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THE RETARDATION OF GROWTH IN *A. AEGYPTI* (L.) LARVAE EXPOSED TO THE VITAL DYES METHYLENE BLUE AND NEUTRAL RED

A Thesis Presented

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INTRODUCTION

Vital dyes have been used widely in biology for many decades. Among the very early uses of dyes in Entomology were attempts to prove the excretory function of the Malpighian tubules by injection of vital dyes (Schindler, 1878). Vital dyes have been used for the selective staining of specific tissues or as general nonselective stains. Vital dyes have been used for the staining of structures like Golgi apparatus, nucleolar and cytoplasmic areas, and nervous tissue.

A recently expanded use for vital dyes involves work with both vertebrates and invertebrates, using dyes as markers in measurements or estimations of population densities and fluctuations (Davis, 1948; Reeves, 1948; New, 1958; Kindel, 1960). Sampling techniques such as the Mark, Release, and Recapture Method have many times depended on the use of vital dyes as markers (Harrell et al., 1968; Lawler et al., 1968).

Peters and Chevone (1968), in screening vital dyes for use as tags, reported retardation of growth and development of mosquito larvae reared in nile blue sulphate (A). If such a trend is demonstrated with other dyes, it may ultimately be significant in two ways. First is its possible future use in insect control. If growth is retarded by vital dyes, treatment would decrease the number of generations and

aid in an integrated control program. There is also the possibility that vital dyes might affect fecundity and viability of eggs produced by treated mosquitoes. The second implication, of more immediate importance, lies in the physiological or behavioral changes in larvae marked with dyes. This is of primary importance when considering dyes as internal tags because techniques such as the Mark, Release, and Recapture Method assume the normal redistribution of animals.

The primary reference and motivating force for this study was the work performed by Peters and Chevone (1968). In searching for an adequate marking agent for mosquito larvae, they found evidence of retardation of growth due to vital dyes. They reported that a 0.2 ppm solution of nile blue sulphate was able to retard the growth of first and second instar larvae. The developmental period was increased by 9 to 11 days. Normal growth was reported to resume 4 to 6 days after larvae were removed from the dye. Another study which continued the work on retardation in mosquitoes was that of Chevone (M.S. Thesis, Amherst, Massachusetts). This study included a comprehensive review of rearing conditions and nutrition of A. aegypti. In addition, parameters such as density, light, and pH were discussed. A basic description of the physiology and internal anatomy of A. aegypti was also thoroughly detailed. Any discussion of the above material at this time would be repetitious. Working with nile blue sulphate A, Chevone concluded that at a minimum concentration of

0.25 ppm inhibition of larval development occurred. He reported further that oxygen consumption was not affected by the dye. He speculated that the dye inhibited the proper and maximal utilization of larval energy sources. Nile blue sulphate A, a fat-soluble dye, stained the fat body but not the foregut, hindgut, Malpighian tubules, tracheal system, hemolymph, heart wall, or exoskeleton.

Methylene blue was also found to retard growth in Drosophila (David, 1963). The retardation and associated mortality varied according to concentration. The prolongation of development ranged from 17.1 hours (with 1 per cent mortality) to 399.8 hours (with 41.9 per cent mortality). Concentrations of methylene blue ranged from 2.5×10^{-5} to 20×10^{-5} grams. David also reported that the longer it took the larvae to develop, the smaller the imago. Dye was concentrated in the intestinal walls and in the Malpighian tubules. He also reported that there was a marked decrease in the fecundity of dyed females. After 10 days the number of eggs laid by the controls were 4 times greater than that of dyed females. No effect on the longevity of the adults was evident in David's results. David (1955) found that methylene blue's effects on successive generations of Drosophila were cumulative. He reported a greater mortality of larvae and pupae, and slower development in the second generation than in the first. Second generation flies were found to be less vigorous and more susceptible to methylene

blue. He pointed out that the survivors of the F_1 generation, merely because they survived, would be expected to be more resistant to the effects of methylene blue. However, since this did not occur, David speculated that methylene blue was somehow affecting gametogenesis of the F_1 generation and reducing the viability of the F_2 generation.

The histological and histochemical importance of vital dyes is obvious, and also abundantly illustrated in many fields of science. The most recent and growing use of vital dyes is that of markers for vertebrates and invertebrates alike. Traditional tagging methods, for example those used in insects (MacLeod et al., 1957; Gangwere et al., 1964), have come into disfavor for use under differing conditions. For many reasons substitutes for these classical marking methods are becoming necessary. Many marking methods affect the activity and behavior of test animals. Also many methods lack flexibility or are inconvenient to use in the field. Others are expensive and may require too much effort and time. For these and other reasons, researchers have been turning to vital dyes as an "obvious" alternative solution.

Vital dyes have been used in studies on many groups of animals. Using dyes, flight ranges for important disease vectors have been described (Reeves et al., 1948). Estimations of natural populations have been made by using vital dyes (Vernon, 1937; Davis et al., 1948; Feder, 1955; Lawler et al., 1968) or in surveys of population fluctuations (Loeb,

1963). The study of movement and distribution of animals is another area in which dyes have been used (Loosanoff et al., 1947; New, 1958).

The use of vital dyes as a tool in hatcheries has been suggested; e.g., for the premature hatching of eggs (Kelly, 1962). Researchers have also incorporated the use of dyes as markers in control programs such as the sterile-male technique (Harrell et al., 1968; Wave et al., 1963). Finally, Dunn et al. (1951) were among the many who suggested the use of dyes for markers in the study of behavior of animals. As more and more investigators are learning, the use of vital dyes should be tailored to the organism being studied and the specific conditions of an experiment.

LITERATURE REVIEW

Insects and Vital Dyes

One of the earliest uses of vital dyes in insects was Schindler's work proving the excretory function of Malpighian tubules (see Palm, 1952). Many different dyes were used for this study. Later, methylene blue was used to study the central and peripheral nervous systems. Experiments on the role of the Malpighian tubules, using vital dyes, were brought up-to-date by studies in the late 1930's. Dyes were also used to study a great variety of biological and biochemical systems. These included the study of growth of oocytes, anaerobic respiration of Chironomus sp., and other histological and histochemical problems.

There are many examples of the vital staining of insects with a great many types of dyes. Palm (1952) listed 3 methods for the introduction of dyes into an organism: immersion, ingestion, and injection. The following are a few representative examples of vital staining of insects. Kolyer (1966) found that neutral red or nile blue A fed to larvae effectively stained all stages of the white cabbage butterfly, while other dyes failed to impart color to adults. He decided to study the staining ability of vital dyes primarily on two pigmented butterflies, Colias philodice (Lat.) and C. eurytheme (Bois.). One of the dyes used was neutral red. The resultant coloration and toxicity were dependent on the

species. Neutral red caused 30 per cent mortality in Pieris rapae, but no mortality was reported in C. philodice, "when feeding was begun at the proper larval length." Neutral red was reported to have both growth-retarding and toxic effects on C. eurytheme when feeding began too early. Morphological aberrations were also observed and in C. philodice larval growth was retarded. Pupae were found to be malformed and undersized when feeding of larvae began too early. Zacharuk (1963), studying the feasibility of the use of several dyes as internal markers, found no ill effects due to any of 10 dyes reported to be suitable markers. Neutral red was found to be an unsuitable marker, since it caused 80 per cent mortality in the larvae. Activity of the larvae seemed to be normal and mortality low until the molting phase began; it was at this point that high mortality occurred. Three days of feeding dyed foliage, during the fifth instar of the larch sawfly, caused no adverse effects (Heron, 1968). Dyes such as nile blue sulphate and rhodamine B colored all stages of this insect.

Over 60 dyes, including methylene blue and neutral red, were tested in feeding media of larvae of the boll weevil (Gast and Landin, 1966). Methylene blue and neutral red stained the larvae but were excreted within 3 days. Gast et al. (1966) concluded that no effect on longevity, emergence, or fecundity was caused by the one dye that was visible in the adult stage.

Vail et al. (1966) tested fluorescent dyes as possible markers of cabbage looper moths. Among the dyes tested was neutral red extra (sic). All dyes were found to be excreted within a short time, and thus were suggested for use in short-term studies only, such as larval migration. Rhodamine B when fed to adults was reported to be a significantly effective marker; it stained spermatophores. It was suggested that this selective staining could prove important in determining, without using dissection, whether sterile males had copulated with wild females. Larval staining, with fluorescent dyes, of Anopheles quadrimaculatus Say was found to be unsatisfactory (Chang, 1946). Mortalities of 75 to 100 per cent were reported with several dyes. The lowest reported mortality was 46.1 per cent. Adult marking proved to be more effective, although higher mortalities were observed.

Growth retardation of larvae of Culex pipiens at high dosages of radioactive 32P in larval rearing media was observed over long periods (Abdel-Malek, 1961). At certain concentrations, pupation was inhibited by 32P (Abdel-Malek, 1961). Hassett and Jenkins (1951) reported that A. aegypti also is slightly retarded at certain concentrations of 32p (cited from Abdel-Malek). Sharma and Rai (1969) reported retardation of development and growth in A. aegypti (L.) larvae exposed to apholute solutions. Concentrations of 50 ppm delayed pupation and reduced the per cent pupation in day one to 23.5 per cent as compared to 80 per cent pupation

in the controls. At 100 ppm no pupation occurred on day one, and at 200 ppm the pupal period was extended by 13 days.

Weatherbee and Hassell (1938) investigated the retention of dye by adult anophelines stained in the larval stage. Among other dyes, neutral red and methylene blue were used. Staining was performed by immersion, not by feeding. The stain seemed to be most apparent in the neck region, the thoracic area, and the walls of the abdomen. Methylene blue was one of a group of 3 dyes reported to have been retained up to 26 days after emergence. Other stains were found to be less satisfactory, including neutral red.

As early as 1841, silkworms fed during the fourth stadium on dye-covered leaves produced green and pink cocoons (Edwards, 1921). In 1902, experiments on wild and domestic silkworms were performed using methylene blue and neutral red. Those feeding on neutral red-impregnated leaves were reported to have developed normally. Leaves sprinkled with methylene blue did not seem to be eaten as readily as those with neutral red. Retardation of growth was observed in these larvae, and this was concluded to be due to lower palatability of the dyed leaves. Twenty-one coal tar dyes were also tested for their toxicity to silkworms (Campbell, 1932). Of these, 4 basic dyes were found to be toxic. Dyes were injected into the silkworms and some fed to larvae. Neutral red was the only dye that changed the external color of the larvae. Dye fed to third instar larvae accumulated in the hypodermal

tissue. Treated larvae were reported to have behaved normally. Basic dyes, in general, and triphenylmethane dyes, specifically, were found to be toxic to silkworms.

Dyes distantly related to the quinone-imine group have also been found to affect growth (Noland and Baumann, 1949). Azo dyes were tested on nymphs of the cockroach, Blattella germanica (L.). Their investigations indicated that dosages preventing growth and reproduction in rats had no effect on the growth of roaches. Thus roaches were found to be relatively more tolerant of azo dyes. Among the malformations caused in roaches by the azo dyes were Malpighian tubule lesions and a type of adhesion of adjacent tubules. The dosages which caused these lesions (0.2 per cent) caused no effects on growth, survival, or reproduction.

Various effects due to methylene blue were found in grasshopper eggs, depending on the extent of their development (Slifer, 1949). Slifer reported that diapause affected the permeability of eggs to dyes. During the period when diapause was broken, during the early development of the egg, dyes were transmitted through the hydropyle of the egg.

Clark (1953) attempted to investigate the mutagenic activity of a series of basic dyes. He tested the ability of dyes (including methylene blue and neutral red) to induce sex-linked, recessive lethals in Drosophila melanogaster. Methylene blue failed to induce any increase in the frequency of recessive lethals. Neutral red was capable of producing

only 0.33 per cent lethals, or 2 lethals out of 633 chromosomes tested. Clark suggested that one of the possible mechanisms involved was the affinity of the dyes to depolymerized nucleic acids.

The excretion of vital dyes was extensively covered by Palm (1952). He stated that the storage or excretion of dyes was related to their physiochemical properties. These properties included the size of the dye particle, the electric charge of these particles, and the lipoid solubility of the dyes. The absorption of dyes is determined by other parameters of the cell; the permeability of the membrane, the charge of the cytoplasmic parts, and the presence and location of lipoid material. The nature of the environment of the cell, as well as the nature of the dye solvent, was also reported to be significant. Palm stated that the pH of the surrounding media was of minor importance. Basic dyes like methylene blue and neutral red are known to produce granules in the cytoplasm. The basic difference between neutral red and methylene blue is that neutral red granules, formed in the cytoplasm, often represent vacuoles. These dyes were also excreted by Malpighian tubules, integumentary glands, and occasionally silk glands. Pericardial cells take part in resorption, although it is only temporary.

The rate of excretion depended upon the insect, the dye, and the concentration of dye in the blood. Malpighian tubules and pericardial cells complement each other's

activities. A decrease in the ability of the tubules to excrete the dye brings on stronger resorption and more permanent storage in the pericardial cells.

Vital Dyes and Other Organisms

Yeasts. The effects of methylene blue and neutral red on the activity of yeast cells have been examined by several workers. Brosi and Rosenberg (1962) reported that the staining of Saccharomyces cerevisiae with methylene blue is age dependent. Very young cells seem to be extremely sensitive to the dye. Nagai (1962) reported that neutral red was ineffective as an inducer of mutants in yeast. Conversely, methylene blue was known to counteract the respiration deficient mutation inducing action of other chemicals. It was also concluded that neutral red alone neither inhibited growth nor induced respiration deficient mutants. However, when mixed with other substances, it affected the formation of mutants. Whether mutants were induced or repressed was dependent on the partner substance. Other classes of dyes, such as triphenylmethanes and xanthenes, not only induced mutants and suppressed growth but were also toxic (Nagai, 1959). Michaelis and Smythe (1936) reported that neutral red induced oxygen consumption but did not inhibit fermentation. However, methylene blue did cause inhibition of fermentation. It was speculated that this was accomplished by the destruction of enzymes. Massart (1949) reported the inhibition of

yeast by methylene blue and other dyes. The addition of substances like streptomycin, spermin (a crystalline aliphatic base found in yeast) and other substances caused the disappearance of the inhibition. Vital dyes have also been found to affect the growth of higher plants in tissue cultures (Sidorenko, 1967).

Bacteria. A great deal of research has been performed on the effects of methylene blue and neutral red in specific groups of unicellular organisms. It is known that phage-susceptible staphylococci contain an intracellular phage precursor. It was found that this precursor could be inactivated by methylene blue. This photodynamic inactivation occurred under strong light (4000-8000 Å) and did not impair or kill the cell (Krueger et al., 1940). The action of methylene blue on bacteria varies greatly, but in general it has been reported that gram-negative organisms are more resistant than gram-positive organisms (T'ung, 1935). It has also been found that the photodynamic action of methylene blue is partially effective in inactivating diphtheria toxin (Lin, 1935). Petroff et al. (1935) attempted to study the bactericidal effects of over 130 dyes and allied compounds. Gram-positive (10) and gram-negative (71) organisms were tested, and in some cases more than one strain. Thiazine dyes, of which methylene blue is a member, were found to be most effective for gram-positive organisms. Other classes of dyes have also been found to be effective (Petroff et al., 1935; Churchman,

1926).

Oxidation of acetate by Escherichia coli was found to be inhibited by methylene blue, although general oxidation did not seem to be affected; it also seems to prevent oxidative assimilation (Stokes, 1950). The photodynamic effect of dyes also occurs in viruses. Neutral red photodynamically inhibited lesions produced by the tobacco mosaic virus; lesions were reduced from 80 to 8 per cent (Orlob, 1962).

Protozoa. Lower organisms and their vital staining have been an area of research for many years. Ball (1927) investigated the effects of dyes on Paramecium caudatum. Only basic dyes like methylene blue or neutral red were found to stain the cytoplasm of normal, living P. caudatum. Acid dyes failed to stain cytoplasm unless P. caudatum were dead or dying. In all except one of the dyes, death of stained P. caudatum occurred unless they were removed from the dye. Photodynamic exposure to the dye seemed to be more severe on the organism. Koehring (1930) performed extensive experimentation on the reactions and effects of neutral red, primarily in lower organisms. Koehring reported that neutral red has long been considered as completely nontoxic. However, several workers have reported high toxicity of this dye. Koehring suggested that this reported toxicity was due to careless use of neutral red and the failure to use specific concentrations for particular circumstances. Koehring described the staining process in Amoebae, Paramecium, and

Vorticella, among others. Vacuoles formed in these organisms were described as being "a bit of the outside enclosed about by a portion of ectoplasmal membrane and sunk into the cytoplasm." The intensity of the stain in the vacuoles seems to have been dependent on its age since the older vacuoles had a deeper color than the newly formed ones. The intensity of the stain was related to the digestive enzymes contained within the vacuoles. In older vacuoles where enzymes were most concentrated the stain was most intense. The fluid in newly formed vacuoles was primarily external cytoplasmic material and very little enzyme substance. Koehring pointed out that since 1907 it has been known that azine dyes precipitate enzymes. She cited the work of Marston (1923) who reported the inactivation of proteolytic enzymes such as pepsin, trypsin, and erepsin by neutral red. It was also reported that the dye precipitated any lipolytic and diastatic substances found in conjunction with these enzymes. In a comprehensive statement Koehring stated that "wherever in both protozoan and metazoan forms proteolytic enzymes are known to exist in active form, there will be found azine dye staining." Both methylene blue and neutral red are azine dyes, the former a thiazine, the latter a phenazine dye.

Tunicates. Vital dyes have been found to affect some aspect of development of a great variety of animals. Some of these effects have been investigated in ascidians. Lynch (1955) ran a series of 4 experiments on Bugula flabellata

investigating the synergistic and antagonistic inhibition of metamorphosis due to neutral red. He demonstrated the photodynamic induction of metamorphosis by the action of neutral red. It was found that alcohol could completely inhibit metamorphic activity, while potassium cyanide was able only to inhibit it partially. The metamorphosis-inducing activity of low concentrations (1:300,000) of neutral red was found to be greatly inhibited by a 3 per cent solution of alcohol; higher concentrations of dye (1:100,000) greatly decreased the inhibition of alcohol. The inducing action of neutral red was also inhibited by a change in pH. This inhibition was complete at a pH of 5.5 and partial at pH's ranging from 6.5 to 7.0. Lynch (1955) speculated that metamorphic inhibition was related to a type of anaesthesia caused by a reduction in the viscosity of protoplasm. This conclusion would seem to imply that neutral red in some manner affects protoplasmic viscosity.

Other studies have shown the accelerating effect on metamorphosis of tunicates due to neutral red (Bradway, 1936). Some areas of the tunicate seem to stain much deeper than others. Bradway suggested that deeply stained areas mark the location of proteolytic enzymes, and that neutral red accelerated metamorphosis by a catalytic action on those enzymes. Tennent (1935), working with eggs of sea urchins, reported that concentrations of dye found to be ineffective in darkness proved to be highly toxic in the presence of light.

Neutral red was one of the dyes used. Eggs affected by neutral red underwent blister cytolysis (formation of colorless blisters).

Echinoderms. Barron (1929, 1932), in reviewing previous studies, pointed out several facts about methylene blue activity. He reported the importance of methylene blue as a catalyst in biological oxidation. Increased respiration was illustrated by both increased oxygen consumption and carbon dioxide production in red blood cells. Methylene blue was also reported to have increased carbohydrate oxidation. Barron attempted to study the effect of the dye on the respiration of sea urchin and starfish eggs. Oxygen consumption was found to increase to a greater extent in starfish eggs than in sea urchin eggs. It is of interest to note that the action of methylene blue was phased; that is, a period of time elapsed before anything occurred. The increased consumption induced by methylene blue was reported to have been related to carbohydrate oxidation. It is known from previous experimentation that cells during respiration primarily oxidize carbohydrates. The dye is said to act during the first process of the metabolism of carbohydrates; i.e., the anaerobic stage. Methylene blue acts on the easily oxidizable substances produced by the breaking down of the carbohydrates.

Brooks (1947), working with eggs of the sea urchin Urechis caupo and Strongylocentrotus purpuratus, found that both neutral red and methylene blue were able to activate

development. By activation, Brooks meant either the formation of the fertilization membrane or an indication of cleavage. At least partial development was observed with both dyes.

Kojima (1966), experimenting with the sea urchins Temnopleurus toreumaticus, Anthocidaris crossispina, and Pseudocentrotus depressus found that neutral red accelerated cleavage. Unfertilized eggs were stained with neutral red and then returned to sea water, where they remained for several minutes before being fertilized. It was observed that cleavage occurred in the stained eggs before any cleavage in unstained eggs. Cleavage acceleration occurred only in eggs with neutral red granules.

Child (1950) attempted to study the modifications in development caused by methylene blue in the sand dollar Dendroster excentricus and other echinoderms. He reported that staining was so gradual that the earlier stages did not seem to be affected. He noted lower frequencies of occurrence of certain stages of development, or even failure to reach certain stages such as the pluteus. Methylene blue was reported to act photodynamically. Many morphological changes were caused by the action of methylene blue and regional differences related to the animals ventrodorsality were destroyed. The entoderm may tend to dissociate itself. Not all species were found to be equally susceptible to the dye. At very low concentrations (1:300,000) the tissues were able to decolorize

themselves continually. At very high concentrations (1:50,000) of dye, a definite lethality was observed.

Vernon (1937) reported that at concentrations of 1 gram per liter no toxicity was observed, in starfish, over a short period. Feder (1955) concluded that vital dyes had no deleterious effects because stained starfish increased their weight by 30 per cent in 4 months.

Mollusks. Loosanoff and Davis (1947) tested the value of neutral red as a dye marker for oyster larvae. They reported that neutral red did not harmfully affect fertilization or development. Other dyes tested were found to be harmful. Nile blue sulphate stopped development and later killed larvae. It has been reported that dyes like azure B (oxidation product of methylene blue) retard development of the gastrula and blastula stages of the starfish, Marthasterias glacialis (Brice, 1959). This action of methylene blue was also found to be photodynamic. The dye was reported to aggregate in cytoplasmic vacuoles. Loosanoff and Davis concluded that the trochophore larva was the stage that stained best. However, they also recognized the need for determining the effects of concentrations of dye other than those recommended by previous workers.

Fish. Much work has been conducted on the effects of dyes on fish. Sudan black B, although not closely related to the thiazine and phenazine dyes, is representative of the dyes used as fish markers and is a vital dye. Experiments were

performed on several species of fish to test the effects of this dye (Loeb, 1963). These included carp, goldfish, brook trout, brown trout, shiner, and creek chub. The dye was found to be harmful to some of these fish. Carp were found to have been severely affected; loss of weight along with an ulcerated condition of the scales was reported. Also, abnormal growths were observed around the head area and other species were more or less similarly affected. Mortality was found to be higher than in the controls. Higher temperatures were reported to increase the severity of the effects. Loeb admitted the need for a greater effort in the screening of dyes.

Studies on the suitability of several dyes for mass marking of brown trout illustrated the ability of vital dyes to induce hatching (Kelly, 1962). The dyes used were nile blue sulphate, bismark brown, and neutral red. Eggs immersed 6 days before normal hatch were observed to hatch on the third day. No significant difference in mortality was noted. When the number of days was increased to 10, 14, or 25, the results differed. For instance, when immersion was started on the twenty-fifth day, mortality reached 60 per cent by the twenty-first day before expected hatch. In tests lasting 25 days, morphologically abnormal embryos resulted. In addition, tissue decay was observed. Dunn and Coker (1951) also tested the effectiveness of methylene blue as a dye marker by injecting the dye into killifish. Methylene blue was found

to be nonlethal. They noted that in previous work fish were fed dye in the food.

Arnold (1966), in a survey of dyes as markers, reported that there have been many attempts to use methylene blue as a marker. Overall success in using the dye has not been extensive. Reviewing the work on the use of neutral red as a marker, Arnold reported that it has been effective; however, indications are that it can at times be toxic. Trout-perch immersed in neutral red were found to have their gills, operculum, and the ventral fin more deeply stained than other areas (Lawler et al., 1968). It was found suitable in concentrations of 1:30,000 for mark and recapture studies. No remarks were made as to its possible toxicity.

Amphibians. Amphibians have also been used as test animals in experiments determining the effects of vital dyes. Teratogenic effects in amphibian embryos (such as inversion and transposition of the heart and visceral organs), as caused by vital dyes, were studied by Yoshida et al. (1957). Test animals included Bufo vulgaris formosus and Rana temporaria ornativentris. In Rana, edema was found to occur in areas such as the anterior lymph sac, abdominal cavity, head tip, subcutaneous tissue, the optic vesicle, etc. This did not occur in Bufo. The axis of orientation of the heart also seemed to be affected. The location and orientation of the visceral organs were found to be in disarray. Methylene blue was found to affect primarily Rana. The Rana and Bufo eggs

were held in the dye only 5 hours. Among other abnormalities observed were turbidity of the cornea, reversed functional orientation of opercula and spiracles, etc. (Yoshida et al., 1957).

Zorzoli (1946) stated that other workers have reported on the ill effects of dyes such as neutral red on other amphibians (e.g., Amblystoma embryos, Triton taeneatus larvae, etc.). He attempted to study the effects of vital dyes on the eggs of the leopard frog, Rana pipiens. He used neutral red, nile blue sulphate, and bismark brown. The gastrula stage was reported to be the first affected. The dyes caused either complete cessation of growth or continued abnormal development. Exposures of short duration were found to be sufficient to cause abnormalities. Immersion at certain stages was found to be more deleterious than at others. The pH was reported to influence the toxicity of the dyes. It was, therefore, concluded that 3 factors must be considered in order to avoid abnormalities caused by the dyes: (1) concentration of dye; (2) duration of exposure; and (3) stage of development which is exposed.

Franston (1940) attempted to study the photodynamic effect of neutral red on pigmented tissue (i.e., the integument of the red-spotted newt) instead of the unpigmented tissue, tested in previous work. She found that light increased the toxicity of neutral red and produced mitotic alterations. The presence of pigment in tissue was reported to require a

stronger concentration of dye than had been used by previous workers to produce the same effects in nonpigmented tissues. Among the abnormalities caused by the dye were pycnosis, pseudoamitosis, fractional nuclei, cessation of mitotic activity, chromosomal extrusions through the nucleus, disintegration and death of cells.

Mammals. The study of mammals in relation to vital dyes has been performed with many classes of dyes. One of the major contributors to knowledge of the reactions of mammals to vital dyes was New (1958). He attempted to find a series of dyes suitable for use as markers. His Ph.D. work (1955; cited in New, 1958) reported the screening of 100 dyes. The dyes were fed to white mice and rats; no indication of toxicity was reported. He conducted experiments using dyes as markers for mammals in the field. Distribution patterns were recorded for the short-tailed shrew, deermouse, and meadow mouse.

In order to determine the reaction of small mammals to dyed insect material, mammals were given options of dyed and undyed substances (Buckner, 1968) with variable results. Two rodents and 2 out of 4 shrews showed no selectivity toward either dyed or undyed material. The remaining 2 shrews both showed a preference for red-dyed material. One rejected blue-dyed material, while the other did not discriminate blue. Buckner concluded that color selectivity is nonexistent or muted, if present at all. He felt that any color selectivity

was minor, and that it did not present enough of a bias. In experiments on another aspect of dye marking, Kindel (1960) studied their use in marking feces of ruminants. Methylene blue was found to stain sheep feces clearly. Neutral red worked to a lesser degree. The appearance of the dye occurred 24 hours after ingestion of dyed food. Methylene blue was field-tested on elk and found to be effective on this nondomesticated ruminant in the wild.

Birds. Studies on birds that were fed vital dyes have revealed many interesting facts (Wetherbee et al., 1964). At a high dosage (16 g/kg of body weight) of sudan black B, only 25 per cent mortality was observed. At lower dosages (500 mg/kg of body weight), dye-fed females were able to lay eggs; however, 93 per cent of the embryos died. At other dosages treatment with dye seemed to affect hatchability. Some aspects such as ovulation, fertilization, and the fertility of males did not seem to be affected. The authors recommended the use of this dye as an agent for population control of nuisance birds.

Chemical Aspects of Vital Dyes

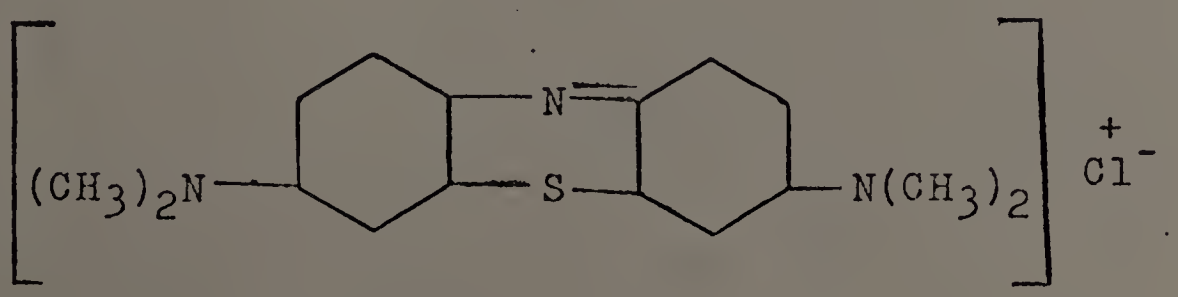
Most dyes are organic compounds which contain a chromophoric and an auxochromic group attached to benzene rings. The color of the dye is attributable to the chromophore and its dyeing property to the salt-forming auxochrome. The auxochrome is also the portion of the dye which gives it

an affinity for fibers and tissues (Conn, 1961).

The most important and most used dyes are synthetics. Synthetic dyes are also known as coal tar dyes, since all are formed by some transformation of substances found in coal tar. Coal tar dyes are organic compounds of the aromatic series since they are derivatives of benzene (Conn, 1961; McClung, 1929). There are only a few inorganic dyes such as iodine, gold chloride, silver nitrate, etc. (Gurr, 1960).

Vital staining is usually referred to as the coloration of a living cell by the ingestion of solid or colloidal particles (McClung, 1929). Vital dyes are divided into basic and acidic dyes. The term "acid" or "basic" dye is based on whether the auxochrome of the dye is anionic or cationic and not on the H-ion concentration. These terms do not truly express the chemical nature of dye solutions. For example, an acid dye like eosin Y forms salt solutions with metallic ions such as sodium. It is the basicity of the sodium cation that determines the basicity of the resultant solution (Conn, 1961).

Methylene blue, also known as Swiss blue, is given a configuration as follows (Gurr, 1960):



It is a basic dye, tetra-methylthionin chloride, and has a molecular weight of 319.851. It is a member of the thiazin subgroup and belongs to the quinone-imine group of dyes. Most of the dyes in this group are primarily important in biological work rather than for textile use. Its ability not to overstain makes it valuable in histological work. Compounds in this group have 2 benzene rings joined by 2 atoms: a sulfur and a nitrogen atom (Conn, 1961).

Methylene blue is reported to be one of the most useful tools in several areas of biology. These include fields such as histology, cytology, histochemistry, parasitology, mycology, microbiology, virology, etc. (Gurr, 1960). It is probably the most used dye, except for hematoxylin (Conn, 1961). Workers such as McClung (1929) and Conn (1961) agree that the priority of methylene blue's importance is based upon its primary uses. First, it is used as a nuclear stain by histologists. Secondly, it is of value to bacteriologists in diagnostic analysis for bacteria (in milk) and for diphtheria. Thirdly, it is important as a nontoxic, vital stain for nervous tissue, due to its tendency to become colorless when the tissue is deprived of oxygen.

The uses of methylene blue, either alone or in combination with other dyes, are numerous. In association with acid dyes, it is an ingredient in stains such as Wright's, Geimsa, Leishman, Jenner, etc. These stains are used in blood and parasite studies. Methylene blue is used for

histological work involving bacteria, leprosy organisms, yeasts, diphtheria, Rickettsia, spirochaetes, algae, Desmid sheaths, bone and cartilage, nervous tissue, splenic and lymphoid tissue, leucocytes, mitochondria, nissl bodies, negri bodies, pancreas paschen bodies, and many others (Gurr, 1960).

Methylene blue is very rarely found in pure form because of the ease with which it oxidizes to its lower homologues. These oxidized forms give the dye its value and effectiveness as a stain or counterstain. These oxidative forms have a higher metachromasy than the pure form, and thus make it a better nuclear stain.

Holmes and French (1926), in their discussion of the oxidation products of methylene blue, point out that early workers quickly realized that polychrome methylene blue arose as a result of oxidation. To some extent this oxidation can occur naturally. However, it can be and is hastened commercially by the addition of alkalies. Many staining formulations require the addition of alkali to hasten oxidation; e.g., Loeffler's methylene blue (Gurr, 1960). Oxidized forms of methylene blue are present in varying degrees at any particular time (Holmes and French, 1926). Because of this, commercial concerns prepare concentrated forms of each of these products. Mild alkaline oxidation results in the formation of azure A, azure B, and methylene violet. Under more vigorous oxidation, methyl thionolin, thionolin, and thionol result. Holmes et al. (1926) indicated a possibility of the

formation of other products not yet known. These products are related to methylene blue by the replacement of differing numbers of methyl groups by hydrogen atoms. Member compounds of the thionin series (the azures) are very strongly basic. Those of the thionolin series are slightly basic and thionol, which has both amino groups displaced by hydroxyl groups, is acidic.

Chart 1 illustrates basic oxidation of methylene blue as given by Holmes and French (1926). Gurr (1960) stated that the major oxidation products of methylene blue vary in their individual staining abilities. Azure A is used with toluidine blue as a stain for the differentiation of metachromatic granules of diphtheria organisms. It is also used as a component in Giemsa stain. These are only 2 of many staining formulations in which azure A is a component. Azure B is said to have no merit in staining (Holmes et al., 1926). However, Gurr felt that it has been proven valuable, combined with other dyes, as a stain for animal tissues, particularly blood. Azure C can be used with eosin Y and orange II as a counterstain to show the presence of bacteria (Holmes and French, 1926). Gurr pointed out reports suggesting its use for staining nucleoli of tumors. Azure I and II and methylene violet are also used in conjunction with other dyes for various histological procedures. Azure I is a hybrid mixture of azure A, B, and C and methylene violet. Azure II is a mixture of azure I and methylene blue. Holmes

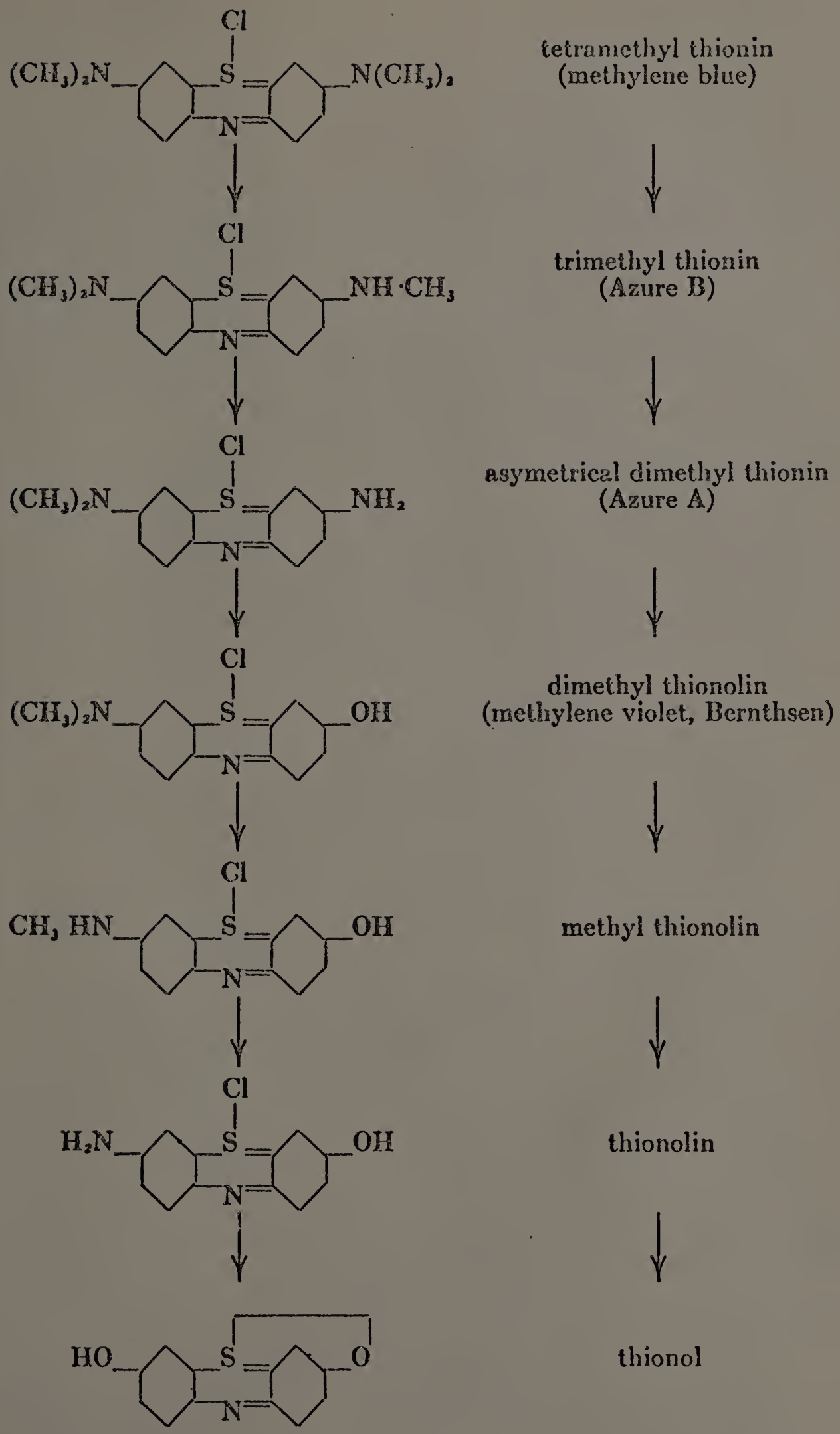
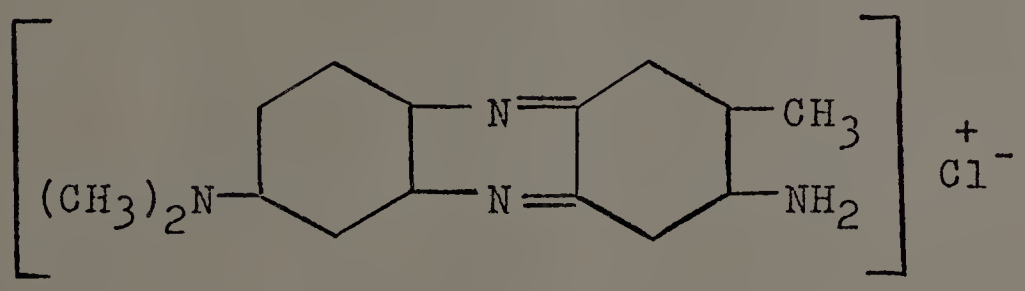


Chart 1.--Alkaline oxidation of Methylene blue

and French (1926) indicated that little data were available on the oxidation of methylene blue under acidic conditions. After running a series of experiments they were able to present this information (Chart 2).

Neutral red is an amino-azin, a derivative of phenazin (a chromogen) by the introduction of one or more amino groups. Neutral red, a weak base, is the chloride of toluylene red and has a molecular weight of 288.775. Gurr (1960) and Conn (1961) give its chemical configuration as follows:



Neutral red has value in biological work for 2 reasons: (1) it is considered to be very nontoxic; (2) it is extremely sensitive to changes of pH. In basic solution, depending on how basic, it changes from a pinkish red color to brown and then yellow. It maintains itself as a deep red in acidic solution, turning blue in extremely acidic solutions (Gurr, 1960; Conn, 1961). It is a component, in combination with other dyes, in special formulae used for staining structures such as the golgi apparatus, nissl granules, and reticulocytes. It is also used in staining bacteria, viruses, and tumor cells. Furthermore, it is often used as a counterstain. The vital staining of living protozoa and other lower organisms is another useful function performed by neutral red.

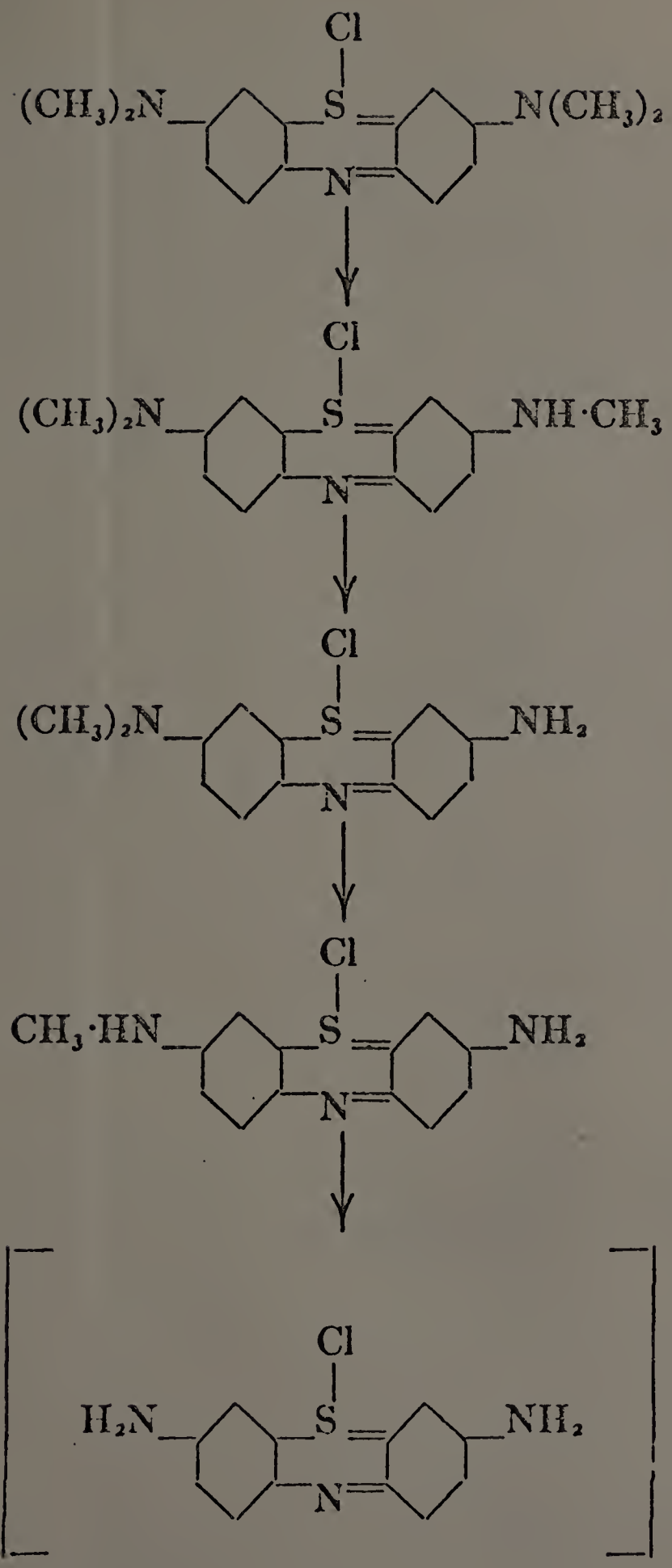


Chart 2.--Acid oxidation of Methylene blue

It is of importance in embryological studies, and was used very early in the history of histology for the study of blood.

The history of the use of vital stains has been discussed previously (Conn and Cunningham, 1932). Examples of several studies from that history will be presented here. The recorded use of vital dyes in living systems included studies in the early part of the eighteenth century. One of these dealt with the observations of newly forming bone with natural coloring substances such as madder (cited from Conn and Cunningham, 1932). Experiments were also performed on the use of vital dyes in nonliving systems. For example, Hill in 1770 reportedly used vital dyes to study twigs. Gerlach (1858) was so successful in demonstrating the use of vital dyes for histological work on fixed material that vital staining was given a secondary direction for some time. In the late 1880's and early 1900's the work of 2 groups of researchers, working on basic and acidic dyes, revived interest in nonhistological uses of vital dyes (see Conn and Cunningham, 1932).

Most of the modern interest in dyes started with the work of Bouffard (1906) and Goldman (1909)(cited from Palm, 1952). With few exceptions, most of the work done earlier was performed with dyes like neutral red and methylene blue. In a few instances dyes such as indigo carmine and lithium carmine were also used. Goldman, using an acid dye, pyrrhol blue, was the first to describe the movement and distribution

of dye throughout the animal body. He also noted that dye granules formed in stained tissues, and felt that formation of granules indicated a cell's secretory function. Later, other researchers reported that the staining capacity of dyes was related to its colloidal state, and that the granules in the tissue were colloidal granulations.

In succeeding years the use of vital dyes was expanded from cytological studies to other areas such as embryological research. Another research area was illustrated by Wislocki's (1917; see Conn and Cunningham, 1932) studies of the growth of the lymphatic system in amphibian larvae. Neutral red, which had earlier been used for the study of leucocytes, began to be used more extensively for the study of blood. For example, it was used for more detailed studies of processes like reticulation and basophilia. Sabin (1920; see Conn and Cunningham, 1932) conducted important experiments on the differentiation of blood cells using neutral red.

The history of the use of vital dyes in insects dates back many years. Palm (1952) reviews the history of the use of vital dyes in insects fairly comprehensively. The following information is taken from that review. Schindler in the late 1800's ran experiments in which he injected insects with dyes like indigo-carmine to prove the excretory function of the Malpighian tubules. In later years, the nervous system of insects was studied with methylene blue (Orlov, 1924; Rehm, 1939; Josting, 1942; cited from Palm, 1952: Nautanen,

1946). Hollander (1922; see Palm, 1952) studied the reactions of pericardial tissue to dyes. Many physiological processes have also been investigated, such as the growth of oocytes and the anaerobic respiration of Chironomus sp. Palm and others in recent years have attempted to develop histological vital staining techniques for insect tissue.

A great deal of work has been done on many aspects of the chemical and physiological effects of vital dyes. In a review of the literature, wide-ranging and very different aspects of the chemistry of these dyes can be found. In order to illustrate the diversity and complexities of these dyes, some of these characteristics will be discussed in the following paragraphs.

Braun and Nemchinskaia (1958) set up a series of experiments to investigate the relationship between acidic and basic dyes and ATP. The assumption that such a relationship existed was based on affinity of these dyes to "protoplasmic constituents" since ATP is a protoplasmic component. The dyes used were methylene blue, nile blue sulphate, neutral red, congo red, and toluidine blue. Braun and Nemchinskaia stated that under normal conditions aggregation of dye in solution increases with increasing concentration of the dye and with addition of neutral salts. This aggregation caused changes in the characteristics of the dye; e.g., absorption spectra and intensity of absorption. Disaggregation seems to be the trend under the influence of ATP. This

is accomplished by the formation of ATP-dye particle complexes. The increased staining power of injured cells is explained by the liberation into the areas around the cell of proteinaceous constituents, thus increasing the dye's tissue affinity. Harris et al. (1958) pointed out that methylene blue acted as an "uncoupling agent and reduced the available ATP." Sandow and Isaacson (1960), reviewing results reported by previous workers, stated that methylene blue was shown to inhibit the activity of ATP in myosin. In their own experiments, methylene blue was found to exert a "suppressive effect on contraction" of frog muscle. It was also found that methylene blue's effect was present only under arc light. It was not known whether this photodynamic effect was oxygen dependent.

Augustinsson (1950), among others, performed experiments demonstrating the inhibitory effects of methylene blue on acetylcholine esterase. Previous workers had illustrated inhibition on many types of acetylcholine esterase. Several compounds with a quaternary ammonium ion, including methylene blue, have been shown to be inhibitory. The mechanism of this inhibition was reported by Augustinsson to be one of competition for the substrate acetylcholine. Methylene blue inhibition was reported to be differential. At a given concentration of methylene blue and a particular concentration of acetylcholine, inhibition was reported to be 12 per cent. At the same concentration of methylene blue and a concentration

of acetylcholine 10 times lower, the inhibition was reported to be 58 per cent; one would expect the reverse of this finding; i.e., the inhibition at a lower concentration of substrate would be expected to be less. The proof of the existence of inhibition was indicated by the upward shifting of the optimum concentration of substrate with increasing concentration of methylene blue. Vialpando et al. (1952), working on the severe hemolytic anemia produced by methylene blue in dogs, found that inhibition of erythrocyte cholinesterase was not the primary cause of the anemia. They reported finding a decrease of 5 million in the erythrocyte count in one week. Reticulocyte values were reported to rise and erythrocytes were found to have greater fragility. Cholinesterase activity decreased at first but then regained high values.

Underhill and Closson (1905) pointed out that because of methylene blue's propensity toward reduction, it was an excellent agent for the study of that process in organisms. Work by many other researchers had already shown that the introduction of methylene blue resulted in the excretion in the urine and feces of several compounds. These included methylene blue, its leuco compounds, and other chromogenic substances. It was found that if these leuco compounds and chromogenic substances were acidified and oxidized the colored dye would reappear. By introducing the dye either intravenously (in cat and rabbit), intraperitoneally (in dog), and orally (in man), in pure form, it was excreted in at least 4

forms: methylene blue, methylene azure, and their leuco compounds. A leuco compound of a dye is the colorless end-product of the reduction of the dye; e.g., methylene blue reduces to leuco methylene blue. These experiments indicated that an animal can both reduce and oxidize methylene blue to some extent.

In experiments on the abnormalities caused by a Vitamin E deficiency, methylene blue was believed to forestall harmful effects from such a deficiency (Dam, 1957). In rats reared on a Vitamin E deficient diet plus methylene blue, the reproductive failure of these animals that was expected from Vitamin E deficiency was not detected. However, diets with no traces of Vitamin E and the same quantities of methylene blue did not counteract abnormal reproduction. Dam theorized that "this must mean that methylene blue retards the depletion by stabilizing traces of Vitamin E otherwise insufficient for protection." He also speculated on other possible protective mechanisms in related abnormalities by using redox dyes and related substances.

Fajer (1957) attempted to check the effects of the addition of methylene blue in a low fat diet fed to mice. Results indicated that in mice of an initial weight of 20 grams, final body weight was not significantly affected. Of all body organs, only the spleen was reported to have been heavier, particularly in males. In male mice initially weighing about 14 grams, there was a reduction in growth rate with

the same effect on the spleen. Fajer concluded that the effects of methylene blue were not related to growth inhibition because of the lack of differences in the initially heavier group.

Based on the knowledge that nile blue stained mouse tumors while other dyes did not, Lewis et al. (1946) studied other oxazine dyes and the closely related thiazine dyes. Results indicated that only nile blue stained and retarded the growth of tumors in mice. He did not find any evidence that methylene blue was able to cause inhibition of tumors.

Gomba et al. (1966) found that neutral red interfered with enzyme activity. They worked with 5 basic dyes, including neutral red, neutral violet, acridin orange, nile blue sulphide, and brilliant cresyl blue, used in 1 to 2 per cent concentrations. Acid phosphatase activity in the juxtaglomerular cells of the kidney in mice had already been established. They found that only with neutral red did enzyme (acid phosphatase) activity cease within 15 minutes. Normal activity did not reappear until 6 hours after the injection of the dye. Activity of the lysosomes; i.e., the acid phosphatase granules of the cells, was also affected. These results were in agreement with the ideas of Koehring (1930). She placed strong emphasis on the direct relationship between neutral red's affinity to tissue and enzyme interaction with the dye. Many other workers have stressed the importance of enzymes in neutral red staining (Marston,

1923; see Koehring, 1930). General trends in the inhibition of enzymes by basic dyes have been studied (Fizhenko, 1967). Gomba et al. speculated that the dye is either combining with or interfering with activities of the enzyme, including resynthesis.

In research on another aspect of these dyes, Haley and Leitch (1953) attempted to study the effects on blood pressure and respiration in rabbits and dogs. Their results indicated that azure A (oxidation product of methylene blue) had a pressor effect, whereas neutral red had a depressor effect, on the blood pressure of the rabbit. In addition, it was reported that azure A increased both the rate and depth of respiration, except at one concentration (10 mg/kg). Neutral red was found to decrease the rate and increase the depth of respiration. The effect on blood pressure of the dog was the same as in the rabbit. Neutral red had the most drastic effects on dogs, since it caused permanent loss of blood pressure; the per cent loss being dependent on the concentration. Haley and Leitch (1953) also indicated that azure A induced blood vessel dilation, thus reducing blood pressure, while neutral red depressed this activity.

Brooks (1933) pointed out that methylene blue was found to act as an antidote to CO poisoning in rabbits. Carbon monoxide was believed to act as a respiratory pigment inactivator. Methylene blue, it was suggested, acted by transferring oxygen until normal respiratory activity resumed.

Methylene blue was found to stimulate respiration of grasshopper embryos (Bodine and Fitzgerald, 1949). It served as a supplementary carrier or hydrogen acceptor, augmenting those normally present and using the same substrate. Results indicated that R Q values of 0.75 were similar to the controls. It was, therefore, reported that the normal respiratory mechanism was not interferred with by methylene blue. Bodine *et al*. (1949) stated that a supranormal respiratory ceiling was reached by adding methylene blue to other carriers present in the system. Therefore, methylene blue, in fact, caused supranormal utilization of the substrate and a subsequent increase in oxygen uptake.

In studies on the respiratory metabolism of the trematode, *Fasciola hepatica* Linn, Grembergen (1948) observed a stimulatory effect of methylene blue on respiration. This stimulation was strong enough to overcome the respiratory inhibition of KCN. Although the use of neutral red as an antiheparin compound was known, Stolarksy and Haley (1951) pointed out that it also can be very toxic. It was reported to cause increased respiration, convulsions, and death due to respiratory and cardiac failure. Neutral red was also found to infiltrate major areas of the body. In some areas damage was observed.

In studies on the effect of methylene blue on cation transport, several additional facts of methylene blue's chemistry were revealed (Harris *et al*., 1958). The blockage of

cation transport was reported to be attributed to neutral red. They reported that the inhibition of cation transport by methylene blue seemed to be merely a surface phenomenon. The photodynamic properties of methylene blue were also demonstrated. The photodynamic oxidation by methylene blue of amino acids (e.g., histidine, tyrosine, and tryptophan) is such an example. At other times its effects were not dependent on its photodynamic qualities (e.g., methylene blue's stimulation of glucose utilization)(Harris et al.). Others (Okuda et al., 1966) have also reported on the disruption of glucose metabolism by neutral red.

A great deal of research has been performed on vital staining at the electron microscopic level. Neutral red is known to form granules. Byrne (1964) attempted to reconcile two opposing opinions on neutral red granule formation. One theory stated that the granules did not already exist in the cell, but formed due to the presence of neutral red itself. In addition, these granules represented condensed golgi substances. The alternative theory stated that neutral red was pathological to many parts of the cell, and thus the granules represented swollen vacuolated mitochondria. Byrne concluded that the former theory was correct. Tanaka (1962) studied neutral red granules and other aspects of vital staining. He indicated that these granules or segresomes were found in many cells stained with the dye. He described these bodies as non-specific granules, some of which were preformed and others

forming when the cell was dyed. He suggests that the misidentification of these granules may have been due to their cristae-like ultrastructure. The vital staining of tissue was reported to consist of the invasion of dyestuff into the cytoplasm and its segregation inside the granules.

MATERIALS AND METHODS

Stock Culture Rearing Techniques

All material used in these experiments originated from a laboratory culture of Aedes aegypti (L.) obtained from Rutgers University and maintained since 1965. Adults of the stock culture were kept in a cage 32" x 18" x 23" enclosed by glass on all sides with the exception of its plywood bottom and cloth-sleeved entrance. The culture cage was kept in a controlled-environment walk-in room. A thermoregulated 1650 W, 120 V Electromode space heater provided a room temperature of 27°C ± 2.0°C. A 115 V. Kenmore automatic humidifier kept the humidity at an average of 60 per cent R.H. A Tork single-pole, single-throw timing motor (Model 7100) maintained the daily light/dark regime set at 10L:14D.

To replenish the culture, eggs were hatched at 25 psi vacuum (Barr and Al-Azawi, 1958) for approximately 30 minutes, using a Gast air and vacuum pump (Model 5KH32EG 550T). Approximately 400 larvae were placed in 9" x 12 " x 2 1/4" enameled pans, each containing 2 liters of Trager's salt solution (Chevone, M.S. thesis, 1968). The larvae were fed daily on dried brewers yeast and kept in the environmental walk-in room. Pupation began in approximately 6 days, with a larval mortality of approximately 10 per cent. Pupae were picked and separated from the larvae by hand. They were then placed in petri dishes with from 250 to 300 pupae per dish. These

were placed in the culture cages for emergence of adults.

Adults emerged in slightly over 2 days. They were fed on a 10 per cent sucrose solution, presented in the soaked cotton plug of an inverted 250 ml polyethylene bottle. Females were blood-fed on a rabbit in a restraining cage 12" x 8" x 3 1/2", composed of a wire mesh cover attached to a plywood bottom. Blood feeding usually was not attempted earlier than 3 days after emergence. Egg laying began 3 days after a blood meal. Successive attempts at blood feeding occurred after a waiting period of at least 3 to 4 days after each oviposition. The egg cones were stored in a dessicator kept at 90 per cent R.H. (maintained by a supersaturated solution of KNO_3). Each egg cone was dated. Viability of these stored egg cones was maintained for many months.

Experimental Techniques

A standard 1:1 (weight:volume) stock solution of methylene blue (Hartman Leddon Company, C. I. No. 52015, 91 per cent dye content) and a 1:1 solution of neutral red (Matheson, Coleman and Bell, C. I. No. 50040, 83 per cent dye content) were used. Stock solutions were prepared with distilled water. Various concentrations of dyes required for experimentation (in parts per million) were prepared and kept in 1000 ml separatory funnels. The concentrations of dyes required for specific experiments were prepared as needed. Excessive evaporation, causing increases in concentration

due to storage, was thus avoided. Concentrations of dyes were prepared with Trager's solution, since it comprised the growth medium for the controls.

In experiments involving the effects of various dyes on larvae, eggs of A. aegypti were vacuum-hatched, using a Gast air and vacuum pump (Model 5KH32EG 550T). Strips of filter paper with eggs, from dated egg cones, were placed in petri dishes with Trager's solution. These eggs were then hatched under a 25 psi vacuum for a maximum of 30 minutes. Four hundred larvae were placed in each of three 9" x 12" x 2 1/4" enamel pans, each with 800 ml of Trager's solution. Due to the very harsh and deleterious effect of certain dye concentrations on the very early stages, at least one instar elapsed before placement of the larvae into the dyes. From 15 to 39 hours after hatch, larvae from all 3 pans were condensed into one pan. In this way the larvae were randomly transferred into dishes from one population and from clean Trager's. The larvae were transferred into 100 mm x 20 mm petri dish covers. The petri dish covers constituted the environmental rearing unit for the experiments involving dye effects on larvae. Eighty ml of dye or plain Trager's (for the controls) and 40 larvae were placed in each dish (Peters et al., in prep.). The dishes and the enamel pans were kept in a Percival controlled environment chamber (Model E-57) with temperature settings averaging 80°F and 90 per cent R.H. The light/dark regime was set at 12L:12D.

Feeding the larvae in the dishes was done on a specific schedule for A. aegypti (Peters et al., in prep.) which suggested the following regime for 40 larvae/80 ml per petri dish: Day I - 0.1 mg/ml, Day II - 0.25 mg/ml, Day III - 0.40 mg/ml, Day IV - 0.80 mg/ml, Day V, etc. - 0.50 mg/ml. Feeding was undertaken volumetrically, using a 10 mg dried Brewer's yeast: 1 ml Trager's solution ratio. For example, the 0.1 mg/ml for Day I called for in the above feeding schedule would require the use of 0.8 ml of the 10:1 yeast suspension.

Larvae were transferred daily to fresh solutions. The transfer of larvae from an old to a new solution was accomplished by the use of a 70 mm diameter by 80 mm long short-stem funnel. The tapered end of the funnel was covered with fine knit cloth and larvae plus liquid were pipetted into the funnel; only the larvae remained in the funnel. In this way only larvae were transferred out of the funnel into each dish and each dish still contained 80 ml of liquid. In addition, practically no contaminating liquid was transferred with the daily change.

In experiments attempting to illustrate the retardation of larval growth by an increase in larval period and a decrease in rate of pupation, observations were made of the number of larvae pupating every 4 or 8 hours, depending on the experiment. Pupae were picked at each interval and placed in vials; one for each of 4 replicates per treatment.

The number pupating in each dish (replicate) at each interval was recorded. Sex ratios of resulting pupae for each replicate were also noted. At the end of each experiment pupae were separated by sex and dried in an oven for one hour at 100°C. Dry weights of males and females for each replicate were recorded, using a Mettler single-pan balance.

An experiment was also conducted attempting to determine whether or not methylene blue treated yeast suspensions were distasteful to larvae. It was considered possible that retardation of growth might have been caused by lack of feeding due to unpalatable food. Larvae were reared in 100 x 20 mm petri dishes. Four replicates for each of 6 concentrations of methylene blue were used. For each replicate 40 larvae were placed in 80 ml of Trager's salt solution and reared to the fourth instar. At this time the larvae were placed in dye for 6 hours after which they were fed. After 2 hours they were counted, dried in an oven for one hour at 100°C, and weighed on a Mettler single-pan balance.

Experiments were also conducted attempting to investigate effects of 3 dyes--methylene blue, nile blue, and neutral red--on various instars. Procedures were similar to those used to determine retardation of growth. After initial pupation in any treatment, recordings were made every 4 hours of the number of pupating larvae.

The statistical analysis of data on mortalities, sex ratios, and pupal and larval weights involved the use of a

standard one-way analysis of variance. Analysis of data on growth retardation involved the use of a one-way nested analysis worked out by Dr. R. A. Damon, Biometrician at the University of Massachusetts (Chevone, 1968).

RESULTS AND DISCUSSION

In a series of 8 experiments, the retardation of growth of larvae of A. aegypti (L.) exposed to both methylene blue and neutral red has been clearly demonstrated. A simplified and overall illustration of this retardation with both dyes can be seen in Table 44. Clearly, the number of hours required for 50 per cent or more of the larvae to pupate increases dramatically from control to the highest concentration of dye. With methylene blue the hours for at least 50 per cent pupation range from 12 to 16 for the controls and from 56 to 80 for 2.5 ppm, the highest concentration tested. Exposure to neutral red resulted in even greater prolongation of development. The retardation at one point was approximately 10 times that of the control (Experiment VI). In all experiments attempting to illustrate the retardation of growth, the criteria used were delay in onset of pupation and relative rates or speed of pupation.

Three concentrations of methylene blue were used in Experiment II: 0.5, 2.5, and 4.5 ppm. In Experiments III and IV, 5 concentrations were used: 0.5, 1.0, 1.5, 2.0, and 2.5 ppm. Retardation for all the above experiments, as indicated by the onset of pupation relative to controls and the rates at which the larvae pupated, is illustrated in Tables 45, 46, and 47. Experiment II was run as a preliminary experiment attempting to find the approximate range of concentrations

that might cause retardation. Observations on the number of larvae pupating were taken every 4 hours for 100 hours. The graph of the results for Experiment II (Figure 1) shows, as do all 6 graphs, 3 important effects of the dyes. These are indicated in the legends of all the graphs and are essentially the initiation of pupation, the relative speed of pupation, and the level of mortality for each concentration and the control. Analysis of the retardation data indicated not only that there was a highly significant difference in the results but that each concentration was significantly different from each of the others. Retardation data obtained from Experiments III and IV are graphically pictured in Figures 2 and 3. Experiment III was run for 184 hours with observations every 4 hours. Experiment IV ran for 192 hours with observations every 8 hours. Figures 2 and 3 again show 3 major features of the graphs illustrating the effects of methylene blue. Analysis of variance and Duncan's multiple range test of the data on pupation rates obtained from Experiments III and IV were similar (Tables 35 and 36).

Unexpected results have occasionally been obtained in this study. Both with methylene blue and neutral red, inconsistent results were obtained from identical experiments. This is illustrated by the extreme variation between replicates within treatments. An aspect of this variation is illustrated by the differences in mortalities between Experiments III and IV (Tables 3 and 4), even though identical

concentrations were used. It is believed that part of the answer to the unexpected variability lies in the unstable nature of the dye. In addition, it is now believed that part of a very routine laboratory procedure may very well have caused differing results. For all experiments, 40 larvae were counted out and placed in each petri dish. In some experiments there were up to 24 dishes. Lack of manpower inevitably delayed the feeding of these newly hatched larvae until all counting was completed. It has been suggested that small differences in the time which lapsed between hatching and feeding may have affected the results. This effect can be either direct, causing higher mortality or other changes, or indirect by increasing the susceptibility of the larvae to the dye.

Experiment V, exposing larvae to 5 and 10 ppm neutral red, was a broad-scale experiment, the pupation data of which are graphically presented in Figure 4. The experiment lasted for 100 hours with observations every 4 hours. Prolongation of the larval period for this experiment is presented in Table 48. Experiment VI, graphically illustrated in Figure 5, tested 5 concentrations of neutral red: 5, 6, 7, 8, and 9 ppm. Table 49 presents data showing the degree of retardation in rates of pupation for each of the above concentrations. The experiment ran 336 hours with observations every 8 hours. Similarly, Experiment VII involved the testing of various concentrations of neutral red: 3, 4, 5, 6, and 7 ppm. This experiment ran for 352 hours with observations every 8 hours.

Analyses of the data for Experiments V, VI, and VII are presented in Tables 37, 38, and 39.

Other parameters were quantitatively tested in an attempt to measure the effects of neutral red and methylene blue. These included mortalities, the percentage of females, and the average pupal weights of males and females. The data on these 3 aspects are presented in Tables 1-7 and 51-59. As can be seen from the analyses of variances and range tests on the mortality figures (Tables 9-16), differing results present a complex situation. No trend is obvious enough to formulate any definitive conclusions. No significant differences were found in the percentages of female pupae from larvae exposed to either neutral red or methylene blue (Tables 17-23). In general, as seen in Tables 24-28, neutral red and methylene blue did not seem to affect the average male pupal weights. On the other hand, the average female pupal weights did seem to be highly significantly affected by larval exposures to both neutral red and methylene blue.

Experiment IX was an attempt to determine if there are any differences in the amount of food (yeast suspension) that larvae will ingest when placed in various concentrations of dye. No significant differences in average larval weights were found (Table 40).

Differing exposures to dyes as a function of time and instars were also investigated. Larvae were exposed to methylene blue, neutral red, or nile blue during the third, fourth,

or both the third and fourth instars in Experiments X, XI, and XII (Tables 60, 61, and 62). The feeding schedule used was one in which the lengths of the instars were known (Peters et al., in prep.). The exposures were, therefore, 24 hours (the third or the fourth instars) and 48 hours (the third and fourth instars). In all 3 experiments the actions of the dyes were similar to each other but significantly different from the controls (Tables 41, 42, and 43). The effects of exposure to later instars seemed to be less severe than they were to earlier instars. In fourth instar larvae exposed to the 3 dyes, the rates of pupation were significantly different, but only to the 95 per cent confidence level; third instar exposures showed significance to the 99 per cent level. Exposure to the 3 dyes lasting through both third and fourth instars resulted in a significant difference to the 99 per cent level. The F value of 12.83 was substantially larger than the 7.33 value for dye exposure over the third instar alone. Similarly, one can say that length of exposure may have a key role in the effects of the dyes, since the duration of instars was known; the third or fourth instars lasted for approximately 24 hours while the third and fourth instars lasted for approximately 48 hours.

As one can ascertain from both the literature and the present study, methylene blue and neutral red are far more complex and their effects far less predictably nontoxic than has been assumed. The use of dyes based on the assumed lack

of toxicity is unwarranted. Even if the abnormalities reported in the literature were exceptions rather than the rule, it would not justify the neglect of thorough study of a dye's effects before implementing its use in living systems. It is to be hoped that further research involving the use of vital dyes will be preceded by a thorough investigation of its behavior, both on the organismic and cellular levels. Thorough investigation is required, since workers such as Noland and Baumann (1949) have reported harmful internal effects that are not immediately noticeable due to the lack of associated external abnormalities. Preoccupation with the activity of vital dyes within cells may seem pointless. However, vital dyes have been reported to act not only at the cellular level but also at the molecular level. Failure to investigate the role of vital dyes in experiments may place a bias in the data so as to completely invalidate the conclusions drawn.

In addition to the role of vital dyes in laboratory experiments, consideration must be given to field studies in which dyes are used as animal markers. In surveys and field studies in general, basic behavioral and physiological assumptions are made. Whether the survey is an attempt to measure a population, follow the movement of individual animals, or map out the distribution of a group of animals, the researcher assumes that the marker in no way alters normal behavior and physiology. One can no longer validly assume that this is

universally true with vital dyes.

Reports discussed in the literature review suggest that the effects of vital dyes vary greatly, and that dyes can affect a great variety of organ systems. Methylene blue was reported to act on several biologically important substances such as ATP and acetylcholinesterase. Also it was found to undergo reduction and oxidation within organisms. Its ability to oxidize amino acids and stimulate glucose utilization has also been documented. Still another aspect of its behavior is its ability to counteract the activity of mutation-inducing substances. The activity of neutral red can be said to be similarly widespread. The animal system which neutral red and methylene blue are consistently reported to act on is the respiratory system. Both neutral red and methylene blue are reported to stimulate and induce respiratory activity. The severity of this behavior is illustrated by a report stating that neutral red's activation of respiration led to respiratory and cardiac failure (Stolarksy and Haley, 1951).

No organism can be assumed to lack sensitivity to vital dyes unless proven immune to its harmful effects. Lower organisms such as bacteria and protozoa have been reported to be affected by vital dyes such as neutral red. Tunicates, echinoderms, molluscs, and fish are some of the other groups in which abnormalities, due to vital dyes, have been reported. In amphibian embryos some of these

abnormalities are perhaps most obvious and clearly illustrated (Yoshida et al., 1957). Even higher organisms such as birds have been found to be harmfully affected by dyes.

Studies ascertaining either the suitability or the effects of vital dyes in insects have not been very numerous or extensive. Most of the experimentation has involved the suitability of dyes (including fluorescent dyes) as internal and external markers of larvae and adults. Most studies have found that vital dyes in varying degree would serve as appropriate markers. Those that were reported to be unsuitable were condemned primarily on the basis of high mortality (Zacharuk, 1963). Other criteria for rejection of dyes included short retention time. In studies where a vital dye was proclaimed to be effective and adequate as a marker, an effort was usually made to point out that feeding the dye occurred during the final stages of the animal's immature life. Early feeding in some instances was reported to produce drastically different results (Kolyer, 1966; Heron, 1968).

Retardation of growth in mosquitoes has been accomplished with a few substances other than vital dyes. These include radioactive 32p in larval rearing media (Abdel-Malek, 1967) and the chemosterilant apholate (Sharma and Rai, 1969), both used on A. aegypti (L.). There have been only a few cases reported on retardation in insects which were directly attributable to neutral red or methylene blue. Perhaps the earliest of these was the report of retardation of growth in

silkworm larvae with both neutral red and methylene blue (Edwards, 1921). The dyes were sprinkled on the leaves, and it was concluded that the retardation of growth might very likely be due to unpalatable qualities of the dye-sprinkled leaves. This suggested the possibility that unpalatability of the dyes might be the mechanism by which A. aegypti (L.) larvae were being retarded. This, therefore, was the motivation for running an experiment to determine the taste selectivity of the larvae to methylene blue.

Kolyer (1966) reported the retardation of growth in Lepidoptera with brilliant cresyl blue and neutral red, although it is not self-evident in his data. His tables and graphs did not clearly indicate or illustrate any obvious evidence of growth retardation. What they did show, and quite clearly, was the formation of dwarfed or undersized adults due to larval exposure to dyes. Another study has reported the retardation of growth in Blatella germanica nymphs exposed to azo dyes (Noland and Baumann, 1949). Even though this retarding effect was caused by dyes only distantly related to the thiazine and azine dyes, it is worthy of discussion. It is important because it reported that concentrations of dye not causing any externally obvious and clearly noticeable effects (such as retarded growth, abnormal reproduction, etc.) nevertheless caused lesions and adhesions in the Malpighian tubules.

The studies most clearly related to this thesis, and

of greatest importance were those by Peters and Chevone (1968), Chevone (1968), and David (1963). Originally, the growth retardation of A. aegypti (L.) was reported in experiments involving screening of several marker dyes (Peters and Chevone, 1968). The developmental retardation due to nile blue, pointed out in the above paper, was reaffirmed and its exact nature investigated in later work (Chevone, 1968). Finally, work very similar to the present study attempted to ascertain the effects of methylene blue on the growth and development of Drosophila (David, 1963). In a series of studies, David extensively investigated all aspects of the effects of methylene blue. Many of his results are very similar to those found in our study. His theory that methylene blue affected gametogenesis and altered the susceptibility of succeeding generations is noteworthy.

It is possible that this dye may be altering normal genetic balance, perhaps indirectly, thereby increasing variability within the population. This speculation is made because in working with this dye (as well as with neutral red) it becomes more and more apparent that these dyes do not produce effects of equal severity from individual to individual for any given concentration. It is because of this great variability of effects that one tends to suspect one of two possibilities. Either the dye is acting on a biological or physical factor within the environment of the larvae, or it is acting on a variable within the larvae which varies among

individuals. Observations which tend to invalidate the former theory and strengthen the latter are that in a replicate of any one concentration (usually the higher concentrations of dye) one finds an abnormal number of different instars; e.g., firsts, thirds, fourths, or pupae. Usually no more than two instars are normally found within an untreated dish, or if an additional instar occurs it is found in extremely small numbers. Although normal variation of any biological system upon which the dyes may act may differ only slightly among the individuals of a population, this variation may become greatly magnified as a function of time or continual exposure to the dye and age or instar of the larvae. An analysis of variance of the experiments determining retardation due to dyes also shows significant differences between replicates of the same concentrations, illustrating perhaps this variability. This theory of variability is speculation and perhaps should not be made for lack of complete and extensive experimental evidence.

It would be hazardous to suggest the use of a vital dye as a control agent for insects. It is not only economically unfeasible but highly impractical to employ vital dyes to retard growth and thereby decrease the number of generations per season. It is perhaps more reasonable and realistic to believe that with further research the mechanism by which retardation occurs can be more precisely delineated. With this knowledge its full potential and control capabilities

can then be realized and exploited. Probably of most immediate importance is the recognition that the value of vital dyes should always be tempered by the knowledge of its limitations. Perhaps there is significance merely in challenging an assumption traditionally held and promoted. Ideas which have been inculcated and have become traditionally accepted without being contested are difficult to invalidate. Hopefully, results reported by others and those reported in this study are sufficient to merit hesitation before future indiscriminate use is made of vital dyes. And so the belief held for many years that vital dyes by definition are nontoxic should not, in the light of what is now known, be blindly accepted. Instead the effects of vital dyes should be thoroughly examined and properly tailored to each experimental situation. Vital dyes have always been useful, and perhaps now they will be even more useful.

CONCLUSIONS

As a result of the experiments conducted, the following conclusions have been reached:

1. Retardation of growth in A. aegypti (L.) larvae was caused by nile blue sulphate A, methylene blue, and neutral red.

2. Exposure to methylene blue and neutral red affected length of development, rate of pupation, and larval mortality of populations of A. aegypti (L.).

3. Female pupal weights seem to have been adversely affected, generally, while male pupal weights were not.

4. Retardation of growth was not caused by the rejection of dyed food under the conditions of my experiments.

5. Methylene blue, neutral red, and nile blue were most severe in their action on larval exposures of two instars, as opposed to only one instar. They were also more severe on earlier instars than on later instars.

6. The use of vital dyes requires prior investigation as to possible effects on the parameters to be studied.

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APPENDIX

TABLES AND FIGURES

TABLE 1.--Larval mortalities of A. aegypti (L.) reared to pupation in methylene blue solutions for Experiment I

	Dye concentrations			
	0 ppm	0.5 ppm	1.0 ppm	1.5 ppm
Dead	3	10	1	2
Live	157	150	159	158
Total[a]	160	160	160	160
$\bar{X}$% Mort.	1.9	6.3	0.6	1.3

[a]Total number in 4 replicates.

TABLE 2.--Larval mortalities of A. aegypti (L.) reared to pupation in methylene blue solutions for Experiment II

	Dye concentrations			
	0 ppm	0.5 ppm	2.5 ppm	4.5 ppm
Dead	4	11	11	93
Live	116	109	109	27
Total[a]	120	120	120	120
$\bar{X}$% Mort.	3.3	9.2	9.2	77.5

[a]Total number in 3 replicates.

TABLE 3.--Larval mortalities of A. aegypti (L.) reared to pupation in methylene blue solutions for Experiment III

	Dye concentrations					
	0 ppm	0.5 ppm	1.0 ppm	1.5 ppm	2.0 ppm	2.5 ppm
Dead	4	6	8	35	60	110
Live	156	154	152	125	100	50
Total[a]	160	160	160	160	160	160
$\bar{X}$ Mort.	2.5	3.8	5.0	21.9	37.5	68.8

[a]Total number in 4 replicates

TABLE 4.--Larval mortalities of A. aegypti (L.) reared to pupation in neutral red solutions for Experiment IV

	Dye concentrations					
	0 ppm	0.5 ppm	1.0 ppm	1.5 ppm	2.0 ppm	2.5 ppm
Dead	10	12	4	17	14	12
Live	150	148	156	143	146	148
Total[a]	160	160	160	160	160	160
$\bar{X}$ Mort.	6.3	7.5	2.5	10.6	8.8	7.5

[a]Total number in 4 replicates.

TABLE 5.--Larval mortalities of A. aegypti (L.) reared to pupation in neutral red solutions for Experiment V

	Dye concentrations		
	0 ppm	5 ppm	10 ppm
Dead	5	7	64
Live	155	153	96
Total[a]	160	160	160
$\bar{X}$% Mort.	3.1	4.4	42.5

[a]Total number in 4 replicates.

TABLE 6.--Larval mortalities of A. aegypti (L.) reared to pupation in neutral red solutions for Experiment VI

	Dye concentrations					
	0 ppm	5 ppm	6 ppm	7 ppm	8 ppm	9 ppm
Dead	12	75	86	66	131	142
Live	148	85	74	94	29	18
Total[a]	160	160	160	160	160	160
$\bar{X}$% Mort.	6.9	45.6	52.5	58.8	77.5	88.8

[a]Total number in 4 replicates.

TABLE 7.--Larval mortalities of A. aegypti (L.) reared to pupation in neutral red solutions for Experiment VII

	Dye concentrations					
	0 ppm	3 ppm	4 ppm	5 ppm	6 ppm	7 ppm
Dead	6	2	3	6	22	89
Live	154	158	157	154	138	71
Total[a]	160	160	160	160	160	160
$\bar{X}$% Mort.	3.8	1.3	1.9	3.8	13.8	55.6

[a]Total number in 4 replicates.

TABLE 8.--Larval mortalities of A. aegypti (L.) reared to pupation in neutral red solutions for Experiment VIII

	Dye concentrations			
	0 ppm	3 ppm	4 ppm	5 ppm
Dead	5	44	60	109
Live	155	116	100	51
Total[a]	160	160	160	160
$\bar{X}$% Mort.	3.1	27.5	37.5	68.1

[a]Total number in 4 replicates.

TABLE 9.--Anova of mortality data for Experiment I found in Table 1

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	78.125	26.0417	2.5641 ns
Within groups	12	121.875	10.1563	
Total	15	200.000		

TABLE 10.--Anova and Duncan's multiple range test of mortality data for Experiment II in Table 2

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	11180.7292	3726.9097	53.0049**
Within groups	8	562.5000	70.3125	
Total	11	11743.2292		

**Indicates significance at the 99% level in this and all other tables.

Duncan's multiple range test

Concentration in ppm	0	0.5	2.5	4.5
Mean	3.33	9.16	9.16	77.50

TABLE 11.--Anova and Duncan's multiple range test of mortality data for Experiment III found in Table 3

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	13676.3021	2735.2604	39.2406**
Within groups	18	1254.6875	69.7049	
Total	23	14930.9896		

Duncan's multiple range test

Concentration in ppm	0	0.5	1	1.5	2	2.5
Mean	2.50	3.75	5.00	21.875	37.50	68.75

TABLE 12.--Anova of mortality data for Experiment IV found in Table 4

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	149.2188	29.8438	1.2502 ns
Within groups	18	429.6875	23.8715	
Total	23	578.9063		

TABLE 13.--Anova and Duncan's multiple range test of mortality data for Experiment V found in Table 5

ANOVA

Source	df	SS	MS	F ratio
Between groups	2	4007.2917	2003.6458	12.0470**
Within groups	9	1496.8750	166.3194	
Total	11	5504.1667		

Duncan's multiple range test

Concentration in ppm	0	5	10
Mean	3.125	4.3750	42.500

TABLE 14.--Anova and Duncan's multiple range test of mortality data for Experiment VI found in Table 6

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	16278.1250	3255.6250	8.6060**
Within groups	18	6809.3750	378.2986	
Total	23	23087.5000		

Duncan's multiple range test

Concentration in ppm	0	5	6	7	8	9
Mean	6.875	45.625	52.500	58.750	77.500	88.750

TABLE 15.--Anova and Duncan's multiple range test of mortality data for Experiment VII found in Table 7

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	8998.9583	1799.7917	29.2023**
Within groups	18	1109.3750	61.6319	
Total	23	10108.3333		

Duncan's multiple range test

Concentration in ppm.	3	4	0	5	6	7
Mean	1.250	1.875	3.750	3.750	13.750	55.625

TABLE 16.--Anova and Duncan's multiple range test of mortality data for Experiment VIII found in Table 8

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	8689.0625	2896.3542	14.6535**
Within groups	12	2371.8750	197.6563	
Total	15	11060.9375		

Duncan's multiple range test

Concentration in ppm	0	3	4	5
Mean	3.125	27.500	37.500	68.125

TABLE 17.--Anova of data on the per cent females for Experiment I

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	294.7601	98.2534	1.5633 ns
Within groups	12	754.1959	62.8497	
Total	15	1048.9559		

TABLE 18.--Anova of data on the per cent females for Experiment II

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	488.8215	162.9405	2.1275 ns
Within groups	8	612.6882	76.5860	
Total	11	1101.5097		

TABLE 19.--Anova of data on the per cent females for Experiment III

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	857.3451	171.4690	1.7559 ns
Within groups	18	1757.7552	97.6531	
Total	23	2615.1003		

TABLE 20.--Anova of data on the per cent females for Experiment IV

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	214.2979	42.8596	0.6326 ns
Within groups	18	1219.4887	67.7494	
Total	23	1433.7866		

TABLE 21.--Anova of data on the per cent females for Experiment V

ANOVA

Source	df	SS	MS	F ratio
Between groups	2	13.3935	6.6968	0.0675 ns
Within groups	9	892.4921	99.1658	
Total	11	905.8856		

TABLE 22.--Anova of data on the per cent females for Experiment VI

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	1033.8655	206.7741	0.7256 ns
Within groups	18	5129.5012	284.9723	
Total	23	6163.3667		

TABLE 23.--Anova of data on the per cent females for Experiment VIII

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	560.6893	186.8964	1.6056 ns
Within groups	12	1396.8622	116.4052	
Total	15	1957.5516		

TABLE 24.--Anova of data on average male pupal weights for Experiment II

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	0.0477	0.0159	2.1140 ns
Within groups	8	0.0602	0.0075	
Total	11	0.1078		

TABLE 25.--Anova of data on average male pupal weights for Experiment IV

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	0.0098	0.0020	0.5128 ns
Within groups	18	0.0690	0.0038	
Total	23	0.0788		

TABLE 26.--Anova of data on average male pupal weights for Experiment V

ANOVA

Source	df	SS	MS	F ratio
Between groups	2	0.0422	0.0211	2.5572 ns
Within groups	9	0.0743	0.0083	
Total	11	0.1166		

TABLE 27.--Anova of data on average male pupal weights for Experiment VIII

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	0.0157	0.0052	1.7520 ns
Within groups	12	0.0358	0.0030	
Total	15	0.0515		

TABLE 28.--Anova and Duncan's multiple range test of data on average male pupal weights for Experiment VI

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	0.1491	0.0298	12.4280**
Within groups	18	0.0432	0.0024	
Total	23	0.1923		

Duncan's multiple range test

Concentration in ppm	9	8	6	7	5	0
Mean	0.1103	0.2042	0.2522	0.2562	0.3080	0.3607

TABLE 29.--Anova and Duncan's multiple range test of data on average female pupal weights for Experiment IV

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	0.0773	0.0155	6.2319**
Within groups	18	0.0447	0.0025	
Total	23	0.1220		

Duncan's multiple range test

Concentration in ppm	1	2.5	0.5	1.5	2	0
Mean	0.4967	0.5007	0.5330	0.5365	0.5892	0.6600

TABLE 30.--Anova and Duncan's multiple range test of data on average female pupal weights for Experiment V

ANOVA

Source	df	SS	MS	F ratio
Between groups	2	0.1076	0.0538	35.0434**
Within groups	9	0.0138	0.0015	
Total	11	0.1214		

Duncan's multiple range test

Concentration in ppm	10	5	0
Mean	0.3472	0.5320	0.5610

TABLE 31.--Anova and Duncan's multiple range test of data on average female pupal weights for Experiment VI

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	0.5183	0.1037	10.3815**
Within groups	18	0.1797	0.0100	
Total	23	0.6980		

Duncan's multiple range test

Concentration in ppm	9	8	7	6	5	0
Mean	0.1658	0.2775	0.3682	0.3697	0.5022	0.6210

TABLE 32.--Anova and Duncan's multiple range test of data on average female pupal weights for Experiment VIII

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	0.1882	0.0627	8.1189**
Within groups	12	0.0927	0.0077	
Total	15	0.2809		

Duncan's multiple range test

Concentration in ppm	5	4	3	0
Mean	0.3680	0.4150	0.5690	0.6330

TABLE 33.--Anova of data on average female pupal weights for Experiment II

ANOVA

Source	df	SS	MS	F ratio
Between groups	3	0.0470	0.0157	0.8087 ns
Within groups	8	0.1549	0.0194	
Total	11	0.2018		

TABLE 34.--Anova and Duncan's multiple range test of data on pupation rates for A. aegypti (L.) larvae exposed to methylene blue in Experiment II

ANOVA

Source	df	SS	MS	F ratio
Treatments	3	6275.02	2091.67	165.08**
Reps:treat.	8	102.34	12.79	0.75 ns
Error	330	5554.22	16.83	
Total	341			

Duncan's multiple range test

Concentration in ppm	0	0.5	2.5	4.5
Mean	4.83	10.12	14.41	19.14

TABLE 35.--Anova and Duncan's multiple range test of data on pupation rates for A. aegypti (L.) larvae exposed to methylene blue in Experiment III

ANOVA

Source	df	SS	MS	F ratio
Treatments	5	21716.08	4343.21	143.34**
Reps:treat.	18	545.46	30.30	1.78**
Error	708	11996.81	16.99	
Total	731			

Duncan's multiple range test

Concentration in ppm	0	0.5	1	1.5	2	2.5
Mean	3.40	6.72	8.34	10.01	18.23	21.65

TABLE 36.--Anova and Duncan's multiple range test of data on pupation rates for A. aegypti (L.) larvae exposed to methylene blue in Experiment IV

ANOVA

Source	df	SS	MS	F ratio
Treatments	5	6368.16	1273.63	86.34**
Reps:treat.	18	265.53	14.75	2.84**
Error	864	4481.19	5.19	
Total	887			

Duncan's multiple range test

Concentration in ppm	0	0.5	1	1.5	2	2.5
Mean	2.33	3.41	5.10	6.13	8.71	9.85

TABLE 37.--Anova and Duncan's multiple range test of data on pupation rates for A. aegypti (L.) larvae exposed to neutral red in Experiment V

ANOVA

Source	df	SS	MS	F ratio
Treatments	2	4597.13	2298.56	77.49**
Reps:treat.	9	266.94	29.66	2.59**
Error	392	4482.93	11.43	
Total	403			

Duncan's multiple range test

Concentration in ppm	0	5	10
Mean	4.61	5.66	12.98

TABLE 38.--Anova and Duncan's multiple range test of data on pupation rates for A. aegypti (L.) larvae exposed to neutral red in Experiment VI

ANOVA

Source	df	SS	MS	F ratio
Treatments	5	23319.59	4663.92	115.73**
Reps:treat.	12	483.68	40.30	1.48 ns
Error	320	8686.02	27.14	
Total	337			

Duncan's multiple range test

Concentration in ppm	0	5	6	7	8	9
Mean	2.64	10.96	18.31	21.60	24.10	26.44

TABLE 39.--Anova and Duncan's multiple range test of data on pupation rates for A. aegypti (L.) larvae exposed to neutral red in Experiment VII

ANOVA

Source	df	SS	MS	F ratio
Treatments	5	16535.84	3307.16	65.37**
Reps:treat.	18	910.74	50.59	3.86**
Error	808	10591.61	13.10	
Total	831			

Duncan's multiple range test

Concentration in ppm	0	3	4	5	6	7
Mean	2.57	3.59	6.91	9.12	13.00	16.65

TABLE 40.--Anova of data on average larval weights from Experiment IX which determined the palatability of dyed food

ANOVA

Source	df	SS	MS	F ratio
Between groups	5	0.0083	0.0017	1.7669 ns
Within groups	18	0.0170	0.0009	
Total	23	0.0253		

TABLE 41.--Anova and Duncan's multiple range test of data on pupation rates of A. aegypti (L.) larvae exposed to methylene blue, neutral red, and nile blue during the fourth instar (or 24 hrs.) in Experiment XI

ANOVA

Source	df	SS	MS	F ratio
Treatments	3	588.44	196.14	3.31*
Reps:treat.	12	710.54	59.21	2.24**
Error	566	14923.80	26.36	
Total	581			

*Indicates significance at the 95% level in this and all other tables.

Duncan's multiple range test

	Dye			
	Control	Methylene blue	Neutral red	Nile blue
Concentration in ppm	0	2.5	7	2.5
Mean	7.08	8.76	9.40	9.68

TABLE 42.--Anova and Duncan's multiple range test of data on pupation rates of A. aegypti (L.) larvae exposed to methylene blue, neutral red, and nile blue during the third instar (or 24 hrs.) in Experiment X

ANOVA

Source	df	SS	MS	F ratio
Treatments	3	1237.96	412.65	7.33**
Reps:treat.	12	674.79	56.23	2.23**
Error	535	13472.03	25.18	
Total	550			

Duncan's multiple range test

	Dye			
	Control	Neutral red	Nile blue	Methylene blue
Concentration in ppm	0	7	2.5	2.5
Mean	7.08	8.05	9.90	10.89

TABLE 43.--Anova and Duncan's multiple range test of data on pupation rates of A. aegypti (L.) larvae exposed to methylene blue, neutral red, and nile blue during the third and fourth instars (or 48 hrs.) in Experiment XII

ANOVA

Source	df	SS	MS	F ratio
Treatments	3	2065.90	688.63	12.83**
Reps:treat.	12	643.97	53.66	1.98*
Error	569	15389.46	27.04	
Total	584			

Duncan's multiple range test

	Dye			
	Control	Nile blue	Neutral red	Methylene blue
Concentration in ppm	0	2.5	7	2.5
Mean	7.08	10.65	11.53	11.80

TABLE 44.--Number of hours (to the nearest observational interval) for 50% or more pupation[a]

Experiment No.	Dye	Concentrations in ppm	Hours for 50% or more pupation
II	Methylene blue	0	16
		0.5 ppm	36
		2.5 ppm	56
		4.5 ppm	76
III	Methylene blue	0	12
		0.5 ppm	24
		1.0 ppm	32
		1.5 ppm	36
		2.0 ppm	68
		2.5 ppm	80
IV	Methylene blue	0	16
		0.5 ppm	24
		1.0 ppm	40
		1.5 ppm	48
		2.0 ppm	64
		2.5 ppm	72
V	Neutral red	0	16
		5.0 ppm	20
		10.0 ppm	52
VI	Neutral red	0	16
		5.0 ppm	80
		6.0 ppm	136
		7.0 ppm	160
		8.0 ppm	176
		9.0 ppm	200
VII	Neutral red	0	16
		3.0 ppm	32
		4.0 ppm	48
		5.0 ppm	72
		6.0 ppm	96
		7.0 ppm	128

[a]Pupation hour zero in the control denotes initiation of pupation.

TABLE 45.--Pupation rates (in %) for A. aegypti (L.) larvae exposed to methylene blue solutions in Experiment II[a]

Concentration in ppm	Rep.	Hours after initial pupation: 12	24	36	48	60	72	84	96
0	1	38.5	84.6	87.2	94.9				
	2	31.6	84.2	94.7					
	3	33.3	87.2	92.3					
0.5	1	0	10.8	48.6	78.4	97.3			
	2	0	11.4	48.6	74.3	85.7	91.4		
	3	0	29.7	54.1	72.9	86.5	94.6		
2.5	1	0	2.8	22.2	41.7	75.0	83.3	86.1	88.9
	2	0	0	8.3	30.6	52.8	77.8	83.3	91.7
	3	0	0	18.9	35.1	48.6	64.9	81.1	91.9
4.5	1	0	0	0	0	12.5	18.8	25.0	43.8
	2	0	0	0	12.5	12.5	17.5	50.0	75.0
	3	0	0	0	0	0	0	33.3	33.3

[a]Data were originally recorded at 4-hour intervals but are condensed above for convenience of presentation. Pupation in this table has been recorded as percentages up to at least 90% (to the nearest observational interval). Values well above 90% are not recorded because stragglers tend to mask the effects being studied.

TABLE 46.--Pupation rates for A. aegypti (L.) larvae exposed to methylene blue solutions in Experiment III[a]

Concentration in ppm	Rep.	Hours after initial pupation								
		12	24	36	48	60	72	84	96	108
0	1	45.9	91.8							
	2	66.6	100.0							
	3	65.0	97.5							
	4	72.5	95.0							
0.5	1	5.0	55.0	82.5	92.5					
	2	2.6	61.5	87.2	94.9					
	3	10.3	58.9	82.1	87.2	97.4				
	4	13.9	61.1	86.1	97.2					
1	1	0	50.0	68.4	97.4					
	2	0	28.6	65.7	82.9	97.2				
	3	2.5	25.0	75.0	97.5					
	4	2.6	30.8	58.9	79.5	94.9				
1.5	1	0	9.4	59.4	87.5	93.8				
	2	0	12.0	56.0	76.0	88.0	96.0			
	3	0	3.6	25.0	67.9	92.9				
	4	5.0	40.0	60.0	87.5	97.5				
2	1	0	0	0	8.3	29.2	50.0	70.8	83.3	87.5
	2	0	0	4.0	20.0	40.0	56.0	68.0	92.0	
	3	0	0	0	10.4	34.5	62.1	72.4	86.2	93.1
	4	0	4.8	4.8	33.3	52.4	66.7	80.9	85.7	85.7
2.5	1	0	0	0	0	28.6	35.7	50.0	71.4	85.7
	2	0	0	0	0	38.5	46.2	61.5	69.2	76.9
	3	0	0	0	8.3	16.7	33.3	41.7	58.3	83.3
	4	0	0	0	10.0	20.0	40.0	40.0	50.0	60.0

[a]Data were originally recorded at 4-hour intervals but are condensed above for convenience of presentation. Pupation in this table has been recorded as percentages up to at least 90% (to the nearest observational interval). Values well above 90% are not recorded because stragglers tend to mask the effects being studied.

TABLE 47.--Pupation rates (in %) for A. aegypti (L.) larvae exposed to methylene blue solutions in Experiment IV[a]

Concentration in ppm	Rep.	Hours after initial pupation: 16	32	48	64	80	96	112
0	1	82.1	94.9					
	2	73.7	97.4					
	3	81.6	97.4					
	4	60.0	91.4					
0.5	1	24.3	94.6					
	2	36.1	86.1	97.2				
	3	13.9	88.9	97.2				
	4	18.4	86.8	97.4				
1	1	0	47.5	82.5	92.5			
	2	0	50.0	75.0	100.0			
	3	10.5	65.8	97.4				
	4	2.6	36.8	78.9	92.1			
1.5	1	0	15.2	60.6	96.9			
	2	0	5.4	43.2	81.1	97.3		
	3	0	39.5	76.3	97.4			
	4	0	11.4	62.9	91.4			
2	1	0	2.9	32.4	58.8	88.2	97.1	
	2	0	2.7	16.2	51.4	78.4	91.9	
	3	0	0	11.8	52.9	70.6	82.4	91.2
	4	0	2.5	20.0	55.0	87.5	97.5	
2.5	1	0	0	0	20.5	51.3	69.2	89.7
	2	0	2.6	17.9	64.1	84.6	89.7	97.4
	3	0	0	8.8	26.5	55.9	85.3	94.1
	4	0	0	2.9	42.9	71.4	85.7	91.4

[a]Data were originally recorded at 8-hour intervals but are condensed above for convenience of presentation. Pupation in this table has been recorded as percentages up to at least 90% (to the nearest observational interval). Values well above 90% are not recorded because stragglers tend to mask the effects being studied.

TABLE 18.--Pupation rates (in %) for A. aegypti (L.) larvae exposed to neutral red solutions in Experiment V[a]

Concentration in ppm	Rep.	Hours after initial pupation 12	24	48	60	72	84	96	108
0	1	38.5	84.6	87.2	94.9				
	2	31.6	84.2	94.7					
	3	25.6	87.2	97.4					
	4	33.3	87.2	92.3					
5	1	30.8	84.6	94.9					
	2	22.5	67.5	95.0					
	3	18.9	70.3	89.2	94.6				
	4	8.1	40.5	89.2	89.2				
10	1	0	0	21.2	57.6	66.7	75.8	90.9	
	2	0	0	3.7	22.2	59.3	74.1	81.5	92.6
	3	0	0	0	7.7	84.6	84.6	84.6	84.6
	4	4.3	13.0	21.7	39.1	52.2	78.3	82.6	86.9

[a]Data were originally recorded at 4-hour intervals but are condensed above for convenience of presentation. Pupation in this table has been recorded as percentages up to at least 90% (to the nearest observational interval). Values well above 90% are not recorded because stragglers tend to mask the effects being studied.

TABLE 49.--Pupation rates for A. aegypti (L.) larvae exposed to neutral red in Experiment VI[a]

Concentration in ppm	Rep.	Hours after initial pupation: 16	32	48	96	128	144	176	224	240	256	272	288
0	1	69.4	91.7										
	2	81.6	92.1										
	3	67.6	91.9										
	4	50.0	92.1										
5	1	0	0	10.0	90.0								
	2	0	0	28.6	85.7	100.0							
	3	0	0	10.5	73.7	92.1							
	4	0	0	6.3	71.9	87.5	90.6						
6	1	0	0	0	45.5	54.5	72.7	81.8	90.9				
	2	0	0	0	20.8	66.7	75.0	79.2	83.3	91.7			
	3	0	0	0	3.6	21.4	50.0	82.1	82.1	92.9			
	4	0	0	16.7	41.7	66.7	75.0	91.7					
7	1	0	0	0	0	25.0	50.0	65.0	80.0	90.0			
	2	0	0	0	11.8	29.4	41.2	58.8	82.4	88.3	94.1		
	3	0	0	0	0	38.5	46.2	61.5	69.2	76.9	84.6	84.6	92.3
	4	0	0	0	0	12.5	37.5	68.8	87.5	100.0			
8	1	0	0	0	0	0	22.2	77.8	88.9				
	2	0	0	0	0	15.4	23.1	46.2	84.6	92.3			
	3	0	0	0	0	0	20.0	40.0	60.0	60.0	60.0	80.0	100.0
	4	0	0	0	0	0	50.0	50.0	50.0	50.0	50.0	100.0	
9	1	100% mortality											
	2	0	0	0	0	20.0	20.0	40.0	80.0	80.0	100.0		
	3	0	0	0	0	0	0	50.0	50.0	75.0	100.0		
	4	0	0	0	0	0	0	11.1	55.6	77.8	77.8	77.8	77.8

[a]Data were originally recorded at 8-hour intervals but are condensed above for convenience of presentation. Pupation in this table has been recorded as percentages up to at least 90% (to the nearest observational interval). Values well above 90% are not recorded because stragglers tend to mask the effects being studied.

TABLE 50.--Pupation rates for A. aegypti (L.) larvae exposed to neutral red in Experiment VII[a]

Concentration in ppm	Rep.	Hours after initial pupation											
		16	32	48	64	80	128	144	160	176	192	208	224
0	1	61.5	89.7	92.3									
	2	61.5	94.8										
	3	81.5	97.3										
	4	61.5	89.7	92.3									
3	1	5.0	82.5	97.5									
	2	12.5	67.5	100.0									
	3	12.8	76.9	94.8									
	4	35.0	90.0										
4	1	0	20.0	67.5	77.5	92.5							
	2	0	12.5	52.5	77.5	90.0							
	3	0	15.7	55.2	76.3	92.1							
	4	0	28.2	76.9	84.6	92.3							
5	1	0	7.5	27.5	50.0	75.0	95.0						
	2	0	0	8.1	35.1	70.2	91.8						
	3	0	0	5.1	38.4	69.2	89.7	94.8					
	4	0	18.4	52.6	73.6	94.7							
6	1	0	0	0	3.3	20.0	76.6	80.0	86.6	86.6	86.6	96.6	
	2	0	0	2.6	18.4	50.0	81.5	92.1					
	3	0	0	0	9.1	30.3	78.7	84.8	90.9				
	4	0	0	0	13.5	37.8	81.0	91.8					
7	1	0	0	0	0	6.6	46.6	66.6	73.3	86.6	100.0		
	2	0	0	0	0	0	25.0	50.0	62.5	62.5	75.0	81.2	93.7
	3	0	0	0	7.4	11.1	59.2	77.7	88.8	92.5			
	4	0	0	0	8.3	8.3	66.6	83.3	91.6				

[a]Data were originally recorded at 8-hour intervals but are condensed above for convenience of presentation. Pupation in this table has been recorded as percentages up to at least 90% (to the nearest observational interval). Values well above 90% are not recorded because stragglers tend to mask the effects being studied.

TABLE 51.--Data from Experiment I on per cent females and mortalities of mosquito larvae exposed to methylene blue

Concentration in ppm	Rep.	Per cent females	Per cent mortality
0	1	41.02	2.50
	2	46.15	2.50
	3	66.66	2.50
	4	41.02	0.00
0.5	1	52.63	2.50
	2	50.00	12.50
	3	55.55	10.00
	4	57.50	0.00
1.0	1	51.28	2.50
	2	40.00	0.00
	3	60.00	0.00
	4	55.00	0.00
1.5	1	40.00	0.00
	2	46.15	2.50
	3	46.15	2.50
	4	37.50	0.00

TABLE 52.--Data from Experiment II on per cent females, mortalities, and pupal weights of mosquito larvae exposed to methylene blue

Concentration in ppm	Rep.	Per cent females	Per cent mortality	Avg. wt/pupa in mg	
				Male	Female
	1	53.84	2.50	.5777	.5428
0	2	36.84	5.00	.3166	.5642
	3	43.58	2.50	.3227	.5235
	1	59.45	7.50	.3333	.5381
0.5	2	54.28	12.50	.3062	.5263
	3	37.83	7.50	.3391	.3143
	1	52.77	10.00	.3411	.5157
2.5	2	44.44	10.00	.3200	.5125
	3	48.64	7.50	.3105	.4777
	1	43.75	60.00	.3000	.4142
4.5	2	25.00	80.00	.1333	.1100
	3	33.33	92.50	.2500	.6000

TABLE 53.--Data from Experiment III on per cent females and mortalities of mosquito larvae exposed to methylene blue

Concentration in ppm	Rep.	Per cent females	Per cent mortality
0	1	47.22	7.50
	2	53.84	2.50
	3	52.50	0.00
	4	47.50	0.00
0.5	1	42.50	0.00
	2	48.71	2.50
	3	38.46	2.50
	4	42.85	10.00
1.0	1	50.00	5.00
	2	51.42	12.50
	3	57.89	0.00
	4	47.36	2.50
1.5	1	46.87	20.00
	2	28.00	37.50
	3	51.85	30.00
	4	58.97	0.00
2.0	1	50.00	40.00
	2	50.00	35.00
	3	67.85	27.50
	4	40.00	47.50
2.5	1	35.71	65.00
	2	14.28	65.00
	3	50.00	70.00
	4	40.00	75.00

TABLE 54.--Data from Experiment IV on per cent females, mortalities, and pupal weights of mosquito larvae exposed to methylene blue

Concentration in ppm	Rep.	Per cent females	Per cent mortality	Avg. wt/pupa in mg Male	Female
0	1	37.14	12.50	.2944	.6400
	2	50.00	5.00	.1000	.6421
	3	44.73	5.00	.3619	.6823
	4	51.28	2.50	.3714	.6769
0.5	1	48.64	7.50	.3277	.5555
	2	40.54	7.50	.3263	.5533
	3	38.88	10.00	.2700	.4714
	4	39.47	5.00	.3250	.5533
1.0	1	45.00	0.00	.2950	.4611
	2	45.00	0.00	.2727	.4555
	3	42.10	5.00	.3095	.5250
	4	34.21	5.00	.3217	.5461
1.5	1	39.39	17.50	.3411	.5615
	2	54.05	7.50	.3062	.5200
	3	36.84	5.00	.2045	.4857
	4	28.57	12.50	.3347	.5800
2.0	1	61.76	15.00	.3461	.6095
	2	32.43	7.50	.3478	.6083
	3	57.14	12.50	.3571	.6050
	4	42.50	0.00	.3130	.5352
2.5	1	46.15	2.50	.3619	.4055
	2	38.46	2.50	.3666	.5733
	3	47.05	15.00	.3058	.5750
	4	50.00	10.00	.2875	.4500

TABLE 55.--Data from Experiment V on per cent females, mortalities, and pupal weights of mosquito larvae exposed to neutral red

Concentration in ppm	Rep.	Per cent females	Per cent mortality	Avg. wt/pupa in mg Male	Female
0	1	53.84	2.50	.5777	.5428
	2	36.84	5.00	.3166	.5642
	3	48.71	2.50	.3200	.6157
	4	43.58	2.50	.3227	.5235
5	1	39.47	2.50	.3173	.5266
	2	62.96	0.00	.3052	.5631
	3	54.28	7.50	.4875	.4789
	4	35.13	7.50	.3375	.5615
10	1	48.48	17.50	.2705	.3937
	2	62.96	32.50	.2800	.3647
	3	46.15	67.50	.2285	.3166
	4	31.57	52.50	.2153	.3166

TABLE 56.--Data from Experiment VI on sex ratios, mortalities, and pupal weights of mosquito larvae exposed to neutral red

Concentration in ppm	Rep.	Per cent females	Per cent mortality	Avg. wt/pupa in mg Male	Female
0	1	50.00	10.00	.3444	.5666
	2	47.36	5.00	.3550	.6277
	3	44.44	7.50	.3800	.7250
	4	55.26	5.00	.3647	.5660
5	1	60.00	75.00	.3000	.5666
	2	57.14	82.50	.2333	.3250
	3	52.77	5.00	.3470	.6105
	4	38.70	20.00	.3526	.5083
6	1	54.54	72.50	.2400	.4307
	2	59.09	37.50	.2333	.3333
	3	33.33	27.50	.2111	.4000
	4	33.33	72.50	.3250	.3777
7	1	45.00	50.00	.2454	.3777
	2	31.25	57.50	.2909	.4600
	3	23.07	67.50	.2100	.2660
	4	66.66	60.00	.2800	.3700
8	1	55.55	77.50	.1750	.3600
	2	46.15	67.50	.2428	.3000
	3	40.00	87.50	.2000	.1500
	4	50.00	77.50	.2000	.3000
9	1	-----	100.00	-----	-----
	2	20.00	87.50	.1750	.1000
	3	75.00	90.00	.1000	.2300
	4	33.00	77.50	.1666	.3333

TABLE 57.--Data from Experiment VII on mortalities of mosquito larvae exposed to neutral red

Concentration in ppm	Rep.	Per cent mortality
0	1	2.50
	2	2.50
	3	5.00
	4	5.00
3	1	0.00
	2	0.00
	3	2.50
	4	2.50
4	1	0.00
	2	0.00
	3	5.00
	4	2.50
5	1	0.00
	2	7.50
	3	2.50
	4	5.00
6	1	25.00
	2	5.00
	3	17.50
	4	7.50
7	1	62.50
	2	57.50
	3	32.50
	4	70.00

TABLE 58.--Data from Experiment VIII on per cent females, mortalities, and pupal weights of mosquito larvae exposed to neutral red

Concentration in ppm	Rep.	Per cent females	Per cent mortality	Avg. wt/pupa in mg Male	Female
0	1	41.02	2.50	.3391	.6250
	2	52.50	0.00	.3526	.6095
	3	48.57	7.50	.4333	.6647
	4	58.97	2.50	.3687	.6347
3	1	50.00	25.00	.3066	.5466
	2	54.16	35.00	.3454	.5615
	3	47.05	15.00	.3555	.5312
	4	69.23	35.00	.3878	.6388
4	1	26.31	0.00	.3833	.5700
	2	57.14	47.50	.2444	.2916
	3	38.88	55.00	.2272	.3428
	4	36.84	47.50	.3500	.4571
5	1	28.57	62.50	.2300	.2000
	2	58.33	70.00	.2800	.4142
	3	44.44	77.50	.3400	.4250
	4	42.85	62.50	.3500	.4333

TABLE 59.--Data from Experiment IX testing the palatability of yeast suspensions dyed with methylene blue

Concentration in ppm	Rep.	Avg. wt/larva in mg
0	1	.1333
	2	.1435
	3	.1333
	4	.1162
0.5	1	.1236
	2	.1350
	3	.1153
	4	.1175
1.0	1	.1243
	2	.1250
	3	.1189
	4	.1324
1.5	1	.1378
	2	.1307
	3	.1225
	4	.1205
2.0	1	.1256
	2	.1350
	3	.2648
	4	.1289
2.5	1	.1051
	2	.0975
	3	.1333
	4	.0650

TABLE 60.--Raw data from Experiment X of pupation rates of A. aegypti larvae exposed to nile blue, neutral red, and methylene blue during the third instar (or for 24 hours)

		Dye															
		Control				Nile blue				Neutral red				Methylene blue			
Observational interval	Conc. in ppm / Rep.	0				2.5				7				2.5			
		A	B	C	D	1	2	3	4	5	6	7	8	9	10	11	12
1		0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
2		0	2	3	2	0	0	0	0	0	0	1	0	0	0	1	0
3		2	5	4	4	0	0	0	0	0	0	1	2	0	0	0	0
4		4	4	3	4	0	1	1	0	3	0	1	8	2	0	2	0
5		9	5	2	1	0	3	0	0	4	3	4	5	2	2	3	0
6		2	7	3	8	1	1	4	6	5	11	6	2	7	5	5	1
7		7	5	3	4	3	8	4	7	6	5	3	5	8	4	2	3
8		3	0	0	3	4	4	6	6	8	2	3	4	3	1	1	2
9		2	3	2	1	5	6	2	3	5	3	2	3	1	3	2	2
10		3	1	2	3	4	5	2	1	3	3	2	1	2	2	1	3
11		3	0	1	0	2	3	5	0	2	1	1	2	1	3	2	3
12		1	1	1	1	3	5	3	0	1	1	1	1	1	4	3	3
13		1	1	1	2	3	1	0	2	1	2	1	0	2	2	3	1
14			1	2	0	0	0	1	1	0	1	1	1	0	1	2	1
15			0	2	0	2	1	4	1	1	1	0	0	0	1	0	2
16			1	1	0	2	0	1	1	0	1	0	1	0	1	1	4
17			0	1	0	0	1	0	0	0	1	1	0	0	0	1	1
18			0	0	0	2		0	0	1	2	0	1	0	1	0	2
19			0	0	0	0		0	0			1	0	0	1	2	2
20			0	0	0	1		0	0				0	0	1	0	2
21			0	1	0			0	1				0	1	0	1	
22			0		0			0					0		0	2	
23			0		0			0					0		2		
24			0		0								1				
25			1		1												

TABLE 61.--Raw data from Experiment XI of pupation rates of A. aegypti (L.) larvae exposed to nile blue, neutral red, and methylene blue during the fourth instar (or for 24 hours)

		Dye															
		Control				Neutral red				Nile blue			Methylene blue				
Observational interval	Conc. in ppm / Rep.	0				7				2.5			2.5				
		A	B	C	D	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
1		0	0	2	2	3	0	0	0	0	0	0	0	0	0	1	1
2		0	2	3	2	7	0	1	0	2	0	0	2	0	1	2	2
3		2	5	4	4	3	2	1	3	2	2	1	1	3	2	5	4
4		4	4	3	4	5	4	4	3	2	2	4	4	7	2	4	5
5		9	5	2	1	3	5	5	9	3	2	3	6	1	2	3	4
6		2	7	3	8	0	3	3	4	6	4	5	3	5	6	3	3
7		7	5	3	4	3	0	0	2	3	2	1	2	1	2	3	4
8		3	0	0	3	0	3	1	1	1	2	4	3	1	1	1	0
9		2	3	2	1	0	4	1	0	1	3	1	1	2	2	3	2
10		3	1	2	3	1	3	0	1	1	1	2	1	1	3	1	2
11		3	0	1	0	1	1	1	0	6	1	3	0	2	0	3	1
12		1	1	1	1	3	1	3	5	0	2	4	0	3	6	0	4
13		1	1	1	2	0	1	1	1	4	6	3	2	1	1	3	2
14			1	2	0	2	0	3	3	0	1	1	1	0	0	2	2
15			0	2	0	1	4	2	0	1	0	2	0	1	2	0	1
16			1	1	0	0	0	1	1	1	1	1	5	0	0	0	0
17			0	1	0	0	1	5	1	1	4		2	1	2	0	0
18			0	0	0	0	3	0	1	0	0		2	3	0	0	1
19			0	0	0	1	0	2	1	2	1		1	0	1	0	
20			0	0	0	0	1	1	0		0		2	0	1	0	
21			0	1	0	1	0	1	1		0		0	1	1	1	
22			0		0		0	0	0		0		0	1	0		
23			0		0		0	1	0		0		0	1	1		
24			0		0		0		1		0		1				
25			1		1		1		1		1						

TABLE 62.--Raw data from Experiment XII of pupation rates of A. aegypti (L.) larvae exposed to nile blue, neutral red, and methylene blue during the third and fourth instars (or for approximately 48 hours)

		Dye															
		Control				Neutral red				Nile blue				Methylene blue			
Observational interval	Conc. in ppm Rep.	0				7				2.5				2.5			
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1		0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
2		0	2	3	2	0	0	0	0	0	0	0	0	0	0	0	0
3		2	5	4	4	0	0	0	2	0	0	0	0	0	0	1	1
4		4	4	3	4	2	0	0	0	0	0	0	0	0	1	1	4
5		9	5	2	1	2	1	5	4	0	4	1	1	2	1	3	7
6		2	7	3	8	4	5	4	2	1	4	2	7	2	2	3	1
7		7	5	3	4	6	4	2	7	4	6	4	2	4	3	3	3
8		3	0	0	3	3	5	3	5	4	7	2	1	0	5	2	0
9		2	3	2	1	2	2	1	2	3	5	6	5	1	4	1	1
10		3	1	2	3	1	1	2	1	5	4	2	6	2	4	1	1
11		3	0	1	0	1	0	1	0	3	0	5	3	3	4	4	1
12		1	1	1	1	0	4	0	3	3	1	6	1	4	2	4	3
13		1	1	1	2	2	2	2	2	1	1	1	1	3	5	1	3
14			1	2	0	1	0	1	1	1	0	4	0	1	0	1	0
15			0	2	0	3	2	4	2	2	1	0	3	1	0	2	1
16			1	1	0	1	1	2	0	0	1	0	0	1	1	3	0
17			0	1	0	2	1	0	2	2	1	0	3	3	2	0	2
18			0	0	0	0	1	4	2	3	1	0	1	3	2	0	0
19			0	0	0	1	2	2	2	4	1	0	0	2	1	3	2
20			0	0	0	1	2	0		0	0	2	0	0	0	1	3
21			0	1	0	0	0	1		2	0	1	1	2	0	1	
22			0		0	0	0	0			1		0	0	1	2	
23			0		0	0	1	2					0	0	0		
24			0		0	3	1	0					0	3	1		
25			1		1	2		1					2	1			

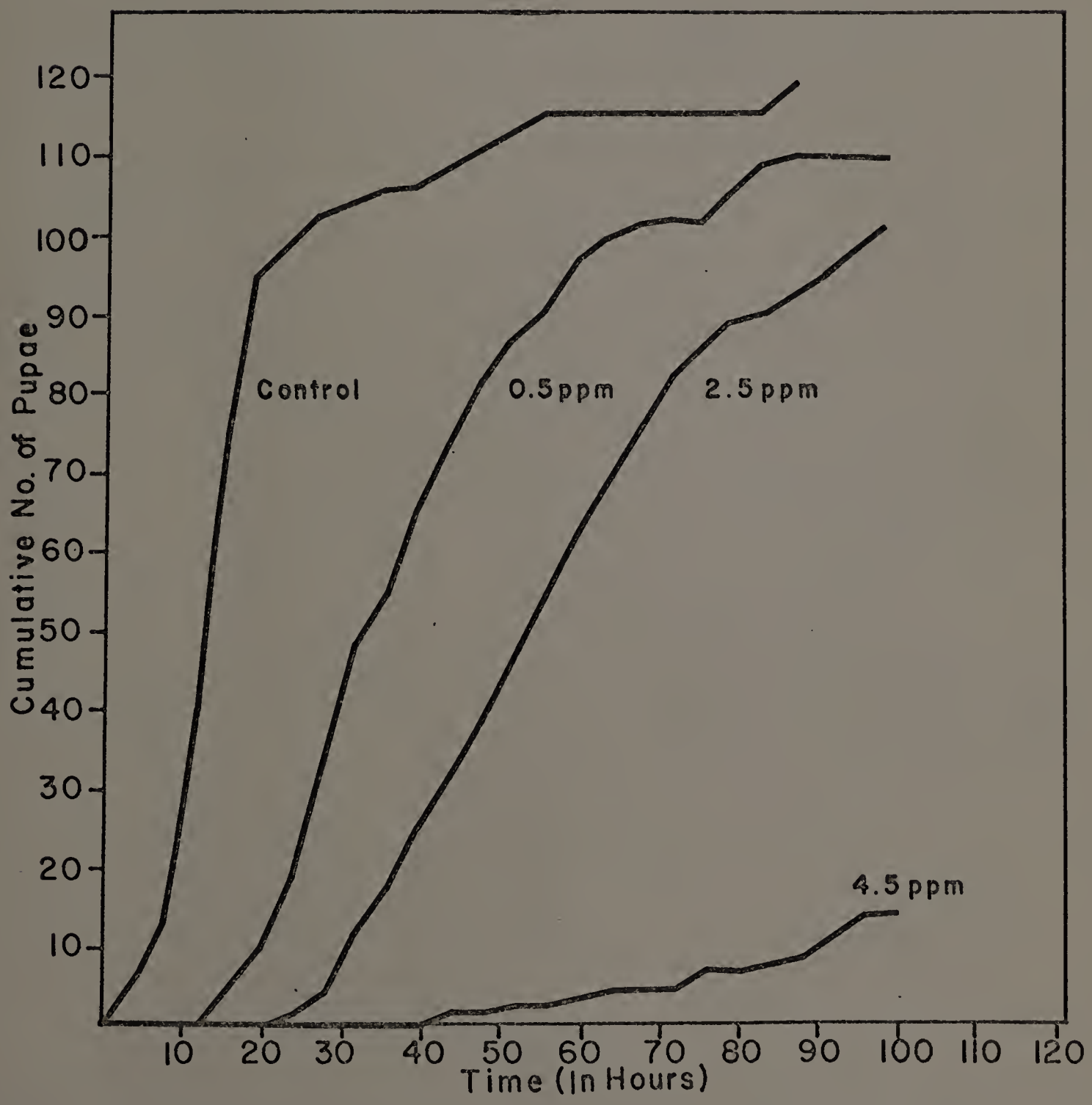

Fig. 1.--Rates of pupation of A. aegypti (L.) larvae exposed to methylene blue in Experiment II. Relative delay of the onset of pupation is indicated by the origin of lines at the abscissa. Mortalities are indicated by the heights of the lines.

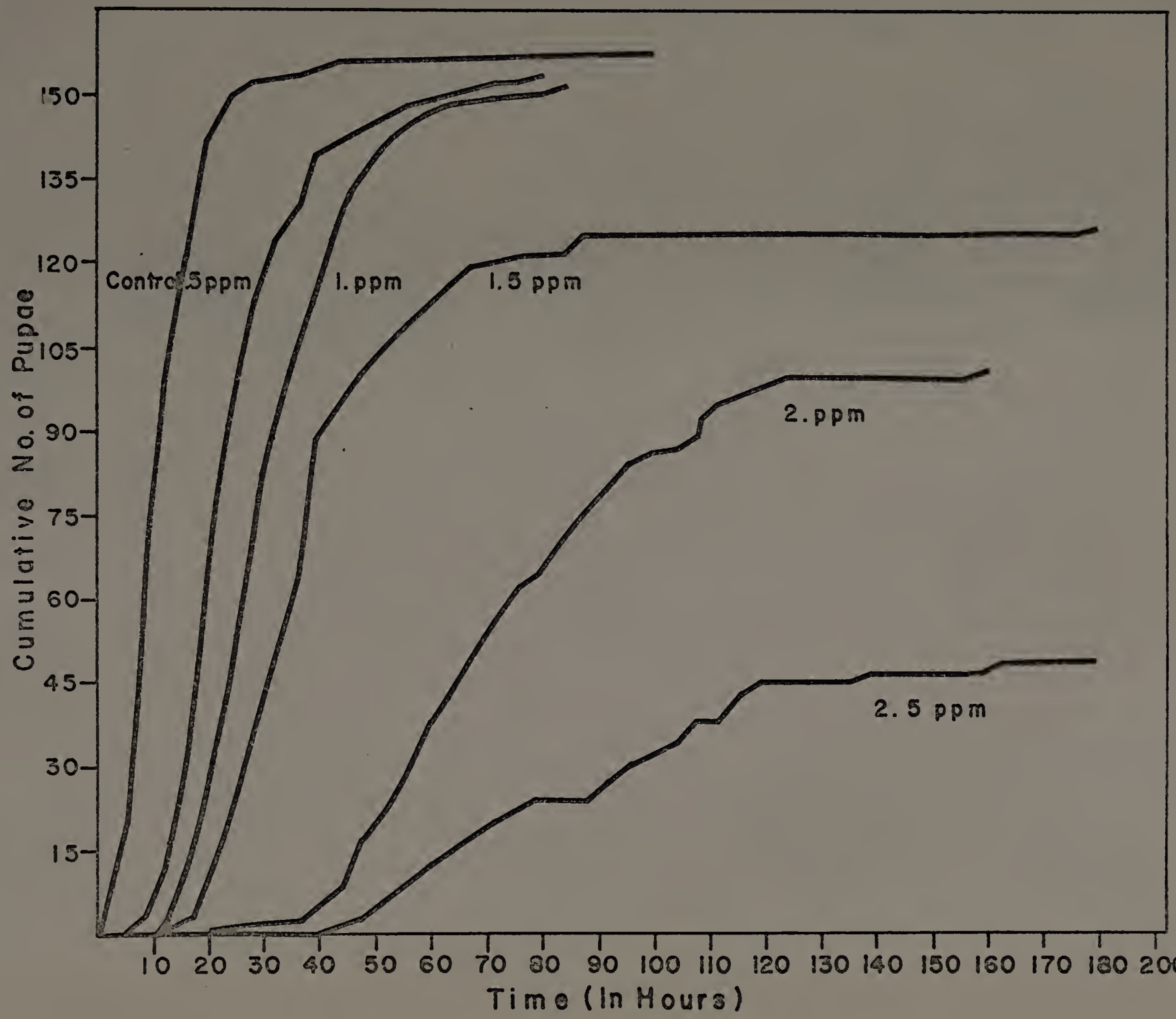

Fig. 2.--Rates of pupation of A. aegypti (L.) larvae exposed to methylene blue in Experiment III. Relative delay of the onset of pupation is indicated by the origins of lines at the abscissa. Mortalities are indicated by the heights of lines.

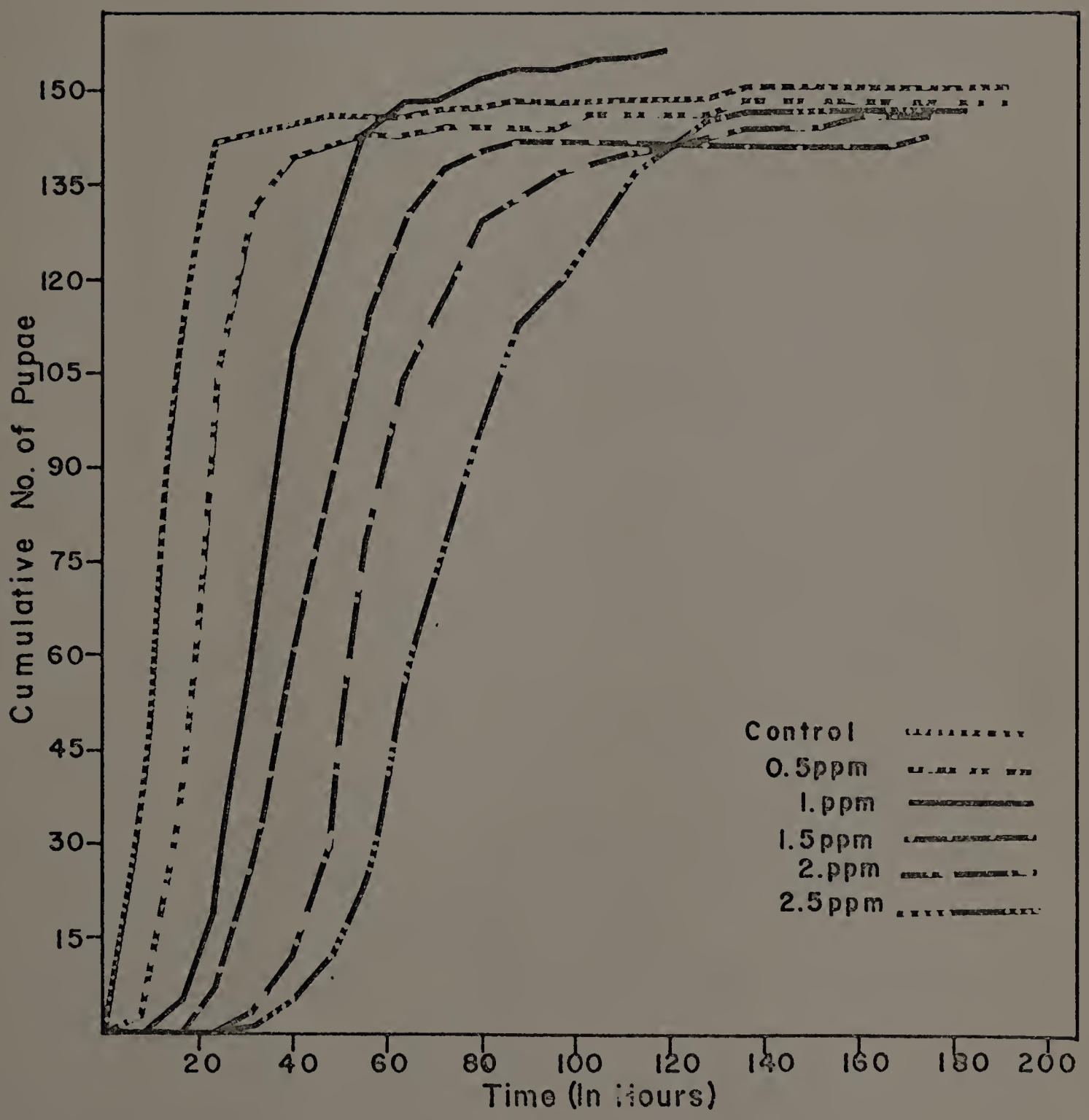

Fig. 3.--Rates of pupation of A. aegypti (L.) larvae exposed to methylene blue in Experiment IV. Relative delay of the onset of pupation is indicated by the origins of lines at the abscissa. Mortalities are indicated by the heights of the lines.

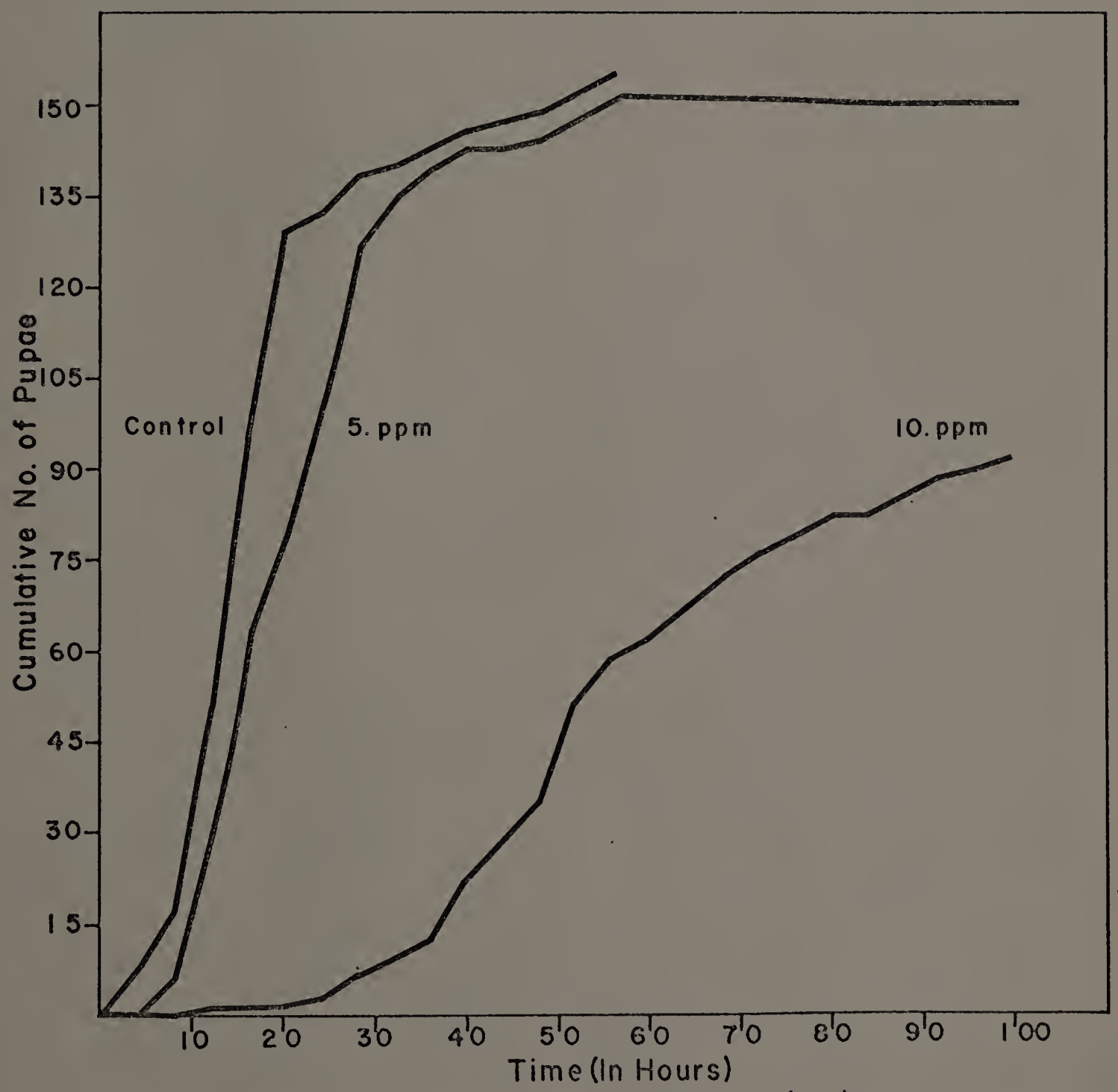

Fig. 4.- Rates of pupation of A. aegypti (L.) larvae exposed to neutral red in Experiment V. Relative delay of the onset of pupation is indicated by the origins of the lines at the abscissa. Mortalities are indicated by the heights of the lines.

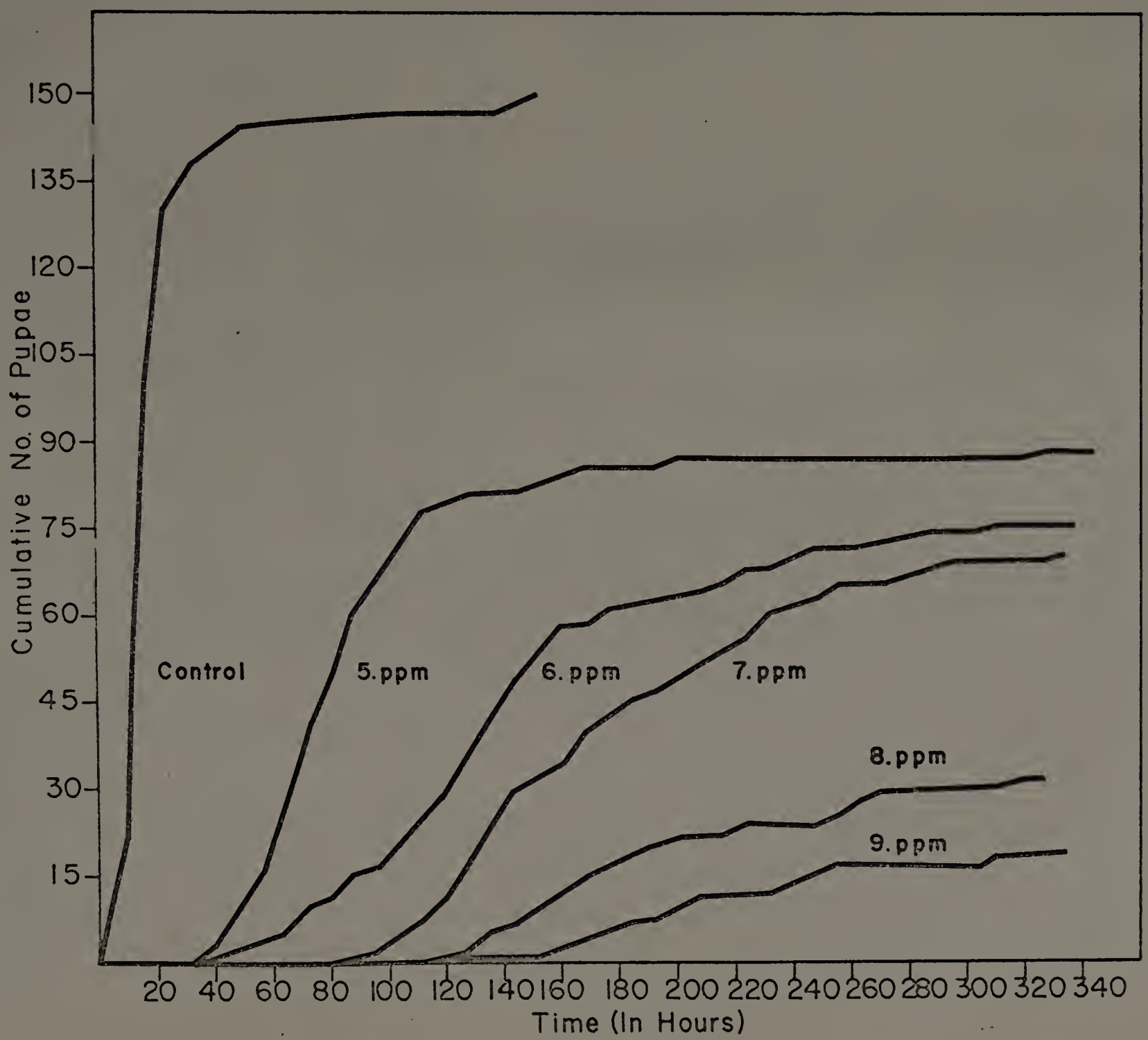

Fig. 5.--Rates of pupation of A. aegypti (L.) larvae exposed to neutral red in Experiment VI. Relative delay of the onset of pupation is indicated by the origin of the lines at the abscissa. Mortalities are indicated by the heights of lines.

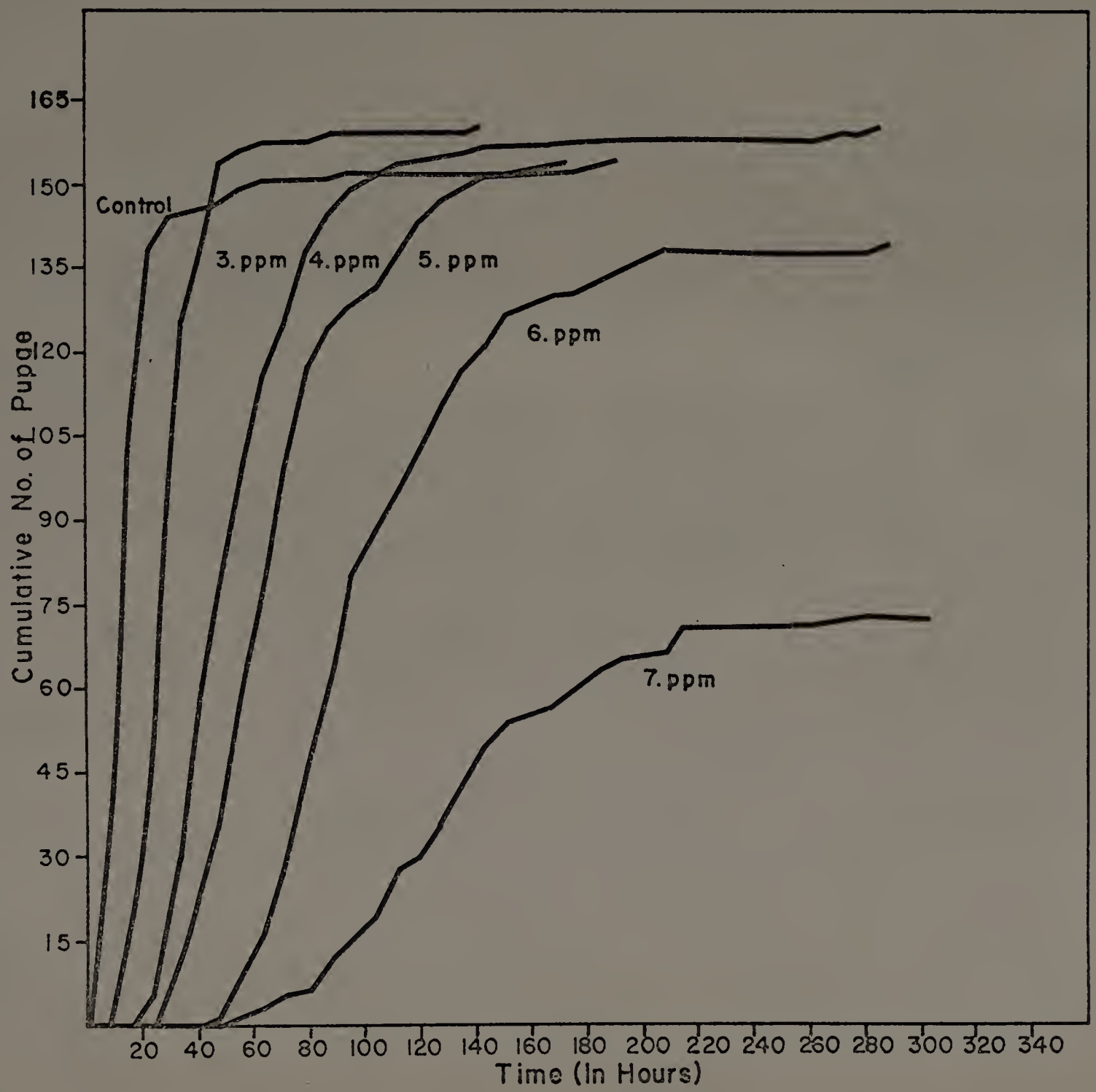

Fig. 6.--Rates of pupation of A. aegypti (L.) larvae exposed to neutral red in Experiment VII. Relative delay of the onset of pupation is indicated by the origin of the lines at the abscissa. Mortalities are indicated by the heights of lines.