epithelium reacted very weakly. The gill epithelium, structurally similar in both rainbow and speckled trout larvae, did not have the same enzyme localization. In speckled trout, large light reacting cells were interspaced between groups of intense reacting cells (fig. 13). The reverse situation was found in rainbow trout. Figure 14 shows the very strong reaction found in the liver and in the cells of the submucosal glands of the stomach of old larvae. At the junction of the dorsal and ventral myotomes, along the mid-lateral line of the body, the layer of cutaneous muscle was interrupted by a small mass of cells forming the "lateral line" nerve. These cells gave a weak reaction which was in sharp contrast to the strong reaction of the neighbouring muscle cells (fig. 15). This area was also found in old rainbow trout larvae, but was poorly developed in young larvae.

Organophosphate treatment apparently did not affect the

overall activity of the enzymes but the distribution within the cells was abnormal. This was especially evident in muscle cells. Strong clumping, in swollen muscle cells (fig. 16), were frequently seen, similar to what was observed in rainbow trout.

Aggregation of grains into small "rosettes" was also apparent in longitudinal sections of fibers showing a fairly normal histological structure (fig. 17).

ii - Cytochrome oxidase.

Although much weaker than the dehydrogenases and diaphorase, cytochrome oxidase had the same pattern of distribution. The activity decreased in the following order: cutaneous muscle, red fibers, mesonephric duct, intestinal mucosa, mesonephric tubule, neural tube. A very slight inhibition was sometimes noted in treated larvae but the inhibition was irregular and could not be related to the dose.

iii - Phosphorylase.

Control sections (no glucose-1-phosphate) when stained with iodine following incubation, were negative. Sections incubated with complete medium containing 20% alcohol gave a positive reaction. In order to verify the technique, the phosphorylase reaction was first performed on rat muscle: similar sections were stained for succinic dehydrogenase (SDH) and for phosphorylase. With SDH, the small fibers gave a strong reaction while the large fibers stained weakly. The phosphorylase reaction gave the opposite distribution: strong in large fibers and weak in small fibers.

Cross sections of body muscle of adult zebra fish showed two types of fibers: small and large. Both reactions for SDH and phosphorylase were strong in small fibers and weak in large fibers (fig. 22).

In cutaneous muscle, iodine staining gave a strong purple coloration indicating the presence of amylose; in muscles situated more mesiad, the reaction was weaker and reddish indicating the presence of glycogen. In trout larvae the reaction was always grayish blue, with the following distribution (fig. 18, 19 and 20):

Cutaneous muscle of mid-body: medium.

Cutaneous muscle (dorsal and ventral parts): weak.

Mid-lateral muscle (intermediate red fibers): weak.

Dorsal corinalis (medial fibers): strong.

White muscle: very weak.

Neural tube (lateral): medium.

Kidney tubules and duct: no reaction.

Optic lobes (acellular): medium.

Retina (inner layer): strong.

Ocular muscle: strong.

When the sections were stained with the PAS reaction following incubation, the localization of enzyme activity was determined after careful comparison between control sections and sections incubated with complete medium. (In the control slides, the acellular part of the neural tube, the notochord, mesonephric duct, intestine, cutaneous muscle and cartilage stained positively (fig. 21).) In the sections incubated with complete medium, an increase in the color intensity showed that enzyme activity was present in the same tissues and in the same proportions as that revealed by iodine staining. In muscle fibers, the enzyme activity was located at the periphery of the fiber and between the myofibrils (fig. 23).

Table 6.8 compares the enzyme activity of untreated larvae

with treated larvae (iodine staining). Inhibition was evident in larvae treated with 100 ppm of Phosphamidon but more pronounced in larvae treated with 30 ppm of Dylox. With Dylox, reduced activity could be observed with a small dose of 5 ppm, while similar inhibition with Phosphamidon occurred only with a dose of 30 ppm.

TABLE 6.8 Phosphorylase activity* in old Salvelinus fontinalis larvae after 24 hr treatment with Phosphamidon or Dylox.

TISSUES	CONTROL	DYLOX 30 ppm	PHOSPHAMIDON 100 ppm
Cutaneous muscle	+++	+	+
Neural tube	++	±	+
Ocular muscle	++++	+	++
Optic lobes	++	±	±
Retina	+++	±	±

* ± to ++++ signifies the intensity of activity from very weak (±) to strong (++++).

Inhibition of phosphorylase was also noted when the PAS reaction was performed after incubation. The color intensity of the PAS reaction in sections incubated respectively with and without substrate was compared to the color intensity of all other sections, and numerical values were attributed to express differences between sections. The results were as follows:

Untreated, no substrate:	2
Untreated, with substrate:	5
Dylox 30 ppm, no substrate:	1
Dylox 30 ppm, with substrate:	3
Phosphamidon 100 ppm, no substrate:	1
Phosphamidon 100 ppm, with substrate:	1.5

In larvae treated either with Dylox or Phosphamidon, the amount of glycogen or PAS positive material in structures where the enzyme phosphorylase was known to be present, was less than in untreated larvae. With Dylox, there was an increase of PAS positive material after enzymatic reaction, but this increase was less than that in normal larvae. With Phosphamidon, the increase was very slight.

iv - Acid phosphatase.

Strong activity was found in the luminar border of the olfactory pit epithelium, especially on the medial side (fig. 24). In the brain (fig. 24 and 25) and neural tube (fig. 26) the reaction appeared as small discrete spots in the regions of low cellular density. A similar reaction was found in the inner plexiform layer of the retina (fig. 25). Similar but smaller discrete dots were found along nerve fibers (fig. 26). In the kidney, the presence of several dots gave the general impression of a medium reaction. The reaction appeared as stellar, oval or crescent-shaped dots between the small tightly-packed cells of the stroma. In the tubules, several oval dots were found either between the cells or on the luminar or basal margins of the cells (fig. 27). A similar reaction, but less precise, was found between the cells of the liver. The apical border of the cells bordering blood sinuses showed some red elongated streaks. The round

structure of the lateral line contained two or three discrete large dots. In several organs such as pineal body, gills, pseudogills, muscle and stomach, fine small dots were irregularly dispersed between the cells.

Treatment did not bring any change in the activity of the enzyme, even in structures which can be quite damaged such as gills, pseudogills and muscles.

v - Alkaline phosphatase.

With the azo dye method, control sections (incubated without substrate) gave a diffuse and faint yellow coloration which could be readily distinguished from a genuine reaction. With Gomori's technique, there was a general light brownish staining, except for bone, teeth, perichondrium and some cartilage which stained black. Muscle nuclei also stained but lightly. With both techniques, a strong positive reaction was present in the following structures: dorsal portion of pineal body (fig. 31), ventro-medial and ventro-lateral epithelium of the olfactory pits (fig. 30), outer nuclear layer of the retina (fig. 37), submucosa of the pharynx (fig. 33), oesophagus, stomach and intestine, striated border of the intestine and mesonephric tubules (fig. 34 and 35) and the lining of the blood sinuses of the liver. In cartilage, the reaction was irregular: the large round chondrocytes usually gave a strong reaction, but were sometimes negative, while the matrix, adjacent to stained chondrocytes, could give a strong reaction. A medium strength reaction was observed in large cells of the gill arches (fig. 36), along the capillaries of the pseudogills (fig. 36) and in the glomeruli of the kidney (fig. 34). The ventricle of the heart (fig. 32), the neural tube and medulla, the ganglia and the lateral line gave a light reaction. A medium reaction in the

submucosal glands of the stomach was obtained only with the azo dye technique.

With the Gomori's technique (fig. 29), the striated border of the mesonephric duct stained strongly in the region of the stomach (fig. 34), however, at the level of the intestine, there was no reaction (fig. 35).

When tissues were stained with the azo dye technique, some cells deeply stained brick red. These cells were not revealed by any other staining technique. Two or three of them were seen in the ventral part of the midbrain. In the epidermis, they were often associated with goblet cells. They were more numerous in the epidermis of the pharynx and in the underlying connective tissue, or at the periphery of the muscle in the pharynx region (fig. 28). They were absent in sections of the body region. Their shape was variable and one portion of the cytoplasm usually stained deeper than the rest while the nucleus did not stain. The staining was increased if the incubating medium was let stand for a few minutes before being placed on the sections. These cells could not be identified by their morphology.

Treatment with Dylox or Phosphamidon did not bring any change in the activity of the enzyme; in damaged muscle, the round and central nuclei gave a light reaction with the Gomori's technique but this was not a genuine reaction since it was also present in sections incubated without substrate.

vi - 5'nucleotidase.

Since adenylic acid is hydrolyzed by both 5'nucleotidase and alkaline phosphatase and α-glycerophosphate only by alkaline phosphatase, localization of the enzyme 5'nucleotidase can be determined by

comparing sections incubated with both media. 5'nucleotidase would be found in areas showing a reaction with adenylic acid but no reaction or a weak reaction when incubated in the presence of α-glycerophosphate.

The reaction was generally weaker when sections were incubated with adenylic acid. The localization of the reaction was the same except in the aorta where a weak reaction was obtained. No reaction was obtained in the aorta with α-glycerophosphate.

vii - ATPase.

Sections incubated without substrate showed a general diffuse light gray staining except for cartilage and bone which were brown.

A strong positive reaction was found in the pharynx submucosa (fig. 40) and in striated muscles. Large white fibers stained more deeply than did small fibers (fig. 42) and cutaneous muscle fibers (fig. 39). Epithelial cells of the pseudogills gave a slightly less intense reaction (fig. 41). In the heart ventricle (fig. 40), the reaction was uniform but weak, while in the atrium it was irregular: at times weak and at others strong. In the gills (fig. 40), the enzyme was absent, except possibly at the margin of the epithelial cells where a weak and irregular reaction was sometimes found.

In treated larvae (Dylox 30 ppm and Phosphamidon 100 ppm) the reaction was modified in abnormal muscle fibers. Abnormally swollen fibers gave a very intense staining, while abnormal small and dense fibers in which there was a fusion of myofibrils gave a reaction weaker than normal (fig. 42).

viii - Cholinesterase.

The distribution of cholinesterase was similar to that found in rainbow trout larvae. When DFP was used as inhibitor for specific

cholinesterase in the incubating medium, no activity of non-specific cholinesterase was detected. In larvae treated with Phosphamidon and Dylox, a strong AChE inhibition normally present in the nervous system and in the end-plates was observed.

D. Biochemical measurements of cholinesterase activity.

The results of ChE activity measured by the methods of Hestrin and Abou-Donia and Menzel are given in Figures 44-49. At first sight, the results from one method seemed to differ from the results obtained by the other. Thus, for instance, a decrease in activity with low doses seemed to occur faster with the method of Abou-Donia and Menzel than with the Hestrin's method (fig. 44), and recovery seemed to occur faster when measured with the Hestrin's method (fig. 44 and 46). However, it was also evident that these differences were not errors due to manipulation, since the curves had similar shapes and the same differences were found in the different groups. For instance, both methods showed that recovery of activity was faster with Dylox (fig. 46) than with Phosphamidon (fig. 44).

Measurements made with the Hestrin's method showed that there could be considerable accumulation of acetylcholine, since negative values were obtained. Such negative values are possible if one remembers that in the Hestrin's method, the activity is calculated from measurements of the residual acetylcholine. Observable accumulation of acetylcholine occurred when ChE activity went below a critical threshold of 15-20% as measured by the method of Abou-Donia and Menzel (fig. 44 and 49). This threshold value was obtained only when activity after the first 8 hr of treatment was already below 40% (compare fig. 44, 45

and figure 46, 47). The methods did not aim at measuring the amount of acetylcholine accumulated in the head of the larvae resulting from the ChE inhibition, so that calculations will yield only very approximate values. Increase in color due to the accumulation of acetylcholine in head homogenate can correspond (fig. 49) to an increase of 30% of the amount originally present (4 µM in 1 ml). Thus, the amount of acetylcholine accumulated in the tissue would be of the order of 0.02 µM/mg of tissue.

Examination of Figures 44-49 shows that in young larvae, inhibition occurred mainly during the first 16 hr when treated with a low dose (5 and 10 ppm) of Phosphamidon or Dylox. When treated with a higher dose, important inhibition was already evident after 8 hr and there was little change thereafter. Recovery from Phosphamidon occurred much slower than it did from Dylox treatment. The dose seemed to have very little influence with either compound. Recovery was almost complete after five days and normal activity was maintained until day 12.

With old larvae, the curves are incomplete because of the high mortality which occurred. With Phosphamidon, the curves are similar to the ones seen in young larvae. High doses reduced activity to less than 20% but recovery was faster than in young larvae. With Dylox, (Hestrin's method) inhibition seemed to occur faster than it did in young larvae especially with low doses. Recovery was observed in only one case and it was faster than the recovery following Phosphamidon treatment.

In all cases, the lowest activity observed with the method of Abou-Donia and Menzel varied between 10 and 20%. Comparison of the

percentage of ChE activity with external symptoms such as the loss of equilibrium, the jump or the long-lasting contraction produced in response to a touch stimulus, showed that a direct correlation did not apply to larvae of both ages. Thus, young larvae lay on their sides only after a prolonged period of strongly reduced ChE activity (activity lower than 20% for at least 8-16 hr). With older larvae, loss of equilibrium also occurred under the same conditions with low doses. With a higher dose, equilibrium was lost as soon as the activity went down to 20%, or to 30% in the case of Dylox.

The correlation of ChE activity with the reaction to a touch stimulus was also different in young and old larvae. In young larvae treated with Phosphamidon, a long-lasting contraction of the body was induced when ChE was reduced to 15-20% or 35%. With Dylox, young larvae having only 25% ChE activity could either show hyperactivity, jump or long-lasting contraction, depending upon the time elapsed from the beginning of treatment. With old larvae, such reactions occurred sooner although the ChE activity was not reduced to such an extent.

The only general conclusion from such comparisons would be that in older larvae, symptoms occurred sooner and at a level of ChE activity higher than that seen in young larvae.

E. Ultra-structure of masseter muscle.

i - Young larvae.

a) Normal muscle.

Myofibrils had an average diameter of .9 μ and showed the typical banding pattern (fig. 50). In the dense and broad A band the thick myosin filaments were running parallel and some filaments appeared

to be joined two by two by some short thick filaments (fig. 55). The H zone, in the center of the A band, was narrow (0.08 μ) and contained an obvious M line (0.05 μ) (fig. 50). The thin (0.22 μ) I band was light and contained three distinct darker zones: the central, well-defined Z line (fig. 50 and 55) having a typical zigzag structure which appeared as being formed by the terminal thickening of the thin actin filaments; the two distinct N bands found on each side of the Z line, in which several small cross filaments were seen (fig. 55). The sarcomere length was 1.5 μ. In cross section of myofibrils the typical hexagonal pattern of the fine filaments surrounding a central thick filament was seen. The triads were located at the level of the Z line of each myofibril (fig. 50, 53 and 55). The central element appeared as a single oval tubule, having a dark thick wall and running perpendicular to the long axis of the myofibrils (fig. 55 and 63). On each side of the transverse tubules, one irregular vesicle (terminal cisterna) was seen to be continuous with an irregular longitudinal tubule. The longitudinal tubules coming from two adjacent triads met at the H zone and seemed to be associated with a very fine transverse tubule running at the level of the M line. The diameter of the longitudinal tubules decreased as it approached the H zone (fig. 53). The longitudinal tubules contained an amorphous material of irregular density and some discrete big granules. Similar granules, presumed to be glycogen granules, were seen in larger numbers outside of the longitudinal tubules, but closely associated with them (fig. 53 and 55). A few rod-shape or oval mitochondria were squeezed between myofibrils and were associated with an abundant endoplasmic reticulum and glycogen granules. They had numerous cristae of irregular diameter and a dense matrix.

Much more numerous than the intermyofibrillar mitochondria, the subsarcolemmal mitochondria were round and they often formed accumulations (fig. 63).

b) Effect of organophosphate on muscle ultra-structure.

The most striking feature of the muscle fibers of larvae treated with either Phosphamidon or Dylox was the transformation of the compactly arranged myofibrils into a sponge-like tissue due to the presence of numerous vesicles of various sizes (fig. 51, 52, 54, 56 and 57).

Most often, the vesicles were present between the myofibrils and appeared to originate from the longitudinal tubular system. In some areas, the transverse tubule at the level of the Z line was normal but the adjacent terminal cisternae were swollen into vesicles (fig. 54 and 57), squeezing the myofibrils. When the vesicles were small, the rest of the longitudinal tubular system associated with abundant glycogen granules appeared fairly normal at the level of the central portion of the A band. However, very often the vesicles were so large that they occupied the entire distance from the Z line to the M line; they thus formed wide rows of vesicles separating the myofibrils (fig. 51 and 56). In such a case, the transverse tubules were difficult to see and often appeared as small tubules squeezed between the membranes of the large vesicles, either along the full width of the vesicles or near the myofibrils. It must be emphasized, that although they varied in size and shape, the vesicles in a row had the same relationship with the banding pattern of the myofibrils as the former longitudinal tubules. Each vesicle was enclosed by a membrane and usually extended from the level of the Z line to the level of the M line. There were

always membranes separating adjacent vesicles at the level of the M line thus indicating that the longitudinal tubules were not continuous over the M line (fig. 65). Usually vesicles were also separated from one another at the level of the Z line, but in several cases no membranes were present at the level of the Z line (fig. 51). Smaller round vesicles could be found within the large ones. Very few glycogen granules were seen in the proximity of the large vacuoles which contained fine, irregular beaded fibrillar material (fig. 51 and 65).

In other areas, disorganization of the tissue was more pronounced and large islands of unoriented vesicles replaced the myofibrils (fig. 52). Similar vesicles could be found in the subsarcolemmal cytoplasm (fig. 51 and 54). Disorganization and vacuolization of the tissue were more pronounced in the muscle of larvae treated with Dylox.

Mitochondria were also affected by the treatment. They were more numerous than in the control and somewhat smaller, suggesting duplication by fission. Their distribution was uneven. In some areas, they were found at irregular intervals between myofibrils (fig. 56). Other areas were completely devoid of them, while in other regions, several mitochondria of various shapes and sizes, sometimes distorted, were grouped in a disorganized fashion (fig. 52 and 57), which was in sharp contrast to what was seen in untreated larvae. They were always associated with large vacuoles (fig. 51, 52 and 56). In Dylox treated larvae, the cristae were numerous and the matrix was relatively dense. In Phosphamidon treated however, several mitochondria had regions devoid of cristae (fig. 52 and 59). Subsarcolemmal mitochondria were also more numerous, more irregular in shape and often closely packed

together and associated with large vesicles.

Most of the myofibrils had a fairly normal structure, but the I band was slightly wider. The H band and M line were more obvious while the Z line and the N bands were more diffused (fig. 51, 52 and 57). Some myofibrils however, were seriously affected. The I band was wide and contained disorganized fibrillar material (fig. 58 and 61). The typical zigzag pattern of the Z line was replaced by an irregular dark band. In the A band, the myosin filaments appeared swollen and unequally spaced. They were associated with granular material (fig. 58 and 60).

ii - Old larvae.

a) Normal muscle.

The ultra-structure of muscle of untreated larvae was identical to the one of younger larvae.

b) Dylox treated.

Some regions of the muscle had a spongy appearance. This appearance differed from the one seen in younger larvae in two ways. First, it was much less extensive and vesicles formed from the swelling of the longitudinal tubular system were smaller and their limiting membrane was much less clearly defined (fig. 65). Second, the myofibrils were sometimes separated from one another by empty spaces. They had a fairly normal structure, but the I band was wider than in the control (compare fig. 65 and 55). Breakage of the fibers was not uncommon and occurred along the N band (fig. 66). Both intermyofibrillar and subsarcolemmal mitochondria often had an irregular surface and contained fewer cristae, small and broken, in a clear matrix (fig. 65).

Other regions of the tissue showed a completely different

structure. The myofibrils were close to one another and were made of tightly-packed filaments while the I band and M line were absent (fig. 67, 68 and 69). Disruption of myofibrils could be associated with small granular material (fig. 70) as in the case of the younger larvae (fig. 58). Finally, other myofibrils were made of a tangled mass of filaments with recurring denser zones (fig. 71).

c) Larvae treated with Phosphamidon.

The structure of the muscle was fairly homogeneous and was characterized by a loosening and longitudinal separation of the myofibrils (fig. 75). The triads at the level of the I band were replaced by irregular enlarged vesicles with folded "voilées" membranes. A small oval tubule persisted at the level of the H line (fig. 72 and 75), but the longitudinal tubules were absent and the myofibrils were separated by clear areas containing irregular fibrillar and granular material (fig. 72 and 74). Very few glycogen granules were present. The myofibrils were fairly normal and the banding pattern was retained. The M line was particularly clear and the H line appeared as three fine parallel lines (fig. 72). Occasional longitudinal splitting of the myofibrils (fig. 75) was seen. The margins of the myofibrils were fuzzy: extremely fine filaments, probably actin filaments since they were continuous with the fine filaments of the I band, were going from the myofibrils into the clear areas found between the myofibrils (fig. 72 and 74). Thick myosin filaments were not present in these clear areas. Both intermyofibrillar and subsarcolemmal mitochondria were abnormal: they were clustered, their surfaces were very irregular and cristae were very few (compare fig. 63 and 64). Some very peculiar vesicles were sometimes seen at the ends of nuclei (fig. 73). These

large vesicles contained irregular smaller vesicles bounded by membranes made of a single unit.

The ultra-structure of muscle from treated larvae was thus quite abnormal.

In young larvae the muscle fibers became spongy due to the presence of numerous vesicles originating from the tubular system and sometimes replacing the tissue.

In older larvae, the presence of vesicles was accompanied by large empty spaces between myofibrils. Disorganization of the banding pattern and of the fibrillar elements as well as distortion and clumping of mitochondria also resulted from treatment with the two organophosphates.

III. DISCUSSION.

A. Enzyme distribution in normal larvae.

The speckled trout larvae on which enzyme localization was performed were older than the rainbow trout larvae used for similar work. New structures were differentiated such as the lateral line hillocks, the ganglion-like cells on the latero-dorsal surface of the posterior hindbrain and the stomachal glands. These were also new sites of high oxidative enzyme activity. The increased activity of the liver appears also associated with further growth.

The localization of LDH, MDH and NADH in the gills was opposite to that found in rainbow trout. This finding was somewhat surprising and casts some doubt on the possible relationship between the presence of high oxidative enzyme content and the function of the

chloride cells, unless one assumes that the majority of the gill epithelial cells in speckled trout are chloride cells.

Although cytochrome oxidase has been studied in a few organs only, it seems that the pattern of distribution parallels that of LDH, MDH and NADH.

The localization of phosphorylase in muscle will be discussed later on. The finding that phosphorylase is present in the brain would indicate that glycogen breakdown can take place in the brain and that the glycolysis is a potential source of energy.

Acid phosphatase was found to be present in the olfactory mucosa, in the white matter of the brain and neural tube and also in the liver and the kidney. This enzyme is often linked with catabolic or degenerative processes, but its function cannot be limited to these, since its presence is associated with the functional stages of the kidney glomerulus and with the final stages of differentiation of the liver (Desalu, 1966; Moog, 1965). Pearse et al. (1963) have suggested that in the developing chick kidney acid phosphatase would play a role in the supply of inorganic phosphate necessary for synthesis. Tewari and Bourne (1962) have found it to be associated with synapses in the cerebellum.

The finding of high activity of alkaline phosphatase in the brush border of kidney tubules and the microvilli of the intestine parallels the results found in other fish species (Ikeda, 1959; Noda and Tachino, 1965) and in mammals (Burstone, 1961; Jervis, 1963; Desalu, 1966). The absence of staining in some segments of the mesonephric duct shows that, as pointed out by Moog (1965), not all segments of the structure acquire the adult enzyme distribution simultaneously. The

present observations are in good agreement with those of Prakash (1960) who made a detailed study of alkaline phosphatase during several stages of development of the steelhead trout. This author, however, has not observed any activity in the stomachal glands while the azo dye technique has shown, in speckled trout, medium activity in these glands. This difference could be due to the fact that our observations were made at the early phase of differentiation of the glands. Ikeda (1959) has shown that alkaline phosphatase is correlated with rapid protein synthesis during initial stage of differentiation. The possibility of an artefact, however, is not excluded since the Gomori's technique did not reveal any activity in these glands. Brown and Millington (1968) have correlated the enzyme activity of the intestinal mucosa of the frog with feeding and absorption of foodstuffs. Such a correlation does not exist in trout since large amounts of enzyme are present long before feeding starts.

The peculiar cells which were revealed by the azo dye technique in the connective tissue appear similar to the mast cells described by Bolton (1933). Prakash (1960) has also shown that mast cells do have high alkaline phosphatase activity.

ATPase activity is slightly higher in large white fibers than in the small fibers or the lateral red fibers. Padykula and Gauthier (1963) have described several kinds of ATPase in the muscle fibers. The ATPase found in speckled trout does not appear to be located in mitochondria, but rather in the sarcoplasm or bound to the myofibrils.

The localization of the various enzymes has shown that the mucosa of the olfactory pits is rich in dehydrogenases and acid and alkaline phosphatases, and that in the retina, cholinesterase, acid and

alkaline phosphatases, phosphorylase and dehydrogenase enzymes are located in different layers. These two structures thus appear interesting from an histochemical point of view and more work should be done to study the relationship between their differentiation and their enzyme content. Very little attention has been given to the morphology and to the function of the pseudogills. Copeland (1952), who first thought that it could be involved in osmotic regulation, thinks that it could be involved in the synthesis of an enzyme which is exported and used in the swim bladder. More recently, Holiday and Parry (1962) have suggested an endocrine function of chromatophore control. The high dehydrogenase and ATPase activities observed in it are certainly of significance and should be further studied.

B. Toxicity of Phosphamidon and Dylox.

The Salvelinus fontinalis larvae, especially the older ones, were found to be much more sensitive to the organophosphates than the embryos. This finding parallels those of Davis (1961), Cairns et al. (1965) and Weisbart (1968) who found that larvae of invertebrates or fishes were more susceptible to poisons and changes in salinity than embryos. Several factors are probably involved in this higher sensitivity. Although the capsule of embryos is permeable to compounds in solution, the capsule certainly slows down the exchanges between the embryo and the water so that the compounds do not reach the embryos as readily as they do in the larvae. In the larvae the gills are also more developed and constitute a free port of entry.

Comparison of the effect of Phosphamidon and Dylox on larvae with that observed on embryos shows an interesting difference. The

toxicity to embryos was greater for Phosphamidon than for Dylox. In larvae, Dylox proved to be more toxic. This change in toxicity cannot be explained at the present time but it probably shows that the mode of action is not the same in both embryos and larvae.

The older larvae were found to be more susceptible than the younger larvae. This is similar to what has been found in rainbow trout; this difference has already been discussed in chapter five (p.178).

The resistance of the larvae to organophosphate treatment is largely influenced by the movement of the water. Much higher mortality occurs if the larvae are either exposed to the insecticides in standing water, or if they are allowed to recover from treatment in standing water. Two factors would be responsible for this difference. First, standing water contains less oxygen and in an effort to compensate for the lack of oxygen, the ventilation depth is increased (Marvin and Heath, 1968) resulting in an increased rate of water movement on the gill surface (Lloyd, 1961) thereby increasing the amount of poison coming in contact with the gills. Second, the yolk circulation, which is the main organ of respiration during embryonic stage, probably has some importance. When the poisoned larvae become immobile, continuous flow of oxygenated water on the body surface provides for some oxygenation of the blood; when the water is not flowing, such oxygenation would be lacking. The apparently higher resistance of speckled trout larvae, as compared to that of rainbow trout larvae, is probably due to this factor since the experiments done with speckled trout larvae were done with a system which allowed for better water flow on the body of the larvae. These results suggest that decreased breathing and eventual asphyxia are factors of importance in causing death.

C. Cholinesterase activity and mortality.

Although no inhibitors were used during the biochemical measurements of ChE activity to distinguish between the activity of the specific (acetyl-ChE) and non-specific (pseudo-ChE) types of ChE, it is assumed that the recorded activity is due only to acetylcholinesterase. This was thought to be justified since with the use of inhibitors for AChE, no activity attributable to non-specific ChE was observed in histochemical staining, and according to several authors non-specific ChE would be either absent or present in insignificant quantity in fish (Lundin, 1958, 1962; Pecot-Dechavassine, 1961; Koelle, 1963).

It is known that the acetylcholine content of the brain rises considerably upon inhibition of cholinesterases. When the results of ChE activity, as measured by the two methods, are compared it is seen that at the end of the treatment the values obtained by the Hestrin's method are not only much lower than those obtained by the Abou-Donia's and Menzel's method but that they are sometimes negative. As already mentioned, these low values would result from the accumulation of acetylcholine in the brain. The discrepancy in the results points to the importance of considering this residual acetylcholine in assessing the ChE activity and this is particularly important if the ChE activity is low. Some of the results reported in the literature (Weiss, 1958, 1961) are probably lower than they should be due to a failure to take this into consideration.

There are two other differences in the results obtained by the two methods: the rate of ChE inhibition during the first 8 hr appears faster when measured with Abou-Donia's and Menzel's (A-D) method than that seen when measured with the Hestrin's (He) method and

the rate of recovery appears slower when measured with the A-D method. In other words, the values observed at 8 hr and 48 hr are lower with the A-D method than with the He method. The reason for this difference is still unknown.

The ChE activity values obtained by the two biochemical methods confirm the results which were obtained in rainbow trout larvae by histochemical methods. For instance, after 16 hr of treatment with 10 and 30 ppm of Dylox, the histochemical method yielded ChE activity of 30% and 20%, while the biochemical method (A-D) yielded 25% and 13%. Although the histochemical method is not per se a quantitative method, this comparison shows that it can give a valuable estimate.

Weiss (1961) has shown that the rate of ChE inhibition recovery is characteristic of both the fish species and the kind of organophosphate. In some species, the recovery is still incomplete after 30 days. Such is not the case in speckled trout as recovery is usually complete on the fifth day (four days of recovery). Thus, the two insecticides Dylox and Phosphamidon do not have a long-lasting action in trout larvae following short exposure.

The level of ChE activity does not appear to be the critical factor in causing death. A low level of activity (10-20%) is present in both young and old larvae poisoned by 10 and 30 ppm (see Plates 6.7, 6.9, 6.11 and 6.12) but the mortality is quite different in young and old larvae: it does not go above 6% in young larvae and reaches 45% in old larvae. When the levels of ChE activity produced by different concentrations in young larvae are examined they are seen to be generally parallel to those found in old larvae. The mortality percentages, however, differ considerably being much higher in older than in young

larvae. Karczmar and Koppanyi (1953) had observed that in urodele larvae, poisoned by DFP, the survival time was dependent on the DFP concentration rather than on the ChE activity level and they have suggested that DFP had both a cholinesterase and a non-cholinesterase action. The results obtained in speckled trout also show that larvae can survive a low level of ChE activity. This is not in agreement with the results of Weiss (1958) who found that death of fish usually occurred when ChE activity was depressed to 60-30% of normal.

Summerford et al. (1953) have suggested that the rate of change of enzyme activity appeared to be as important as the final degree of activity found after organophosphate poisoning. Although we agree that the rate of ChE inhibition can have a certain importance, it does not appear to be the determining factor in these experiments. When larvae are exposed to Phosphamidon the rate of ChE inhibition, as measured by the two methods, was either equal in both young and old larvae or just slightly higher in the old larvae. The mortality, however, differs considerably being much higher in old larvae. In larvae exposed to 50 or 100 ppm of Dylox, the rate of ChE inhibition is not very different in young and old larvae. The mortality, however, reaches 100% at 24 hr in old larvae while it is below 5% in young larvae.

When Tables 6.2, 6.3, 6.5 and 6.6 are examined, one sees that important mortality takes place during the first 24 hr of recovery suggesting that the rate of reactivation of the inhibited ChE could be an important factor. This possibility is ruled out by the fact that reactivation of ChE inhibited with Dylox occurs more rapidly than the reactivation of ChE inhibited by Phosphamidon.

The study of ChE reactivation rate also shows that Dylox does not accumulate in the tissues of the larvae and is not transformed there into DDVP which would inhibit the enzyme. If such were the case, the rapid rate of reactivation within 24 hr would not be obtained. One would rather expect that the inhibition would be prolonged and even increased during the first period of recovery, as it is with indirect inhibitors (Weiss, 1961). Since both Phosphamidon and Dylox contain similar alkyl or basic groups $(Me\text{-}O)_2$ attached to the phosphorus atom and since the rate of reactivation of the enzyme ChE does not depend on the original acidic group but solely on the structure of the basic groups (Heath, 1961), one would expect that the rate of reactivation would be similar in larvae poisoned with Dylox or Phosphamidon. The rate of recovery is lower in Phosphamidon than in Dylox thus suggesting that Phosphamidon would have a tendency to be accumulated in the tissues of the larvae for a short period and phosphorylate again the reactivated ChE. These results are thus parallel to those obtained in speckled trout embryos where it was seen that the inhibiting action of Phosphamidon, long after the end of treatment and at a time where important de novo synthesis of the enzyme took place, was stronger than the inhibiting action of Dylox. We do not know to what extent, if any, both compounds are detoxified in speckled trout larvae. If detoxification occurs, it is possible that the recovery rate from the poisoning with Phosphamidon and Dylox could be related to the different ability of the larvae to detoxify each compound.

In examining the results on mortality it is seen that the mortality is usually proportional to the dose. Two exceptions, however, were encountered where mortality at the end of treatment is higher with

a lower dose (Tables 6.5 and 6.6). It must be noted, however, that at the end of the treatment the larvae of the group where mortality was lower were extremely weak and that during the next 24-hr period a very high mortality rate occurred in the groups treated with higher doses. Thus at the end of the first 24 hr of recovery mortality was proportional to the dose. The difference in mortality observed in these cases might then not be of considerable significance. An hypothetical explanation could be that a stronger dose produces a quick and appreciable decrease in metabolism so that the larvae are quickly and almost completely inactivated thereby preventing them from burning themselves out.

Since the mortality of speckled trout larvae cannot be related to the rate and final level of ChE inhibition and to the rate of ChE reactivation, other factors would then be important in the poisoning of the larvae.

D. Histopathological changes.

Extensive histological damage was found in Salvelinus fontinalis larvae treated with Dylox or Phosphamidon. With the exception of the damage found in the gills, which were more resistant to the action of Phosphamidon, no histological damage could be attributed to one particular insecticide. Damage of the large white muscle fibers of the body appeared to differ in the speckled trout larvae from that found in the rainbow trout larvae as longitudinal splitting of the fibers was much more common in speckled trout while hyaline degeneration was more common in rainbow trout. Histological damage has been extensively discussed in chapter five. In the present discussion,

attention will be drawn to two points: 1) the relationship between the damage and the mortality; 2) the relationship between the damage found in muscle fibers and the histological and histochemical characteristics of the various types of muscle fibers.

Comparison of the extent of histological damage (Table 6.7) with the mortality rates in both young and old larvae shows that there are several similarities between them. At the same concentration one compound produced more extensive damage in old larvae than in young larvae. On the other hand, as already mentioned, both compounds were more toxic to old larvae than to young larvae. Furthermore, in old larvae the damaged produced by Dylox is more important than the damage produced by Phosphamidon. Dylox was also found to be more toxic than Phosphamidon. This parallelism between the mortality and the histological damage strongly supports the idea that tissue damage is an important factor in the death caused by the two organophosphates.

Histological and histochemical staining has shown that four types of muscle are present in the rainbow and speckled trout larvae. The first type is found in the lateral sheet of muscle underlying the epithelium. The fibers are large, give a strong reaction for oxidative enzymes and for phosphorylase and contain a large amount of glycogen. Except for the size of the fibers, these characteristics are similar to those described for lateral red muscle in trout (Boddeke et al., 1959) and in goldfish (Dubowitz and Pearse, 1960). Damage was very seldom observed in these fibers. Such a finding does not appear surprising since this lateral red muscle does not seem suitable to perform considerable work since it is only one-cell thick. Pora and Wittenberger (1960) and Wittenberger (1967) have suggested that the

lateral red muscle of the teleost would have a vegetative rather than contractile function.

In the muscles of the head, of the pectoral region and of the body three types of fibers were recognized. The first type has histochemical characteristics similar to the lateral red muscle but the size of the fiber was much smaller. The other type of fiber has a much larger diameter and is characterized by low oxidative enzyme activity, low phosphorylase activity and a very small content of glycogen. A third type of muscle with characteristics intermediate between those of the small and large fibers is also observed. These results agree with that obtained in other species of fish by Dubowitz and Pearse (1960), Ogata (1958a, 1958b, 1958c), Nishihara (1967).

It must be noted, however, that in trout the mosaic pattern of large enzyme-poor and small enzyme-rich fibers found in mammals and fishes (such as the goldfish and the zebra fish) is not present. The different types of fibers are either arranged in a gradient in one muscle or are found in different muscles.

In mammals and birds, a reciprocal relationship does exist between the amount of oxidative enzyme on the one hand and the amount of phosphorylase and glycogen on the other. This suggested to Dubowitz and Pearse (1960) that the white fiber, rich in phosphorylase and glycogen, would depend mainly on glycolysis as a source of energy while the red fibers would depend mainly on the Kreb's cycle. The red fibers would thus be capable of sustained activity and would be resistant to fatigue and the white fibers would be used mainly for a short burst of activity. This, however, does not apply to fish and it is difficult to explain the type of metabolism prevailing in the large white fibers of

fish.

Because of their high oxidative enzyme content, one would expect that the small fibers would be more resistant to fatigue. Higher resistance to fatigue, however, does not necessarily mean higher resistance to degenerative changes. In mammals, the small red fibers with high oxidative enzyme activity and which are considered as slow contracting fibers and as the most active fibers are the first ones to undergo degeneration after tenotomy or after a dose of Plasmocid (McMim and Urbova, 1962; Standish and Clark, 1964).

In trout larvae, studies have not been made to establish the different physiological characteristics of the various types of fibers, however, they have the same histological and histochemical features as the small red fibers of carp. These were also found to have a slow contraction and a multi-focal type of innervation: two features which make them similar to the slow fiber of the frog (Nishihara, 1967). Assuming that the small red fibers of trout are somewhat analogous to the slow fibers of the frog, a contracture would be expected to occur in the presence of a cholinesterase inhibitor. It is known, indeed, that in the twitch or white fiber excess acetylcholine or a ChE inhibitor do produce a prolonged depolarization of the end-plate membrane so that after a first contraction there is a relaxation of the fiber. In the slow fiber of the frog, however, the situation is quite different. In the presence of the prolonged depolarization the tension and the muscle shortening is maintained thus producing a contracture (Thesleff, 1960).

On the other hand, lactic acid is expected to have risen considerably in the muscle of the larvae. Several authors have found

that in trout the lactic acid content rose considerably during the first few minutes following the beginning of intense muscular activity (Black et al., 1962; Hammond and Hickman, 1966; Stevens and Black, 1966; Wendt, 1967). An important rise in lactic acid content also occurs with hypoxia (Heath and Pritchard, 1965). It has been suggested that retention of lactic acid and depletion of glycogen could be the cause of muscular necrosis produced in spontaneous ischemia or in degeneration induced by a noxious agent but this point still remains uncertain (Tillotson and Coventry, 1950; Field, 1960). Contracture and lactic acid accumulation could be the cause of the very extensive damage found in small fibers.

Two restrictions, however, should be mentioned. First, it has been assumed that because of the similarity with the carp red fiber, the small fibers of trout were slow fibers analogous to the slow fibers of frog. The small fibers of trout also have histochemical similarities with fibers found in the toad Xenopus laevis which are fast fibers and in which no contracture occurs (Lannergren and Smith, 1966). It is also known in mammals that fast fibers which have a higher ChE content are more affected by ChE inhibitors and that in the extremely fast ocular muscles, contracture sometimes happens (Gerebtzoff et al., 1954). Secondly, it has been mentioned that mortality was more related to histological damage than to ChE inhibition. Similarly, it appears that histological damage is more related to the dose of the compound than to ChE inhibition. Over-stimulation of muscle by excess acetylcholine does not seem to be solely responsible for muscle damage since damage is not necessarily associated with low levels of ChE. Further work would be necessary to decide whether the extensive damage found in the small

fibers is related either to a higher sensitivity of the fiber to excess acetylcholine resulting from ChE inhibition or to a high sensitivity to accumulation of lactic acid resulting from the type of metabolism in these fibers or to a higher sensitivity of the fibers to a direct action of the organophosphate or to a combination of these three factors. This problem could probably be solved by the comparison of the effect of acetylcholine and of organophosphates on the structure of different types of muscles in vivo and in vitro.

E. Histochemical changes.

Organochlorine insecticides are known to decrease the activity of oxidative enzymes (Sova, 1966; Petrun and Onikienko, 1965) but organophosphate produces inhibition of oxidative enzymes only when high concentrations are used. The distribution of the enzymes was changed in damaged cells, but no change was observed in the activity of the enzyme. It is possible, however, that biochemical measurements would show a decreased activity in the cells since it was shown that loss of proteins and enzymes from the cells to the plasma occurred in muscle disease (Tyler, 1966) or in muscle fatigue (Papadopoulos et al., 1968).

The in vivo effect of organophosphate on alkaline phosphatase appears variable: Rucinski et al. (1966) have observed decreased activity, while Murphy (1966) recorded increased activity after acute poisoning. Changes in alkaline phosphatase activity and increased activity of acid phosphatase linked to degenerative processes are often associated with muscular abnormalities (Stolk, 1960; Kosmider et al., 1965; Verma and Veiking, 1967). In speckled trout larvae, however, no change in the phosphatases were observed and the change observed with

ATPase appears to be more related to the abnormal structure of the muscle cells than to the degree of activity itself.

Decrease in the glycogen content of nervous tissue and muscle was observed in larvae treated by either Dylox or Phosphamidon. Such a decrease appears normal in the view of the fact that organophosphate poisoning produces hyperactivity of the larvae and tetanic contraction of the muscles. In trout, glycogen disappears very quickly during muscular activity (Black et al., 1962; Hammond and Hickman, 1966; Stevens and Black, 1966).

It is known that in trout muscle, phosphorylase "b" (the inactive form) is converted to phosphorylase "a" (active form) by physical exercise (Yamamoto, 1968). One would then expect a high phosphorylase activity since prior to paralysis there is intense muscular activity. Such was not the case: the enzyme phosphorylase was found to have a lowered activity in larvae treated with Dylox or Phosphamidon. The decreased activity was readily observable with histochemical methods and could be detected in larvae treated with 30 ppm of Phosphamidon or with only 5 ppm of Dylox.

Since alcohol was used in the incubating medium and alcohol destroys the activity of the enzyme transglycosylase which effects the synthesis or the breakage of the α-1,6 glucosidic linkages (branching of the chains) (Pearse, 1960), it is assumed that the observed activity was mainly that of the enzyme phosphorylase (which effects in vitro the synthesis or destruction of the α-1,4 glucosidic linkages). Although there is no doubt about the inhibition of the activity, different results were obtained when the end product of the enzymatic activity, in larvae treated with 100 ppm of Phosphamidon and in larvae treated

with 30 ppm of Dylox, was stained with iodine or with the Periodic Acid-Schiff (PAS) reaction. With the iodine staining, the activity was lower in larvae treated with 30 ppm of Dylox, while with the PAS reaction, activity appeared lower in larvae treated with 100 ppm of Phosphamidon. This discrepancy might be explained by the fact that the PAS reaction is not considered as reliable and as precise as the iodine staining.

Lowered phosphorylase activity does not necessarily mean that the active form of the enzyme is directly inhibited by the organophosphate. In normal muscle the inactive form is transformed into the active form by phosphorylation, the phosphate coming from ATP. It is then quite possible that the decreased activity which is observed is due to the fact that the inactive form is phosphorylated by the organophosphate at the site where normal phosphorylation takes place so that less of the active form can be produced. Inhibition of the inactive form was found to occur by the action of dinitrophenol (Goncalves and Focesi, 1967). A smaller amount of the active form could also result from the inhibition of the enzyme phosphorylase-b kinase which effects the transformation. Which one of the three enzymes is in fact inhibited? This question cannot be answered at the present time. The words "phosphorylase inhibition" should then be understood in their broadest sense. Further work will be necessary to confirm this finding with biochemical methods and to characterize the type of inhibition.

Reported for the first time as being produced <u>in vivo</u> by an organophosphate, the inhibition of phosphorylase appears to be an important finding for several reasons. Except for the inhibition of the aliesterases, the function of which is still unknown, no important

enzymes are known to be inhibited by concentrations of organophosphates equal to the concentrations necessary to inhibit cholinesterase. Phosphorylase has also a key function in the utilization of the reserves of energy and it plays an important role in the metabolism of the cells. It is then quite possible that the inhibition of phosphorylase is an important factor as the cause of death of the organism. Decreased oxygenation of the cells results from brachycardia and partial paralysis of the respiratory muscles. By the inhibition of phosphorylase, the other source of energy (i.e. glycolysis) would also be blocked. In this regard, the high phosphorylase activity in the brain of normal larvae as well as in the respiratory muscle would be of importance. Paralysis during prolonged exposure could result from the lack of energy rather than from the ChE inhibition.

F. The ultra-structure of muscle.

The electron microscope study has shown that in the masseter muscle of trout larvae the structure of the myofibrils, of the endoplasmic reticulum and especially the location of the triads at the Z line level are similar to those of the red pectoral muscle and white abdominal fin muscle of the carp (Nishihara, 1967), of the white body muscle of cod (Bishop and Odense, 1967), of carp and guppy (Partmann, 1968) and of the subcutaneous and truncal muscle of pike (Kilarski, 1966). In other muscles, like the ocular muscle of the pike, one set of triads is found on each side of the Z line (at the junction of the A and I bands) (Kilarski, 1966, 1967). This study has also confirmed the histochemical finding that mitochondria are found in larger numbers under the sarcolemma than between the myofibrils.

Staining of the glycogen had shown that glycogen was present between the myofibrils of the muscle fiber and that the amount of glycogen decreased in muscle of treated larvae. The electron microscope study has shown that abundant granules were present in proximity to the endoplasmic reticulum and that these granules were greatly reduced in number in muscle of treated larvae.

The histochemical reaction for the dehydrogenase enzymes strongly suggested that a clumping of mitochondria occurred in abnormal muscles. The electron microscope showed that this was true and also revealed that the masses of mitochondria were always associated with large irregular membrane-bounded vacuoles. Clumping of mitochondria is not uncommon. It was observed in blastocyst cells treated with Urethane (Battle and McLeese, 1954), in fibers of muscle suffering of trichinosis (Blotna, 1967) or of corticosteroid induced myopathies (Engel, 1966). Variations in size, shape and structure of mitochondria are frequent and they were found to be associated with several conditions such as Urethane treatment (Battle and McLeese, 1954), dedifferentiation (Lentz, 1969), thyrotoxicosis (Frey and Skjorten, 1967), myopathies (D'Agostino et al., 1968; Hulsmann et al., 1967), vitamin E deficiency (Van Vleet et al., 1968), exercise (Laquenz and Gomez-Dumm, 1967), hypoxia (Onishi, 1967), glycolysis (Santalo, 1966). According to Lehninger (1965) changes in the mitochondrial structure is a common sign of cell damage. The changes observed in mitochondria can thus be regarded as non-specific, but the constant association of mitochondria with large vacuoles appears to be more specific.

It is quite possible that abnormalities of the respiratory mechanism would be associated with the abnormal mitochondrial structure.

Dissociation of respiration and phosphorylation has been frequently found to be associated with mitochondrial swelling (Hulsmann et al., 1967; Lipskaya, 1968; Lenkova, 1968).

The most striking feature of the muscle abnormality was the production of large vacuoles from the terminal cisternae of the longitudinal tubular system and the presence of large intermyofibrillar spaces. These vacuoles and intermyofibrillar spaces would correspond to the swelling and hyaline appearance of the fibers in one case and to the longitudinal splitting of fibers in another. Swelling and vacuolization of the sarcoplasmic reticulum is frequent in muscle degeneration resulting from progressive dystrophy (Both et al., 1967), from ischemia (Moore et al., 1956; Stenger et al., 1962; Reznik, 1967) and from Plasmocid administration (Price et al., 1962). The vacuolization and production of intermyofibrillar spaces could be due to an accumulation of water within the structures of the fiber. Gillis (1964a) has shown that tetanization of muscle produces considerable uptake of water by the fibers with a concomittant increase in volume and he has suggested that this would be caused by the accumulation of osmotically active metabolites such as lactate, phosphate and cations in the fiber. The same author (1964b) has shown that an increase of Na^+ and Ca^{++} ions does occur in the fiber which probably resulted from decreased activity (due to a lack of energy) of the mechanisms involved in active transport. This retention of cations in trout larvae muscle is probably due, not only to frequent contraction and tetanization, but also to the action of the organophosphate. Kovacs and Szabo (1966) have shown that cholinesterase inhibitors such as physostigmine and DFP inhibit the extrusion of Na^+ ions normally occurring in frog muscle fiber. Several

authors, reviewed by Koelle (1963), had suggested that acetylcholine and AChE could have a role in active transport since ChE inhibitors were found to modify the membrane permeability of erythrocytes, brain cells, amphibian skin, skeletal muscle and crab gills.

A similarity between the muscular degeneration observed in trout larvae and the degeneration induced by Plasmocid, as seen by the light microscope, has been mentioned in the preceding chapter. Electron microscope studies, however, have revealed that Plasmocid destroyed selectively actin filaments and the Z band (Price et al., 1962). In this regard, the degeneration seen in trout is dissimilar and appears non-specific since disruption affects both actin and myosin filaments.

Muscular degeneration was found to be more important in old than in young larvae. The type of abnormality was also different in both: vacuolization prevails in muscle of young larvae while in old larvae vacuolization was accompanied by wide interfibrillar spaces. These two situations could possibly represent two successive stages of degeneration, but they could also represent two different types of reaction of the fibers in the degenerative process.

As already mentioned, it is still impossible to decide whether we are dealing with a degeneration resulting directly from the action of the organophosphates or with a degeneration resulting from an indirect action. One thing, however, is sure: tissues of larval stages of trout show an unusual sensitivity to the toxic effects of compounds such as Dylox and Phosphamidon.

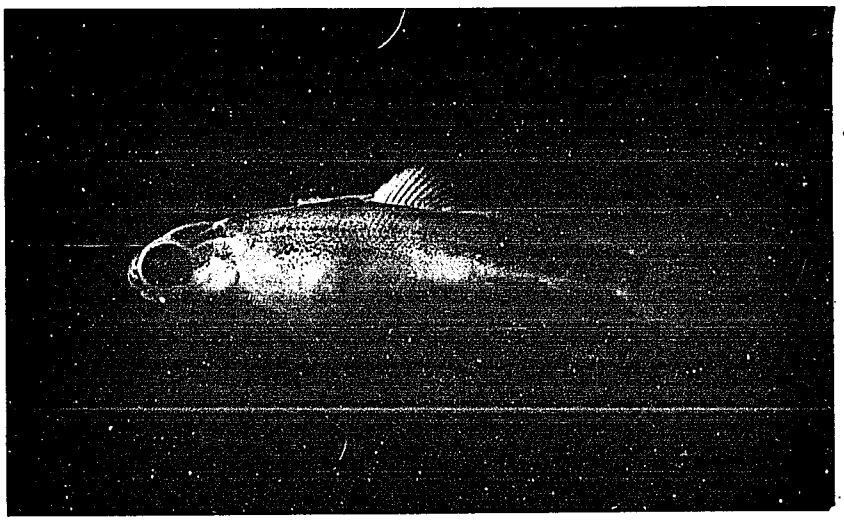


Fig. 1. Young Salvelinus fontinalis larva (12 days old and 17 mm). 4.3 x.

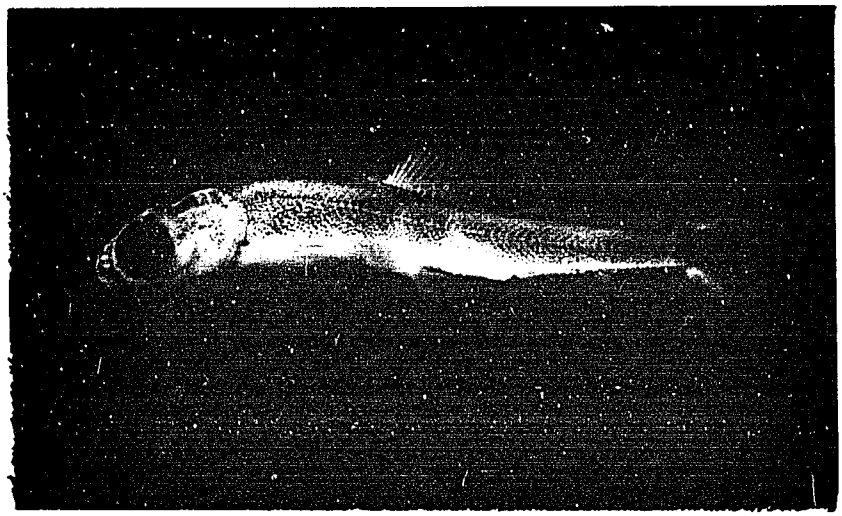


Fig. 2. Old Salvelinus fontinalis larva (34 days old and 21 mm). 4.3 x.

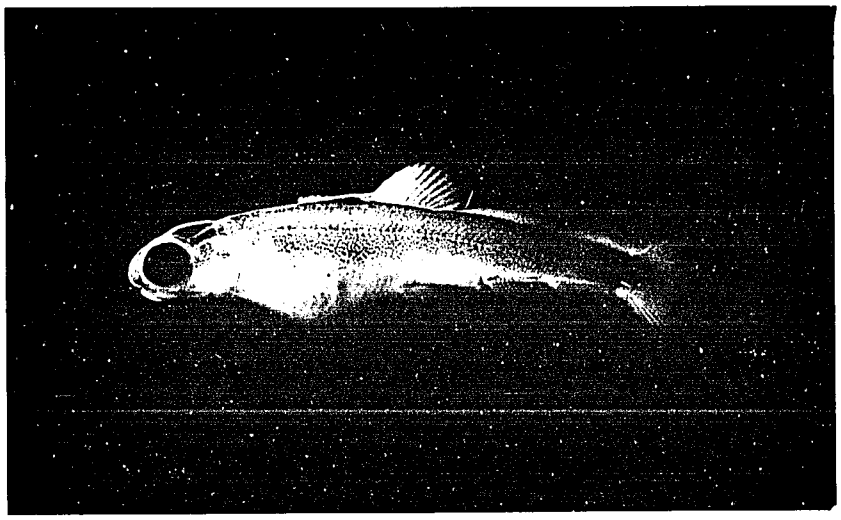


Fig. 1. Young Salvelinus fontinalis larva (12 days old and 17 mm). 4.3 x.

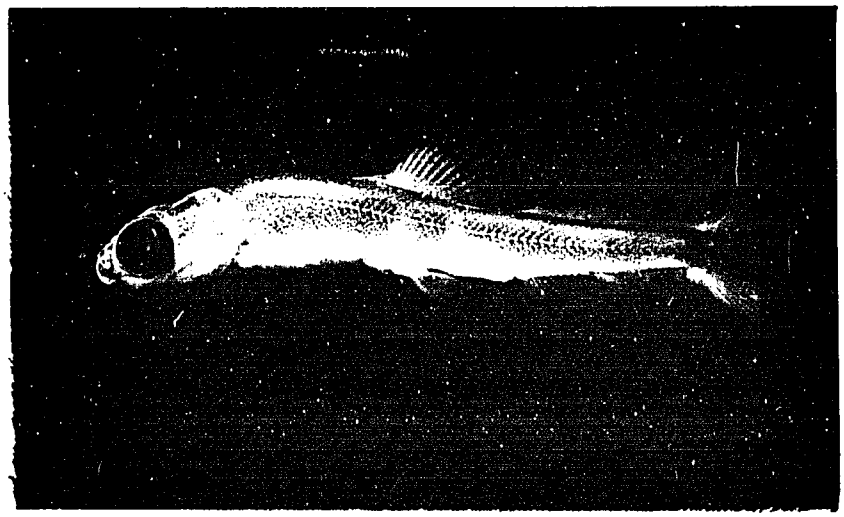


Fig. 2. Old Salvelinus fontinalis larva (34 days old and 21 mm). 4.3 x.

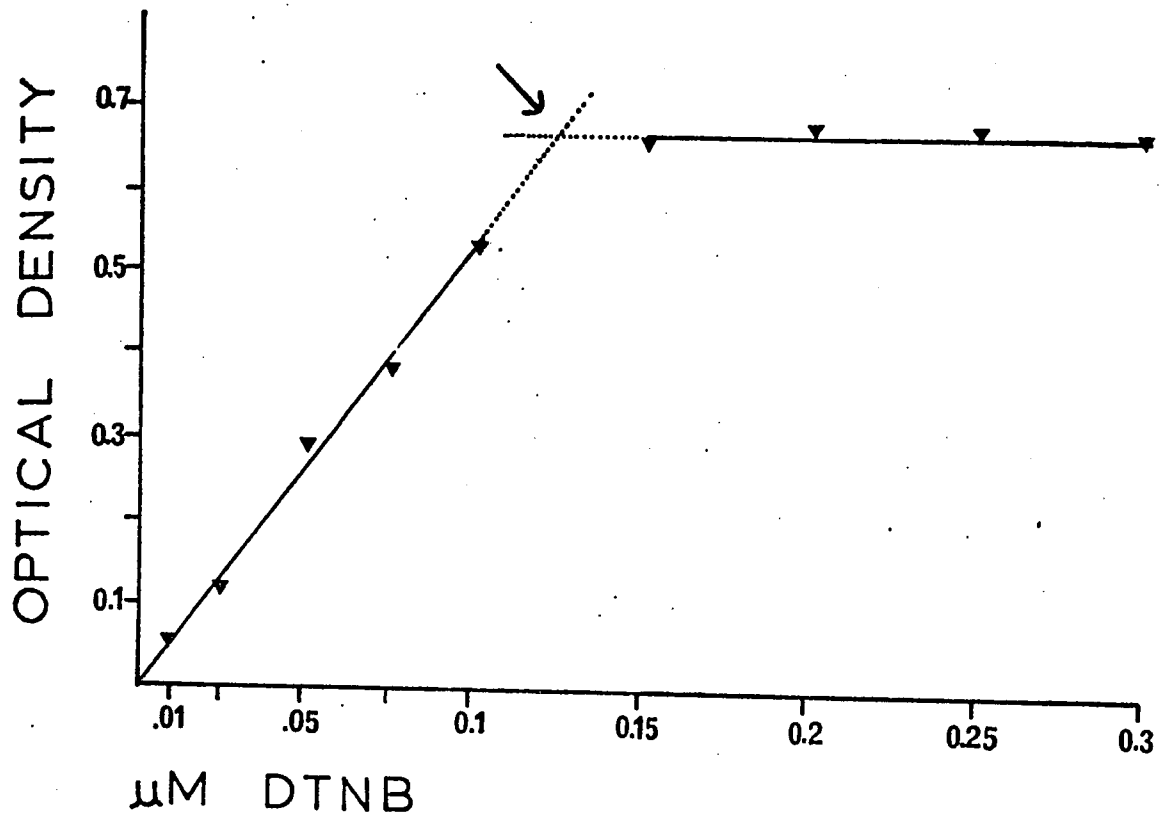


Fig. 3. Optical densities obtained after 30 min of incubation when 6 mg of head homogenate are incubated with 4 mM of acetylthiocholine in the presence of various quantities of DTNB. The amount of DTNB corresponding to the point of intersection of the two lines (indicated by the arrow) is the amount reacting with the product of hydrolysis when the enzyme is the limiting factor.

PLATE 6.1

Histological damage in Salvelinus fontinalis larvae after 24 hr of treatment with organophosphate. Magnification: 500 x.

Fig. 4. Longitudinal section of body muscle (white fibers) of control old larva.

Fig. 5. Longitudinal section of body muscle (white fibers) of old larva treated with 30 ppm of Dylox. Note the longitudinal splitting and branching of fiber bundles.

Fig. 6. Cross section through white fibers of old larva treated with 100 ppm of Phosphamidon showing one focus of swollen glossy fibers and small dense, widely separated fibers.

Fig. 7. Cross section through white fibers of control old larva.

Fig. 8. Cross section through part of the masseter muscle of old larva treated with 30 ppm of Dylox.

Fig. 9. Longitudinal section through young larva treated with 100 ppm of Dylox showing hyperplasia of the skin. Compare with skin in Figure 4.

Plate 6.1

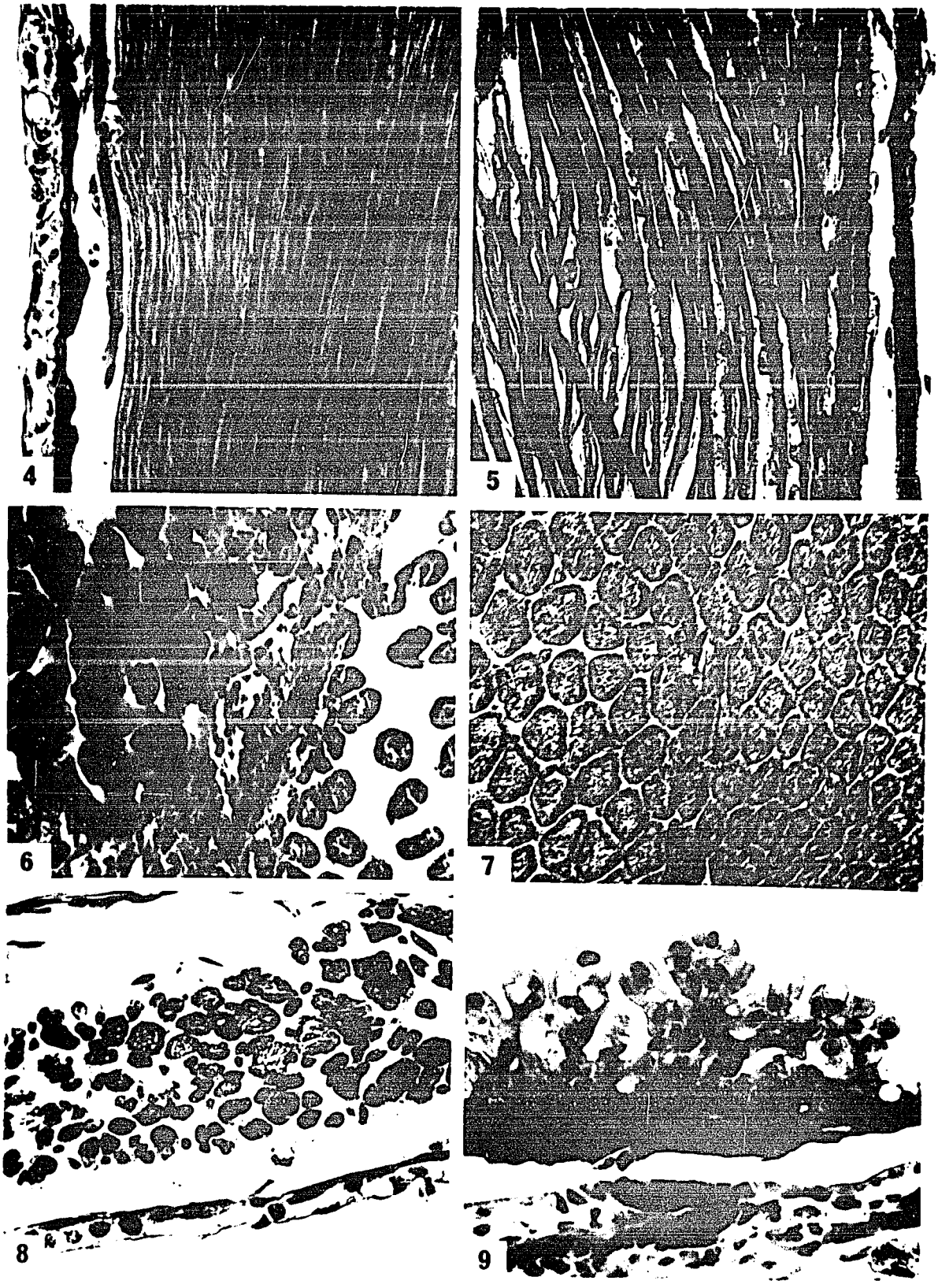


Plate 6.1

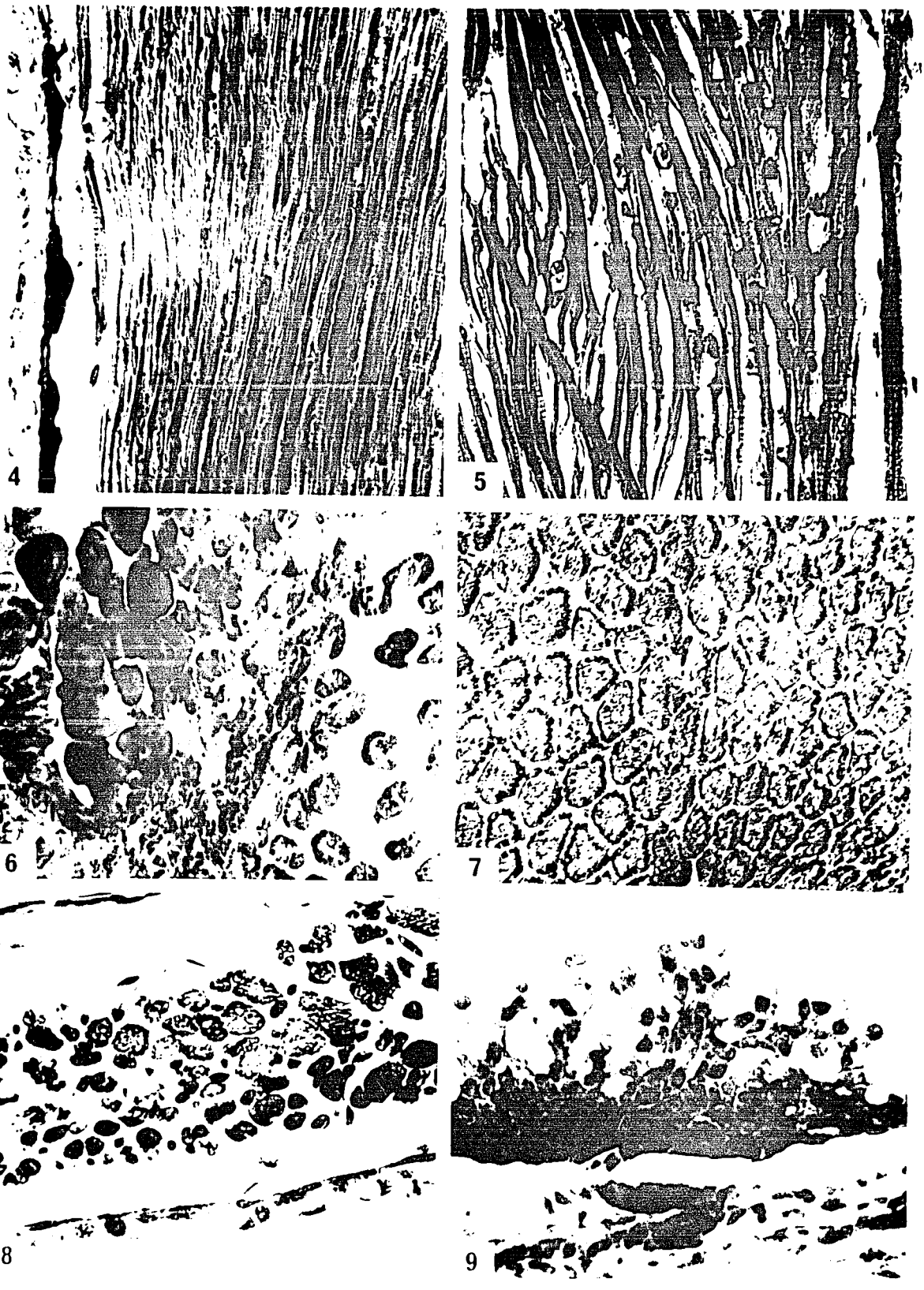


PLATE 6.2

Lactic and Malic Dehydrogenases in old Salvelinus fontinalis larvae.

Fig. 10. Cross section through lateral part of the head showing the epithelium below the eye (lower right) interrupted by sensory-like structures (arrow). LDH. 125 x.

Fig. 11. Cross section through pineal body. MDH. 125 x.

Fig. 12. Cross section through the posterior part of the midbrain showing strong reaction in the lateral layer of cells. (N.B. In a complete section of the head, this layer would be vertical). LDH. 500 x.

Fig. 13. Cross section at the level of the pharynx showing the LDH reaction in the gills. Series of cells giving a strong reaction are interrupted by single cells giving a weak reaction. 125 x.

Fig. 14. Cross section through the stomach and part of the liver. LDH activity is very strong in parenchymal cells of the liver and strong in the submucosal glands. 125 x.

Fig. 15. Cross section through the tail part showing MDH activity in the neural tube, body muscle and mid-lateral structure (arrow). 125 x.

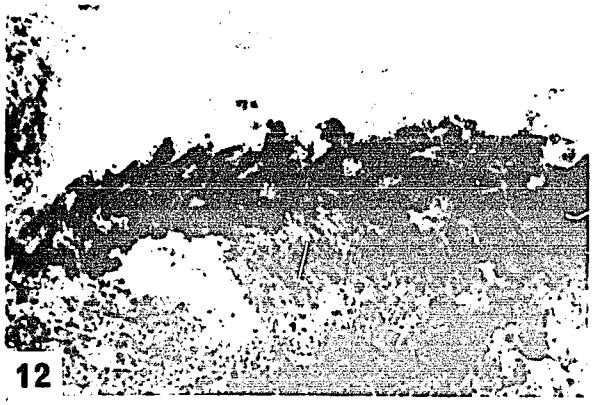
Fig. 16. Cross section through the lateral part of muscle showing swollen cells with clumping of grains. Dylox, 30 ppm. 500 x.

Fig. 17. Longitudinal section through the pharynx muscle showing "rosette" clumping of grains. Phosphamidon, 100 ppm. 1,250 x.

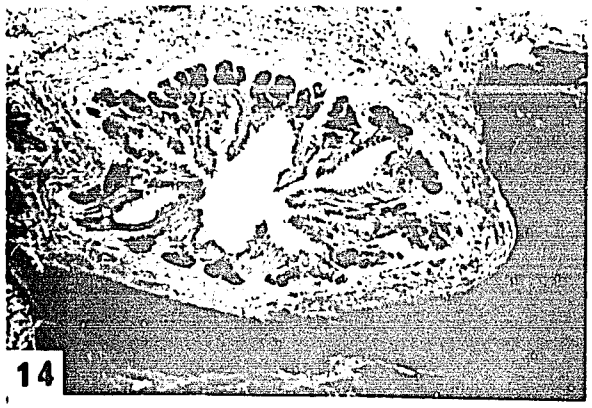
Plate 6.2



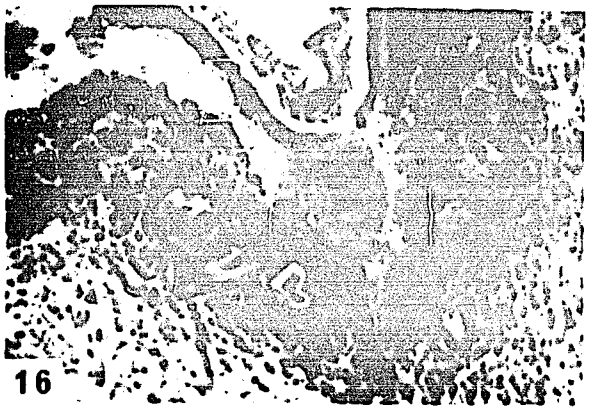












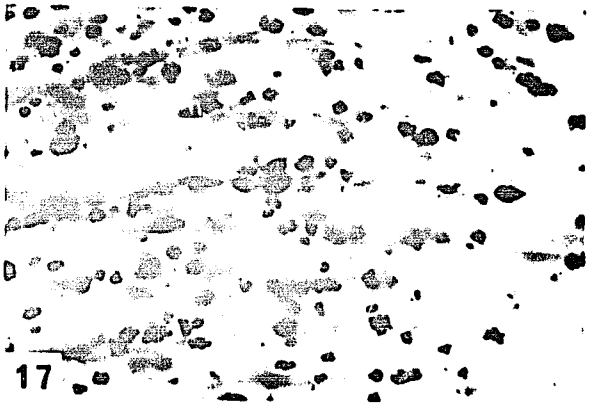
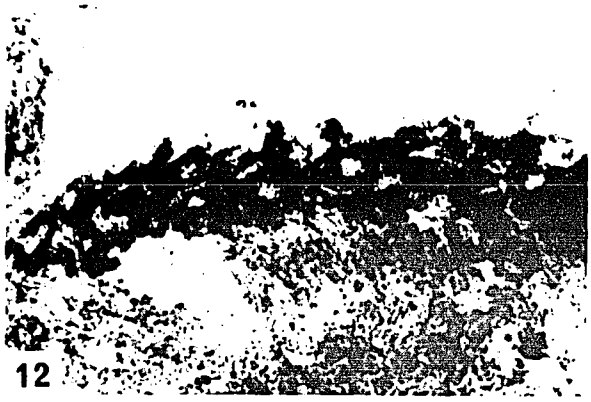


Plate 6.2

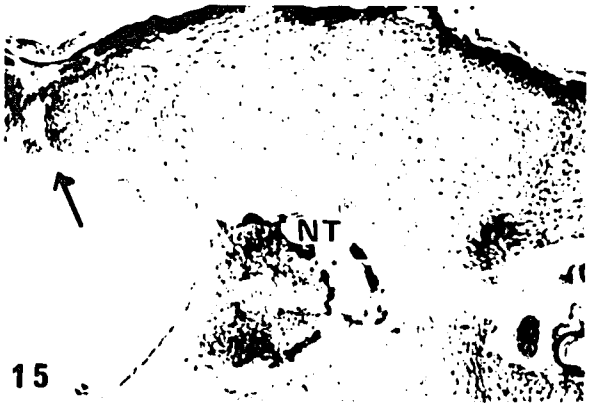














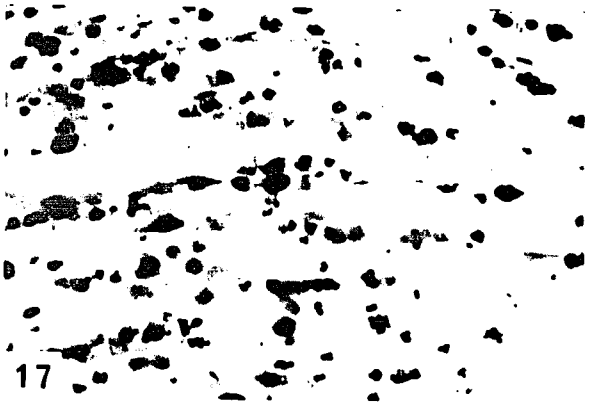


PLATE 6.3

Phosphorylase activity in old Salvelinus fontinalis larvae.

Fig. 18. Cross section through the mid-lateral part of the body: strong activity in the cutaneous muscle and low activity in the adjacent red fibers. Untreated larva; iodine stain. 125 x.

Fig. 19. Cross section through the neural tube. Activity is present in the lateral part. Untreated larva; iodine stain. 125 x.

Fig. 20. Cross section through ventro-medial part of the eye showing a strong reaction in the inner plexiform layer of the retina. Untreated larva; iodine stain. 125 x.

Fig. 21. Cross section through the body showing PAS positive material after incubation with complete medium. NT: neural tube; N: notochord; D: mesonephric duct; In: intestine; SM: cutaneous muscle. Untreated larva; PAS stain. 75 x.

Fig. 22. Cross section through the body of zebra fish showing the difference of phosphorylase activity between the small and large fibers as a verification of the technique; iodine stain. 75 x.

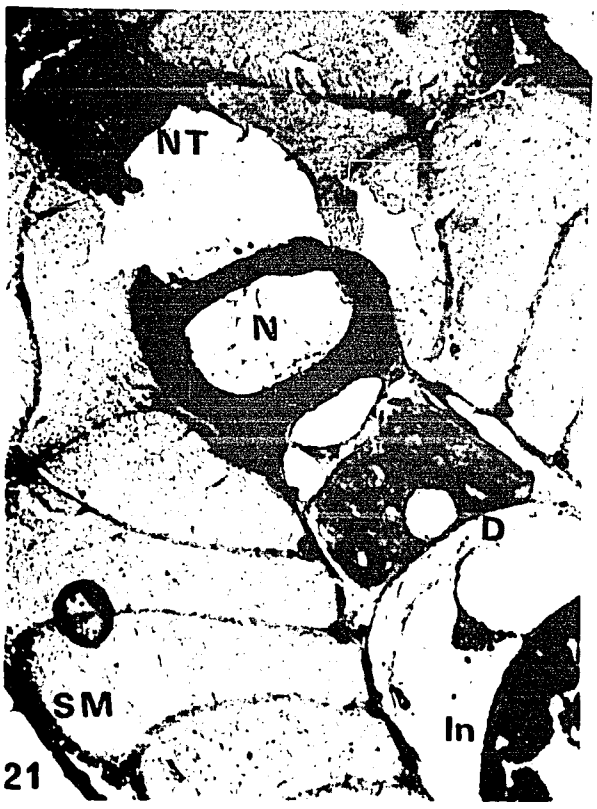
Fig. 23. Cross section through masseter muscle showing localization of the enzyme activity within the fibers. Untreated larva; PAS stain. 500 x.

Plate 6.3

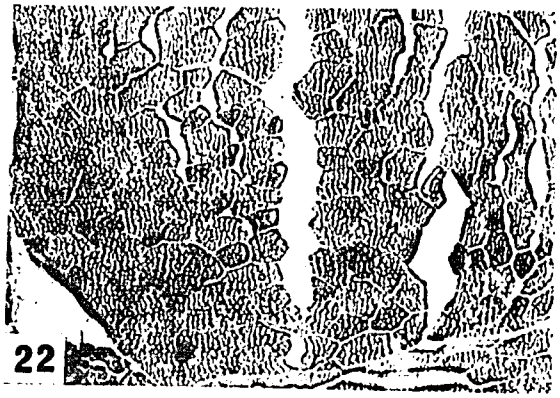

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21


22


23

Plate 6.3

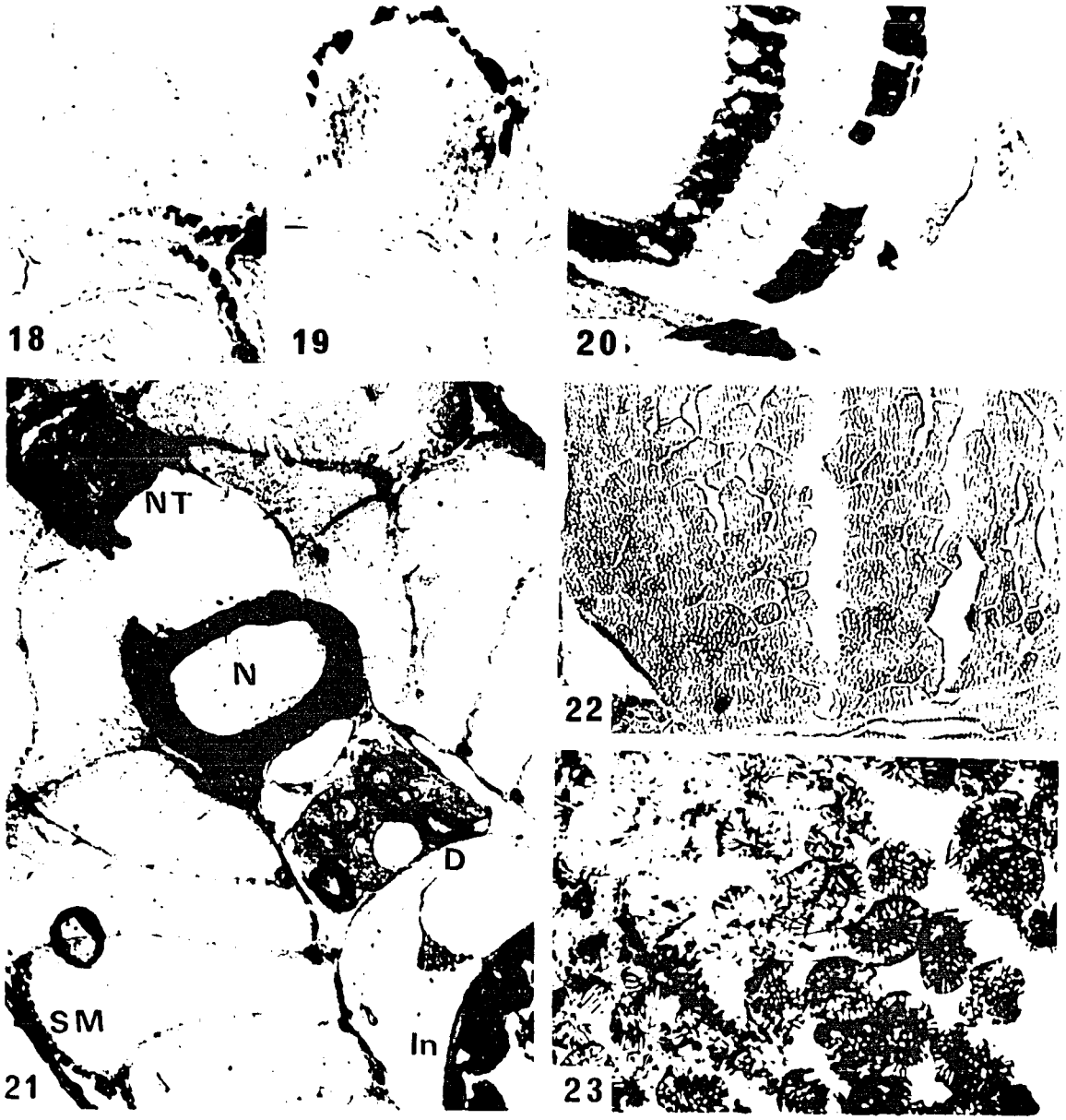


PLATE 6.4

Distribution of Acid and Alkaline Phosphatases in old *Salvelinus fontinalis* larvae.

Fig. 24. Cross section through the head showing the acid phosphatase activity in the olfactory pit epithelium and in the forebrain (FB). Dylox, 30 ppm. 125 x.

Fig. 25. Cross section through the midbrain. The acid phosphatase activity is localized as discrete dots in the midbrain but absent in the densely cellular region of the optic lobes. Untreated larva. 75 x.

Fig. 26. Cross section through the body showing the acid phosphatase activity in the neural tube (NT), along the nerve fibers in the lateral line (arrow). There is very weak activity in the carinalis muscle (CM). N: notochord. Dylox, 30 ppm. 125 x.

Fig. 27. Cross section through the kidney. Three tubules appear darker than the stroma and have areas of strong acid phosphatase activity. Untreated larva. 500 x.

Fig. 28. Cross section through the pharynx. Strong alkaline phosphatase activity is found in the cells scattered in the gill tissue or along the margin of the muscles. Untreated larva; azo dye technique. 500 x.

Fig. 29. Cross section through the stomach showing strong alkaline phosphatase activity in the submucosal connective tissue and medium activity in the mucosal glands. Untreated larva; azo dye technique. 125 x.

Plate 6.4

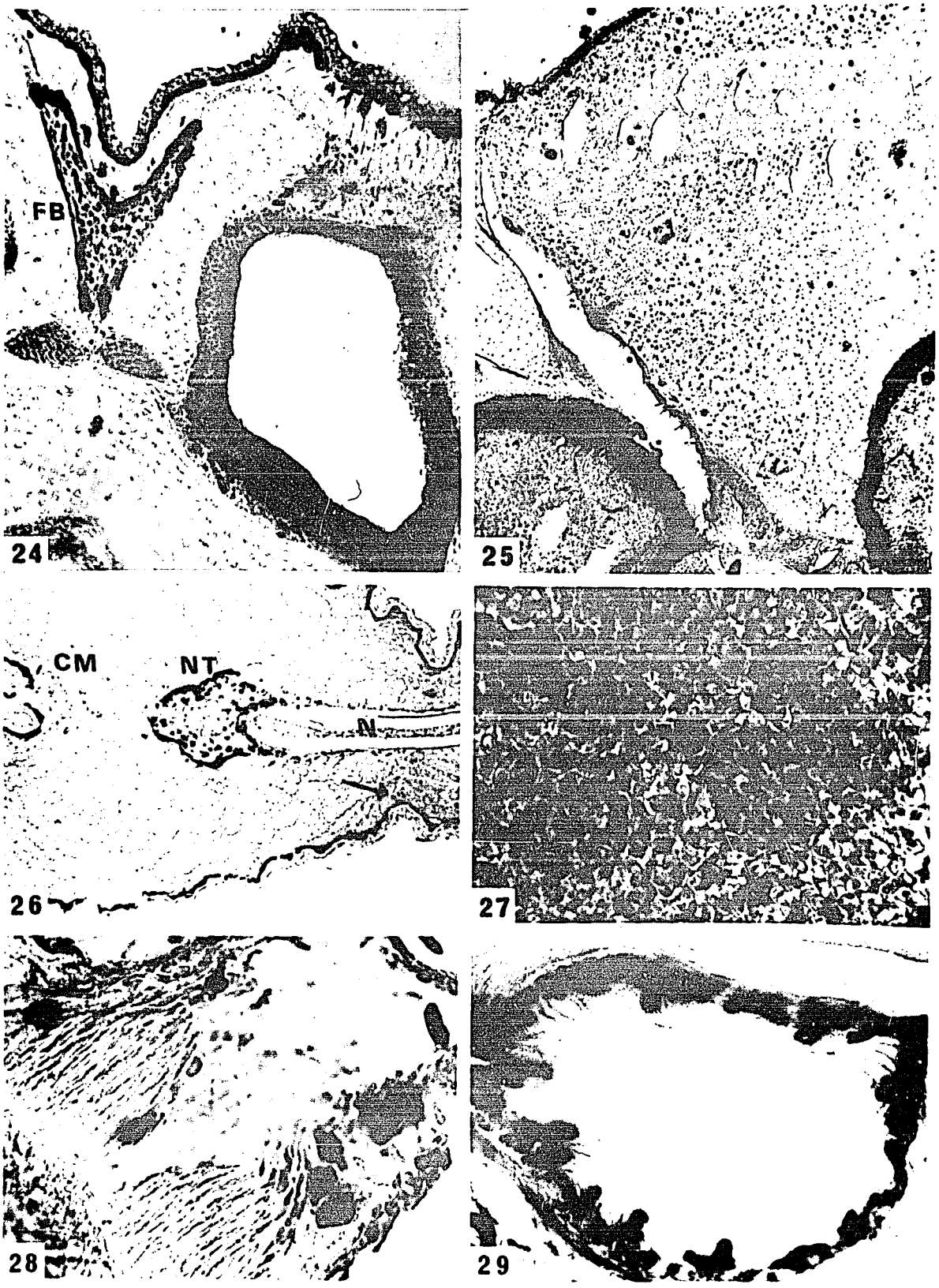


Plate 6.4

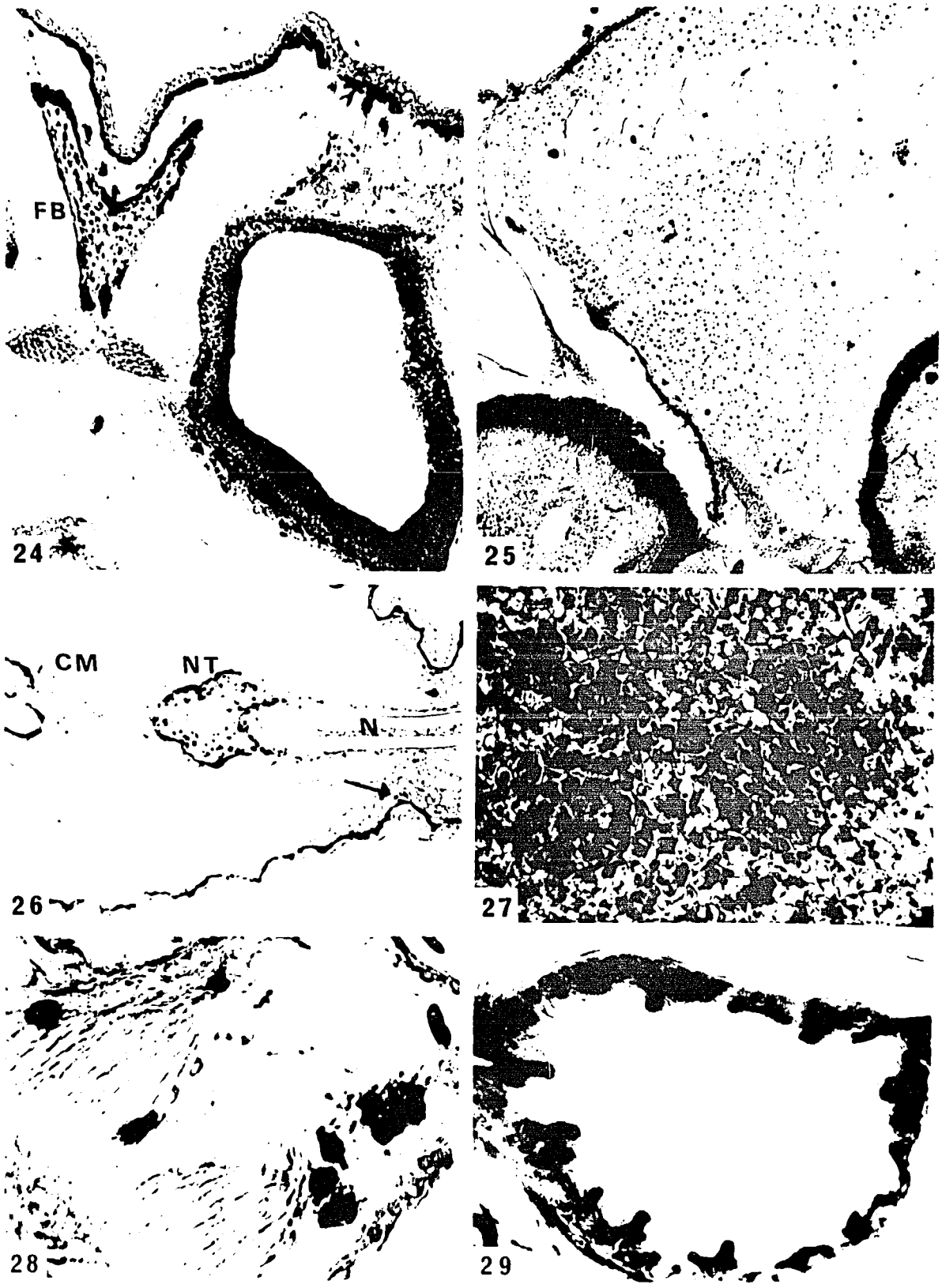


PLATE 6.5

Distribution of Alkaline Phosphatase in old Salvelinus fontinalis larvae.

Figure 32: azo dye technique. Figures 30, 31, 33-37: Gomori's technique. Magnification: 125 x.

Fig. 30. Cross section through the olfactory pits. The enzyme is present on the ventro-medial and ventro-lateral margins of the lumen.

Fig. 31. Cross section through the pineal body. The activity is strong in the dorsal part.

Fig. 32. Cross section through the body at the level of the heart. The activity is medium in the ventricle.

Fig. 33. Cross section through the mouth region: strong activity in the submucosa.

Fig. 34. Cross section through the kidney at the level of the stomach: strong activity in the striated border of tubules and ducts and medium activity in the glomerulus. N: notochord.

Fig. 35. Cross section through the body at the level of the intestine. The reaction is strong in the striated border of tubules and of intestinal epithelium but negative in the striated border of the ducts (arrow).

Fig. 36. Cross section through the head at the level of the pseudogills (PG) and of the gills. The reaction is medium in the large cells within the gill arches (GA) and along the margin of the epithelial cells of the pseudogills.

Fig. 37. Cross section through the retina of the eye: the activity is strong in the outer nuclear layer. The dark outer most layer is the

Plate 6.5



Plate 6.5

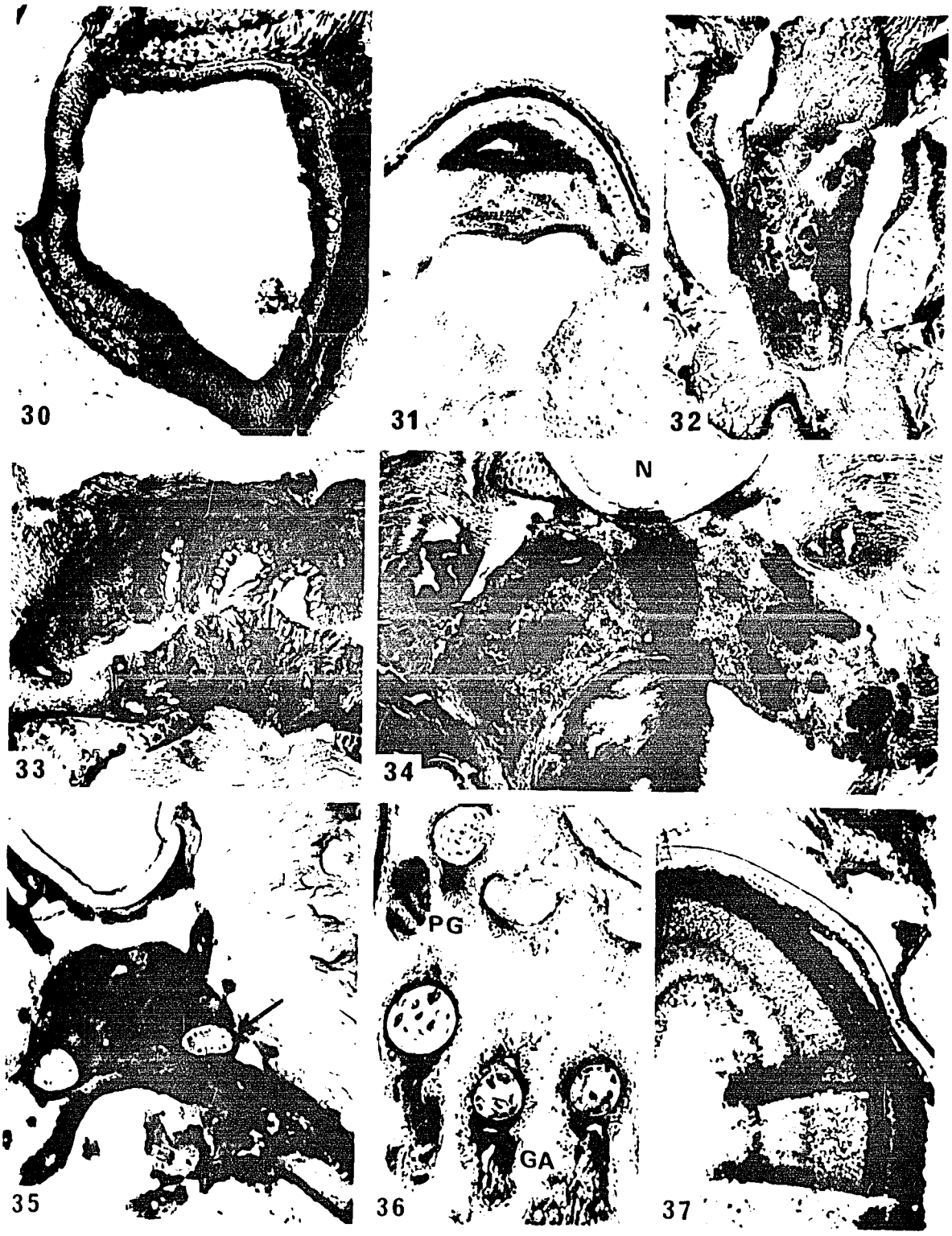


PLATE 6.6

Distribution of Adenosine Triphosphatase in old Salvelinus fontinalis larvae.

Fig. 38. Cross section through the tail part. Control section (no substrate). N: notochord. 75 x.

Fig. 39. Cross section through the tail part. The enzyme activity is stronger in the white fibers than in the cutaneous muscle. N: notochord; NT: neural tube. 75 x.

Fig. 40. Cross section at the level of the heart. The activity is strong in the pectoralis muscle (PeM) and the pharyngeal submucosa but weak in the ventricle (V) and irregular in the auricle (Au) and in the gills (G). 75 x.

Fig. 41. Cross section at the level of the pseudogills. The activity is strong in the epithelial cells. 125 x.

Fig. 42. Cross section through the masseter muscle. The reaction is stronger in the larger fibers. 500 x.

Fig. 43. Cross section through the masseter muscle. Control section (no substrate). 500 x.

Plate 6.6

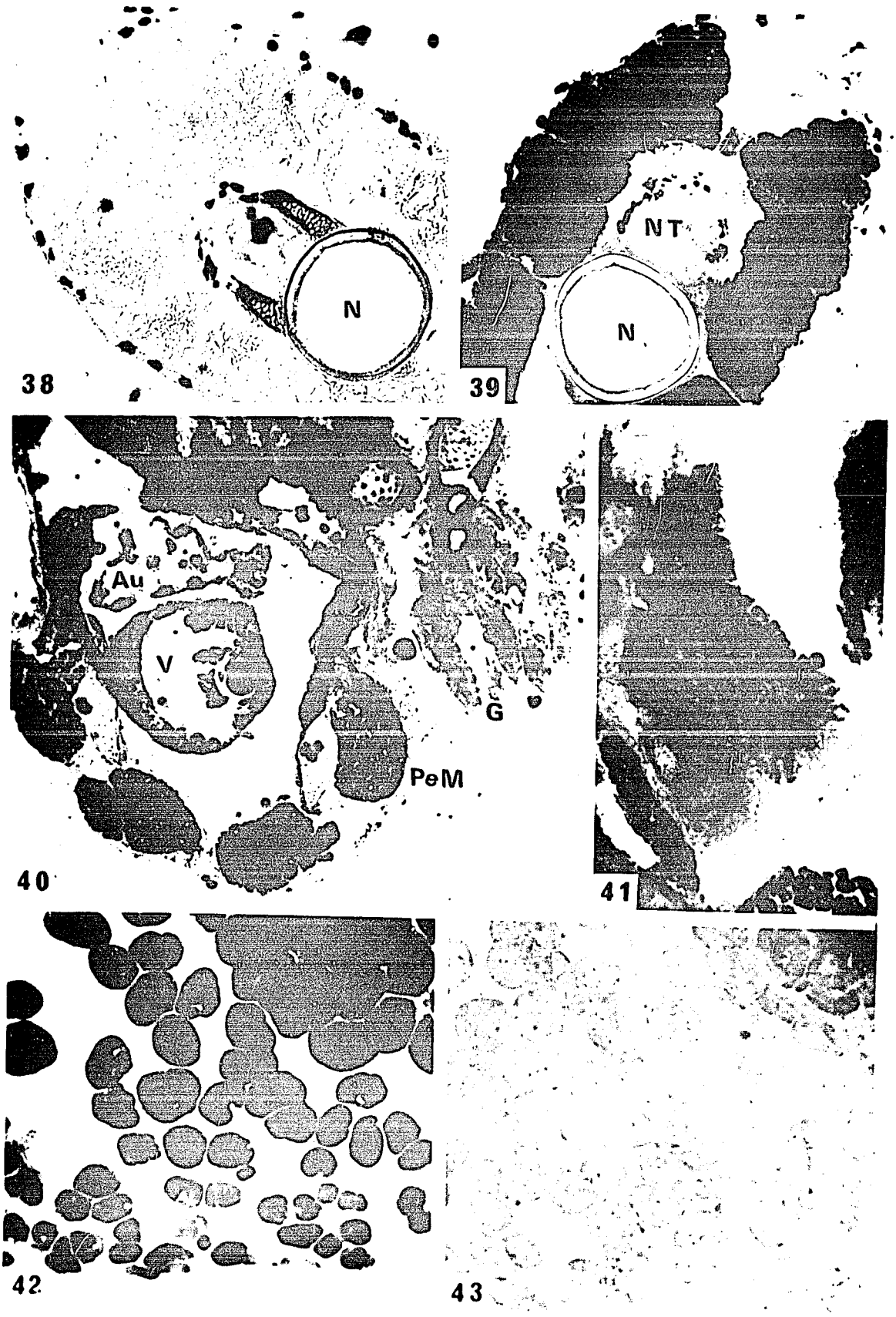


Plate 6.6

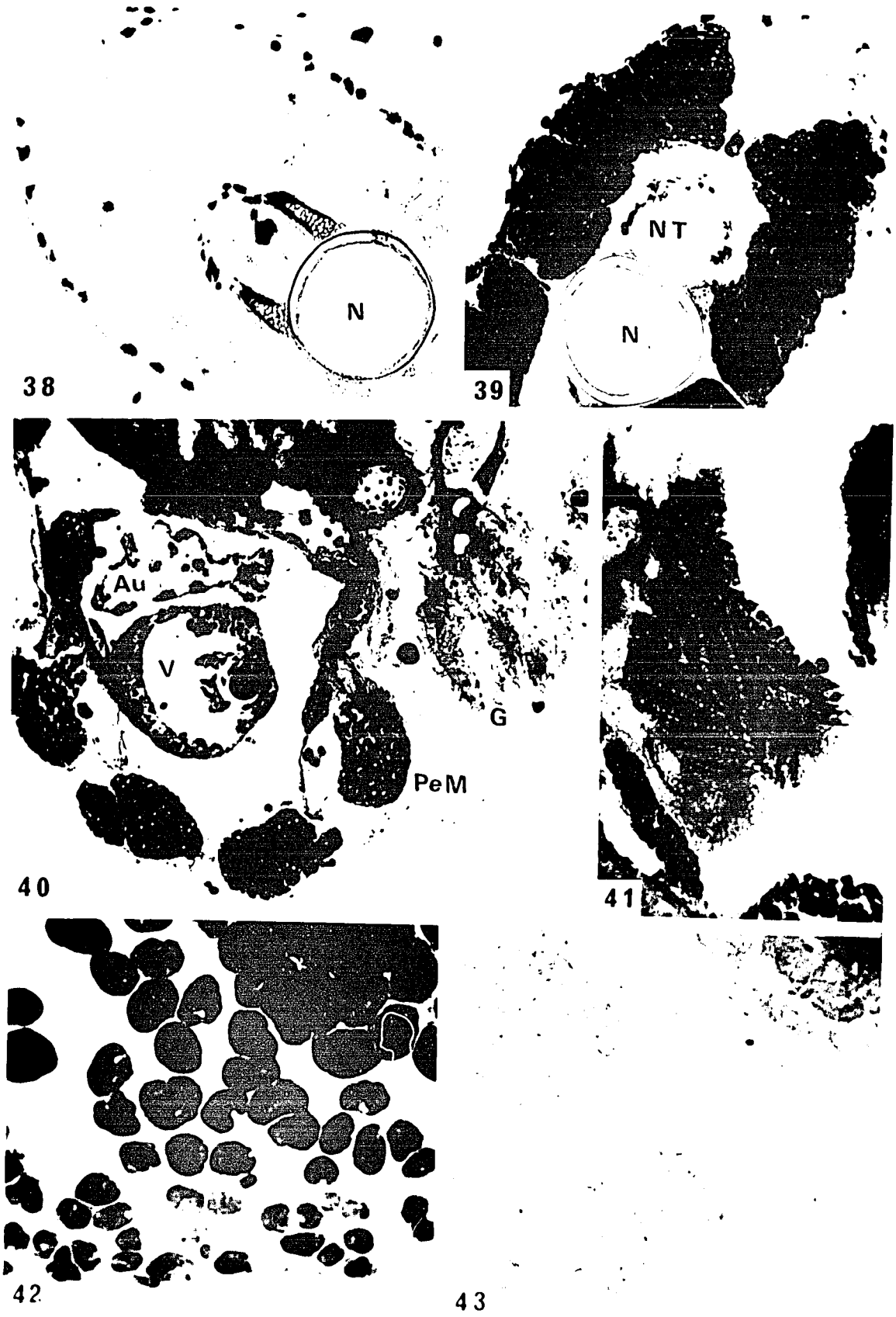


PLATE 6.7

Cholinesterase Activity.

Fig. 44. Percentage activity in young Salvelinus fontinalis larvae treated with Phosphamidon as measured by the Hestrin's (He) method and the Abou-Donia's and Menzel's method (A-D). Treatment lasted for the first 24 hr. Negative percentage activity would be due to accumulation of acetylcholine (see text p. 236).

Plate 6.7

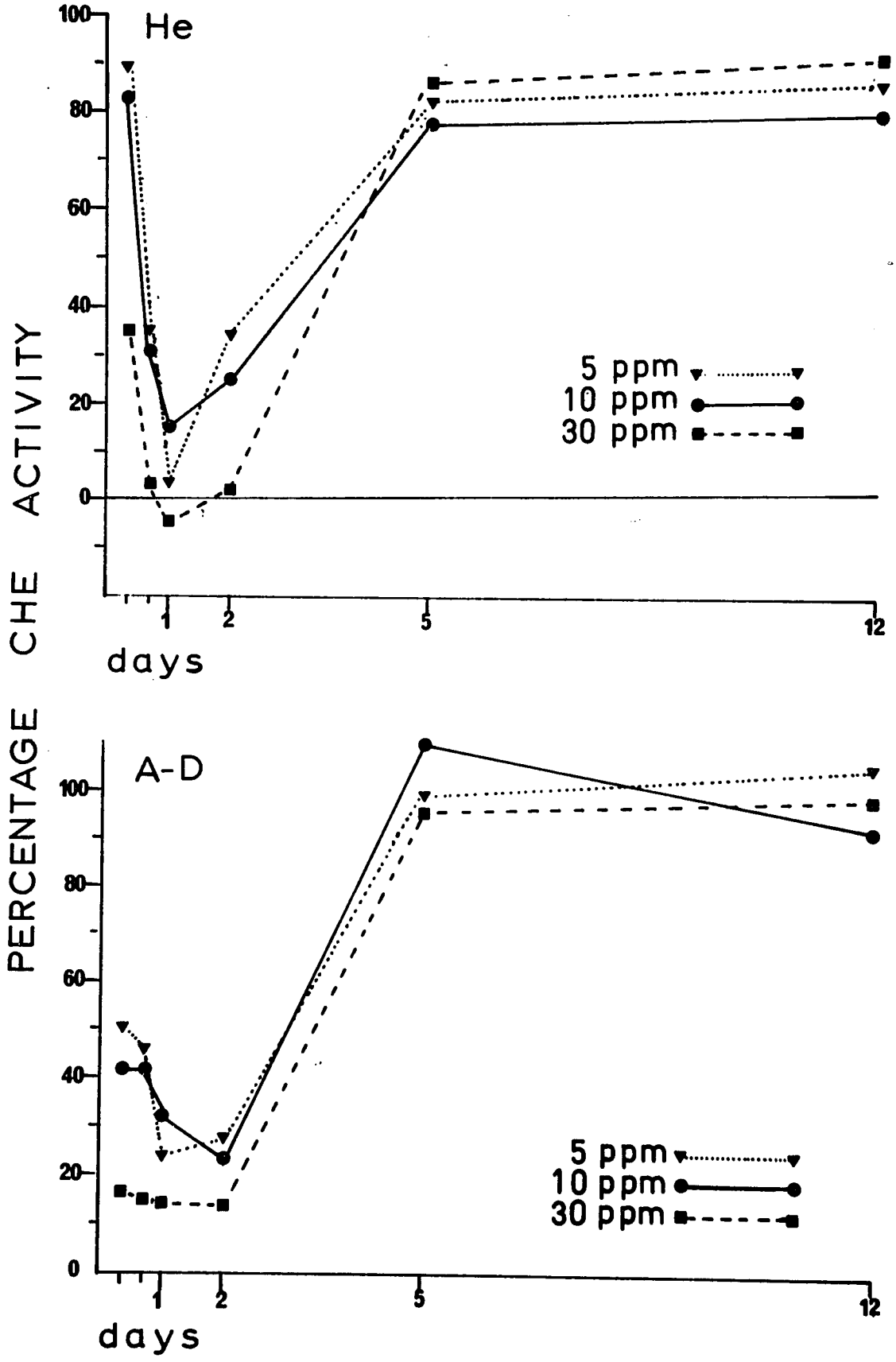

He
PERCENTAGE CHE ACTIVITY
100
80
60
40
20
0
1
2
5
12
days
5 ppm
10 ppm
30 ppm
A-D
PERCENTAGE
100
80
60
40
20
0
1
2
5
12
days
5 ppm
10 ppm
30 ppm

PLATE 6.8

Cholinesterase Activity.

Fig. 45. Percentage activity in young Salvelinus fontinalis larvae treated with Phosphamidon as measured by the Hestrin's (He) method and the Abou-Donia's and Menzel's method (A-D). Treatment lasted for the first 24 hr. Negative percentage activity would be due to accumulation of acetylcholine (see text p. 236).

Plate 6.8

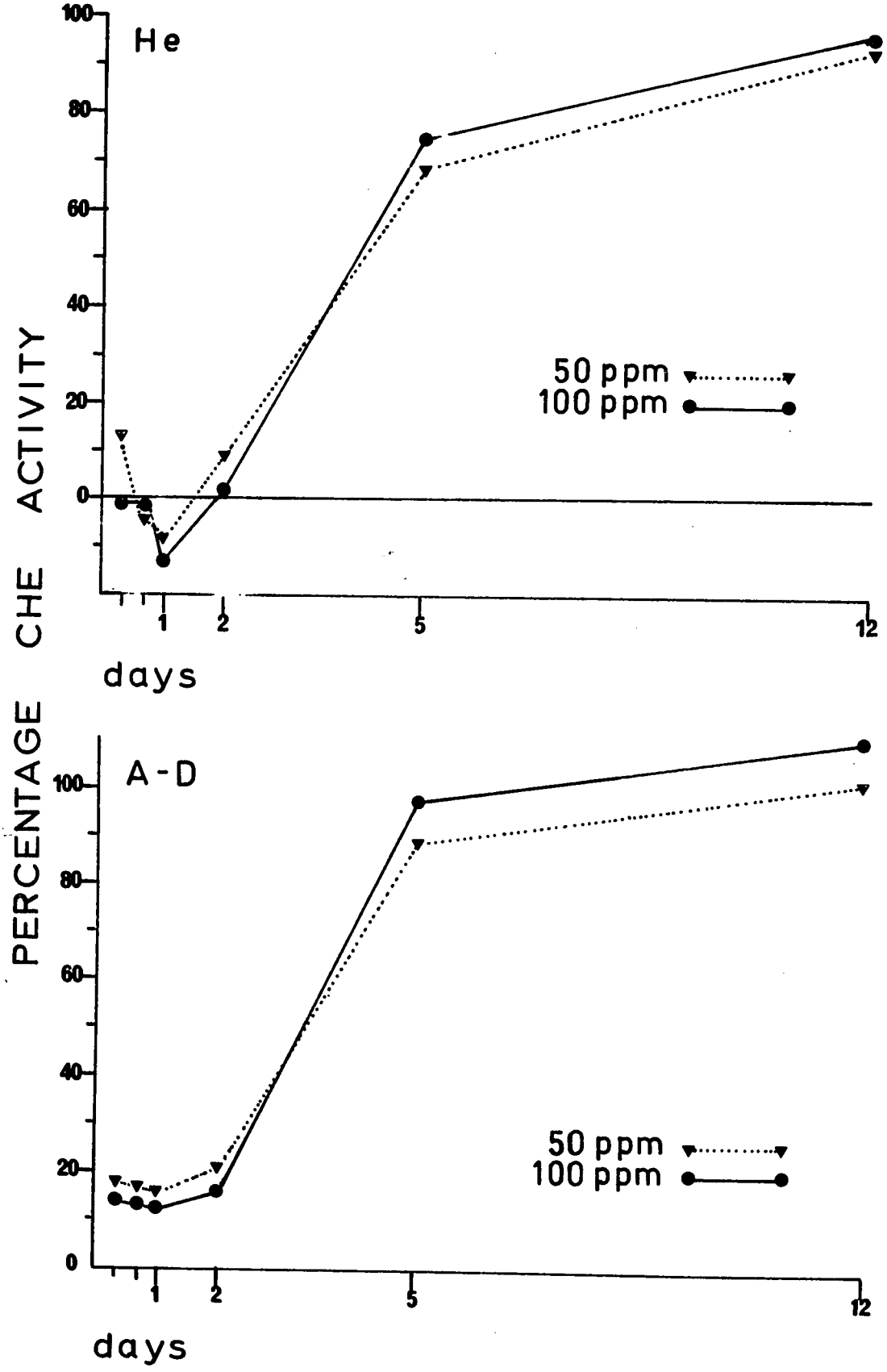

He
PERCENTAGE CHE ACTIVITY
100
80
60
40
20
0
50 ppm
100 ppm
1
2
5
12
days
A-D
PERCENTAGE
100
80
60
40
20
0
50 ppm
100 ppm
1
2
5
12
days

PLATE 6.9

Cholinesterase Activity.

Fig. 46. Percentage activity in young Salvelinus fontinalis larvae treated with Dylox as measured by the Hestrin's (He) method and the Abou-Donia's and Menzel's method (A-D). Treatment lasted for the first 24 hr. Negative percentage would be due to accumulation of acetylcholine (see text p. 236).

Plate 6.9

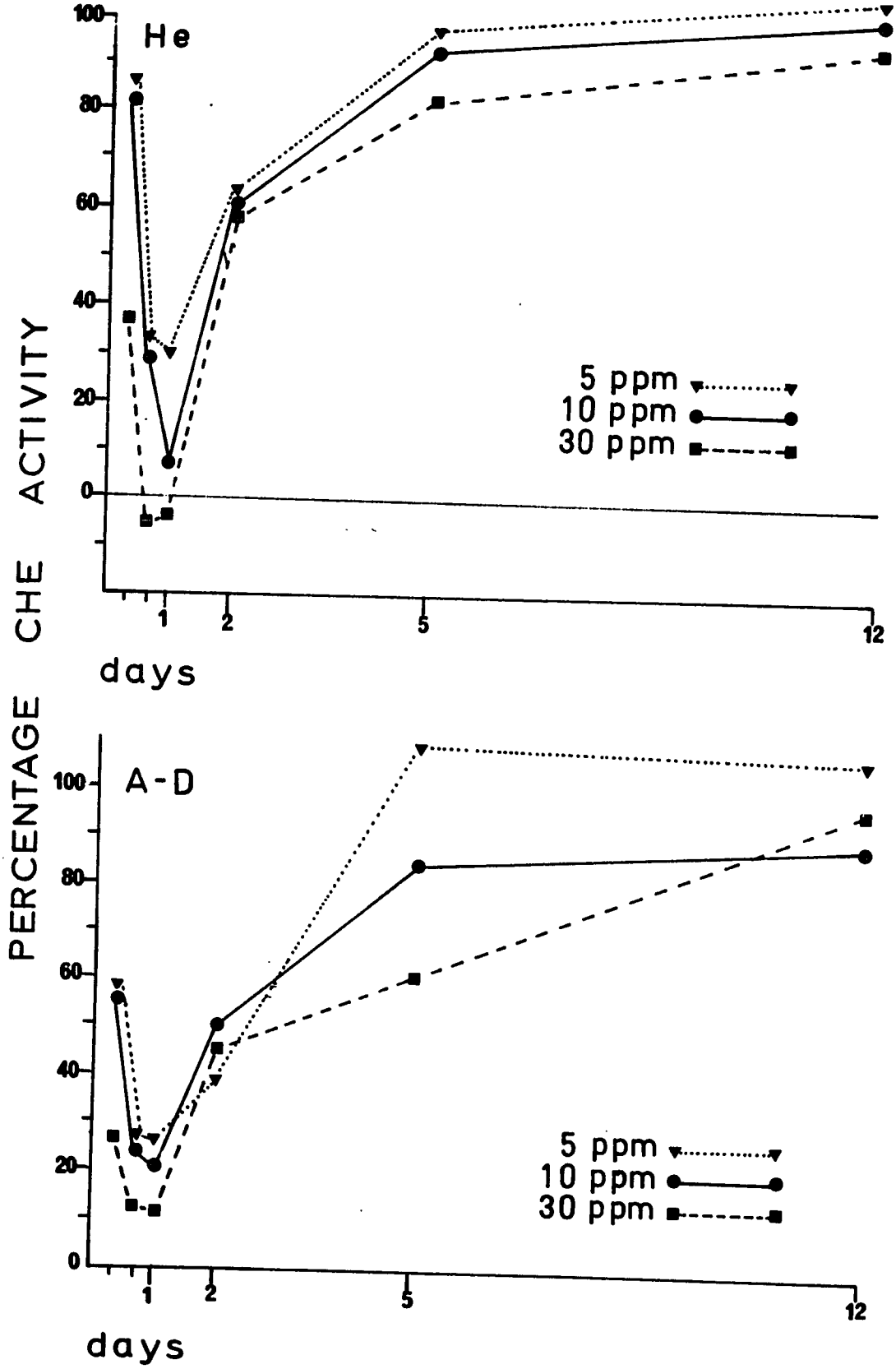

He
PERCENTAGE CHE ACTIVITY
100
80
60
40
20
0
1
2
5
12
days
5 ppm
10 ppm
30 ppm
A-D
PERCENTAGE
100
80
60
40
20
0
1
2
5
12
days
5 ppm
10 ppm
30 ppm

PLATE 6.10

Cholinesterase Activity.

Fig. 47. Percentage activity in young Salvelinus fontinalis larvae treated with Dylox as measured by the Hestrin's (He) method and the Abou-Donia's and Menzel's method (A-D). Treatment lasted for the first 24 hr. Negative percentage would be due to accumulation of acetylcholine (see text p. 236).

Plate 6.10

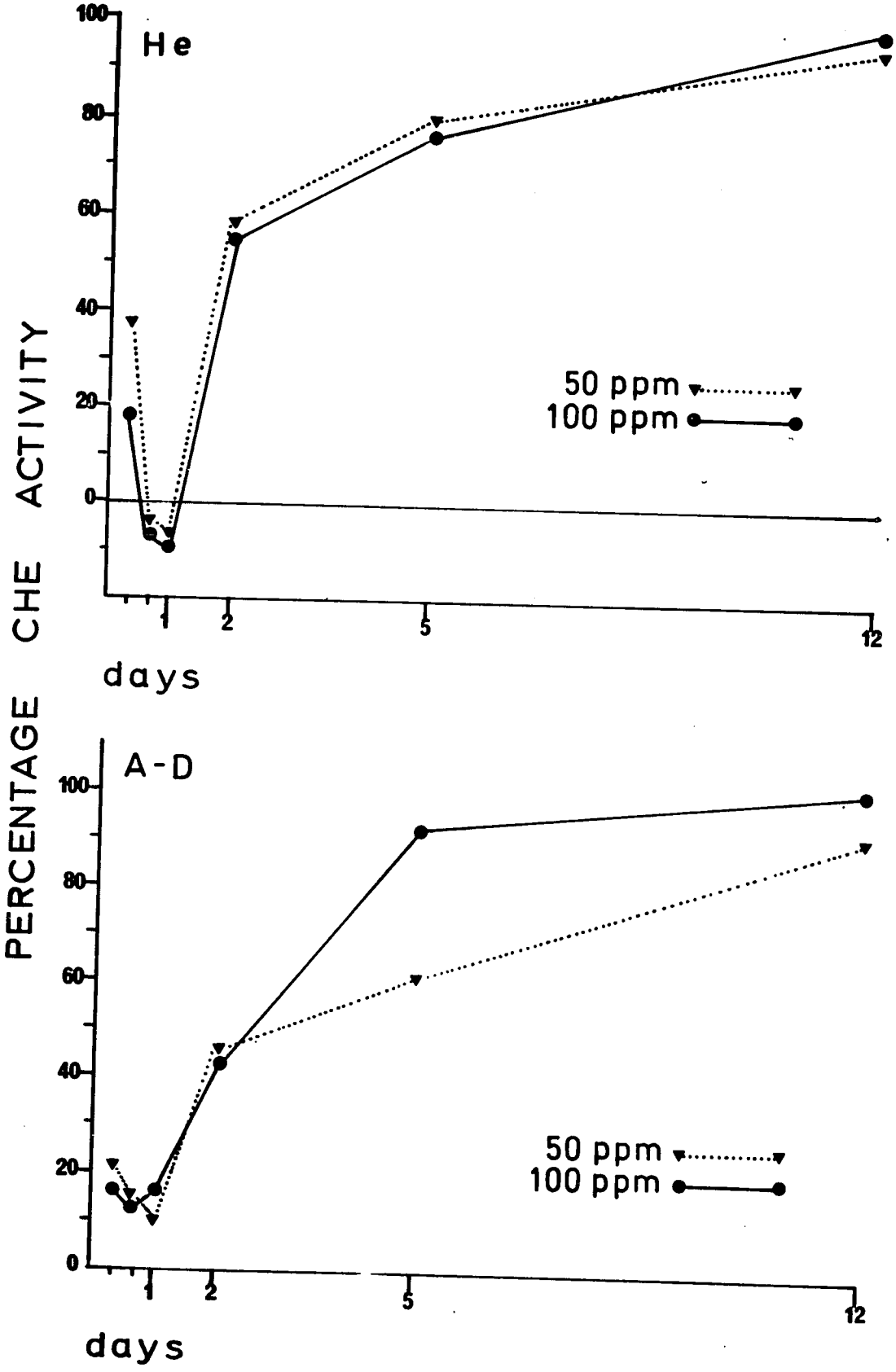

He
PERCENTAGE CHE ACTIVITY
100
80
60
40
20
0
50 ppm
100 ppm
1
2
5
12
days
A-D
PERCENTAGE
100
80
60
40
20
0
50 ppm
100 ppm
1
2
5
12
days

PLATE 6.11

Cholinesterase Activity.

Fig. 48. Percentage activity in old Salvelinus fontinalis larvae treated with Phosphamidon as measured by the Hestrin's (He) method and the Abou-Donia's and Menzel's method (A-D). Treatment lasted for the first 24 hr. Negative percentage would be due to accumulation of acetylcholine (see text p. 236). With concentrations of 30, 50 and 100 ppm, the results are incomplete due to death of all larvae prior to the end of the experiment.

Plate 6.11

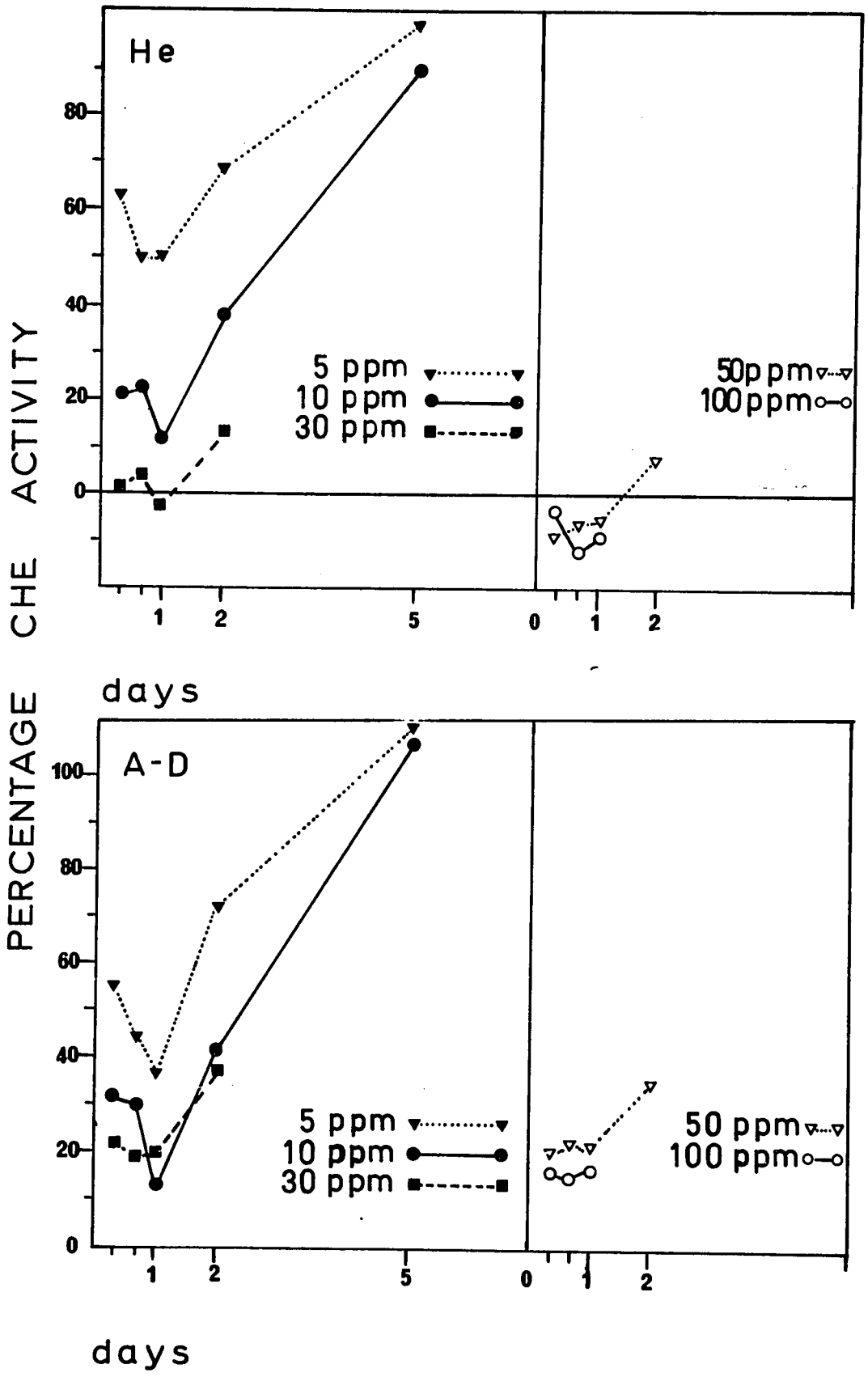

He
A-D
PERCENTAGE CHE ACTIVITY
5 ppm
10 ppm
30 ppm
50 ppm
100 ppm
0
20
40
60
80
100
1
2
5
0
1
2
days
days

PLATE 6.12

Cholinesterase Activity.

Fig. 49. Percentage activity in old Salvelinus fontinalis larvae treated with Dylox as measured by the Hestrin's (He) method and the Abou-Donia's and Menzel's method (A-D). Treatment lasted for the first 24 hr. Negative percentage would be due to accumulation of acetylcholine (see text p. 236). With concentrations of 10, 30, 50 and 100 ppm, the results are incomplete due to death of all larvae prior to the end of the experiment.

Plate 6.12

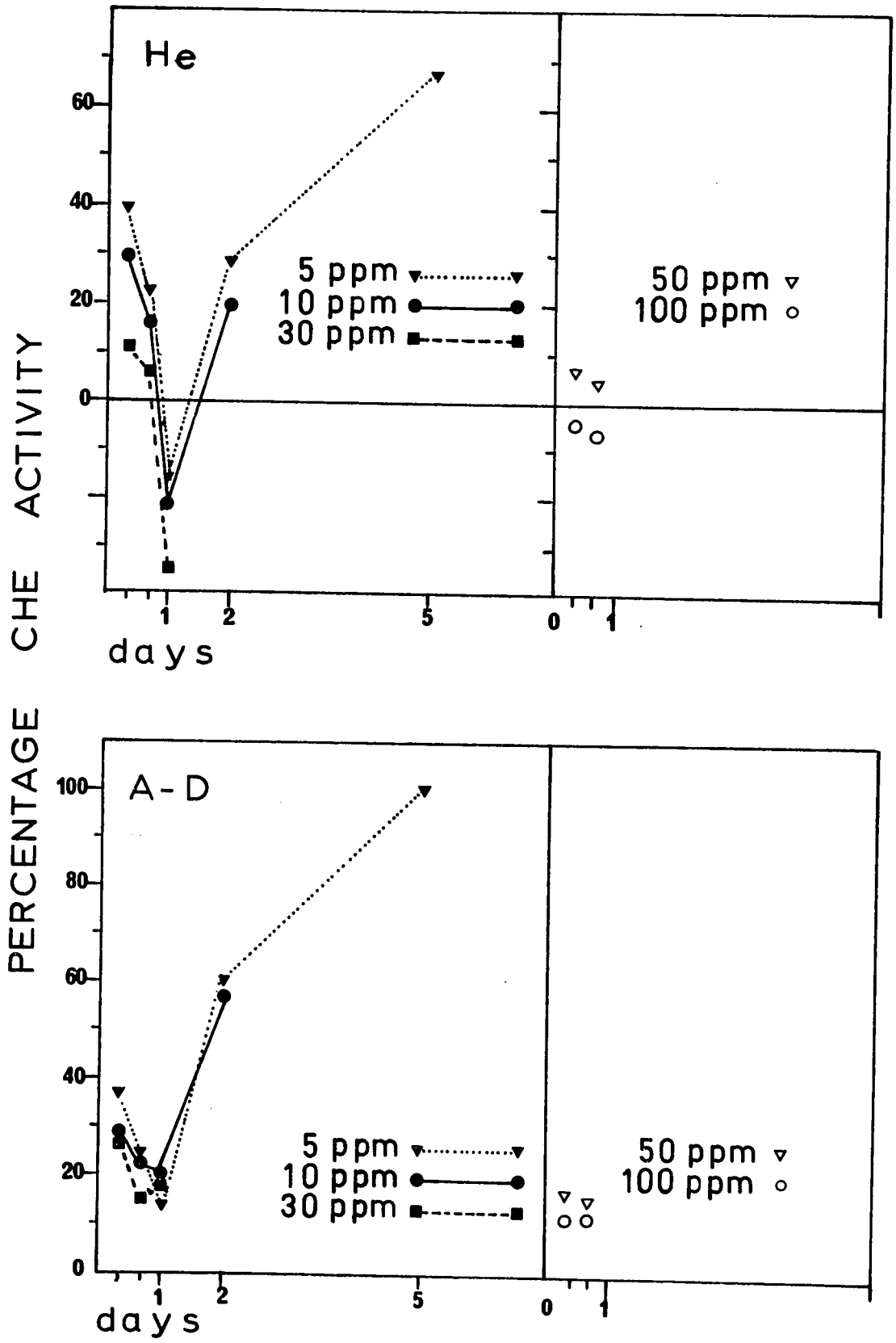

He
A-D
PERCENTAGE CHE ACTIVITY
60
40
20
0
100
80
60
40
20
0
5 ppm
10 ppm
30 ppm
50 ppm
100 ppm
1
2
5
0
1
days

PLATE 6.13

Ultra-structure of the masseter muscle of young Salvelinus fontinalis larvae (longitudinal section), 20,000 x.

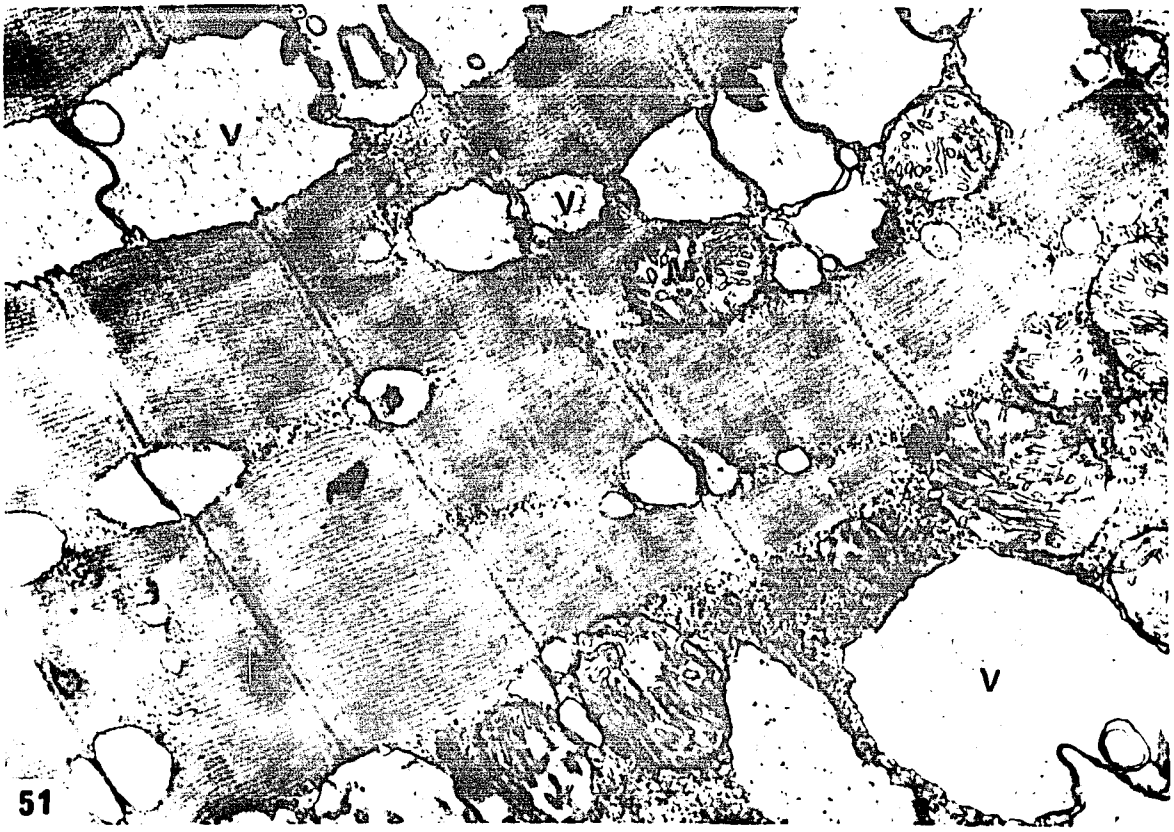
Fig. 50. Normal fiber of a control larva. A: A band; H: H band; I: I band; TR: triad.

Fig. 51. Abnormal muscle fiber. The myofibrils are separated by large vesicles (V). Most of the intermyofibrillar and subsarcolemmal mitochondria (MI) are associated with large vesicles which contain fine beaded filaments. Dylox, 100 ppm for 24 hr.

Plate 6.13

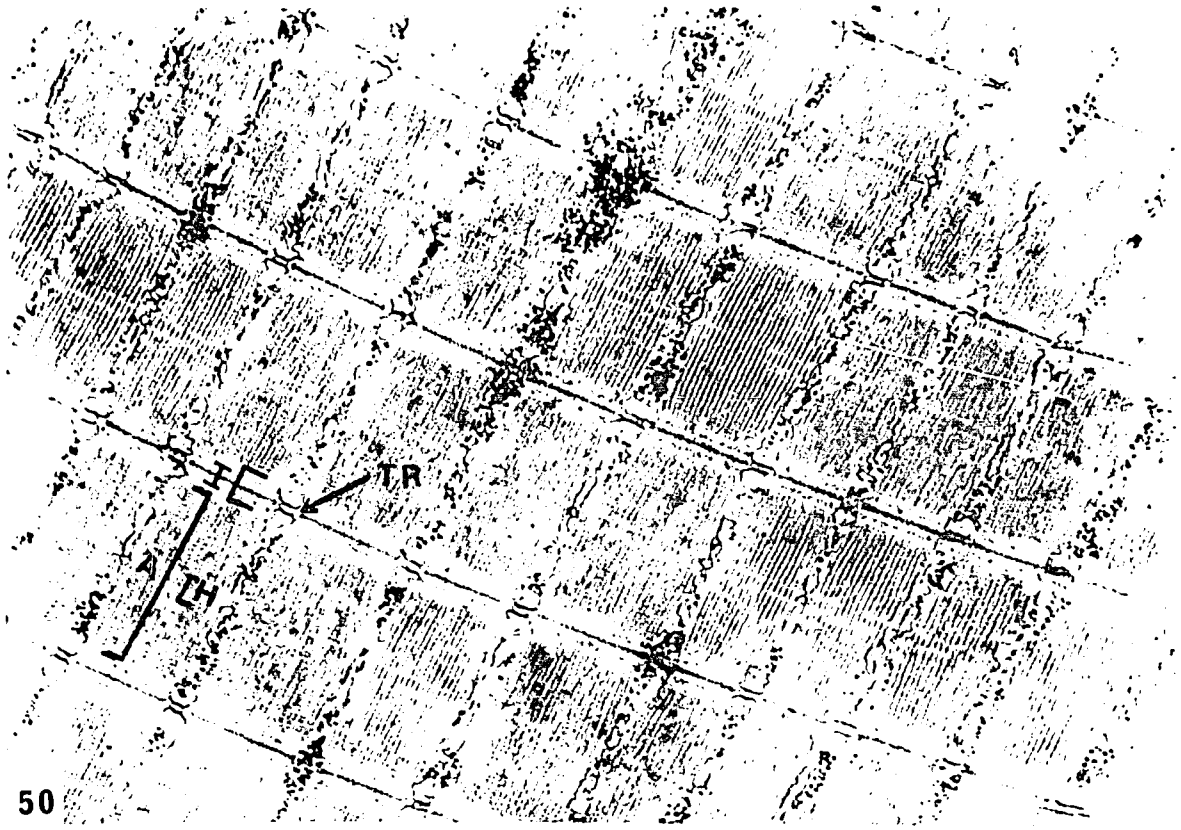


50



51

Plate 6.13



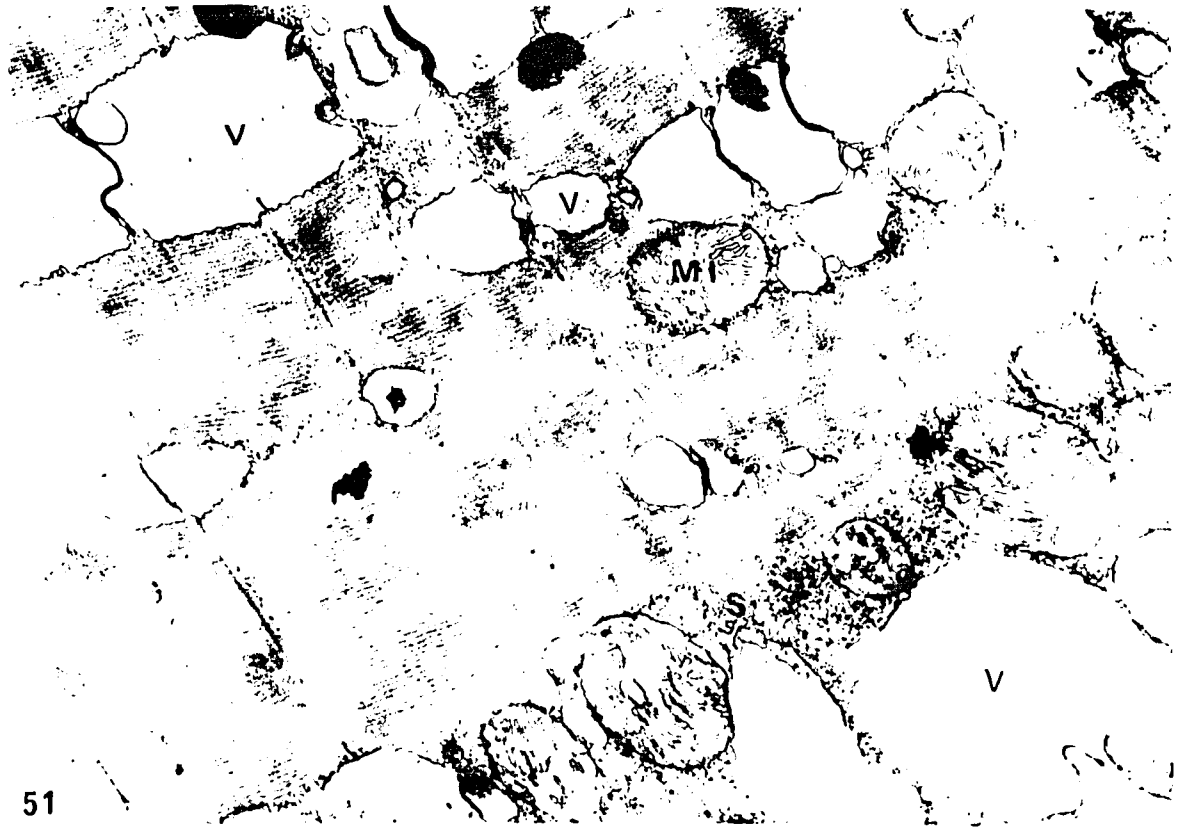


PLATE 6.14

Ultra-structure of the masseter muscle of young Salvelinus fontinalis larvae (longitudinal section).

Fig. 52. Abnormal fiber. The myofibrils are interrupted by a series of large irregular vesicles and mitochondria of irregular shapes are grouped. Dylox, 100 ppm for 24 hr. 16,800 x.

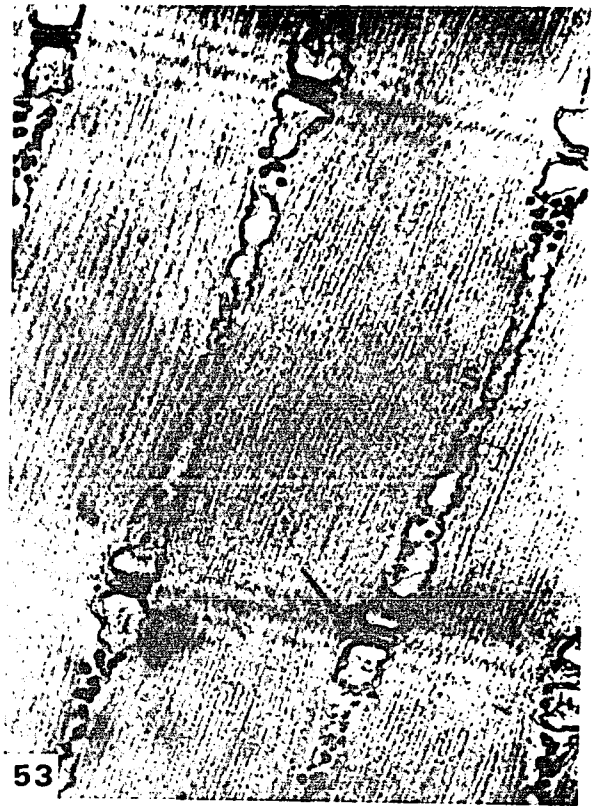
Fig. 53. Normal fiber of a control larva showing the details of the transverse (arrow) and longitudinal tubular systems (LTS) associated with large granules (glycogen). 46,000 x.

Fig. 54. Abnormal fiber. The longitudinal tubular system is swollen into large vesicles (V) at the Z line; large vesicles containing fine filamentous material are present between mitochondria of the peripheral sarcoplasm. Phosphamidon, 100 ppm for 24 hr. 15,300 x.

Fig. 55. Normal myofibril of a control showing the details of the I band and Z line. Note the irregular transverse filaments joining the actin filaments in the N zone (N). 155,000 x.

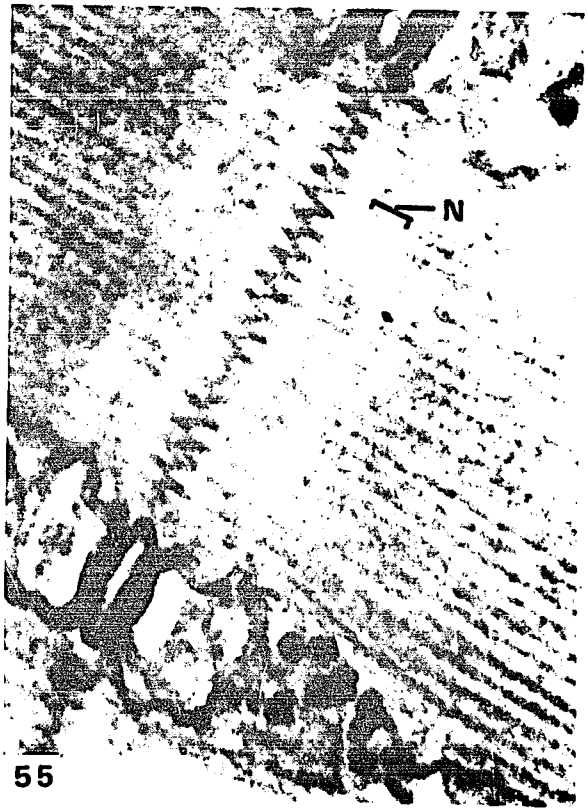
Plate 6.14


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53



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Plate 6.14

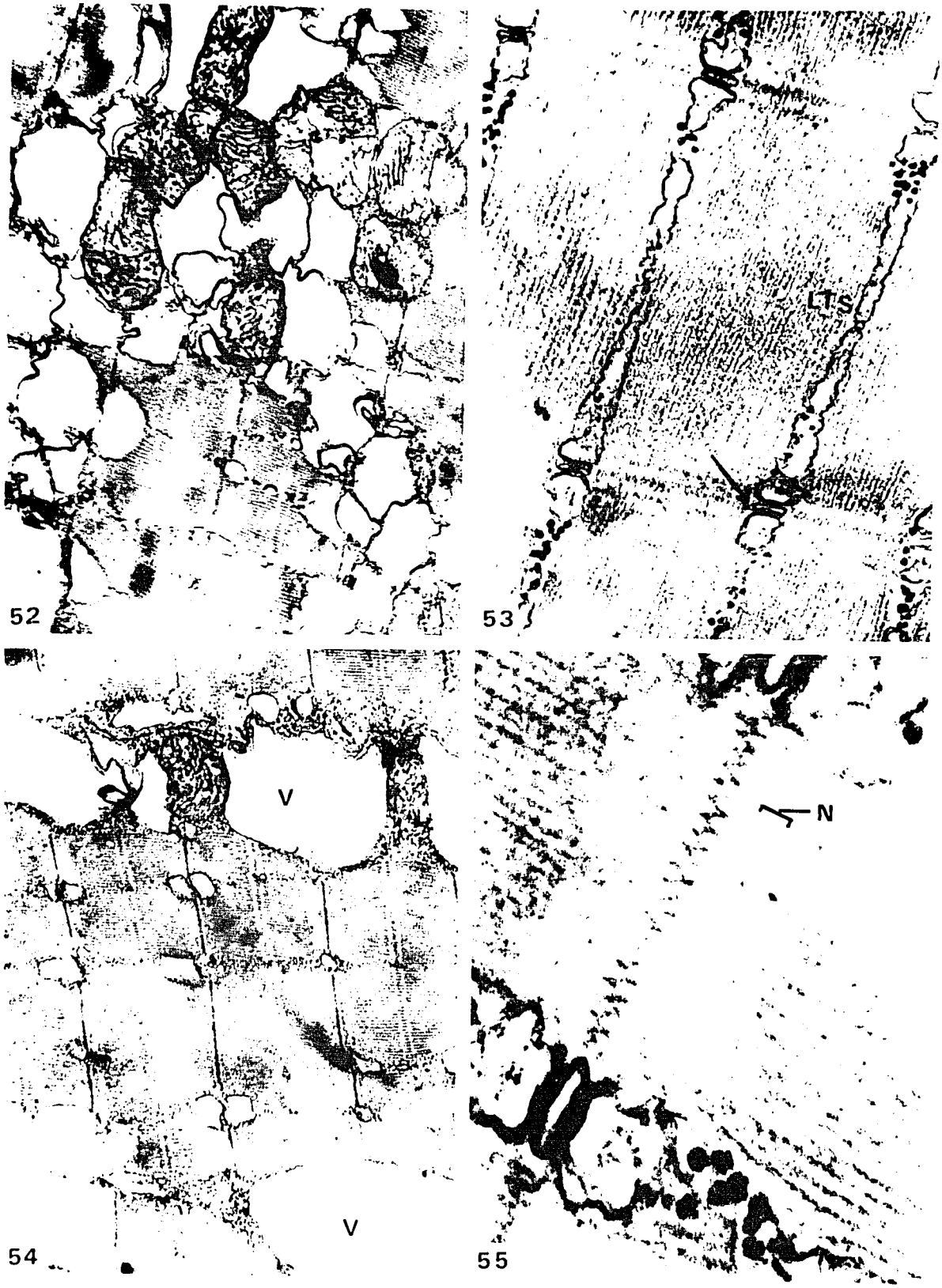


PLATE 6.15

Ultra-structure of the masseter muscle of young Salvelinus fontinalis larvae (longitudinal section).

Fig. 56. Abnormal fiber. Large vacuoles replace the sarcoplasmic reticulum; intermyofibrillar mitochondria are either rounded or more or less squared; the transverse tubular system is no longer visible in this region. Dylox, 100 ppm. 10,000 x.

Fig. 57. Abnormal fiber with mitochondrial aggregation next to large vesicles. Dylox, 100 ppm. 10,000 x.

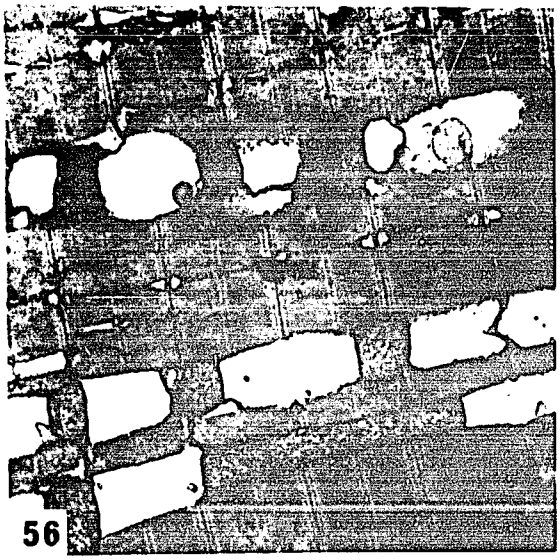
Fig. 58. Abnormal myofibrils showing the details of the I band. The clear zipper-like pattern is lost and the I band is wavy; the boundary between the A and I bands is no longer visible. Phosphamidon, 100 ppm. 60,000 x.

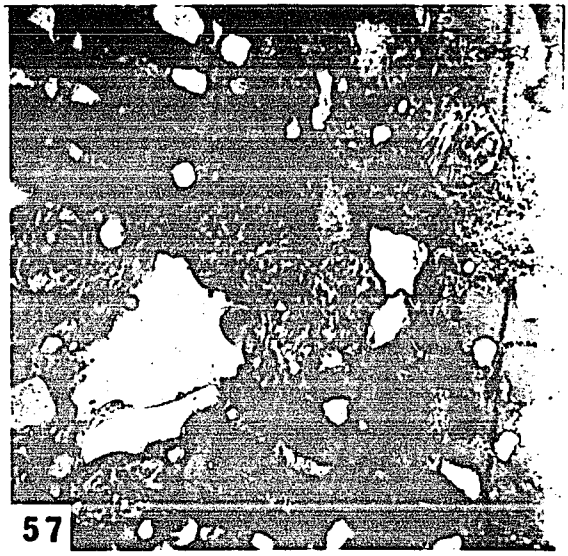
Fig. 59. Abnormal fiber. One mitochondria is abnormally large and some of its portions are devoid of cristae; the I band is also abnormal. Phosphamidon, 100 ppm. 30,000 x.

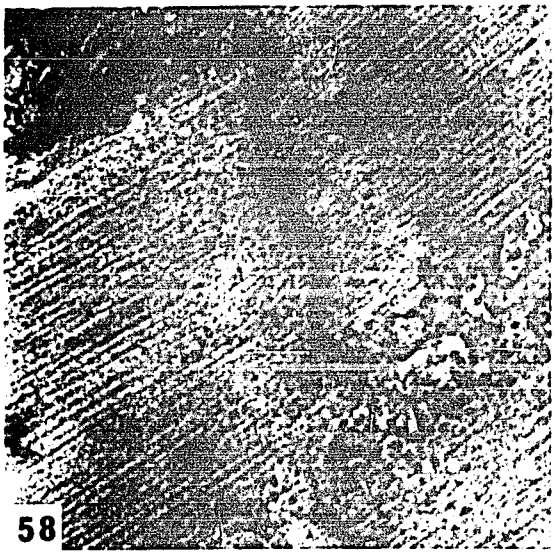
Fig. 60. Abnormal myofibril. No I band is visible and some granular material is associated with the filaments. Phosphamidon, 100 ppm. 60,000 x.

Fig. 61. Abnormal myofibril showing the details of the I band and Z line. The granules normally associated with the sarcoplasmic reticulum are absent. Dylox, 100 ppm. 60,000 x.

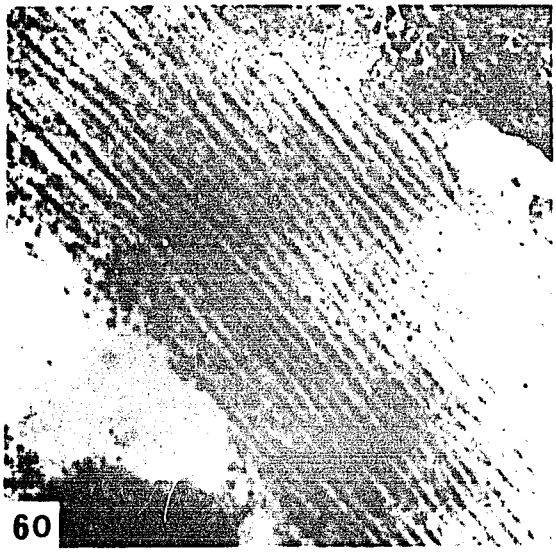
Plate 6.15


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57


58


59


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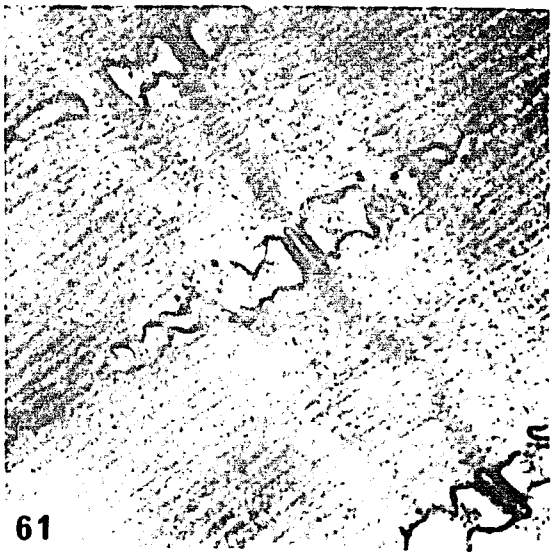
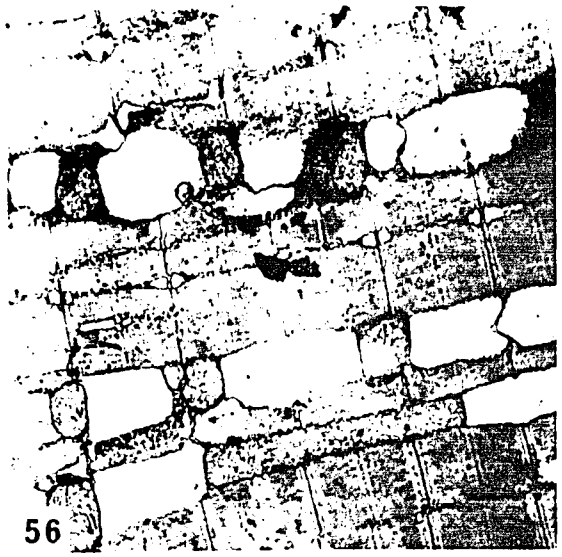
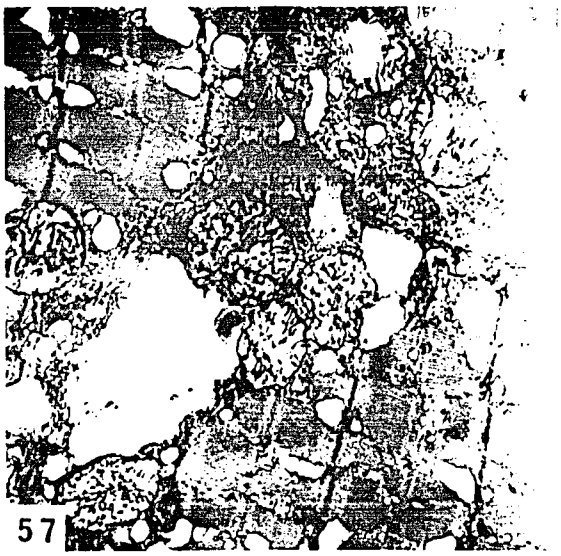
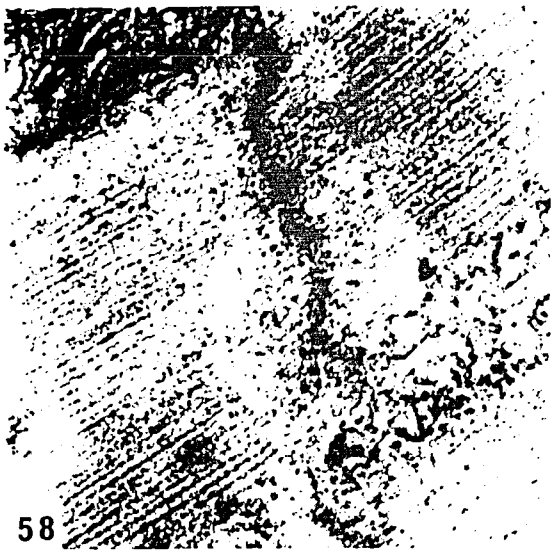
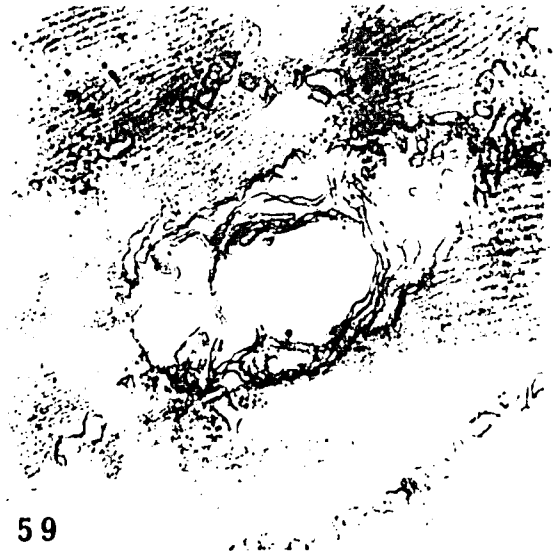

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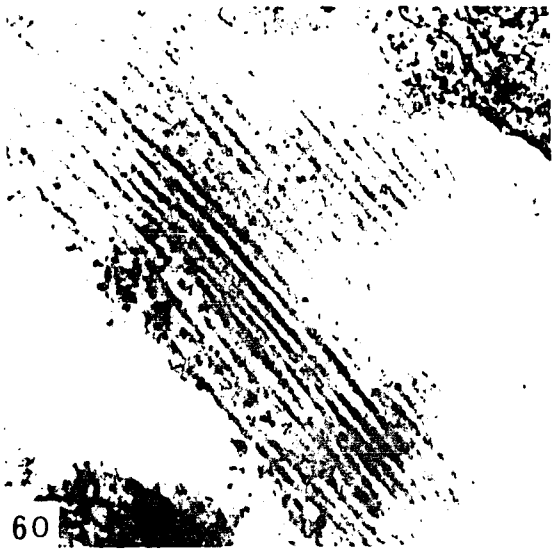
Plate 6.15


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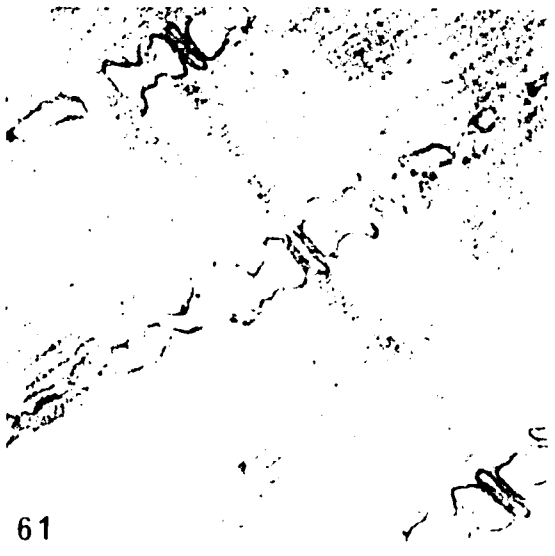

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PLATE 6.16

Ultra-structure of the masseter muscle of Salvelinus fontinalis larvae (longitudinal section).

Figure 62: young larva; Figures 63-65: old larvae.

Fig. 62. Abnormal myofibril showing the details of the I band and the triad. Dylox, 100 ppm. 93,000 x.

Fig. 63. Normal fibers of a control larva showing subsarcolemmal mitochondria. 15,500 x.

Fig. 64. Abnormal fiber. The subsarcolemmal mitochondria have few small broken cristae; the sarcoplasm is scanty and vacuolar. (The dark patch is a staining artefact). Phosphamidon, 100 ppm. 15,500 x.

Fig. 65. Abnormal myofibril. The sarcoplasmic reticulum is swollen into large vesicles. Dylox, 30 ppm. 46,500 x.

Plate 6.16

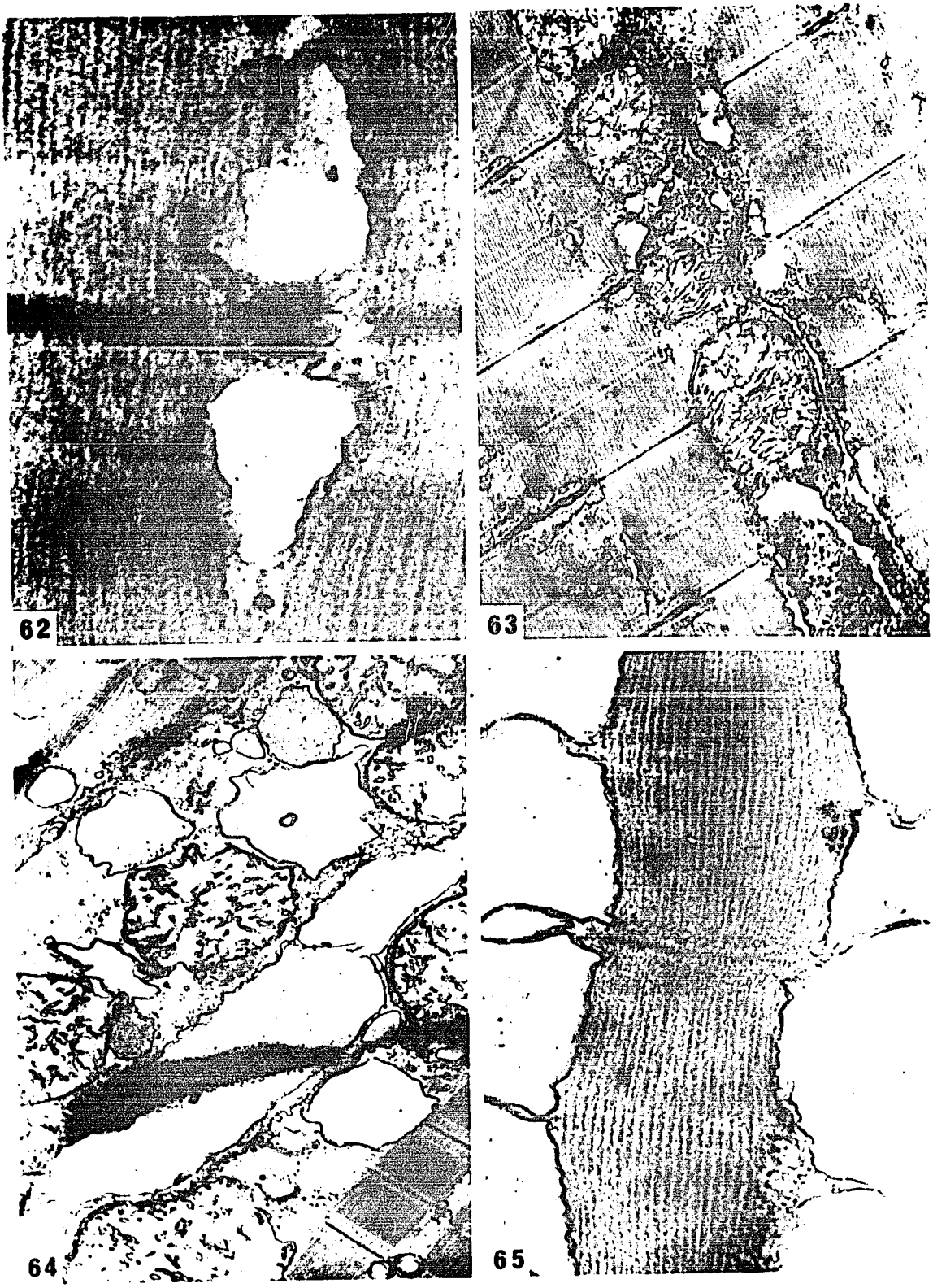


Plate 6.16


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PLATE 6.17

Ultra-structure of the masseter muscle in old Salvelinus fontinalis larvae (longitudinal section).

Fig. 66. Abnormal myofibril showing partial breakage along the N zone in the I band. Phosphamidon, 100 ppm. 60,000 x.

Fig. 67. Abnormal myofibril. The I band with the Z line is almost completely absent. Phosphamidon, 100 ppm. 20,000 x.

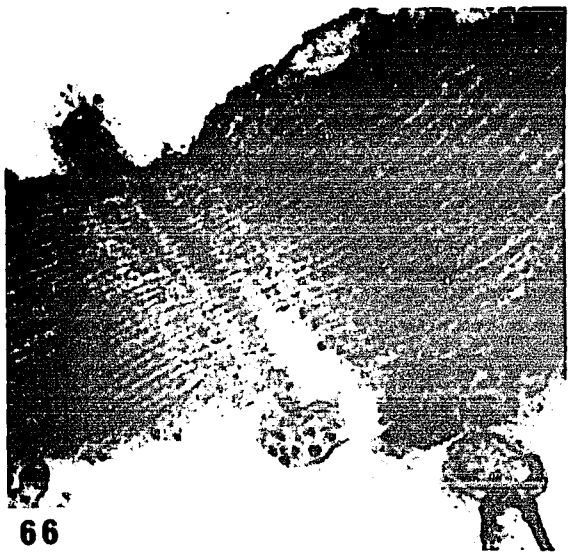
Fig. 68. Abnormal myofibril. The parallel arrangement of the myofilaments is lost in the A band (right) and only a tangled mass of filamentous material is left. Dylox, 30 ppm. 30,000 x.

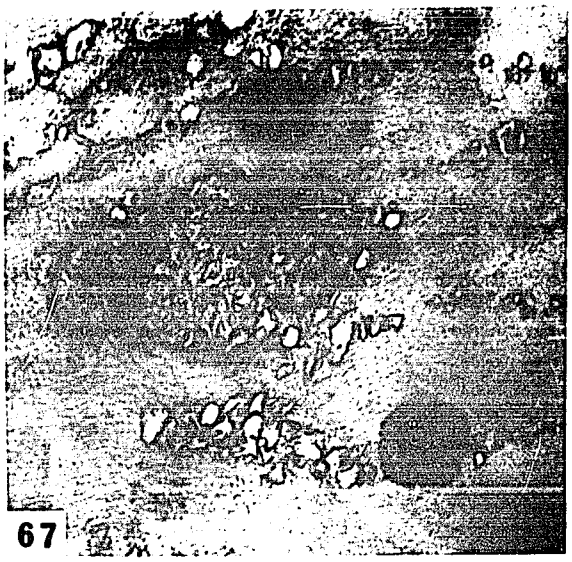
Fig. 69. Abnormal myofibril. The banding pattern is no longer visible. Dylox, 30 ppm. 60,000 x.

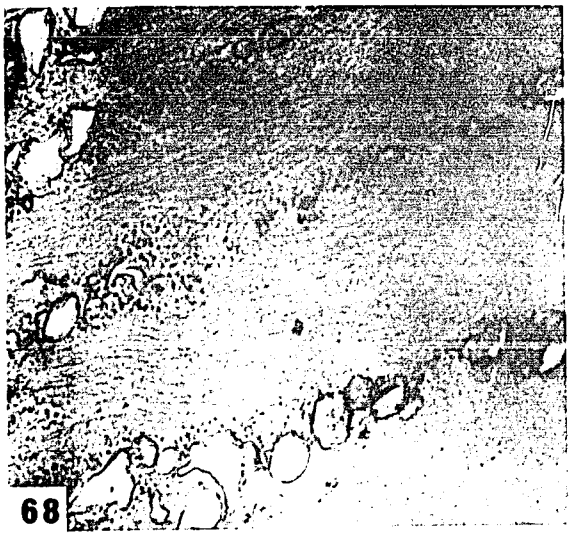
Fig. 70. Abnormal myofibril showing loose disorganization of myofilaments with concentration of material in the former A band. Phosphamidon, 100 ppm. 100,000 x.

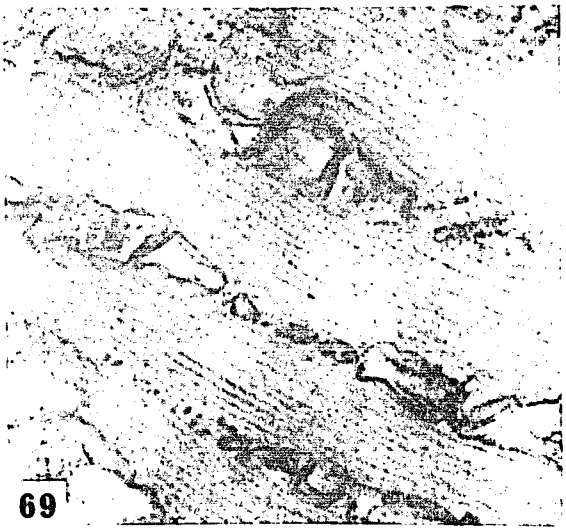
Fig. 71. Abnormal myofibril showing the details of the Z line. The zipper-like structure is lost and the actin filaments are broken and appear as dots. Dylox, 30 ppm. 60,000 x.

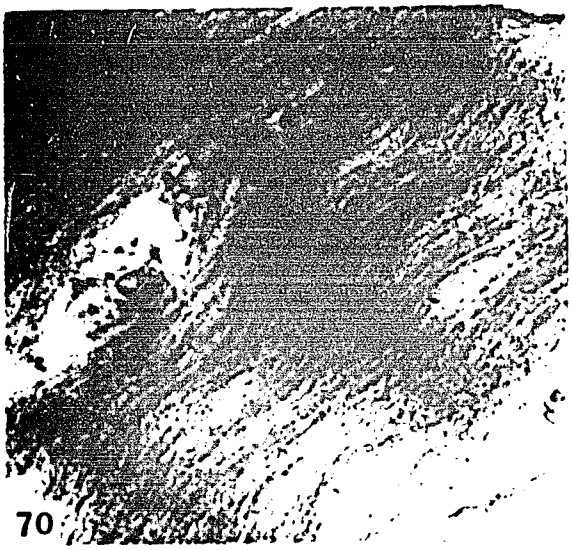
Plate 6.17


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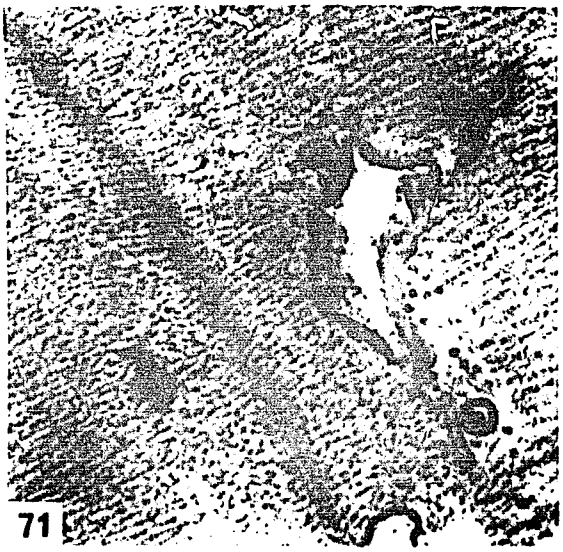
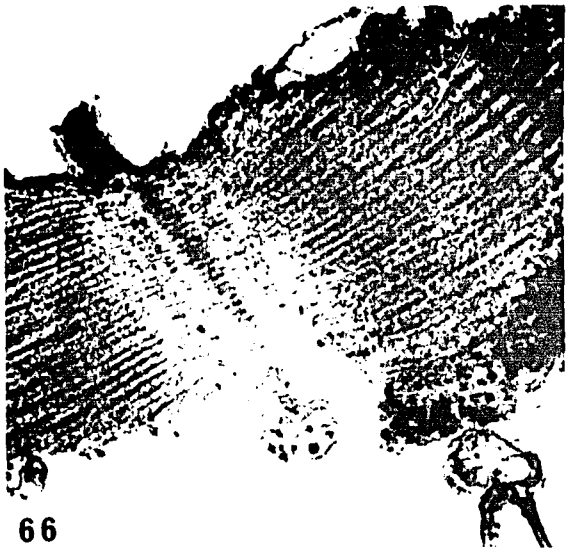
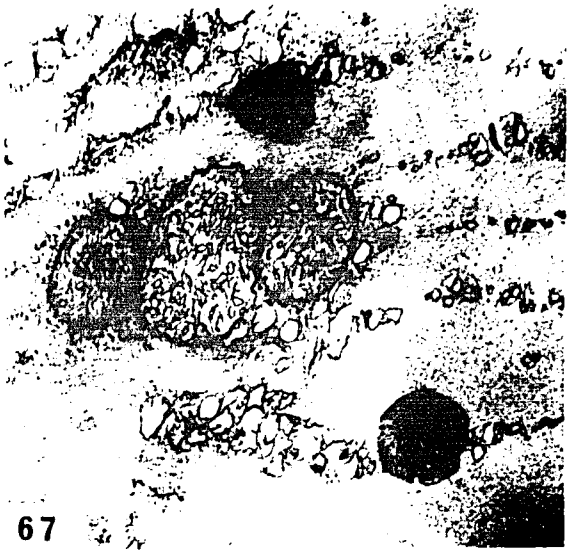
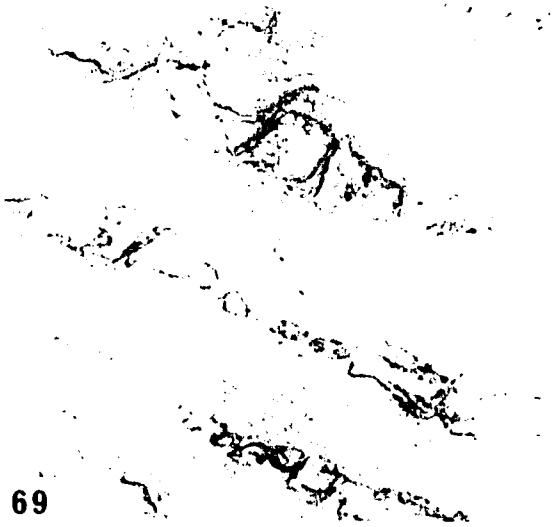

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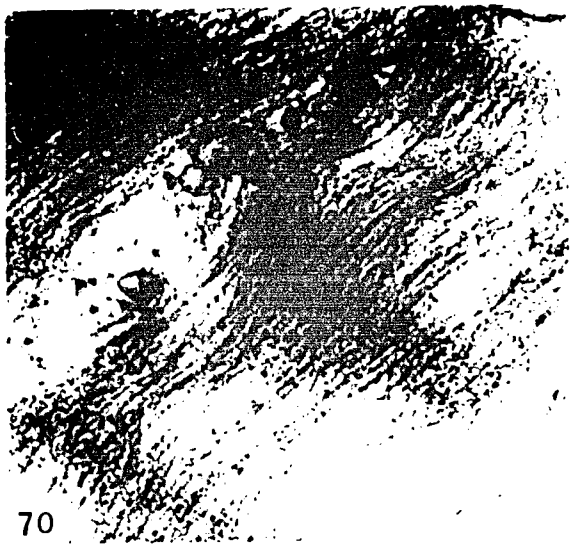
Plate 6.17


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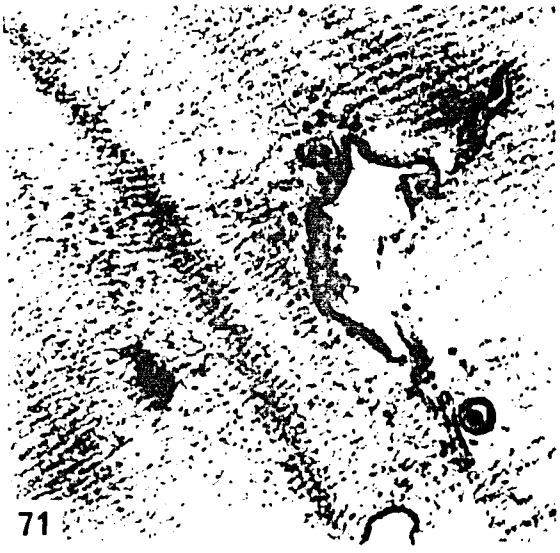

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PLATE 6.18

Ultra-structure of the masseter muscle of old Salvelinus fontinalis larvae (longitudinal section).

Fig. 72. Abnormal myofibrils separated from each other; the sarcoplasmic reticulum is partly desintegrated. Phosphamidon, 100 ppm. 60,000 x.

Fig. 73. Abnormal fiber showing a very large vacuole, next to the nucleus (NU), containing thin-walled vesicles of various sizes and shapes. Phosphamidon, 100 ppm. 47,000 x.

Fig. 74. Abnormal fiber showing the details of the spaces separating the myofibrils. The fine filaments in the empty spaces are seen branching from the main mass of filaments; the thickening of the myosin filament in the M line is evident; actin filaments end at the M line. Phosphamidon, 100 ppm. 95,000 x.

Plate 6.18







Plate 6.18

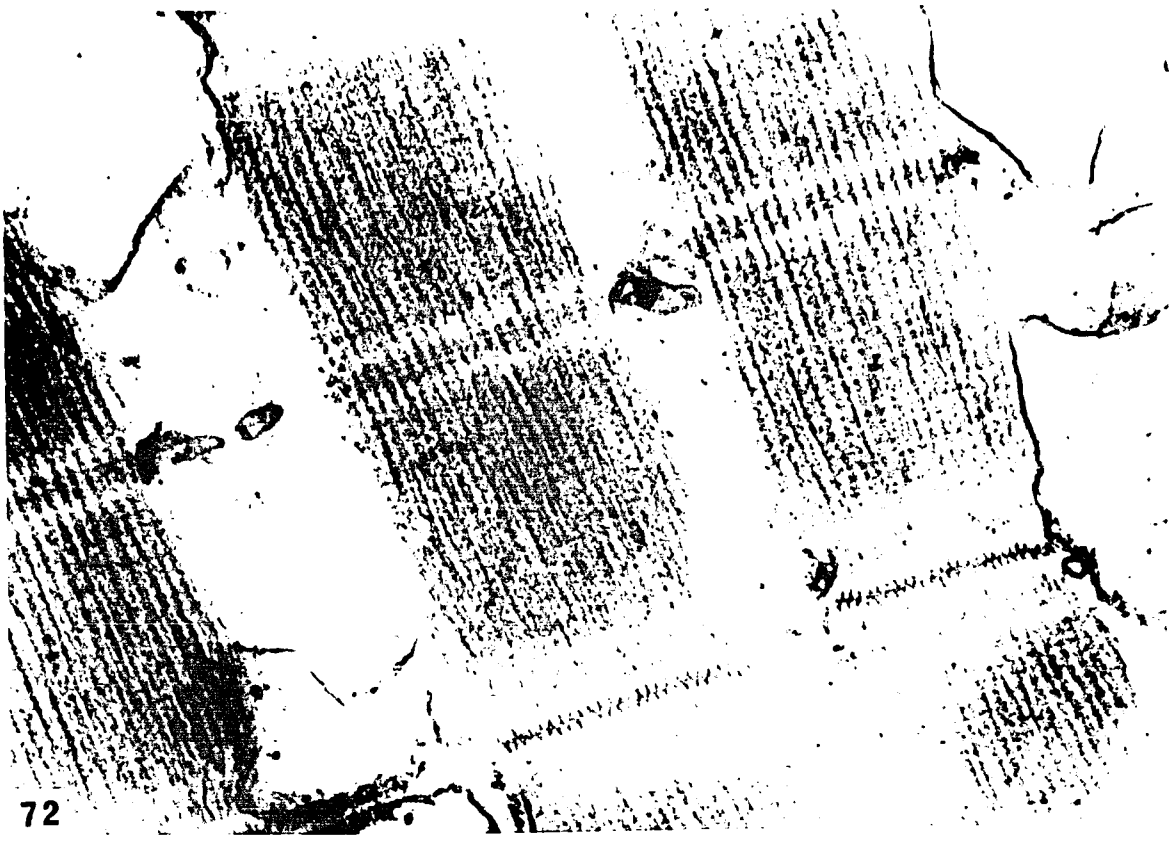
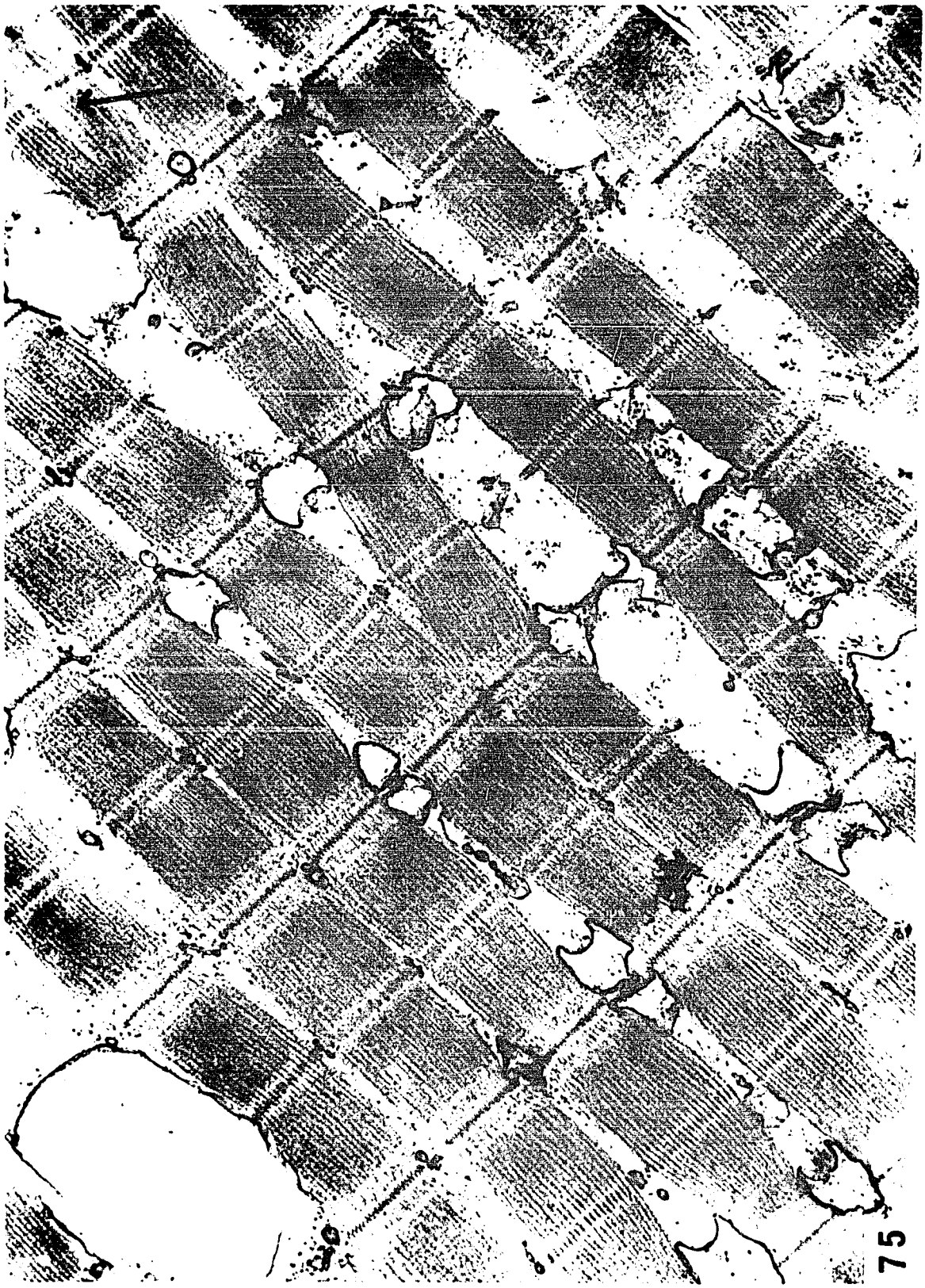
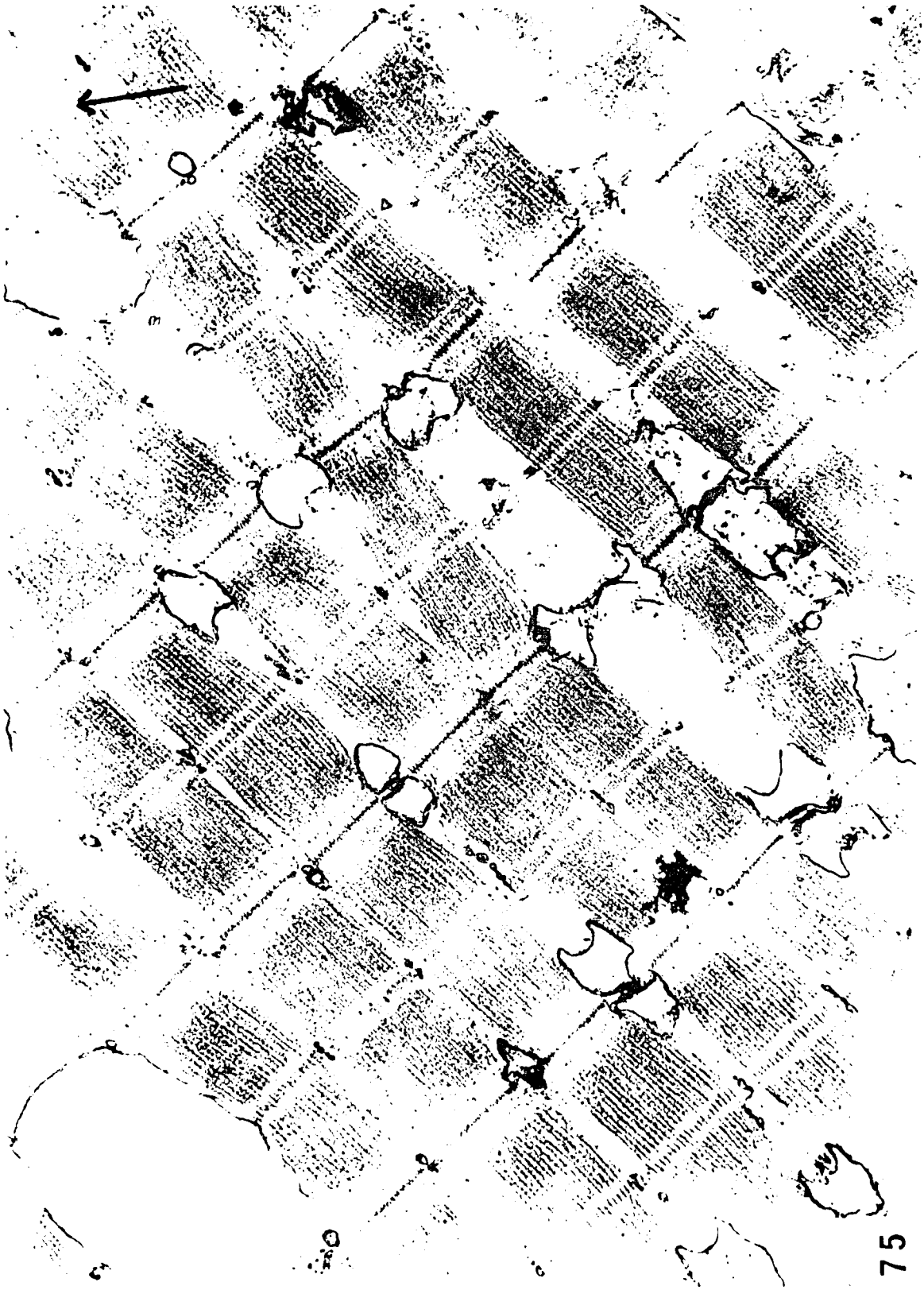






Fig. 75. Ultra-structure of the masseter muscle in old Salvelinus fontinalis larva (longitudinal section). Myofibrils are separated by empty spaces and lateral elements of triads are swollen into irregular vesicles. One myofibril is longitudinally split (arrow). Phosphamidon, 100 ppm. 40,000 x.


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CHAPTER SEVEN

CONCLUSION

1. The two organophosphates Phosphamidon and Dylox had a lethal, a teratogenic and an antigrowth action on the developing eggs of Brachydanio rerio and Salvelinus fontinalis. Phosphamidon was more potent than Dylox and its action was also more immediate than that of Dylox.

2. In Brachydanio rerio, Phosphamidon had an important lethal and teratogenic action if the period of treatment included the first part of gastrulation, even when the period of treatment was of short duration ($5\frac{1}{2}$ hr). This effect was not proportional to the dose but was evident with a threshold concentration of 600 ppm.

3. The most obvious teratologies were: a) reduction in the length of the body axis; b) curved, short and often blobby tail; c) abnormal muscle differentiation; d) reduction and abnormal organization of nervous tissue; e) irregular and reduced heart rate; f) hemostasis; g) edema of the cardiac region and failure of the heart to develop normally; h) capillary fragility and abnormal development of the vascular system manifested by extra vesicular blood.

4. The teratisms resulted from abnormal gastrulation and possibly the effect of the organophosphate on the differentiation of tissues.

5. In Salvelinus fontinalis embryos, Phosphamidon and Dylox were found to have important effects at concentrations of 100 and 200 ppm and the gastrulation period was found to be the most sensitive stage to the lethal, teratogenic and antigrowth action of the organophosphates.

The effects of the organophosphates on trout embryos differed from those in zebra fish in two aspects: a) the effect was more proportional to the dose; b) treatment during stages other than gastrulation resulted in the production of gross teratisms.

6. The main effects of organophosphate treatment during the first half of development were the following: decreased hatching, delayed development, important reduction in the size of the embryo, severe abnormalities of nervous and muscular tissue.

7. The inhibition of ChE is of secondary importance in the production of the abnormalities since these originated at a time when no ChE can be detected. The abnormalities resulted from abnormal gastrulation and the partial inhibition of tissue growth and differentiation. The relationship between the higher sensitivity of the gastrulation period and a possible effect of the organophosphates on the important factors involved in gastrulation such as respiration, protein and RNA synthesis has been discussed. An effect on glycolysis from the inhibition of the enzyme phosphorylase has been suggested as a possible cause of abnormal gastrulation.

8. Histochemical reactions have shown that in Salvelinus fontinalis embryos: a) ChE can be detected for the first time at the 12th day in the hindbrain, in the anterior part of neural tube and in presumptive muscle; b) the enzyme gradually becomes localized in more anterior and posterior parts of the brain and neural tube; c) the bulk of ChE synthesis in the brain occurs during the last six days before hatching; d) the enzyme malic dehydrogenase, at 12 days, has a higher activity in the future white matter of the brain, in the cells which show ChE activity and in the cells along the future myotomal septa; e) at the 19th day, the basic pattern of activity found in the larva is already present.

9. The main effects of organophosphate treatment during the first half of development were decreased hatching, delayed development, important reduction in the size of the embryo and severe abnormalities

of nervous and muscular tissues. The inhibition of ChE would be of secondary importance in the production of these abnormalities.

10. Prehatching treatment did not decrease the percentage of hatching but caused a slight reduction in growth which was compensated for after treatment ended. Prehatching treatment also resulted in earlier hatching and protected the young larvae against the lethal action of the organophosphate. Motility of the embryo did not appear to be critical for normal hatching to occur.

11. The study of Dylox on Salmo gairdneri irideus larvae and of Phosphamidon and Dylox on Salvelinus fontinalis larvae has shown that these organophosphates were lethal in concentrations ranging from 5 to 100 ppm and that old larvae were much more susceptible than younger larvae. In Salvelinus fontinalis, both compounds were much more toxic to larvae than to embryos; Dylox, which was less toxic than Phosphamidon in embryos, was found to be more toxic than Phosphamidon in larvae.

12. Important histopathological changes were observed in treated larvae and these changes were more extensive in older larvae than in young larvae. Stomachal glands, liver, heart, blood, gills, pseudogills, mucosa of the digestive tract and brain were damaged. Severe disruption of the structure of the muscle fibers was also characteristic.

13. The mortality percentages in the treated larvae were more related to the extent of tissue damage than to the rate and final degree of ChE inhibition or the rate of reactivation of the phosphorylated ChE.

14. The small muscle fibers which are rich in oxidative and phosphorylase enzymes suffered more extensive damage than the other types of fibers. The relationship of this damage to the physiological and histochemical properties has been discussed.

15. The study of the muscles from treated larvae with the electron microscope has shown that the damage was associated with clumping of abnormal mitochondria and the production of intermyofibrillar spaces and of membrane-bounded vacuoles originating from a swelling of the longitudinal tubular system. Disruption of the structure of the myofibrils appeared to be non-specific.

16. No significant change was detected in the histochemical activity of the enzymes: cytochrome oxidase, malic and lactic acid dehydrogenases, NADH diaphorase, acid and alkaline phosphatases and ATPase. With some of these enzymes, however, the distribution of the reaction within the cells was modified. A significant change in the activity of the enzyme phosphorylase was observed in treated larvae.

17. Although it is still debatable whether some of the histological damage is due to the direct action of the organophosphates, it appears that the histopathological changes and the inhibition of the enzyme phosphorylase are key factors in the toxic action of the organophosphates studied.

18. Histochemical staining showed that the distribution of dehydrogenases, phosphorylases and phosphatases was similar to other species of fish and other vertebrates. It also revealed that the olfactory mucosa, the retina and pseudogills are of interest from an histochemical point of view and that further work should be done on these tissues in relation to differentiation.

19. The finding of the inhibition of the enzyme phosphorylase by organophosphate can be of importance in developmental biology, since it can be used as an interesting tool in studying the role of this enzyme during development and especially during muscle differentiation.

20. This study also showed that, in the fight for wildlife conservation, several aspects should be studied before a chemical compound is considered as safe since the toxicity of a compound can vary during the different stages of the life cycle of a species. In the use of these compounds the period of application of the insecticide should be considered since the period of application could coincide with a period of high sensitivity in development. It is unlikely that trout embryos could be affected during field application because periods of application do not coincide with periods of embryonic development and because it is unlikely that concentrations of 100 or 200 ppm would be attained after field application of insecticides.

It is quite possible, however, that larvae could be affected because field applications are usually done during late spring or early summer which corresponds to the late larval or beginning of feeding periods, and field application could result in concentrations of 5 or 10 ppm which are deleterious to old larvae. Attention should be drawn also to the fact that the assessment of the lethal action of a compound during field application can lead to erroneous results if the parameter used is the counting of dead fish in the neighbouring regions of application. The deleterious action on larvae could easily be overlooked because larvae are small and not readily observable and because poisoned larvae become the easy prey of big fish.

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APPENDIX I

Numerical and statistical values (homogeneity and X^2-test) of experiments with Brachydanio rerio.

1. TABLE 3.1

		D	Hn	Hct	uH	Total
a) Control.	1)	1	34	0	5	40
	2)	0	38	0	2	40
	3)	0	39	0	1	40
	4)	2	36	0	2	40
	5)	1	37	0	2	40
		4	184	0	12	200

Homogeneity: $p > .40$

b) 100 ppm.	1)	2	37	0	1	40
	2)	1	35	0	4	40
	3)	1	36	0	3	40
		4	108	0	8	120

Homogeneity: $p > .40$
Comparison with control: $p > .10$

c) 200 ppm.	1)	0	37	0	3	40
	2)	2	36	0	2	40
	3)	2	34	0	4	40
		4	107	0	9	120

Homogeneity: $p > .40$
Comparison with control: $p > .40$

d) 400 ppm.	1)	12	23	5	0	40
	2)	7	27	6	0	40
	3)	4	14	2	0	20
		23	64	13	0	100

Homogeneity: $p > .40$
Comparison with control: $p < .01$

e) 600 ppm. Evident: 100% mortality.

f) 800 ppm. Evident: 100% mortality.

2. TABLE 3.2

		D	N	Mi	Ma	Total	H	uH
a) Control.	1)	1	39	0	0	40	39	1
	2)	1	38	1	0	40	39	1
	3)	3	37	0	0	40	37	3
		5	114	1	0	120	115	5

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$

b) 600 ppm, stages 12-23: evident 100% mortality.

c) 600 ppm, stages 20-24.

1)	33	0	1	6	40	1	39
2)	35	0	3	2	40	3	37
	68	0	4	8	80	4	76

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

d) 400 ppm, stages 22-24.5.

1)	10	0	40	0	50	35	15
2)	15	0	35	0	50	30	20
	25	0	75	0	100	65	35

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .20$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

e) 600 ppm, stages 22-24.5.

1)	16	0	24	0	40	24	16
2)	22	0	18	0	40	18	22
	38	0	42	0	80	42	38

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .10$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

f) 400 ppm, stages 23-25.

1)	19	0	21	0	40	19	21
2)	13	0	27	0	40	24	16
	32	0	48	0	80	43	37

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

g) 600 ppm, stages 23-25.

	D	N	Mi	Ma	Total	H	uH
1)	35	0	5	0	40	2	38
2)	39	0	1	0	40	0	40
3)	20	0	0	0	20	0	20
	94	0	6	0	100	2	98

Homogeneity: Dead and abnormal $p > .30$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

3. 36-HR TREATMENT

a) Control.

	Live	Dead	Total	H	uH
1)	39	1	40	35	5
2)	37	3	40	34	6
3)	38	2	40	38	2
	114	6	120	107	13

Homogeneity: Dead $p > .20$
Hatched $p > .20$

b) 400 ppm.

1)	49	1	50	42	8
2)	46	4	50	39	11
3)	49	1	50	37	13
	144	6	150	118	32

Homogeneity: Dead $p > .20$
Hatched $p > .40$
Comparison with control: Dead $p > .20$
Hatched $p < .01$

c) 600 ppm

1)	12	28	40	9	31
2)	19	21	40	15	25
	31	49	80	24	56

Homogeneity: Dead $p > .20$
Hatched $p > .40$
Comparison with control: Dead $p < .01$
Hatched $p < .01$

4. 24-HR TREATMENT, 400 PPM

a) Control.

	D	N	Mi	Ma	Total	H	uH
1)	2	58	0	0	60	55	5
2)	0	58	0	0	58	53	5
3)	1	59	0	0	60	52	8
4)	1	58	0	0	59	51	8
5)	1	58	1	0	60	56	4
	5	291	1	0	297	267	30

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$

b) Stages 4-28.

1)	9	26	5	0	40	11	29
2)	7	24	9	0	40	14	26
3)	7	28	5	0	40	16	24
	23	78	19	0	120	41	79

Homogeneity: Dead and abnormal $p > .20$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

c) Stages 10-34.

1)	1	39	0	0	40	36	4
2)	1	39	0	0	40	34	6
3)	3	37	0	0	40	33	7
	5	115	0	0	120	103	17

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p > .05$
Hatched $p > .20$

d) Stages 24-48.

1)	4	36	0	0	40	36	4
2)	1	39	0	0	40	37	3
	5	75	0	0	80	73	7

Homogeneity: Dead and abnormal $p > .30$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p > .05$
Hatched $p > .40$

e) Stages 72-96.

1)	7	33	0	0	40	32	8
2)	4	34	0	0	38	33	5
	11	67	0	0	78	65	13

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p < .01$

5. TABLE 3.3 (600 ppm, 24 hr)

a) See Control (no. 4 a) p. 324.

b) Stages 12-21.

	D	N	Mi	Ma	Total	H	uH
1)	44	5	5	6	60	14	46
2)	54	1	3	2	60	5	55
3)	47	2	7	4	60	9	51
4)	50	4	2	4	60	6	54
5)	37	0	9	4	50	9	41
	232	12	26	20	290	43	247

Homogeneity: Dead and abnormal $p > .20$
Hatched $p > .20$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

c) Stages 17-22.

1)	2	73	5	0	80	67	13
2)	4	64	12	0	80	55	25
3)	6	67	7	0	80	58	22
	12	204	24	0	240	180	60

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .05$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

d) Stages 18-22.

1)	0	76	4	0	80	74	6
2)	1	78	1	0	80	75	5
3)	2	76	2	0	80	67	13
	3	230	7	0	240	216	24

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .05$
Comparison with control: Dead and abnormal $p > .05$
Hatched $p > .40$

e) Stages 20-23.

1)	1	79	0	0	80	67	13
2)	0	80	0	0	80	74	6
3)	1	79	0	0	80	74	6
	2	238	0	0	240	215	25

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .20$
Comparison with control: Dead and abnormal $p > .40$
Hatched $p > .40$

f) Stages 22-23.5.

	D	N	Mi	Ma	Total	H	uH
1)	4	74	2	0	80	62	18
2)	7	72	1	0	80	61	19
3)	3	77	0	0	80	69	11
	14	223	3	0	240	192	48

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

g) Stages 23-24.

1)	5	45	0	0	50	39	11
2)	2	48	0	0	50	30	20
	7	93	0	0	100	69	31

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .10$
Comparison with control: Dead and abnormal $p < .02$
Hatched $p < .01$

h) Stages 23.5-24.5.

1)	16	42	2	0	60	31	29
2)	10	48	0	0	58	32	26
3)	22	54	4	0	80	36	44
	48	144	6	0	198	99	99

Homogeneity: Dead and abnormal $p > .05$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

i) Stages 24-25.

1)	10	20	0	0	30	19	11
2)	2	18	0	0	20	16	4
	12	38	0	0	50	35	15

Homogeneity: Dead and abnormal $p > .10$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

6. TABLE 3.4

a) See Control (no. 5 a) p. 324.

b) 400 ppm, stages 12-16.

	D	N	Mi	Ma	Total	H	uH
1)	2	58	0	0	60	53	7
2)	4	56	0	0	60	50	10
3)	1	59	0	0	60	55	5
	7	173	0	0	180	158	22

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p > .40$
Hatched $p > .40$

c) 400 ppm, stages 15.5-18.

1)	3	57	0	0	60	53	7
2)	0	60	0	0	60	56	4
3)	2	58	0	0	60	55	5
	5	175	0	0	180	164	16

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p > .40$
Hatched $p > .40$

d) 600 ppm, stages 12-16.

1)	19	11	10	20	60	17	43
2)	5	15	5	15	40	18	22
3)	8	7	8	17	40	11	29
4)	13	17	17	13	60	22	38
5)	23	23	13	21	80	29	51
6)	15	13	16	16	60	15	45
7)	16	9	12	13	50	12	38
	99	95	81	115	390	124	266

Homogeneity: Dead and abnormal $p > .20$
Hatched $p > .20$
Comparison with control: Dead and abnormal $p < .01$
Hatched $p < .01$

e) 600 ppm, stages 15.5-18

1)	2	37	1	0	40	34	6
2)	1	55	4	0	60	54	6
3)	2	37	1	0	40	36	4
4)	3	54	3	0	60	53	7
	8	183	9	0	200	177	23

Homogeneity: Dead and abnormal $p > .40$
Hatched $p > .40$
Comparison with control: Dead and abnormal $p < .05$
Hatched $p > .40$

7. TABLE 3.5

a) Treated with 600 ppm.

Comparison of normal with pierced: Dead and abnormal $p > .40$
Hatched $p > .40$

b) Control.

Comparison of normal with pierced: Dead and abnormal $p > .20$
Hatched $p > .40$

8. EFFECT OF DYLOX

Dose	D	N	Abn	Total	H	uH
Control	2	38	0	40	37	3
100	1	39	0	40	38	2
200	3	37	0	40	36	4
400	2	20	18	40	18	22
600	20	9	11	40	14	26
800	40	0	0	40	0	40
1000	40	0	0	40	0	40

X^2-test.

Dose	D-N-Abn	H-uH
100	> .40	> .40
200	> .40	> .40
400	< .01	< .01
600	< .01	< .01
800	< .01	< .01
1000	< .01	< .01

APPENDIX II

1. Mortality rate during or shortly after treatment: comparison between control and treated.

	Days	Dead	Total	X^2	p
Z	3	54	1763		
	6	91	1689		
	9	72	1564		
	12	51	1452		
	15	45	1384		
A	3	25	594	4.3	< .05
B	6	62	570	> 15	< .01
C	6	51	615	4.6	< .05
D	12	35	535	> 6	< .02
E	15	33	522	> 6	< .02
F	3	123	572	> 10	< .01
G	6	224	415	> 10	< .01
H	6	81	695	> 6	< .02
I	12	91	554	> 10	< .01
J	15	121	518	> 10	< .01
K	3	39	606	> 6	< .02
L	3	206	584	> 10	< .01
M	6	208	644	> 10	< .01
N	12	198	474	> 10	< .01
O	5	185	480	> 10	< .01
P	3	28	542	4.03	< .05
Q	6	157	573	> 10	< .01
R	6	70	619	> 10	< .01
S	9	28	566	.20	> .10
T	15	91	460	> 10	< .01
U	3	46	613	> 15	< .01
U	6	282	557	> 10	< .01
V	6	380	600	> 10	< .01
W	6	265	642	> 10	< .01
X	12	18	472	.20	> .10
Y	15	49	529	> 22	< .01

2. Total mortality at the end of treatment: comparison between control and treated.

Group	Days	Dead	Total	X^2	p
Z	3	54	1763		
	6	146	1763		
	12	274	1763		
	16.5	342	1763		
A	3	25	594	4.3	< .05
B	6	78	585	> 13	< .01
C	6	70	634	4.20	< .05
D	12	114	623	2.55	> .05
E	16.5	121	602	.137	> .40
F	3	123	572	> 10	< .01
G	6	325	509	> 10	< .01
H	6	103	717	> 13	< .01
I	12	179	650	> 10	< .01
J	16.5	259	617	> 10	< .01
K	3	39	606	> 10	< .01
L	3	206	584	> 10	< .01
M	6	444	662	> 10	< .01
N	12	330	612	> 10	< .01
O	16.5	362	601	> 10	< .01
P	3	28	542	5.23	< .02
Q	6	180	596	> 10	< .01
R	6	96	645	> 16	< .01
S	12	75	597	3.21	> .05
T	16.5	221	606	> 10	< .01
U	3	259	613	> 10	< .01
V	6	330	640	> 10	< .01
W	6	280	657	> 10	< .01
X	12	76	560	2.3	> .05
Y	16.5	180	625	> 43	< .01

3. Total mortality at day 26: comparison between control and treated.

Group	Dead	Total	X^2	p
Z	486	1763		
A	171	594	.36	> .40
B	183	585	2.34	> .10
C	155	634	2.50	> .10
D	174	623	.02	> .40
E	138	602	4.96	< .05
F	251	572	> 10	< .01
G	373	509	> 10	< .01
H	206	717	.35	> .40
I	328	650	> 10	< .01

Group	Dead	Total	X^2	p
J	293	617	> 10	< .01
K	304	606	> 10	< .01
L	409	584	> 10	< .01
M	578	662	> 10	< .01
N	492	612	> 10	< .01
O	418	601	> 10	< .01
P	194	542	> 10.2	< .01
Q	299	596	> 10	< .01
R	250	645	> 10	< .01
S	198	597	> 4.72	< .02
T	289	606	> 10	< .01
U	463	613	> 10	< .01
V	548	640	> 10	< .01
W	393	657	> 10	< .01
X	202	560	> 10.5	< .01
Y	317	625	> 10	< .01

4. Total mortality at day 32: comparison between control and treated.

Z	793	1763		
A	239	594	4.02	< .05*
B	336	585	> 5.0	< .05
C	250	634	5.82	< .05*
D	273	623	> 6.0	< .05*
E	226	602	> 5.87	< .05*
F	352	572	> 10	< .01
G	442	509	> 10	< .01
H	414	717	> 10	< .01
I	453	650	> 10	< .01
J	391	617	> 10	< .01
K	404	606	> 10	< .01
L	482	584	> 10	< .01
M	606	662	> 10	< .01
N	538	612	> 10	< .01
O	469	601	> 10	< .01
P	249	542	.15	> .40
Q	402	596	> 8	< .01
R	380	645	> 10	< .01
S	323	595	> 6.27	< .02
T	395	606	> 10	< .01
U	496	613	> 10	< .01
V	598	640	> 10	< .01
W	455	657	> 10	< .01
X	355	560	> 10	< .01
Y	383	625	> 10	< .01

* Better viability than control.

5. Mortality rate at day 32: comparison between control and treated.

Group	Dead	Total	X^2	p
Z	269	1118		
A	60	366	7.49	< .01*
B	130	358	> 10	< .01
C	84	423	3.50	> .05
D	90	408	0.482	> .40
E	81	426	4.45	< .02*
F	71	285	.07	> .40
G	45	89	> 10	< .01
H	188	461	> 10	< .01
I	112	287	> 10	< .01
J	84	278	4.32	< .05
K	74	225	5.92	< .02
L	53	127	> 10	< .01
M	27	62	> 10	< .01
N	36	95	> 6.69	< .01
O	43	153	1.18	> .20
P	45	286	> 7.08	< .01*
Q	87	251	> 8.93	< .01
R	114	348	> 7.62	< .01
S	113	360	> 5.68	< .02
T	51	237	0.87	> .20
U	25	113	.26	> .40
V	31	58	> 10	< .01
W	51	218	.04	> .40
X	137	320	> 10	< .01
Y	60	278	.75	> .40

*Better viability than control.

6. Total mortality at the end of treatment and at day 32: comparison inter-group.

Group	End of treat.	X^2	p	at day 32	X^2	p
2-5 days						
A-F	25/594-123/572	>10	<.01	239/594-352/572	>12.7	<.01
A-K				239/594-404/606	>15	<.01
A-L	25/594-206/584	>10	<.01	239/594-482/584	>10	<.01
F-K	123/572- 39/606	>10	<.01	352/572-404/606	3.38	>.05
F-L	123/572-206/584	>10	<.01	352/572-482/584	> 5.5	<.02
F-P	123/572- 28/542	>10	<.01	352/572-249/542	>12.6	<.01
K-U	30/606-259/613	>10	<.01	404/606-496/613	> 8.41	<.01
L-U	206/584-259/613	> 6.66	<.01	482/584-496/613	.516	>.40
P-U	28/542-259/613	>10	<.01	249/542-496/613	>10	<.01
2-8 days						
B-G	78/585-325/609	>10	<.01	336/585-442/509	>10	<.01
G-Q	325/609-180/596	>10	<.01	442/509-402/596	>10	<.01
Q-V	180/596-330/640	>10	<.01	402/596-598/640	>10	<.01

Group	End of treat.	X^2	p	at day 32	X^2	p
5-8 days						
C-H	70/634-103/717	3.32	>.05	250/634-414/717	>10	<.01
C-M	70/634-444/662	>10	<.01	250/634-606/662	>10	<.01
H-M	103/717-444/662	>10	<.01	414/717-606/662	>10	<.01
H-R	103/717- 96/645	.07	>.40	414/717-380/645	.74	>.20
R-W	96/645-280/657	>10	<.01	380/645-455/657	>10	<.01
8-14 days						
D-I	114/623-179/650	>10	<.01	273/623-453/650	>10	<.01
D-N	114/623-330/612	>10	<.01	273/623-538/612	>10	<.01
I-N	179/650-330/612	>10	<.01	453/650-538/612	>10	<.01
I-S	179/650- 75/597	>10	<.01	453/650-323/597	>10	<.01
S-X	75/597- 76/560	.27	>.40	323/597-355/560	> 4.3	<.05
12.5-18.5 days						
E-J	121/602-259/617	>10	<.01	226/602-391/617	>10	<.01
E-O	121/602-362/601	>10	<.01	226/602-469/601	>10	<.01
J-O	259/617-362/601	>10	<.01	391/617-469/601	>10	<.01
J-T	259/617-221/606	3.92	<.05	391/617-395/606	.56	>.40
T-Y	221/606-180/625	> 5.40	<.05	395/606-383/625	1.99	>.10

7. Hatching and mortality at day 36: comparison of control with each group.

Group	H	D	uH	Total	X^2	p
Z	169	40	16	225		
A	84	8	8	100	5.21	> .10
B	72	62	7	141	7.56	< .05
C	150	25	15	190	1.52	> .40
D	76	25	1	102	0.021	> .40
E	78	38	20	136	8.04	< .02
F	13	40	0	53	> 10	< .01
G	7	15	0	22	> 10	< .01
H	33	72	7	112	> 10	< .01
I	9	61	9	79	> 10	< .01
J	29	69	12	110	> 10	< .01
K	26	54	0	80	> 10	< .01
L	16	28	1	45	> 10	< .01
M	0	8	4	12	> 10	< .01
N	1	8	11	20	> 10	< .01
O	10	69	8	87	> 10	< .01
P	55	31	0	86	3.82*	> .05
Q	23	48	17	88	> 10	< .01
R	44	51	24	119	> 10	< .01
S	25	106	22	143	> 10	< .01
T	4	23	14	41	> 10	< .01

Group	H	D	uH	Total	X^2	p
U	5	19	4	28	> 10	< .01
V	1	12	5	19	> 10	< .01
W	5	19	26	50	> 10	< .01
X	3	71	15	89	> 10	< .01
Y	0	66	33	99	> 10	< .01

*The difference is significant if three classes are considered. This value corresponds to the X^2 when only two classes are considered: hatched and total unhatched (dead and unhatched still alive).

8. Hatching and mortality at day 36: comparison inter-group.

Group	X^2	p
I-S	1.90	> .40
J-T	> 10	< .01
X-X	> 8	< .01
0-Y	> 10	< .01

9. Morphology at day 32: comparison between control and treated.

Group	I	II	III	IV	Total	X^2	p
Z	42	13	2	3	60		
A	10	11	3	4	28	9.95	< .02
B	11	13	4	3	31	10.76	< .02
C	13	8	3	6	30	8.64	< .05
D	19	7	3	0	29	3.32	> .20
E	14	13	2	1	30	6.29	> .05
F	1	7	7	15	30	> 12	< .01
G	1	5	9	13	28	> 12	< .01
H	7	14	1	6	28	> 12	< .01
I	11	12	5	2	30	> 12	< .01
J	14	9	6	1	30	> 12	< .01
K	11	6	9	5	31	> 12	< .01
L	1	4	7	18	30	> 12	< .01
M	0	3	14	12	29	> 12	< .01
N	3	7	10	10	30	> 12	< .01
O	1	14	11	4	30	> 12	< .01
P	13	11	2	4	30	> 10	< .02
Q	11	10	6	3	30	> 10	< .02
R	14	13	3	0	30	> 10	< .02
S	11	10	7	2	30	> 12	< .01
T	2	15	12	1	30	> 12	< .01
U	4	9	7	10	30	> 12	< .01
V	0	2	12	14	28	> 12	< .01
W	3	16	9	1	29	> 12	< .01
X	4	12	11	3	30	> 12	< .01

10. Morphology at day 32: comparison inter-group.

A-B-P-Q $X^2 = 3.0$ $p < .40$

11. Mortality of embryos at day 20 in prehatching treatment.

	H	D	Total
Dylox 200	214	19	233
Control	175	7	182
	389	26	415

$X^2 = 3.279$, $p = > .05$

12. Mortality of larvae at day 20 in prehatching treatment: comparison with control.

	L	D	Total	%	X^2	p
Control	149	23	182	12.6		
Phosphamidon 30	212	25	237	10.5	1.09	> .10
Phosphamidon 100	126	54	180	30	> 12	< .01
Phosphamidon 200	132	36	168	21.4	4.14	< .05
Dylox 100	177	34	211	16.1	.84	> .30
Dylox 200	182	32	214	14.9	.501	> .40

END OF REEL