EFFECTS OF METABOLIC INHIBITORS ON PLANARIAN REGENERATION [1]

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This study describes abnormalities in regenerating planarians induced by inhibitors of carboxylation enzymes. The physiological significance of CO_2 fixation in animals has been in doubt. One way of assessing its importance in normal metabolism would be to inhibit fixation in a suitable test specimen and study the effects on some well-known physiological process in that specimen. This report is concerned with the influence of *p*-chloromercuribenzoic acid (PCMB) and avidin on regeneration of the head in the planarian, *Dugesia tigrina*.

It is known that enzymatic activity which depends on the free active sulfhydryl or thiol group (−SH) can be inhibited by various compounds, resulting in blockage of normal metabolic pathways. PCMB is a typical mercuric, mercaptide-forming inhibitor of sulfhydryl enzymes. One of the enzymes known to require free −SH groups is the malic enzyme, which catalyzes the reversible decarboxylation of l-malate to pyruvate. Hargreaves (1954) worked with the isolated malic enzyme system of pigeon liver and found PCMB concentrations of 8.3×10^{-6} and 1×10^{-4} *M* inhibitory. Hammen and Lum (1962) showed the presence of CO_2 fixation in the planarian *Dugesia tigrina,* in which exposure to C^{14}-bicarbonate led to labeling of malate and other acids of the citric acid cycle. The main pathway was evidently *via* the malic enzyme system. Coldwater (1933) used the nitroprusside reaction on *Planaria maculata,* now known as *Dugesia tigrina,* and found a high concentration of the −SH group in regenerating tissue during the period of most active cellular division. Thus, it seems logical that PCMB could inhibit or alter normal planarian regeneration.

In an organism's intermediary metabolism, two of the possible pathways of CO_2 fixation are *via* (a) the malic enzyme as indicated above, and (b) the propionyl carboxylase system which catalyzes the conversion of propionate to succinate. Halenz and Lane (1960) isolated propionyl carboxylase from bovine liver mitochondria, and showed that there was an associated enzyme-bound biotin, which is necessary for enzymatic activity. Avidin is a protein isolated from raw egg white, which has a specific biotin-binding capacity. Hammen and Lum (1962) demonstrated the utilization of propionate by the planarian, *Dugesia tigrina,* by means of C^{14}-labeling. Thus, by exposing regenerating planarians to avidin, one might expect some adverse effect on regeneration.

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Methods and Materials

The original stock of flatworms, *Dugesia tigrina,* was obtained from the Champlain Biological Service, Glen Gardner, N. J. The worms were maintained in filtered pond water cultures under laboratory conditions (22–27° C.) and were fed twice a week on raw beef liver. To maintain a healthy stock, culture bowls were cleaned and water changed after each feeding. To study regeneration, worms with an average weight of 4 mg. and length of 10–11 mm. were decapitated behind the auricles and sectioned through the pharyngeal region. The anterior section was that region between the head and the pharynx, and the posterior section was that region behind the pharynx. Each worm then yielded two pieces, each with the possibility of regenerating a head.

The water was obtained from a goldfish pond on the Adelphi College campus, which maintained a variety of plant and animal life. After collection the water was filtered and allowed to stand overnight to reach room temperature before use. As Jenkins (1961) and others have shown, the oxygen consumption of planarians declines quite slowly after the first week of starvation, suggesting a relatively stable state of metabolism. Therefore, all experiments were begun six days after feeding.

To study the normal rate of regeneration, daily observations were made, and the stages of regeneration were classified according to Henderson and Eakin

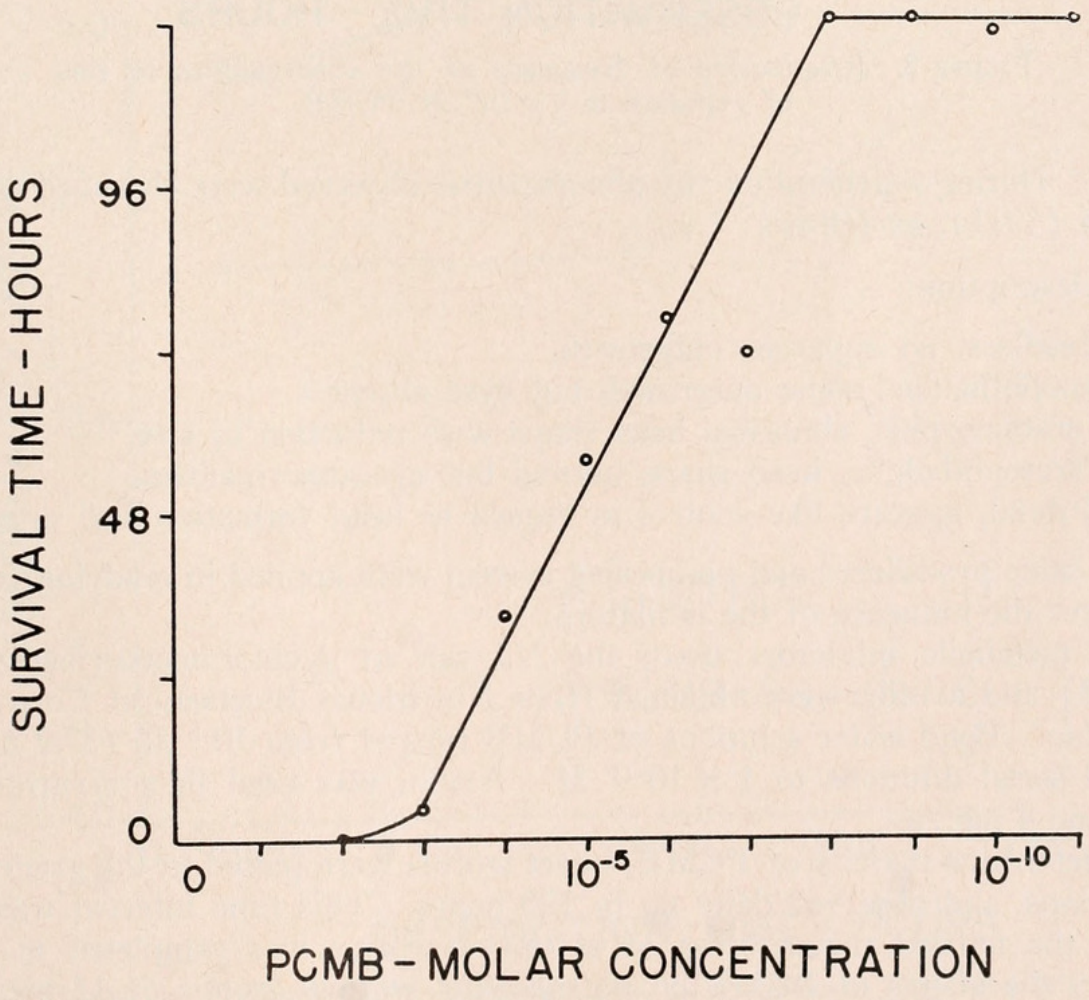

Figure 1. Survival of intact worms in varying concentrations of PCMB.

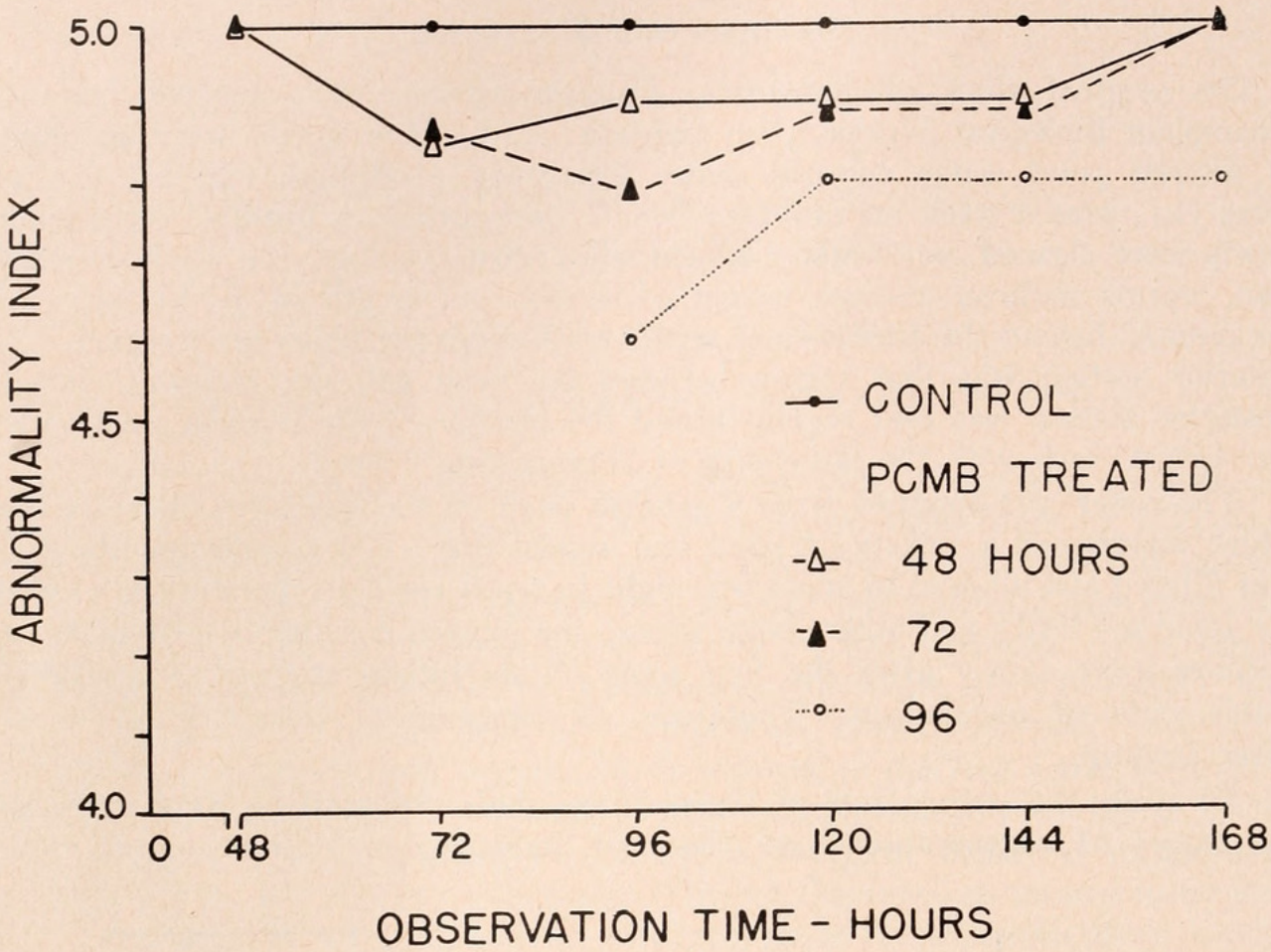

FIGURE 2. Relationship of frequency of eye abnormalities to time of exposure to 1×10^{-8} *M* PCMB.

(1959). During regeneration any abnormalities observed were classified according to Child (1911), as follows:

Index Description

1. Headless, no apparent outgrowth.
2. Anophthalmic, some outgrowth but eyes absent.
3. Teratomorphic, abnormal head shape with reduction in size.
4. Teratophthalmic, head shape normal but eye abnormalities.
5. Normal, appears like control in regard to head formation and eyes.

The same procedures and numbering system were applied in studying regeneration under the influence of the inhibitors.

The metabolic inhibitors used, the Na salt of *p*-chloromercuribenzoic acid (PCMB) and avidin, were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Pond water solutions of PCMB ranged from 10^{-1} *M* (37.9 mg./ml.) by 1:10 serial dilutions to 1×10^{-11} *M*. Avidin was used in concentrations of 0.4, 1, and 4 mg./ml.

To determine toxicity of PCMB, intact worms were placed in the graded series of solutions, and observed daily up to 120 hours. This time interval was chosen because the normal regeneration of sectioned worms was completed in 5 days. To study the effects of PCMB on regenerating worms, groups of 15 pieces each were placed in 1×10^{-8} M, the highest concentration at which lethal effects were

not expected in 120 hours. The exposure times, chosen from preliminary experiments, were 24, 48, 72 and 96 hours after sectioning. After exposure, pieces were removed, washed, and allowed to complete regeneration in pond water. Since that experimental group which was exposed for the longest time had eye abnormalities persisting longer, the 96-hour experiment was repeated, using groups of 30 pieces each.

To study the effect of avidin on regenerating worms, they were placed in solutions of 0.4, 1 and 4 mg./ml. for 24 to 72 hours. A sample of denatured avidin was prepared by heating at 80° C. for 5 minutes. Worms were allowed to regenerate in the two test solutions of avidin and heated avidin along with pond water controls.

Results

Effects of various PCMB concentrations on planarians

In preliminary experiments, the normal regeneration rate of *Dugesia tigrina* was studied in pond water, at room temperature. The anterior and posterior sections regenerated new heads at essentially the same rate, and that regeneration was complete in 5 days.

The survival time of intact worms in various concentrations of PCMB is shown in Figure 1. In concentrations of 10^{-1} and 10^{-2} *M* PCMB, death occurred in a relatively short period of time. The reactions were violent contortions, then

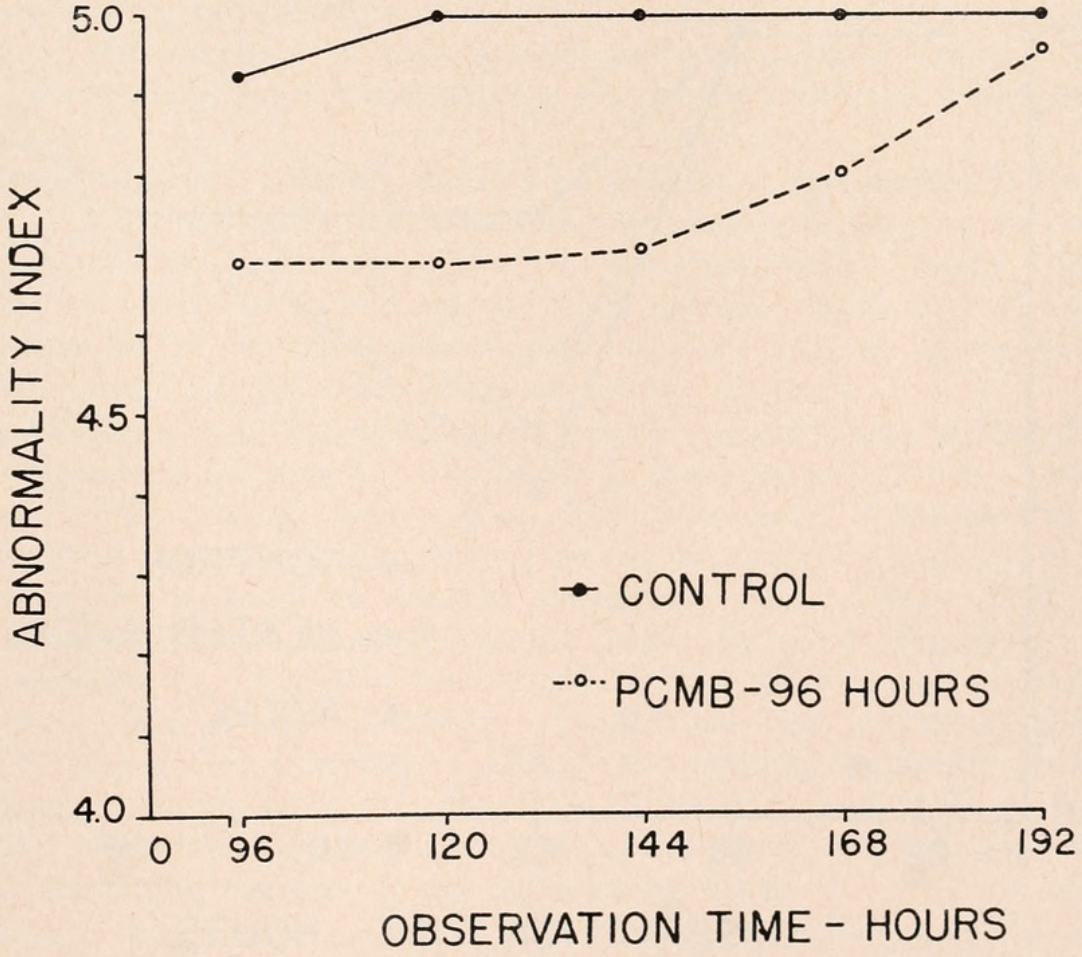

Figure 3. Frequency of eye abnormalities resulting from a 96-hour exposure to 1×10^{-8} *M* PCMB.

complete body paralysis, and finally cytolysis, or cellular disintegration in which the outer cellular layer peels away from the head region first and then proceeds posteriorly along the body.

In concentrations higher than 10^{-8} *M*, the worms did not survive much more than three days (77 hours), while in lower concentrations survival was long enough to observe complete regeneration. When the experiment was stopped at 121 hours, the pond water controls as well as the experimentals in concentrations lower than 10^{-8} *M* were still alive.

The rate of new head regeneration of the experimental pieces in PCMB was found to be essentially the same as in the pond water control group. The most interesting observation was an increased incidence of some form of eye abnormality in all experimental groups, relative to the small number of such defects in the controls. These were in the form of unequal eyes, eyes closer together than normal, eyes further apart than normal, one center eye, or joined eyes, the bridge of which often broke leaving two triangular eyes closer together than normal.

Degree of abnormalities related to duration of exposure

The index values of eye abnormalities resulting from different intervals of PCMB exposure during regeneration are shown by Figure 2. On observation at 168 hours, the experimental animals which had shorter intervals of PCMB exposure appeared identical to the normal controls, indicating correction of de-

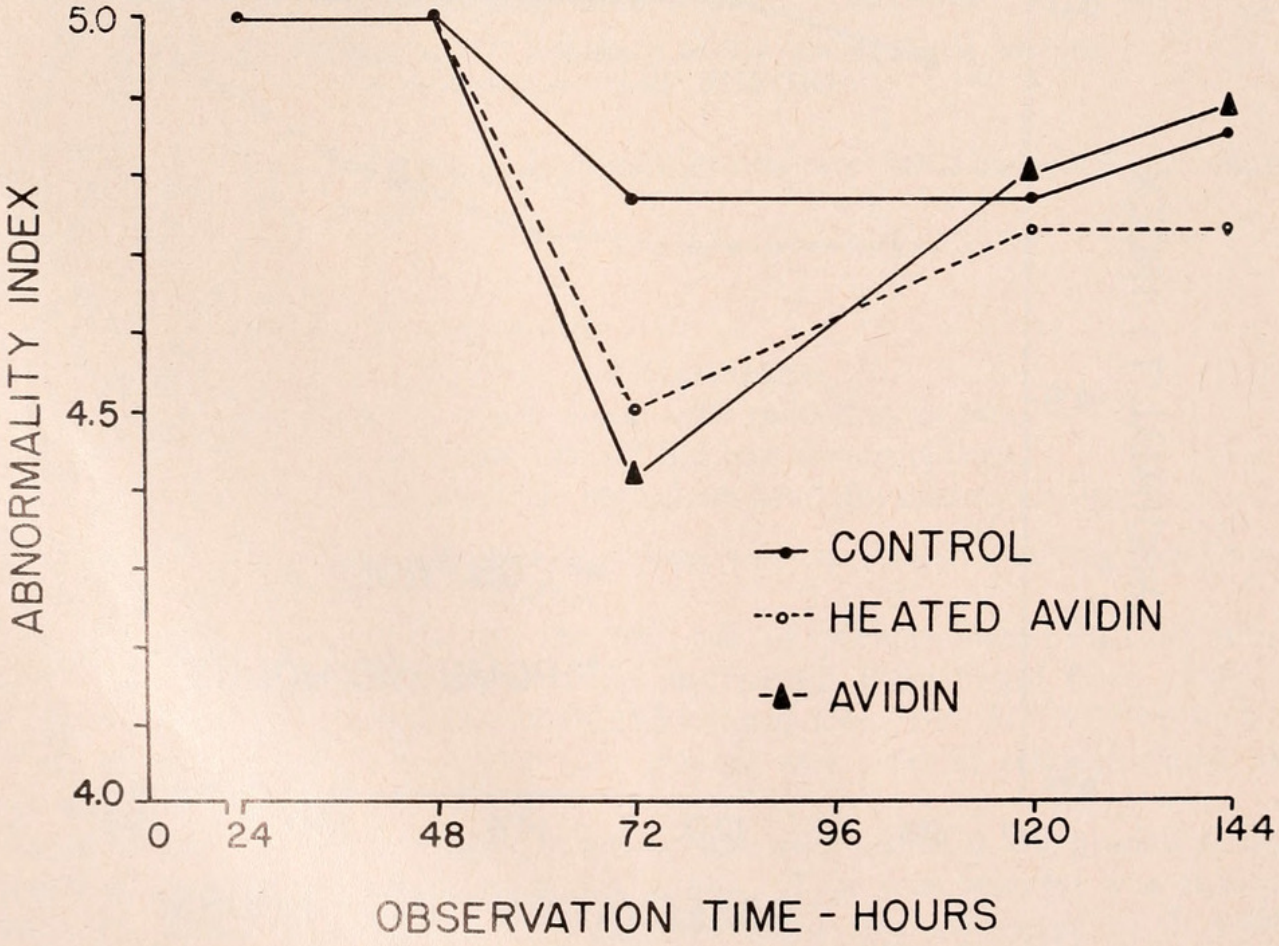

FIGURE 4. Frequency of eye abnormalities resulting from a 24-hour exposure to 1 mg./ml. of heated avidin and avidin.

fects. That experimental group which was exposed for the longest time, 96 hours, had the lowest index number, 4.6, and the most persistent eye abnormalities. A second and larger group was exposed for 96 hours and the results were as shown by Figure 3. In this group the index was first at 4.7, then rose to near 5.0 as the abnormalities tended toward self-correction.

Since it was found in a preliminary experiment that the lower concentration of avidin (0.4 mg./ml.) produced little effect and the higher (4 mg./ml.) had a lethal effect, a concentration of 1 mg./ml. was used with a 24-hour exposure. The results of this avidin experiment are shown by Figure 4. The rate of head regeneration in the test solutions was not significantly different from that of the pond water controls. Some eye abnormalities, mostly joined eyes, were found in the control group, but they tended toward self-correction. Avidin caused the greatest number of abnormalities, index 4.4, while heated avidin had a lesser effect. As in PCMB experiments, there was a tendency to correction of the defects.

Discussion

Influence of PCMB on planarian regeneration related to malic enzyme inhibition

Exposure of worm sections to PCMB solutions for various intervals during their regeneration seemed a promising approach, because Henderson and Eakin (1959) had stated that different enzyme inhibitors are effective at different stages in planarian regeneration. It was found in a preliminary experiment that if intact worms were pretreated with PCMB before sectioning, there was a higher incidence of abnormalities in both control and experimental groups. This occurrence of eye abnormalities was perhaps due to the additional deleterious effect of starvation.

On exposure to high concentrations of PCMB, the worms reacted in the manner described by Murray (1928), who classified the reactions of *Dugesia dorotocephala* to salt solutions as contortions, paralysis, and cytolysis. Hinrichs (1924), in describing the effects of caffein on the same species, stated that disintegration usually starts at the head region and proceeds posteriorly along the body, as a result of the drug having specific action on specific tissues.

Since PCMB is a sulfhydryl-binding agent, it may react with an enzyme which is —SH-dependent for activity, thus causing inactivation. Ochoa, Mehler and Kornberg (1947) isolated from pigeon liver extracts a TPN-specific enzyme which catalyzes the reversible oxidative decarboxylation of l-malate to pyruvate and CO_2 in the presence of divalent manganese ions. Ochoa *et al.* (1947) referred to this TPN-specific enzyme as the "malic enzyme." Hargreaves (1954) studied inhibition by PCMB of the activity of this enzyme, and showed that it requires free —SH groups for activity. PCMB has been reported (Hilton and Smith, 1955) to inhibit a number of sulfhydryl-containing respiratory enzymes of fungal origin, including malic dehydrogenase of the citric acid cycle, so it is clear that PCMB inhibition is not specific to the malic enzyme, but may apply to other sulfhydryl-containing enzymes in the regenerating tissue.

The results of this work show that PCMB can alter normal planarian regeneration. Worm pieces exposed to PCMB for 96 hours during their regeneration had eye abnormalities which persisted longer than those of groups which had

shorter PCMB exposures. Since it is implied by other work that *Dugesia tigrina* possesses the malic enzyme system (Hammen and Lum, 1962), and PCMB is known to inhibit this enzyme, one could conclude that there is a possible relationship between CO_2 fixation and the grossly observable abnormalities in regenerating planarians.

Influence of avidin on planarian regeneration related to propionyl carboxylase inhibition

Barban and Ajl (1951) proved the reversibility of the decarboxylation of succinic acid to propionic acid and CO_2 when they demonstrated the fixation of $C^{14}O_2$, from labeled bicarbonate into succinate by *Propionibacterium pentosaceum.* Lardy and Peanasky (1953) concluded from work with extracts of rat liver mitochondria that ATP and divalent magnesium ions are necessary for the carboxylation of propionate to yield radioactive succinate.

Flavin, Ortiz and Ochoa (1955), in a study of propionate carboxylation by extracts of pig heart, found evidence of an intermediate compound, methylmalonyl-Coenzyme A (CoA), an isomer of succinyl-CoA. Tietz and Ochoa (1959) referred to the enzyme which reversibly catalyzes the conversion of propionyl-CoA to methylmalonyl-CoA as propionyl carboxylase. The enzyme requires biotin for activity, as Halenz and Lane (1960) demonstrated with their avidin studies. Avidin caused inhibition of the propionyl carboxylase enzyme system, leading to a decreased amount of $C^{14}O_2$ fixation with increasing concentrations of avidin. Since avidin pretreated with biotin did not cause inhibition, it can be concluded that the influence of avidin on propionyl carboxylase is biotin-specific. Biotin is also required for the activity of phosphoenolpyruvate carboxylase, which catalyzes the fixation of CO_2 into oxaloacetic acid. In fact, biotin is not known to serve any other function than cofactor in carboxylation reactions.

In this investigation, avidin altered normal planarian regeneration. Worm sections exposed to either avidin or heated avidin formed abnormal eye spots, avidin having a greater initial effect than heated avidin. The reason for an effect of the denatured compound is unknown. Since it has been shown that the planarian utilizes propionate (Hammen and Lum, 1962) and that avidin inhibits the biotin-requiring propionyl carboxylase, the results of the avidin experiments may be interpreted as additional evidence of a possible relationship between CO_2 fixation and grossly observable abnormalities in regenerating planarians.

The work reported is taken from the thesis of Agnes A. Smith, presented in partial fulfillment of the requirements for the Master of Science in the Department of Biology at Adelphi College, Garden City, L. I., N. Y.

Summary

1. In this investigation, treatment of regenerating worms with 10^{-8} *M* *p*-chloromercuribenzoic acid (PCMB) and 1 mg./ml. avidin resulted in the development of defective eye spots, which were scored according to a numbering system based on Child's classification.

2. The rate of regeneration was not significantly altered by treatment with PCMB and avidin in the concentrations used.

3. A direct relationship was found between duration of exposure to PCMB solutions and frequency of abnormalities.

4. These results indicate that inhibitors of carboxylation enzymes are effective in producing defects in planarian regeneration.

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