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A study was then conducted to determine if the enzyme, located previously in the reproductive organs, might be serving as a reserve protein and acting only secondarily as a detoxifying enzyme. The pressure of starvation on unsexed adults was used for this study. There was demonstrated a sharp decline of 55% of the enzyme in the first 24 hours with a 25% dissipation being observed over the next three days. The total percent protein experienced a reduction of about 5% in 24

hours, followed then by a slight increase over the next three days. This data pointed out that the reserve protein idea is quite tenable but further studies must be conducted.

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TITLE PAGE

ANNUAL PROGRESS REPORT

April 1, 1962 - March 31, 1963

Dr. Averett S. Tombes

Clemson College

The role of DDT-dehydrochlorinase in the detoxification mechanism of the naturally resistant Mexican bean beetle, and the influence of certain inhibitors and competitors on its action.

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ANNUAL REPORT

I. INTRODUCTION

The initial progress of this research grant was hindered on two accounts. First, there was a delay in obtaining purchased research equipment and secondly difficulty was experienced in obtaining a suitable research technician. Consequently, this report covers a nine-month period of research (July 1, 1962 - March 31, 1963). The body of this report will be in two parts, accomplishments and planned research, each then subdivided as necessary.

II. ACCOMPLISHMENTS

1. Semi-micro technique for DDT-dehydrochlorinase assay

A technique had to be devised so that a fresh insect sample weighing at least 500 milligrams (100 mg. dry weight) could be prepared for enzymatic evaluation. This required alterations in the technique previously followed (Tombes and Forgash, 1961; Jour. of Ins. Phy. 7:215-223) primarily in the areas of acetone powder preparation and volumes of extracting water. The initial step in this new procedure was the homogenization of a weighed insect sample in 100 volumes of cold acetone in either a ground glass mortar and pestle or a Waring blender. The resulting slurry was poured into a beaker containing a second 100 volumes of cold acetone, stirred for approximately two minutes and then filtered through normal filter paper. This filtered preparation was then removed, transferred to a vacuum desiccator for ten minutes for the removal of all traces of acetone, thus leaving a completely dry powder. The powder was then extracted with 100 volumes of cold glass distilled water for two hours at 2° C., centrifuged at 2° C. for 15 minutes

at 12,500 times gravity and then assayed directly without further treatment. The spectrophotometric portion of the analysis was identical to that described in previous mentioned publication. If a delay in the procedure was necessary, this could be incorporated into the procedure following the evacuation of acetone under partial vacuum. This resulting powder can be retained at a low temperature (-35° C.) for a period of several months.

2. Development of interior growth room for the Mexican bean beetle.

It was necessary to develop a growth room to retain an active colony of Mexican bean beetles throughout the fall, winter and spring thus not having to depend upon field collection or greenhouse supply. An interior growth room with no external light entering was developed with a productive capacity of from 100 to 200 adults per week.

3. Assay for DDT-dehydrochlorinase in male and female.

Based on earlier studies which demonstrated a predominant amount of the dehydrochlorinating enzyme to be present in the adult reproductive organs, the evaluation of the enzyme content within sexed adults was necessary. This study was replicated five times using twenty adults in each enzyme determination. Table 1 presents the data from this experiment. The female, whether gravid or not, is consistently heavier than the male; thus the enzyme value per insect was found to be higher in the female than in the male. The second column under each sex provides more significant data, presenting the micrograms of TDE-dehydrochlorinated per milligram dry weight of the adult body. From the mean value of these columns it is evident that the female has approximately 1½ times the enzyme as the male. Based

on this data, together with the previously reported data showing the high level of enzyme in reproductive organs, one can then reason that the highest content of the enzyme will be found in the female reproductive organs. This reasoning has thus fostered the research concerning the enzyme concentration within the various portions of the female reproductive system.

Table 1. Level of DDT-dehydrochlorinase in the male and female Mexican bean beetle

MALE		FEMALE	
Micrograms TDE-dehydrochlorinated		Micrograms TDE-dehydrochlorinated	
Per insect	Per mg. dry wt.	Per insect	Per mg. dry wt.
146	16.9	244	22.2
100	12.1	290	25.5
138	19.9	211	22.0
113	15.5	260	23.4
72.3	10.2	232	21.6
Mean 114	Mean 14.9	Mean 247	Mean 22.9

The question of what role this enzyme might be performing within these organs is immediately asked and sponsors speculation as to whether the protein fills a functional, dynamic biochemical role or serves as a reserve source of protein. This speculation thus leads to the research which is discussed in the next section.

4. Influence of starvation on DDT-dehydrochlorinase, total body protein, fresh and dry body weight.

The level of the enzyme throughout the larval, pupal and adult stages of the Mexican bean beetle is shown in Figure 1 (Tombes and Forgash, 1961). During the first week following emergence from the pupal period the initial decline and the subsequent increase in enzyme level prompted questioning as to the direction this level might take when the insect was subjected to starvation.

Thus, three questions were asked concerning the change in the enzyme level during starvation: would it increase, would it remain the same or would it decrease? In the first place an increase would designate that the enzyme would be serving an essential biochemical role with the removal of food not hindering its synthesis. Second, if the level did not deviate, the synthesis of the enzyme would be dependent upon the intake of food. More specifically, the enzyme available at the initiation of starvation was sufficient for necessary biochemical process and was not being utilized as a substitute for dietary protein. Third, if the enzyme level declined, the necessity of the enzyme in the organism undergoing a stress of this nature would be questioned, and this necessity of the enzyme would be related to the rate of decline in enzyme content.

The first experiment in this series is that shown in Figure 2 where the emerging adults were immediately removed from all food. This resulted in enzyme concentration reduction from 30 micrograms of TDE-dehydrochlorinated per insect to about 2 micrograms within sever days. When food was replaced, the enzyme concentration increased within 10 days to a normal level of 70 micrograms per insect. In Figure 3 a similar starvation study utilizing week-old adults demonstrates that the enzyme level can be reduced initially in four

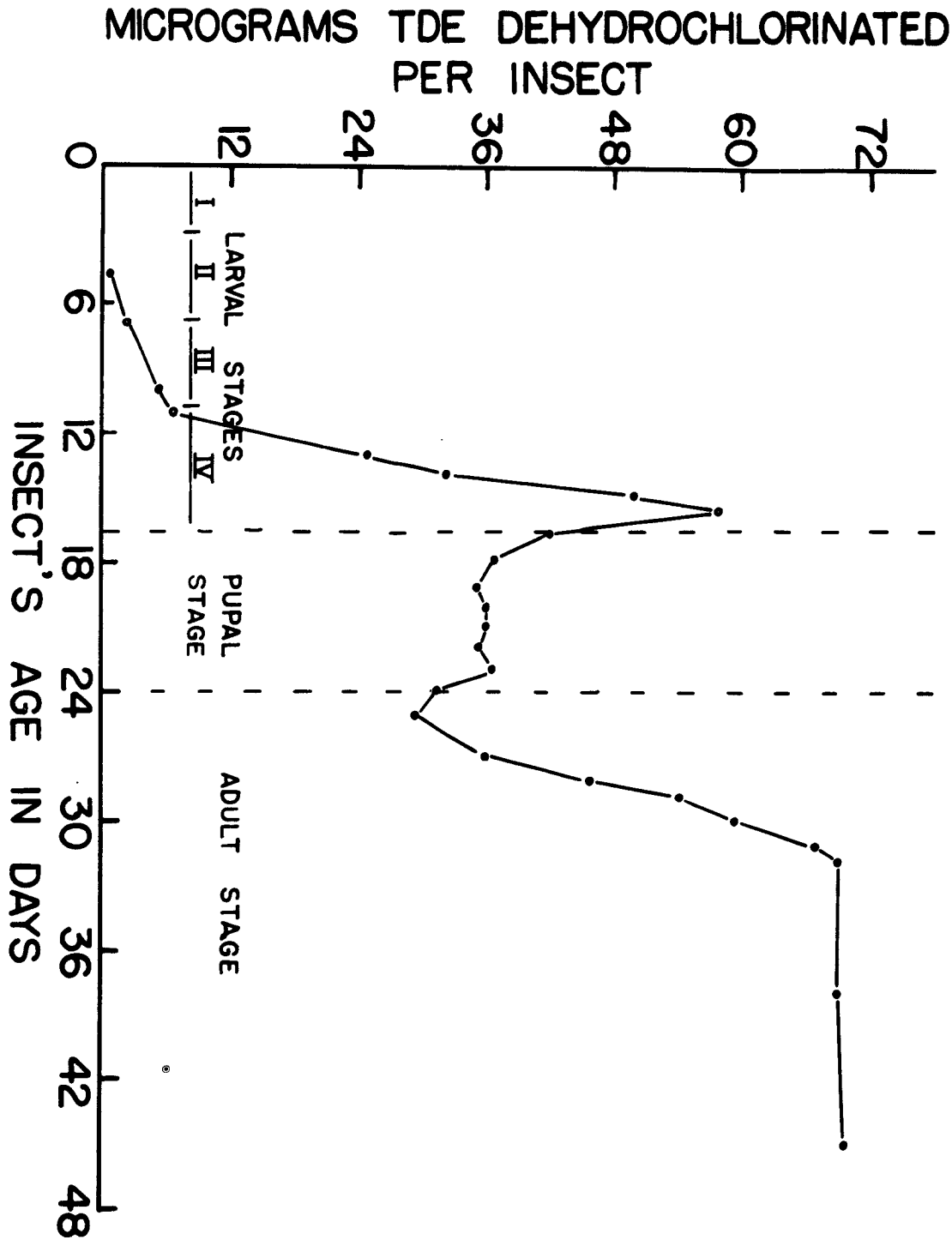

FIGURE 1. Levels of DDT-ase in the Mexican bean beetle

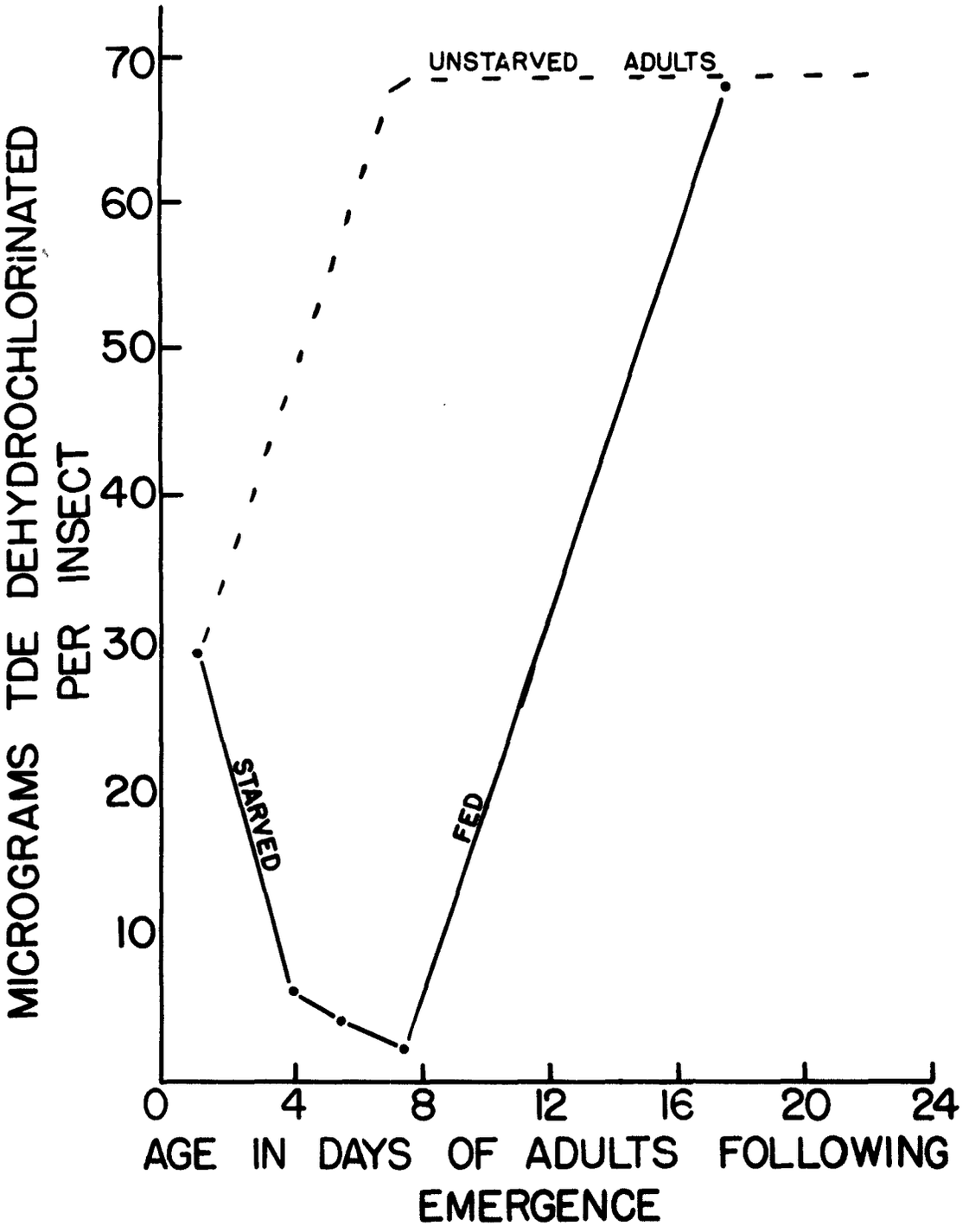

FIGURE 2. Influence of starvation on DDT-ase in the Mexican bean beetle

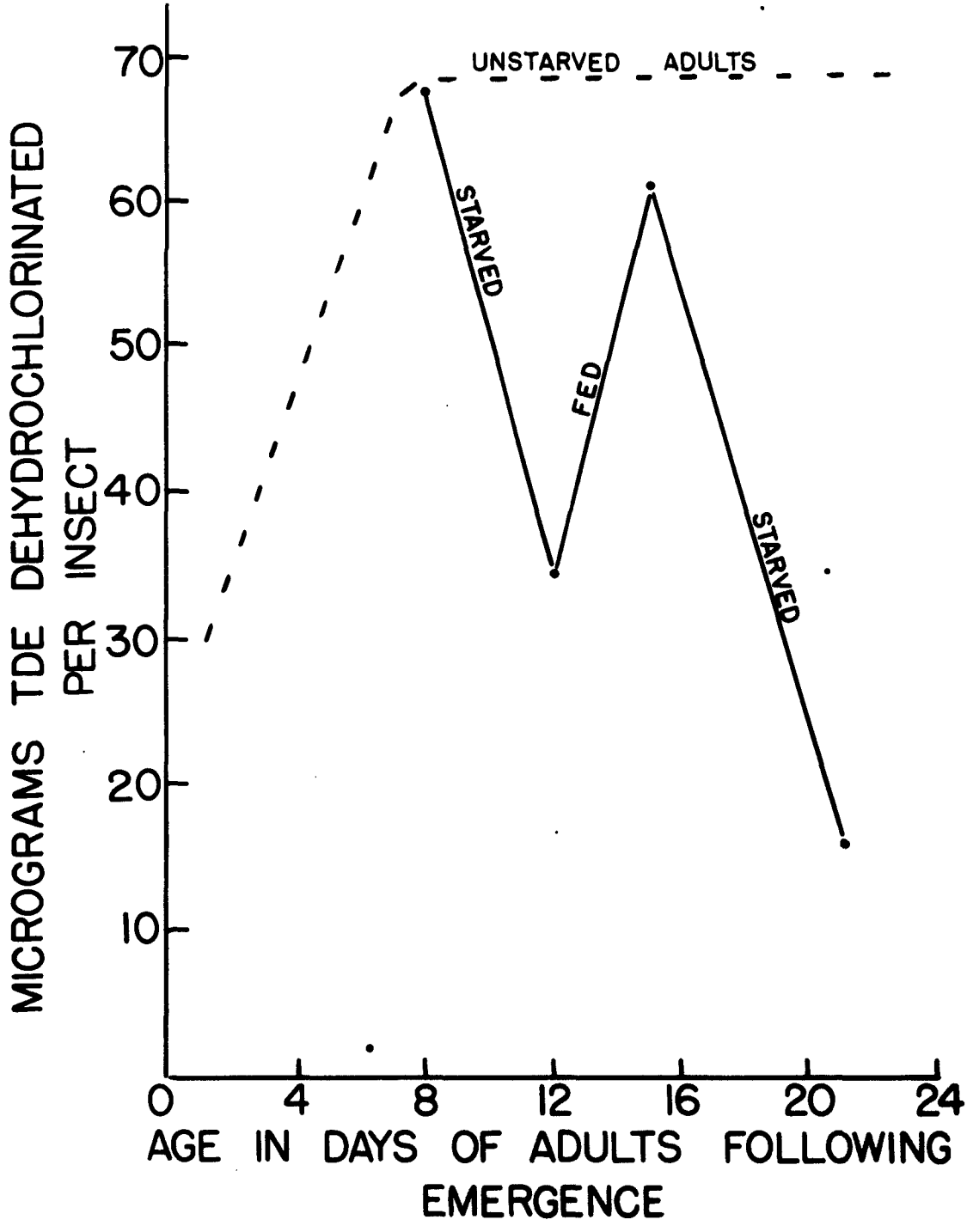

FIGURE 3. Influence of starvation on DDT-ase in the Mexican bean beetle

days, returned to a near normal level in three days and reduced the second time in the same population within six days.

It is thus evident that the enzyme level is definitely under the influence of the nutritional condition of the insect. When the level of enzyme and protein content of week-old adults is measured daily over a four-day period of starvation, the results are observed in Figure 4. This demonstrates the labile characteristic of the enzyme, which during the first 24 hours under starvation is reduced 55%. During the next 24 hours there is a slight increase followed by a constant decline over the next 2 days. During the same four-day period of time the percent protein is reduced but later shows an increase returning to near its original level. This third and fourth day increase does not reflect a conversion of other body materials into protein but rather an increase in the percentage of protein due to the preferential reduction of other body constituents as in the utilization of the carbohydrates and fats. In Figure 5 the changes in the fresh and dry weights are shown during the same experiment. The fresh weight experienced a 20% weight decline from 40 mg. to 32 mg. The dry weight at the same time was reduced 25% from an initial level of 8.25 mg. to a low level at the four day interval of 6.25 mg.

Thus it can again be stated that the level of the DDT-dehydrochlorinase enzyme, predominantly located in the reproductive organs of the female Mexican bean beetle, is closely related to the intake of food. The evidences point strongly to the theory that this

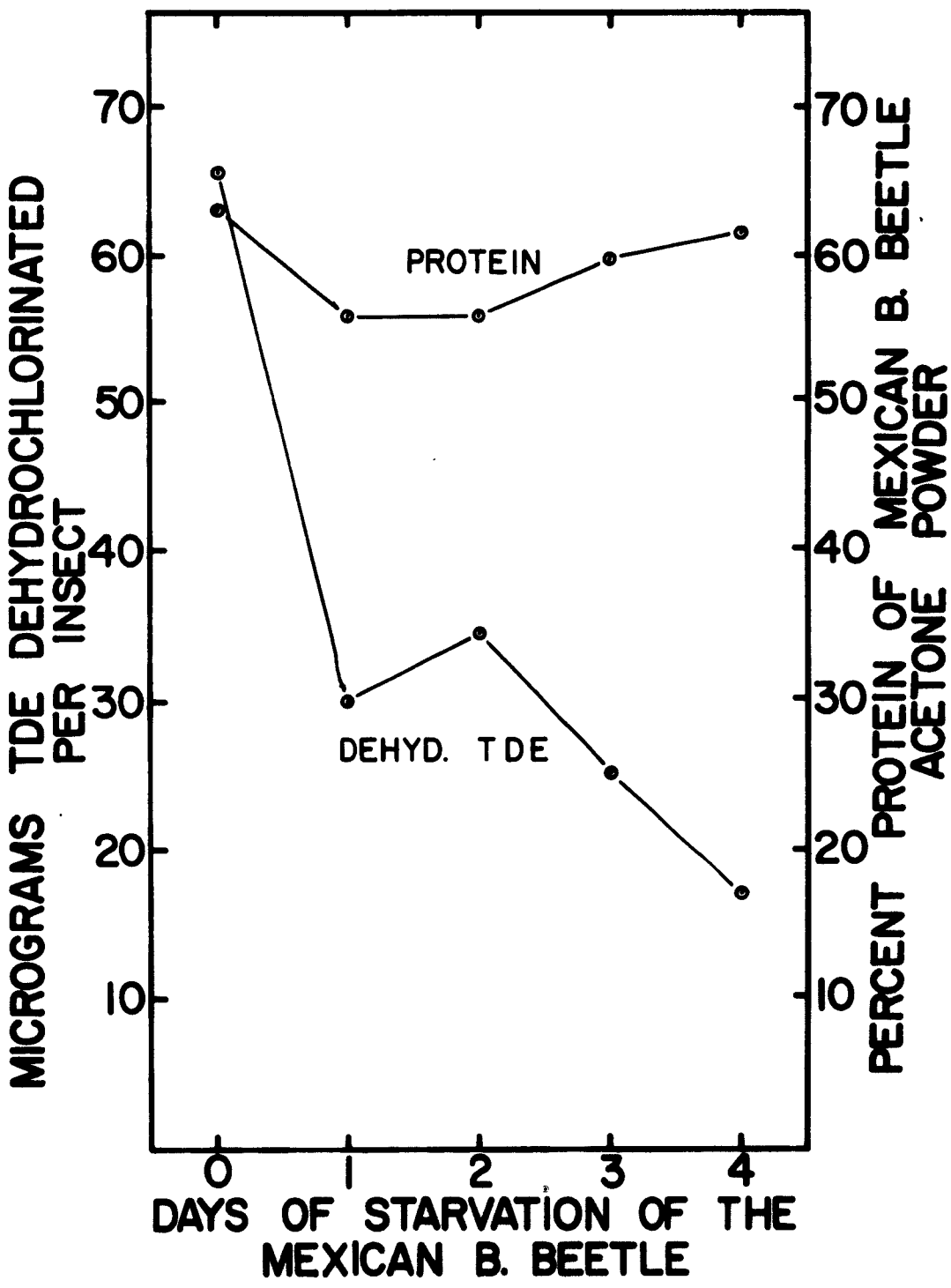

FIGURE 4. Influence of starvation on DDT-ase and protein content in the Mexican bean beetle

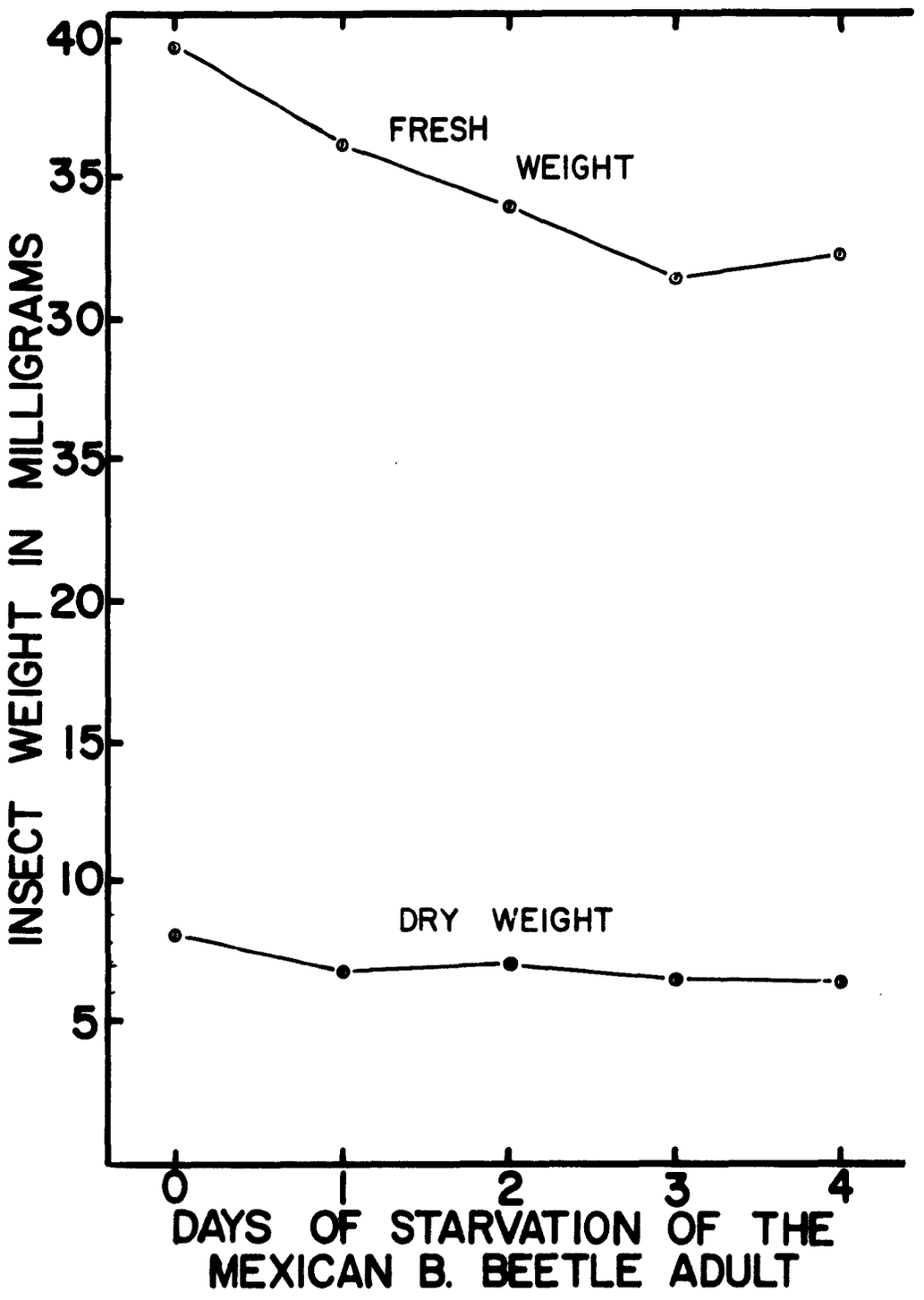

FIGURE 5. Influence of starvation on fresh and dry weight of the Mexican bean beetle

enzyme serves as a reserve protein, metabilized preferentially by the insect under the stress of starvation. This would make it analogous to the role of the plasma proteins in the blood of vertebrates.

5. Techniques for the partition of female reproductive organs.

If a more critical evaluation of the enzyme location within the female reproductive organs was to be made, then techniques had to be developed for the removal of such structures for their biochemical evaluation. This technique involves the dissection and removal of two portions of the reproductive system. The first are the intact ovaries and the second are the lateral and common oviducts with the accessory glands. These tissues are collected as rapidly as possible and frozen immediately to wait analyses as described earlier.

6. Techniques for dehydrogenase assay

The procedures have been developed for the determination of the dehydrogenase activity within the Mexican bean beetle using the Thunburg procedure. This is a deviation from the standard procedure that is found in Umbrite's text entitled Manometric Procedures in Tissue Metabolism (Burgess and Co., Minn., Minn.).

The rate of metabolism, determined by measuring the activity of the dehydrogenase and cytochrome oxidase systems, will be determined on the reproductive organs both under normal conditions with a high DDT-dehydrochlorinase level, under starvation stress with low DDT-dehydrochlorinase level and under the stress of condition of hibernation. An evaluation will thus be possible as to the comparative activity of the detoxicating and metabolism systems.

III. FUTURE RESEARCH

1. Assay for DDT-dehydrochlorinase within structures of the female reproductive system.

This enzyme has been found at the highest concentration within the reproductive organs of the female Mexican bean beetle. An evaluation of the various structures of this system will be conducted to determine where the enzyme might be concentrated and from the data add to the information as to its function. The reproductive organs would be divided in half, the anterior portion with ovaries and component ovarioles, and the more posterior region consisting of the lateral and common oviducts with accessory glands attached. The principle problem to overcome in this portion of the research will be the amount of tissue needed to enable the performance of necessary biochemical tests. As stated above, a technique has been devised for this dissection procedure; thus it is anticipated that this procedure will be carried out with sufficient replicates.

The determination of the enzyme in any of the eggs will not be performed until the eggs have been deposited on a leaf by the female. It is anticipated that known aged eggs will be accumulated and the enzyme determined giving data for the eggs at 8-hour periods in their development following oviposition.

2. Assay for DDT-dehydrochlorinase on cellular particles.

It is anticipated here that the reproductive tissues will be homogenized in a ground glass pestle and then spun at various speeds in the refrigerated centrifuge to give factional centrifugation of the homogenate. Homogenization will take place in an ice jacketed

mortor and pestle using sucrose as a buffering medium. The first centrifugation will be at 700 x G for 10 minutes which will spin down nuclei and intact cells. The supernatant will then be re-centrifuged at approximately 5,000 x G for 10 minutes forcing down the nitochondria. The supernatant from this centrifugation will be recentrifuged again at a speed of 24,000 x G for 10 minutes forcing down additional mitochondria. The final centrifugation will be at 54,000 x G for 60 or 120 minutes which will leave only soluble materials and extremely small particles within the final supernatant. Enzymatic determinations will be run on the supernatant and also the sediment after each of the centrifugation procedures. It is hoped from this that some evaluation as to the position of the DDT-dehydrochlorinase enzyme within the cell might be obtained.

3. Evaluation of DDT-dehydrochlorinase as a dehydrogenating system.

Samples of reproductive organ tissue removed from adult female beetles undergoing either starvation or hibernation stress will be prepared for several biochemical assays. The level of the detoxicating systems of DDT-dehydrochlorinase, as outlined earlier, and the level of metabolism as demonstrated by the dehydrogenase and cytochrome oxidase activity will be determined. It is known that the dehydrochlorinating enzyme is reduced under such environmental stress but what effect of this condition will have upon metabolism, or the effect of the DDT-dehydrochlorinase reduction on the intermediary metabolic enzyme systems is not known and will thus be determined.

4. Attempt to increase resistance and DDT-dehydrochlorinase level.

This aspect of the grant research is being conducted by Mr. Joseph E. Bumgarner as a Master of Science thesis problem. The three objectives for this research are listed below:

(1) To attempt to influence the level of resistance in the Mexican bean beetle (Epilachna varivestis) through artificial selection pressures. DDT-dehydrochlorinase and the LD 50 values will be determined for each generation.

(2) To correlate any change in body composition of the Mexican bean beetle with a change in the level of resistance.

(3) To determine whether the enzyme DDT-dehydrochlorinase acts as an oxidase or a reductase in reaction.

5. Evaluation of certain biochemicals as inhibitors and competitors for the DDT-dehydrochlorinase reaction.

A complete review of the procedures and biochemicals for this portion of the research has not been completed. However, it is anticipated that inhibitors such as many of the heavy metals and biochemicals such as iodoacetatic, benzaldehyde, ornithine, and tetracycline will be included in an evaluation of this type.

IV. SUMMARY

The enzyme DDT-dehydrochlorinase has been under investigation for the past nine months with an over-all objective of studying its natural physiological function in the Mexican bean beetle. Previous studies had demonstrated its location to be predominantly in the reproductive organs of the adult with lesser amounts in the other organs of the body.

The first series of experiments conducted under this grant, following the development of a semimicro analytical method, was to determine the relative quantitative values of the enzyme in the male and female. The work demonstrated a two to three times greater amount in the female over that in the male. This evaluation was on a per milligram dry weight basis thus taking into consideration the larger female body.

A study was then conducted to determine if the enzyme, located previously in the reproductive organs, might be serving as a reserve protein and acting only secondarily as a detoxifying enzyme. The pressure of starvation on unsexed adults was used for this study. There was demonstrated a sharp decline of 55% of the enzyme in the first 24 hours with a 25% dissipation being observed over the next three days. The total percent protein during the same period experienced a reduction of about 5% in 24 hours, followed then by a slight increase over the next three days. This data pointed out that the reserve protein idea is quite tenable but further studies must be conducted before any positive statement in this direction can be made.

The establishment of methods for the biochemical determination of several dehydrogenases and for the removal of component parts eggs, ovaries, oviducts and accessory glands of the female's reproductive organs for assay has been completed.

It is requested that the final written report be delayed in preparation until June 1964, sixty to ninety days following the financial termination of this grant.

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