NUTRITIONAL AND METABOLIC STUDIES ON THE BLOWFLY, PHORMIA REGINA (MEIG.)

by

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TO MY WIFE

TABLE OF CONTENTS

	Page
INTRODUCTION	1
Part I Nutrition	1
Part II Metabolism	8
MATERIALS AND METHODS	14
Part I Nutrition	14
Part II Metabolism	18
Preparation of sarcosome fraction	19
Preparation of soluble enzyme	20
Preparation of acetone powder extracts	21
Manometric methods and chemical determinations	22
RESULTS AND DISCUSSION	24
Part I Nutrition	24
Part II Metabolism	41
Citric acid cycle	41
Glycolysis	50
Pentose cycle	52
Polyphenol oxidase	57
SUMMARY	59
Part I Nutrition	59
Part II Metabolism	61
BIBLIOGRAPHY	63

LIST OF TABLES

	Page
Table I	15
Table II	33
Table III	35
Table IV	37
Table V	40
Table VI	48
Table VII	51

LIST OF FIGURES

	Page
Figure 1	25
Figure 2	27
Figure 3	29
Figure 4	31
Figure 5	36
Figure 6	42
Figure 7	44
Figure 8	45
Figure 9	54
Figure 10	55
Figure 11	56

NUTRITIONAL AND METABOLIC STUDIES ON THE BLOWFLY, PHORMIA REGINA (MEIG.)

INTRODUCTION

PART I NUTRITION

The class Insecta includes a great number of organisms with widely diversified habitats and nutritional requirements. For many years man has tried to reduce the insect menace using both biological and chemical methods. This battle is still going on without any noticeable demonstration of man's superiority.

One approach, which although not new, is receiving increasing attention. This is the study of the nutritional needs of the different insect species. The influence of nutritional level upon insect populations is well documented (16, pp.399-401) and thus a knowledge of the dietary needs offers a more fundamental approach to insect control. Specific nutritional requirements may suggest possible antimetabolites which can be used for control.

Many insects differ from the higher forms of life, in that their habitats differ greatly between immature and mature stages. For example, the mosquito, an aquatic organism during larval life, is terrestial during adulthood. Uvarov has pointed out in his review of early nutritional work (51, pp.309-320) that marked differences

are found in the nutritional requirements of immature and mature insects. Thus, while in no case can an insect develop on a protein free diet, it is possible to maintain adults on a carbohydrate and water diet. It is of significance however, that some protein source apparently is essential for the adult if it is to reproduce. In an early study on houseflies, Glasser (12, pp.383-412) demonstrated that bouillon or blood serum had to be included in the diet of the adult before eggs were produced.

Recently Rasso and Fraenkel (39, pp.636-645) studied the effect of adult diet on ovarian development and egg production in Phormia regina. Their results showed that the ovaries did not develop on a sucrose-water diet. Addition of different proteins to the diet resulted in oviposition. The use of liver as the protein source yielded better results than any other diet so far tested.

Much of the early work in insect nutrition was concerned with qualitative analyses of the food and the insect bodies. Indeed many studies are still being directed along these lines (2, pp.30-41; and 38, pp. 573-580) but much is also being done toward the goal of obtaining chemically defined diets capable of supporting growth and reproduction.

The first species reared on a chemically defined medium was Drosophila melanogaster in 1946 (44, p.540).

The medium consisted of a mixture of amino acids equivalent to hydrolysed casein. Sucrose was included as the carbohydrate source. Salts, cholesterol, ribonucleic acid, biotin, choline, folic acid, nicotinamide, pantothenic acid, para-aminobenzoic acid, pyridoxine, riboflavin and thiamine also were included. Agar was used to produce a solid medium. In this report the authors' stated, "Racemic mixtures have high toxicity" and indicated that mostly L-alpha amino acids were used in the medium.

In a later report (45, pp.657-658) Schultz and Rudkin indicate that with certain mutant strains of D. melanogaster dietary changes have a marked effect as is evidenced by their statement, "Deficiency of folic acid, of biotin, of the unidentified yeast residue factors as well as sub-lethal concentrations of the toxic D-amino acids serine and alanine all enhance the variegation" (at the white locus). In a second report Rudkin and Schultz (40, pp.652-653) show further evidence of the variation of dietary requirement with mutant strains. One mutant cannot develop to maturity on a mixture of 11 amino acids as the nitrogen source, yet prospers on casein hydrolysate. With other strains the reverse is true. When such differences are noted within one species it seems evident that although there is a broad similarity of nutritional requirements among

insects, the exact requirements of any one species can only be determined by direct experiment.

Hinton et al. (21, pp.335-353) further examined the nutrients required by Drosophila. Their studies indicated that the larvae required thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, inositol, biotin, folic acid, choline and cholesterol. Development was obtained on a mixture of 13 amino acids which included glycine, glutamic acid and cystine in addition to the ten essential for rat growth. However the inclusion of five others--L-proline, L-hydroxyproline, L-aspartic acid, L-alanine and L-tyrosine--permitted better growth. These authors found that D-serine at a level of 1.26 mg. per milliliter of medium was toxic to this organism causing death prior to pupation. Part of this toxicity was overcome by the inclusion of ribonucleic acid in the diet.

The toxic effect of D-serine is not universal in invertebrates. Kidder and Dewey (29, pp.425-432) working with the ciliate Tetrahymena geleii found that DL-serine when added to the culture medium was actually stimulatory. Recently House (24, pp.331-341) reported a chemically defined medium adequate for rearing Pseudosarcophaga affinis, the dipterous parasite of the spruce bud worm. This diet contained DL-serine at a level exceeding eight

percent of the total amino acid mixture without any deleterious effect.

Nutritional requirements of the yellow fever mosquito, Aedes aegypti have been well established (49, pp.1211-1223). The amino acids required for development include glycine, L-leucine, DL-isoleucine, L-histidine, L-arginine, L-lysine, L-tryptophane, DL-threonine, DL-phenylalanine and DL-methionine. The essentiality of valine was in doubt. It is of interest that a tryptic digest of casein supported growth whereas a papain digest was not as good. An acid hydrolysate proved completely inadequate, even after removing chloride and supplementing with amino acids. When L-cystine was absent from the diet, adults were formed but failed to escape from the puparium (14, pp.379-387).

Several of the water soluble vitamins have been shown to be essential. These are thiamin, riboflavin, pantothenic acid, nicotinic acid, biotin and folic acid. The latter seems to be directly associated with pupation and is most critical in the last instar (49, pp.1211-1223).

Three species of the Dipteran family, Calliphoridae, have been used in nutritional work. In early studies there was much controversy whether fly larvae could develop in the absence of microorganisms (6, pp.1-81). This question was settled by Michelbacher and coworkers

(34, pp.109-131) in 1932 with the development of a method for rearing Lucilia sericata under aseptic conditions on an artificial medium. This diet was composed of casein, yeast powder, butter fat or cod liver oil, salt mixture, cystine and agar. It is interesting that addition of cystine was found necessary to permit adult emergence. Later, Brown (7, pp.895-902) replaced the lipid fraction of the above diet with lanolin, omitted cystine and obtained satisfactory development.

The black blowfly Phormia regina was used in the treatment of osteomyelitis and early nutritional studies were conducted in an attempt to develop satisfactory laboratory rearing techniques (17, pp.51-56). More recently with the emphasis changing to insecticide work, this species has proven to be a fine test organism. Hill et al. (20, pp.213-216) reported a means of rearing this species under aseptic conditions on a simple artificial medium. The diet is essentially that used by Brown for rearing Lucilia sericata (7, pp.895-902). Details concerning the essential nutrients for these two species are lacking.

Kadner and La Fleur (27, pp.129-136) worked with the related species Phaenecia sericata and established the essentiality of thiamine, riboflavin, pantothenic acid, pyridoxine and nicotinic acid in the diet. Cholesterol and choline also proved to be necessary.

However, there appears to be no information concerning the amino acid requirements in any of these species. As yet, no one has reared these species on a chemically defined diet nor elucidated the essential amino acids.

To date relatively few insect species have been reared on purified diets. Studies so far indicate that thiamine, riboflavin, nicotinic acid and pantothenic acid are required by most insects, the essentiality of other vitamins being a function of the species (1, pp.29-30). Cholesterol seems to be the one lipid universally required among insects. Although there is considerable variation in the indispensable amino acids, those required by insects approximate those required by the growing rat. These findings strongly support the concept of the unity of biochemistry.

The object of this study was to compound a chemically defined diet which would support development of the black blowfly, Phormia regina from egg to adult under aseptic conditions. The elucidation of the essential metabolites for this insect was a second objective.

INTRODUCTION

PART II METABOLISM

Studies of oxidative metabolism in insects are scattered and no effort appears to have been made to study completely the processes in any one species. Perhaps there are two reasons for lack of information in this field. First, much work remains to be done in vertebrate and microbial metabolism and thus attention has not yet been diverted to the insect world. Second, owing to the difficulty of obtaining insects in large quantities, they have not been attractive for metabolic work. Further the insect has a heterogenous structure and the practical problem of obtaining sufficient tissue of any one type for study offers a formidable obstacle. In spite of these problems some basic work has been done on insects, notably Keilin's studies of insect cytochromes (28, pp.312-338).

Much of the early work on metabolism dealt with respiratory quotients and the changes in oxygen uptake that were associated with metamorphosis. Hitchcock and Haub studied the gas exchange in later stages of development of Phormia regina (22, pp.17-25). They showed that as the larvae begin to pupate the oxygen consumption rises to a maximum, falls off after pupation is completed and then rises prior to emergence, giving a

typical U-shaped curve. This has been observed by other investigators working with several holometabolous organisms. Copious ammonia release was noted during late larval and early pupal life. The ammonia excretion declined after pupation, paralleling the decrease in oxygen consumption, but did not rise again. Concerning these observations they remark, "This leads us to conclude that protein metabolism plays a much more important part in the formation of the pupal case and the early stages of pupation than it does in the later phases of the process and the emergence of the adult."

Although this type of study gives an overall indication of what is occurring, no details of the processes involved can be obtained. Gilmour went further in a study on the meal worm Tenebrio molitor (13, pp.93-100). He measured the gas exchange and also analysed for formation of lactic acid and disappearance of fat and carbohydrate in the body. The accumulation of lactic acid and development of an oxygen debt under anaerobiosis was demonstrated. However there was insufficient lactic acid formed to account for all the carbohydrate disappearing.

The work of Barron and Tahmisian (4, pp.57-76) can be considered a milestone in the study of carbohydrate metabolism in insects. In this study the leg muscles of adult American cockroaches were dissected out and the fibers teased apart. The oxidative activity of these

separated fibers was measured manometrically. The release of CO_2 from a bicarbonate buffer, using glucose as substrate with concomitant production of lactic acid, was evidence for glycolytic activity of the tissue. However, the CO_2 evolved far exceeded the lactic acid formed and no evidence of pyruvate formation was obtained. Oxidation of citrate, α-ketoglutarate, malate and succinate suggested citric acid cycle activity. Further evidence for this process was the inhibition of glucose oxidation by malonate and reversal of inhibition by fumarate.

The observations on glycolysis were confirmed by Humphrey (25, pp.323-325) except that the production of pyruvate was also detected. Working with the grasshopper Migratoria locusta, Humphrey and Siggins (26, pp.353-359) demonstrated the presence of glycolytic enzymes. Glycogen, glucose, fructose, G-1-P[1], G-6-P[1] and FDP[1] all gave rise to lactic and pyruvic acids. Inhibition of acid formation was observed in the presence of oxygen, similar to the Pasteur effect that is known to occur in vertebrates.

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[1] The following abbreviations used throughout this report:
AMP - adenosine monophosphate
ATP - adenosine triphosphate
DPN - diphosphopyridine nucleotide
FDP - fructose diphosphate
G-1-P - glucose-1-phosphate
G-6-P - glucose-6-phosphate
SH - sedoheptulose
TPN - triphosphopyridine nucleotide
TPP - thiamin pyrophosphate (co-carboxylase)
Tris - trimethylolaminomethane
TTZ - triphenyltetrazolium chloride

Spirtes (47, p.251) reported the presence of Krebs cycle enzymes in Drosophila melanogaster in 1951. Watanabe and Williams (52, pp.675-689) were the first to demonstrate oxidative activity in the sarcosome fraction of insect muscle. They obtained sarcosome preparations from P. regina thoraces by differential centrifugation and demonstrated the presence of succinoxidase, α-glycerophosphate dehydrogenase, malic dehydrogenase and pyruvic dehydrogenase. In addition they obtained a high titer of cytochromes. In a subsequent report (53, pp.71-90) these authors indicated that the type of suspending medium used had a great effect on the morphology of the sarcosomes.

Sacktor, using methods very similar to those above, isolated mitochondria from house flies. In a series of studies with this fraction he demonstrated the presence of an ATPase (41, pp.371-387), malic dehydrogenase, $DPNH^+$ oxidase, cytochrome C oxidase and malic- and succinic-cytochrome C reductases (42, pp.349-365). He also observed oxidative phosphorylation, with P:O ratios up to 1.7 (43, pp.343-359).

Lewis and Slater (33, pp.207-217) have also demonstrated oxidative phosphorylation with insect sarcosomes. They used the blowfly Calliphora erythrocephala and obtained P:O ratios up to 2.0. They showed that the phosphorylation mechanism is very sensitive and that it is

easily lost during preparation of the enzyme system.

In a recent study with the pea aphid, Macrosiphum pisi, by Newburgh and Cheldelin (35, pp.37-46) evidence for citric acid cycle activity has been obtained. Of more importance however, is the demonstration of an active pentose cycle in these organisms. This constitutes the first known report of this metabolic pathway in insects.

It thus appears that the pathways of carbohydrate degradation in insects are similar to those established in the vertebrates. It is interesting to note, however, that very little work has been done on the metabolism of immature stages. That differences in metabolism do exist in these different stages, especially in holometabolous forms seems evident. Chefurka and Williams (9, pp.562-563) have shown that in the pupal stage alone of the Cecropia moth there is a conversion of flavin adenine dinucleotide in the diapausing pupa to the mononucleotide with the onset of adult development. Similarly Dennell (10, pp.79-110) has shown a decided rise of tyrosine and tyrosinase in the blood of a flesh fly just prior to pupation.

Observations such as these suggest that important differences in metabolic pattern at different stages of development probably are widespread, if not universal among insects. With the emphasis now placed on mode

of action of organic insecticides, a knowledge of these differences may be fundamental to adequate control. Consequently it was decided to study the pathways of carbohydrate degradation in different stages of development of one species, Phormia regina.

MATERIAL AND METHODS

PART I NUTRITION

The insects used throughout this study were the progeny of a wild population trapped in the Corvallis vicinity. Succeeding generations of flies have been reared on a synthetic medium, modeled after Hill et al. (20, pp.213-216). To maintain the bulk colony the newly formed pupae were placed in screen and wood cages where emergence occurred. These adults were maintained on sucrose cubes and water. When oviposition was desired, either fresh liver or fresh kidney was placed in the cage. Oviposition usually followed within 24 to 48 hours. The colony was maintained in a constant temperature rearing room at 80 $\pm 2^{\circ}$F.

The final composition of a number of diets used in the nutritional studies is given in Table I.

The final medium was compounded by combining three separate fractions. The first fraction consisted of a solution of the different salts in distilled water. A cholesterol suspension containing 50 mg/ml. as described by House (24, p.333) constituted the second fraction. The third, a mixture of the appropriate dry ingredients--casein, yeast extract, agar, carbohydrate and tryptophane--formed the dietary variable.

TABLE I

Composition of Diets used in Nutritional Studies with *P. regina*

	A	B	C	D	E	F	G	H	I	J
Vitamin free casein	29	26	23	19	23	19	23	19		
Yeast extract	3	3	3	3	3	3	3	3	3	3
Glucose			6	10						
Sucrose					6	10				
Soluble starch							6	10		
Ensyme hydrolysed casein									29	
Acid hydrolysed casein										29
Agar	1	1	1	1	1	1	1	1	1	1
Water	67	70	67	67	67	67	67	67	67	67
Supplements (mg./gm.)										
DL-tryptophane									5.8	5.8
Cholesterol	0.67	0.78	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
NaCl	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
KCl	0.013	0.015	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013
$CaCl_2$	0.013	0.015	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013
$NaHCO_3$	0.013	0.015	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013
$NaH_2PO_4 \cdot H_2O$	0.35	0.41	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
K_2HPO_4	0.30	0.35	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30

In preparing the diets, the liquid components, formed by combining the salts solution and cholesterol suspension were first introduced into the container. The dry components were then added and thoroughly mixed. The medium described by Hill et al. (20, pp.213-216) contained lanolin. However cholesterol at a level of 0.67 mg./gm. of medium has proved to be an adequate substitute.

For bulk rearing, diet B proved to be the best one tested. One hundred grams of this diet was mixed in a 500 ml. Erlenmeyer flask and was sterilized at 16 pounds/sq.in. steam pressure for 15 minutes. This quantity of medium permitted development of approximately 400 blowflies.

In nutritional studies six-inch culture tubes were used as growth chambers. The appropriate components were placed in the tubes which were then covered with aluminum caps and sterilized as described above. After removal from the autoclave, the tubes were set at a 30 degree angle and allowed to cool. In all studies, except where otherwise indicated, six grams of medium were placed in each tube.

When a component was tested at a series of different concentrations it was added as an aqueous solution. Diet A was used as the basal ration in all such studies. To maintain a constant composition, half quantities of a double strength solution of salts and cholesterol were

used. Water was added as necessary to the correct final concentration.

Sterilization of the eggs was accomplished by first placing the fresh egg masses in a two percent NaOH solution until the eggs separated (about 15 minutes). The NaOH was then decanted off, and the eggs were rinsed twice with 70% ethanol to remove the remaining base. They were then immersed in 70 percent ethanol for fifteen minutes. At this time they were transferred aseptically to the culture tubes on the tip of a small alcohol-sterilized camel's hair brush. Approximately five eggs were placed in each tube. The tubes were incubated in a 30°C. water bath and the development was recorded by measuring the larval length.

MATERIALS AND METHODS

PART II METABOLISM

In metabolic studies on the adult fly the insects were reared in bulk, using methods already described. For convenience in handling, lots of about 100 pupae were placed in quart jars, using the apparatus described by Hill (20, pp.213-216), where emergence occurred. In studying Krebs cycle activity in adults the flies were maintained on a 20 per cent sucrose solution. However when adult flies, allowed to feed on this solution, were used in studying pentose cycle activity the sugar content interfered seriously with the colorimetric assays used in determining hexose formation. Consequently when the flies were to be used in this type of work, only water was supplied.

Enzymatic studies were conducted on third instar and prepupal forms. (In this report prepupa is used to designate the mature, non-feeding larval form prior to formation of the pupal case). The feeding larvae were obtained for this work by removing them manually from the growth medium. The prepupae were more easily obtained for in this stage they search for a dry environment in which to continue development. Advantage was taken of this habit and the insects were collected in

dry sand after having escaped through a screen bottomed container.

The eggs used in these studies were obtained as described previously.

Preparation of Sarcosome Fraction

The adults were placed in a cold room, 5°C., for about 30 minutes before dissection. With this treatment they were sufficiently inactive to permit easy handling. The heads and abdomen were removed and the thoraces were saved for enzyme preparation. When the required number of insects was dissected, the thoraces were ground gently with an ice cold mortar and pestle. The suspending medium, 0.9% KCl and 0.001 M versene, was gradually added to the brei until a final ratio of four milliliters of suspending medium per gram of thoraces was obtained.

The resulting suspension was pressed through eight layers of cheese cloth to remove large particles and cuticular debris. The "filtrate" was then centrifuged five minutes at 10,000 x g. and the supernatant fluid discarded. The brownish pellet remaining in the bottom of the tube was washed three times by resuspending in the KCl-versene solution and centrifuging for five minutes at 10,000 x g. The washed sarcosomes were then diluted to the required volume with the suspending medium. Microscopic examination of this material showed a uniform

population of spherical particles indicative of sarcosomes. All operations were conducted at a temperature between 0 and 5°C.

With larvae, the same method of preparation was used as described above, with one exception: the larvae were not dissected but instead the whole animal was homogenized. The brei which resulted was much more viscous and did not pass through the cheese cloth as readily. After centrifugation a much heavier layer of fatty material was noted on the surface of the supernatant liquid. Microscopic examination of the final preparation revealed a uniform population of particles, morphologically similar to adult sarcosomes.

Preparation of Soluble Enzymes

The fat layer was removed from the surface of the supernatant liquid after centrifugation of the adult homogenate at 10,000 x g. for 20 minutes. The supernatant liquid was then dialysed at 0°C. for varying periods of time, against 2 liters of 0.02 molar Tris[1] buffer at pH 8.0.

The same procedure was used for preparing the soluble enzymes of larvae and prepupae. Polyphenol oxidase activity was very noticeable in the prepupal preparations for the soluble fraction, which was usually a yellowish

opalescent solution when freshly prepared became black after two hours dialysis.

Only a small quantity of eggs was available for metabolic studies. Consequently methods were used which permitted conservation of the material. The eggs were homogenized in a Potter-Elvhjem glass homogenizer with the KCl-versene solution. This preparation was centrifuged at 20,000 x g. for 30 minutes. The supernatant fraction was dialysed as described above and the small amount of solid matter discarded.

Preparation of Acetone Powder Extracts

Acetone powders were prepared by blending the insects (prepupal or adult) in a Waring blendor with 10 volumes of acetone (-20°C.) for two minutes, and filtering the resulting powder. The acetone powder was blended again in five volumes of acetone, recovered by filtration, dried *in vacuo*, and stored in a deep freeze. Cell-free extracts were prepared as needed from the acetone powder. One gram of powder was homogenized with 10 mls. of 0.02 M tris buffer, pH 8.0. The homogenate was extracted at 0°C. for thirty minutes, and centrifuged at 20,000 x g. The supernatant was then dialyzed against two liters of 0.02 M tris buffer, pH 8.0 for three hours prior to use in the enzymatic studies.

Manometric Methods and Chemical Determinations

Manometric studies were conducted using a conventional Warburg apparatus. In oxygen consumption studies, pure oxygen was used as the gas phase. To accomplish this the Warburg flasks were placed on the manometers and oxygen was passed through the system for four or five minutes during which time the system was shaken.

TTZ[1] was used as the electron acceptor in some of the oxidative work. The reaction mixture was made up to one milliliter in a four-inch test tube, then incubated at the desired temperature in an evacuated desiccator. The reaction was stopped by the addition of five milliliters of acetone, the precipitate removed by centrifugation and the optical density of the supernatant fluid was read on a Bausch and Lomb colorimeter at 550 mμ. The quantity of substrate oxidized was determined by referring to a standard curve, which had been constructed by reducing known amounts of TTZ with excess $Na_2S_2O_4$.

Experiments on glycolytic enzymes were conducted manometrically according to the method of LePage (32, pp. 1009-1020). The reaction was run under anaerobic conditions with 5% CO_2, 95% nitrogen as the gas phase. The system included a bicarbonate buffer, FDP[1] as substrate, fluoride to inhibit enolase and pyruvate as the hydrogen acceptor for reduced DPN[1] generated by triose phosphate

dehydrogenase. CO_2 evolution, measured manometrically, indicated acid production. The quantity of lactic acid formed was thus a direct measure of the anaerobic oxidation which occurred. Lactic acid was determined by the method described by Umbreit et al. (50, p.192). FDP was determined by the resorcinol method (50, p.191).

Studies on pentose cycle activity were conducted by observing the ribose disappearance and the formation of sedoheptulose and hexose sugars. The reaction mixture was incubated in a water bath at 37°C. At appropriate times, one milliliter aliquots were removed and added to an equal volume of 10% trichloroacetic acid. The precipitate was removed by centrifugation and the amounts of compounds present in the supernatant fluid were determined. Pentose was assayed by the Meijbaum orcinol method as described by Umbreit et al. (50, p.191). Sedoheptulose and hexoses were measured by the method of Axelrod et al. (3, pp.619-634) on a Bausch and Lomb Spectronic 20.

The method of Weichselbaum (54, pp.40-49) was used for all protein determinations on the enzyme preparations.

RESULTS AND DISCUSSION

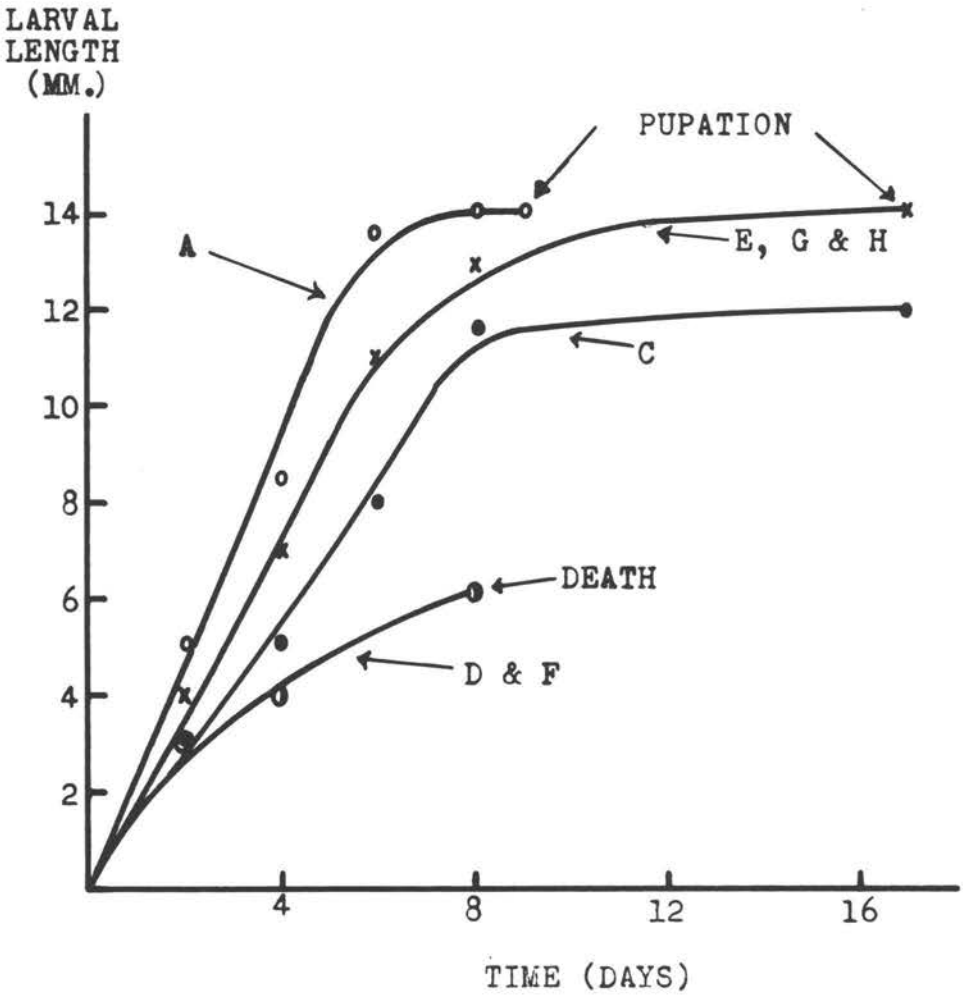
PART I NUTRITION

The standard diet, A, to which all others were compared is shown in Table I. Blowflies reared on this medium reached larval maturity in eight days and emerged as adults in 16 days.

The substitution of glucose for casein in the diet resulted in a retardation in development. When diet C was used (six per cent glucose) growth was reduced approximately 15%. When glucose constituted 10% of the medium (Diet D) mortality of young underdeveloped larvae was very high. In the presence of glucose, the normally light colored medium became brown during sterilization. This type of reaction has been reported by Lemonde and Bernard (31, pp.71-79) to result in a diet toxic for Tribolium confusum. Possibly this inhibition in blowfly stems from the same cause.

As a check on this observation, diets which included sucrose and soluble starch were also tested (Diets E, F, G and H). These results are shown in Figure 1. Because neither starch nor sucrose have reducing power, no browning occurred. It is of interest to note that of the diets containing six per cent carbohydrates, the glucose medium gave the poorest growth. Sucrose at the same level allowed more rapid development and less mortality. When

Figure 1



Effect of replacing casein by different carbohydrates in the diet on P. regina development[2].

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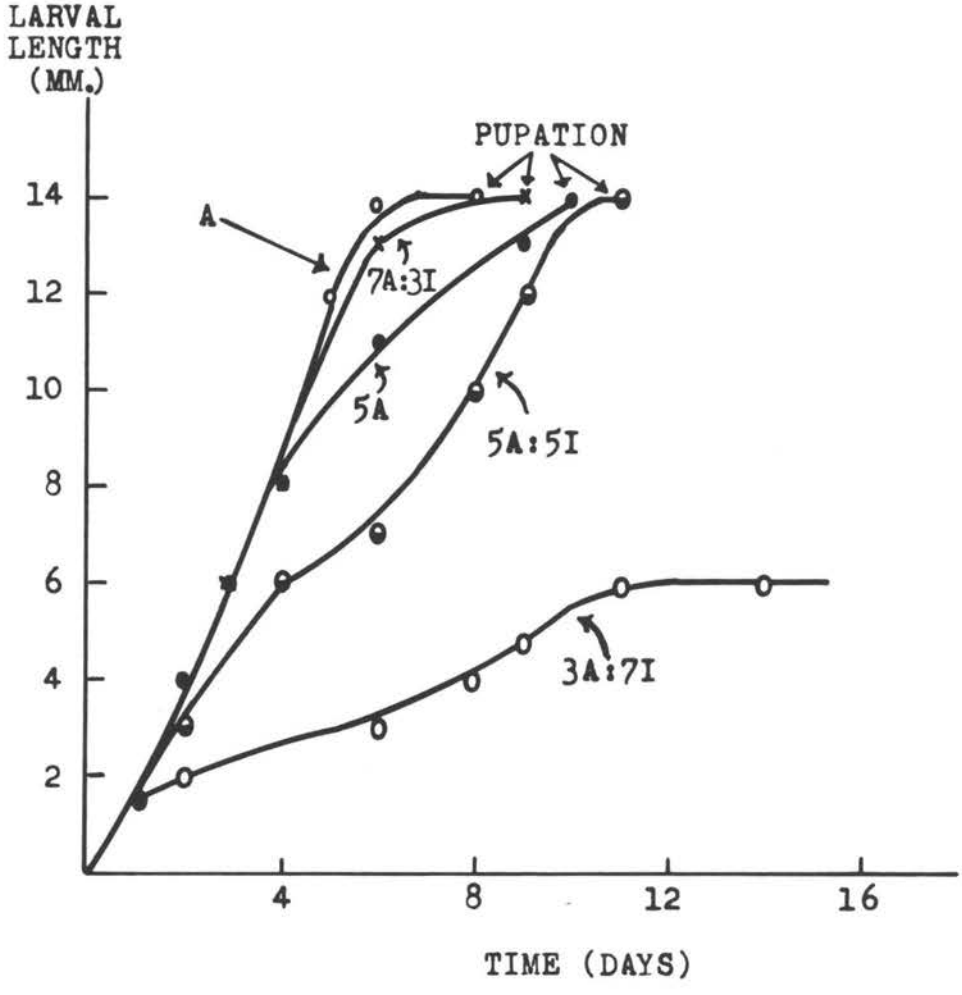
[2] See Table I for composition of diets.

starch was used, the rate of development was similar to that on sucrose. At the ten per cent carbohydrate level only starch permitted growth. Both glucose and sucrose were inhibitory, and caused death of immature larvae. These findings suggest that perhaps some other factor besides the browning is responsible for mortality. As will be discussed more fully later, osmotic effects in the medium may be important.

In an effort to more closely approximate a chemically defined medium for this organism, hydrolysed casein was tested, both as acid and enzyme digests (diets I and J). Development on these media was very poor. This suggested that either some component was released in concentrations that were toxic, or that some essential peptides were broken down. Diets A and I, were combined in different proportions in an effort to overcome this inhibition. As shown in Figure 2, growth equivalent to that on the standard ration, A, was obtained only when 70 per cent or more of diet A was present. Further, the presence of diet I in the mixed medium was actually inhibitory. Better growth was obtained when the casein content of diet A was reduced by half, than when equal quantities of diets A and I were present. Similar results have been reported by Stokstad (48, pp.42-48) with growing chicks.

Figure 2



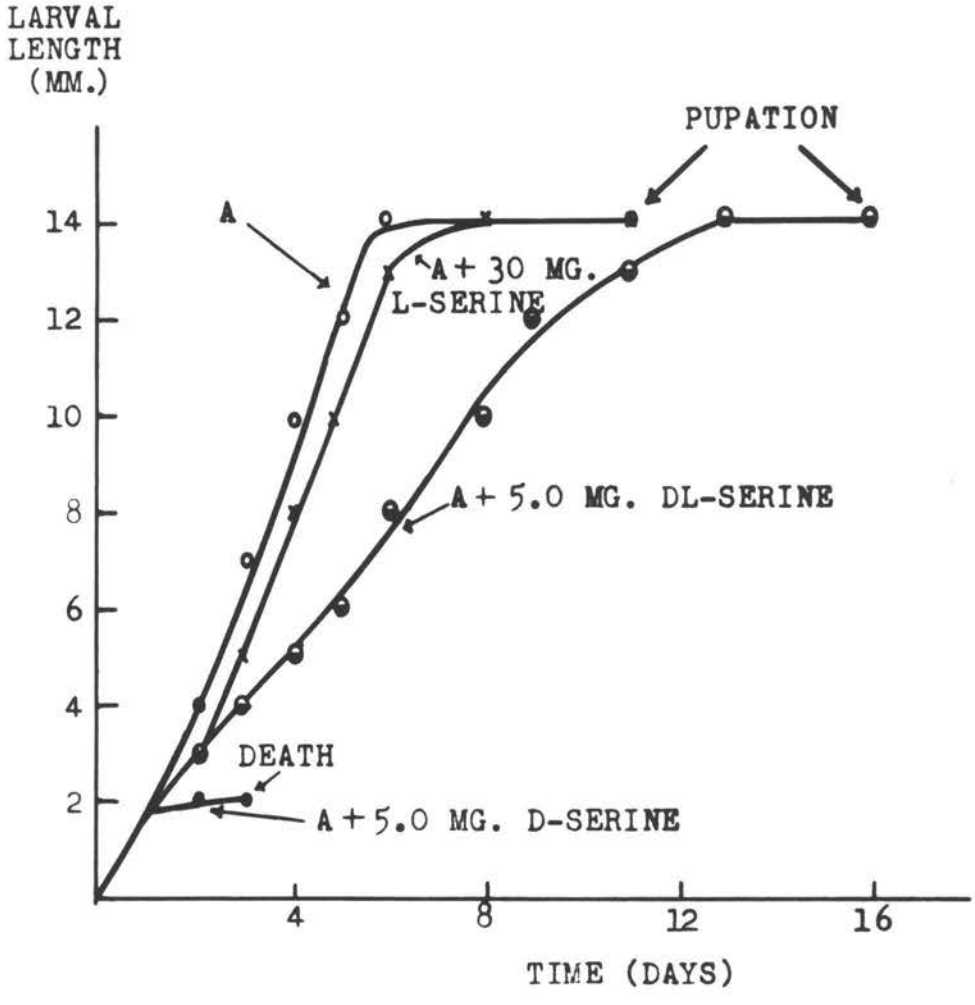
Growth of P. regina on diets containing different proportions of whole and enzymatically hydrolysed casein[2].

This suggested that perhaps some factor was released in such concentrations as to be inhibitory. Snell (46, pp.66-73) showed that glycine, β-alanine, DL-serine and DL-threonine were all inhibitory to the microorganism Streptococcus lactis. This inhibition was reversed by increasing the pyridoxine content of the medium or by addition of DL-alanine.

With rats, administration of DL-serine by stomach tube caused kidney damage, according to Fishman and Artom (11, pp.288-292). These authors concluded that the D-isomer was not metabolized and was the causal agent. Further, D-serine is known to be toxic to Drosophila (21, pp.335-353). In view of these observations glycine, DL-serine, DL-threonine, L-tyrosine and L-glutamic acid were tested for inhibitory action.

In these tests the respective amino acids were added to culture tubes containing 6 gms. of diet A. Addition of 100 mg. L-glutamic acid per tube appeared to be inhibitory. No inhibition was noted when glycine, DL-threonine or L-tyrosine were included at the rate of 50 mg. per tube. In contrast, 10 mg. of DL-serine per tube was enough to completely inhibit larval growth and caused death shortly after hatching. When the serine isomers were tested individually, it was found that only the unnatural D-form was inhibitory. These results are shown in Figure 3.

Figure 3



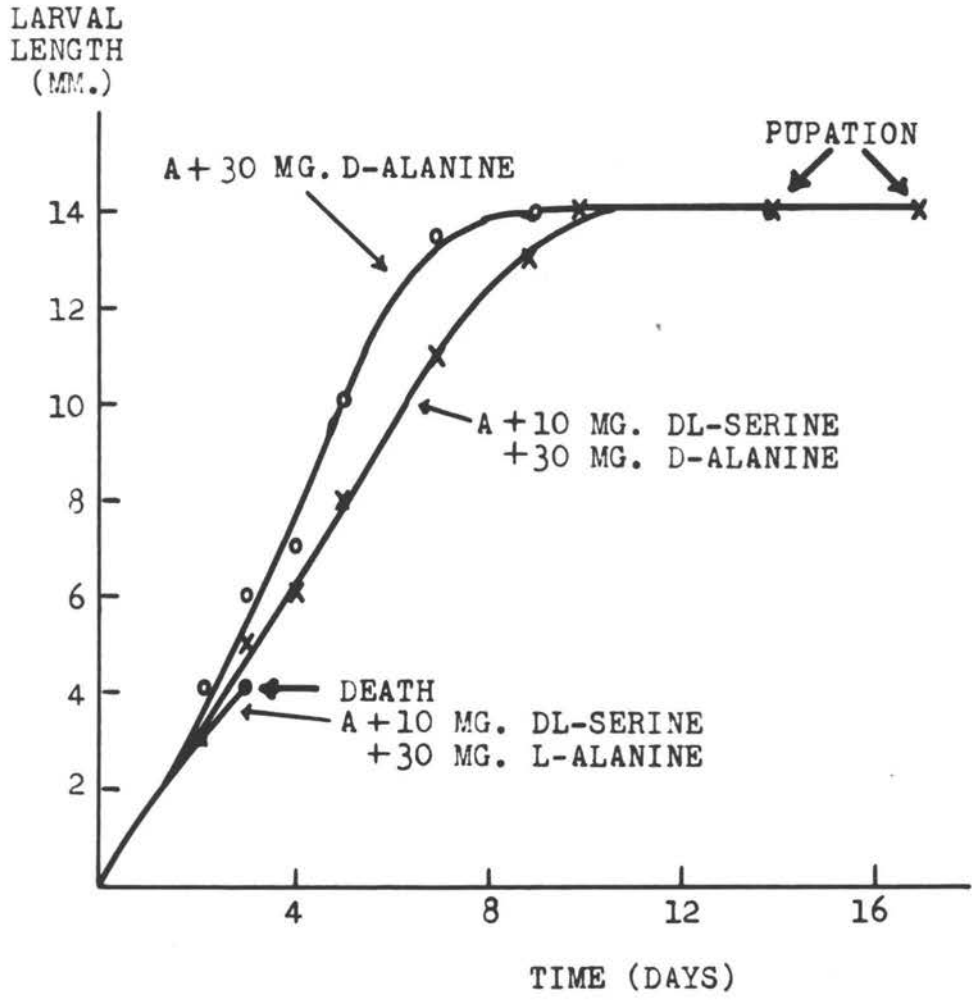
Effect of addition of D-, L-, and DL-serine to standard diet on P. regina development[2].

In view of the well known competition between serine and alanine, the latter was tested as a reversing agent. It was found that 60 mg. of DL-alanine could reverse the inhibitory effect of 10 mg. of DL-serine, and that the alanine effect was given only by the D-isomer. These results are shown in Figure 4. As was to be expected, the effective concentrations of the D-amino acids were one-half of those required with the racemic mixtures.

The fact that serine toxicity is conferred by the unnatural D-isomer rules it out as the toxic component of enzyme hydrolysed casein, and probably also of acid-hydrolyzed casein, since these digests should yield only L-amino acids. It seems safe to conclude that some other factor or factors, as yet unknown, is causing the inhibition of growth on the enzyme hydrolysed casein diet. That this same factor is responsible, in part at least, for the inhibition noted on the acid hydrolysed casein diet seems probable.

These findings suggest that the essential nature of alanine in diets for the German cockroach as reported by Hilchey (19, pp.203-219) may reside in this competition. He noted that serine was essential for the development of males. More striking than the reduced growth caused by the serine deficiency was the inhibition of development caused by the omission of alanine. He used the racemic mixture in both instances. It may be that the

Figure 4



Reversal of D-serine inhibition in P. regina diet by D-alanine[2].

D-serine and D-alanine in the medium were at such levels that the potential inhibition was suppressed. However, when the alanine was removed, the inhibitory effects were noted. If this is true, D-alanine must be a much more effective reversing agent in the cockroach than is the case with the blowfly.

The inhibitory effect of the dipeptide, glycyl-DL-serine was tested by addition of 30.8 mgms. to the standard assay tube. This corresponds to a molar concentration twice that at which DL-serine is known to completely suppress development. However, no retardation in larval development was noted. In this same experiment the effect of acetyl-DL-alanine as a reversing agent was tested. To the standard assay tube containing 10 mg. of DL-serine was added 88 mgms. of this N-substituted amide (corresponding to 60 mg. of DL-alanine). As has been indicated previously this amount of DL-alanine is sufficient to completely reverse the inhibitory effects of 10 mg. of DL-serine. However with this level of N-acetyl-DL-alanine only slight reversal was noted. These results are shown in Table II.

These findings suggest that the free amino group on both D-alanine and D-serine is essential for union with the active enzyme surface. It is also indicative of the fact that the dipeptides fed in Table II were

TABLE II

Effect of Glycyl-DL-Serine as a Growth Inhibitor, and of N-Acetyl Alanine in Reversing D-Serine Inhibition

	Larval Length (mms.)				
Days after seeding:	2	3	4	6	9
Diet A	4	7	10	14	14
Diet A + 10 mg. DL-Serine	3	dead	-	-	-
Diet A + 30.8 mg. Glycyl-DL-Serine	4	7	10	14	14
Diet A + 10 mg. DL-Serine + 88 mg. N-acetyl alanine	3	4	4	4	5
Diet A + 10 mg. DL-Serine + 60 mg. DL-alanine	4	6	9	13	14

degraded to the amino acid level at a very slow rate, if at all. In contrast to the findings of Kihara et al. (30, pp.791-800) the peptides were less effective in the diet than are the free amino acids. Based on the work of Olivard and Snell (37, pp.203-214) and others one might speculate that D-serine interferes in the alanine racemase system or in some system closely related to it. As yet, however, there is no information bearing on the site of action of this D-serine inhibition.

In the initial studies with a chemically defined medium, the diet was modeled after that described by House (24, pp.331-341). Certain modifications were made and a diet was finally compounded which permitted development from egg to adult under aseptic conditions. The composition of the diet is given in Table III.

In the preparation of the amino acid mixture, appropriate quantities of the eighteen amino acids were weighed out on an analytical balance, placed in a mortar and ground together thoroughly. Seventy-five mg. of this mixture and 50 mg. agar were added to each culture tube.

Individual vitamin solutions were freshly prepared at such concentrations that addition of one-tenth milliliter of the solution sufficed for two milliliters of medium. The three remaining components were combined in solution form immediately prior to use. The double

TABLE III

Composition of Chemically Defined Diet for *P. regina*

Amino acids	Concentration (mg/ml)	Vitamins	Concentration (μgm/ml)
DL-Alanine	2.58	Thiamin	5
L-Arginine	1.06	Riboflavin	15
DL-Aspartic acid	3.06	Pyridoxine	15
L-Cystine	0.36	Inositol	100
L-Glutamic acid	5.48	Nicotinic acid	15
Glycine	0.70	Choline chloride	100
L-Histidine	0.70	Ca pantothenate	15
L-hydroxyproline	0.48	p-Aminobenzoic acid	15
DL-Isoleucine	3.06	Folic acid	5
L-Leucine	2.80	Biotin	0.025
L-Lysine	1.76		
DL-Methionine	1.88		
DL-Phenylalanine	2.80		
L-Proline	2.00		
DL-Threonine	1.88		
DL-Tryptophane	0.94		
L-Tyrosine	1.64		
DL-Valine	3.30		

	Concentration (mg/ml)
Ribonucleic Acid	1.0
Cholesterol	1.0
Inorganic Salts	2.4
Agar	25.0

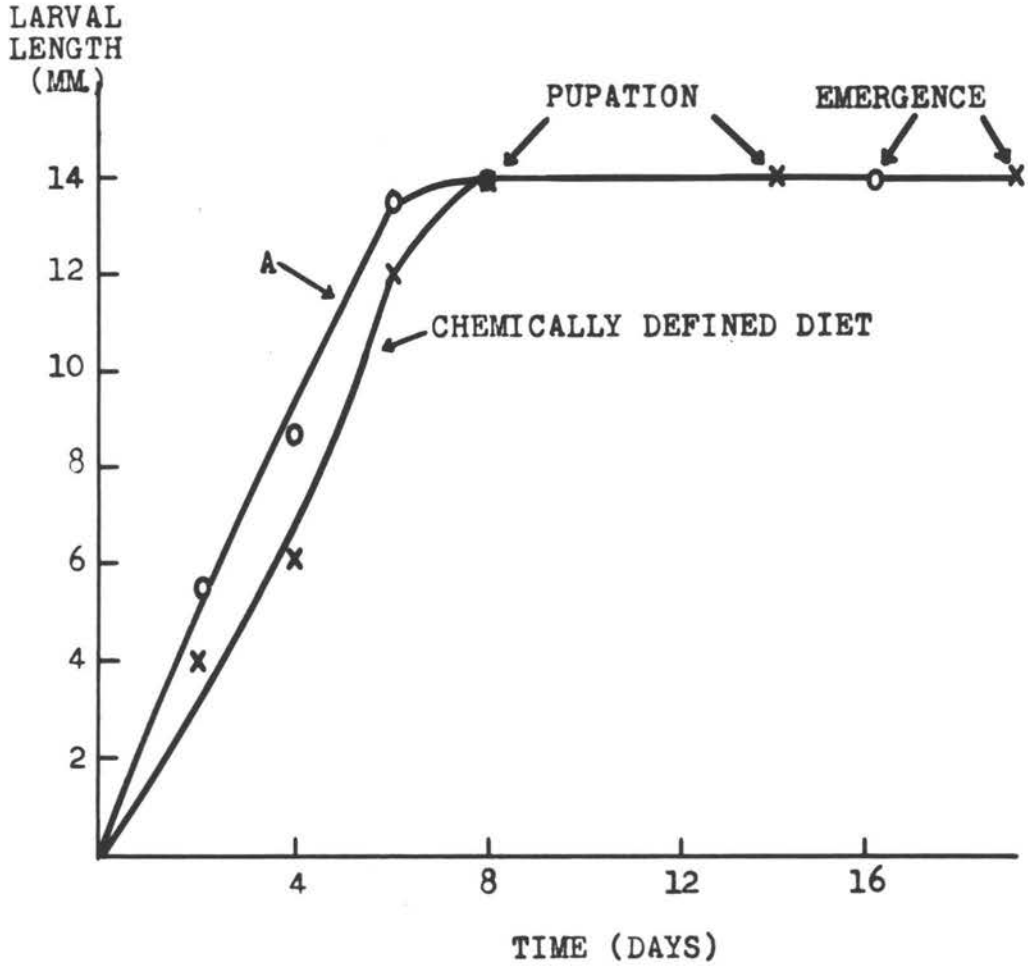
strength salts solution used in previous experiments was used as the mineral source. The stock cholesterol suspension used was prepared as described by House (24, p.333). The ribonucleic acid solution was freshly prepared for each experiment. It was prepared by dissolving 200 mg. of yeast ribonucleic acid in four milliliters of 2N NaOH. One milliliter of the solution, consisting of 44 parts salts solution, two parts cholesterol suspension, and four parts ribonucleic acid solution, was added to each culture tube.

The contents of the tubes were thoroughly mixed by shaking prior to sterilization at 16# pressure for 15 minutes. After removal from the autoclave, slants were prepared as described previously. The final pH was approximately 5.6. If the pH deviated greatly from this value the agar hydrolysed and the medium failed to solidify.

The adequacy of the chemically defined diet is demonstrated in Figure 5. Although larval growth compared favorably with that on the standard casein diet, it is evident that metamorphosis was considerably delayed.

In an effort to determine which amino acids were essential for this insect, single amino acids were deleted from the mixture. No other amino acid was added to the medium as a nitrogen replacement, therefore slight variations existed in the total nitrogen contents of these diets. These results are shown in Table IV.

Figure 5



Comparison of P. regina development on standard and chemically defined diets.

TABLE IV

Development of P. regina on Purified Diets when Single Amino Acids are Omitted (Experiment concluded after 26 days)

Diet	Days from seeding to: Death	Pupation	Emergence
Complete*	-	14	19
Complete + DL-Serine	4	-	-
A. Essential Amino Acids			
Complete - L-Arginine	4	-	-
" - L-Histidine	4	-	-
" - L-Leucine	4	-	-
" - L-Lysine	4	-	-
" - DL-Phenylalanine	4	-	-
" - DL-Threonine	4	-	-
" - DL-Tryptophane	6	-	-
" - DL-Valine	4	-	-
B. Effect of omission of other Amino Acids			
Complete - DL-Alanine	-	14	19
" - L-Glutamic Acid	-	19	-
" - Glycine	-	13	-
" - L-Hydroxyproline	-	19	-
" - DL-Methionine	-	14	-
" - DL-Aspartic Acid	23	-	-
" - L-Cystine	19	-	-
" - DL-Isoleucine	23	-	-
" - L-Proline	26	-	-
" - L-Tyrosine	21	-	-

* Diet shown in Table III.

Based on this work, the amino acids can be tentatively divided into three groups: inhibitory, essential and effects indefinite. DL-serine was the only amino acid which was inhibitory. When it was included in the diet at the rate of 7.0 mg. per 75 mg. of amino acid mixture all larvae died while small and immature. Eight amino acids were immediately established as being essential. These are L-arginine, L-histidine, L-leucine, L-lysine, DL-phenylalanine, DL-threonine, DL-tryptophane and DL-valine. When any one of this group was omitted from the diet, the larvae died shortly after hatching without having increased appreciably in size. The indespensability of the remaining amino acids in the diet is still in doubt. Removal of DL-alanine from the diet had no deleterious effect. Omission of DL-aspartic acid, L-cystine, DL-isoleucine, L-proline or L-tyrosine prolonged larval life but all insects died prior to pupation. When L-glutamic acid, glycine, L-hydroxyproline or DL-methionine was omitted pupation occurred but no emergence was noted. However, until further work is done on this group of amino acids, a final tabulation of the essential ones must be delayed.

The nitrogen content of the chemically defined diet was only about one tenth that of the casein diet. It seemed likely that better growth could be obtained if the nitrogen content in this artificial medium was increased. However, when the amino acid mixture was

used at a level of 886 mg. in each tube which is comparable to the nitrogen content of diet A, complete inhibition of larval development resulted. Different levels of the amino acid mixture were tested and it was found that concentrations in excess of 60 mg. per milliliter were inhibitory.

Two possible explanations of this inhibition are, first, since some amino acids were included as racemic mixtures, one or more of the unnatural isomers may have been toxic at the higher concentrations; second, the high osmotic pressure of the medium may have caused the inhibition. Support for this concept is indicated by the observation that enzymatically hydrolysed casein is inhibitory yet none of the unnatural amino acids should be present. The effect of sugars on the diet also supports such an hypothesis. As will be recalled, sucrose caused no browning, yet retarded development. Furthermore, sucrose is more inhibitory than starch on a weight basis.

Experiments to determine which components are essential in the vitamin mixture were conducted using a casein-agar-ribonucleic acid stock ration. The composition of this ration is shown in Table V.

TABLE V

Composition of Ration for Testing for Essential Vitamins

Vitamin Free Casein	(30 grams)
Agar	(1 gram)
Ribose nucleic acid	(0.5 gram)

To the assay tubes were added all vitamin solutions except the one being tested. Two milliliters of double strength inorganic salts solution containing two mg. cholesterol per milliliter were added to each tube. Two grams of the stock ration were then added with thorough mixing. Sterilization and subsequent treatments of these diets were identical to those described earlier.

Results from this study showed that thiamin, nicotinic acid, pantothenic acid and choline were all required. The other six vitamins appeared to be non-essential, but this needs to be established rigorously with a completely purified diet, since it is possible that trace amounts of vitamins were present in the casein used. In addition, the eggs may have contained enough of these vitamins to sustain growth during the first generation, as observed by Gordon (15) in studies of cockroach nutrition.

RESULTS AND DISCUSSION

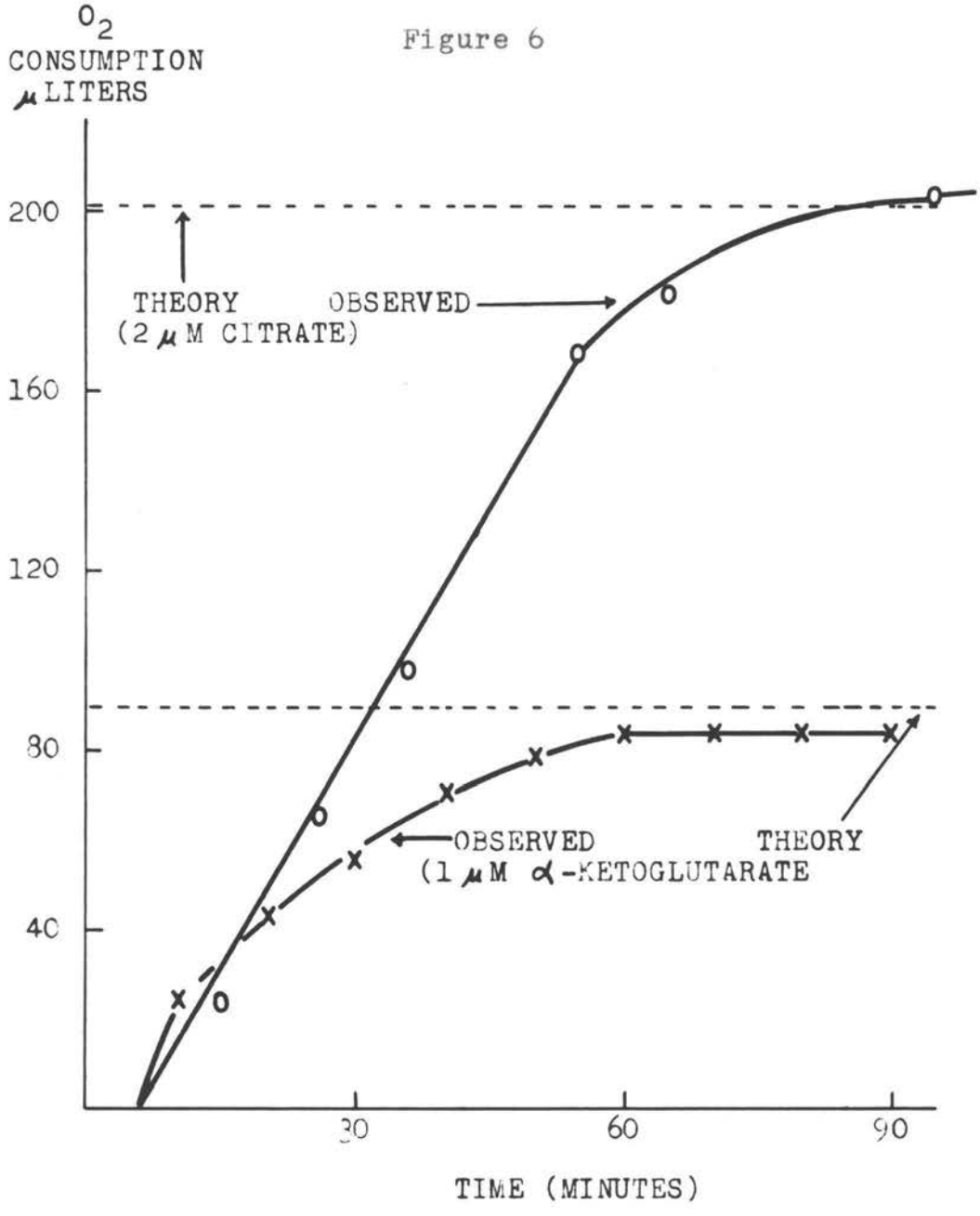
PART II METABOLISM

Citric Acid Cycle

It is recognized that insects such as the blowfly must be capable of very high oxidative rates. Watanabe and Williams (52, pp.675-689) were the first to demonstrate that the sarcosomes were chemically and enzymatically similar to mammalian mitochondria. Sarcosome fractions were prepared from adult blowflies using methods similar to those of Watanabe and Williams except that 0.001 M versene was included in the 0.25 M sucrose or 0.9% KCl suspending medium. The oxidative capacities were improved by the addition of versene but no differences were noted between the sucrose and KCl preparations. Sarcosome fractions were obtained from isolated thoraces and also from heads and thoraces combined. No oxidative differences were observed between the two types of preparation, but a pigment contained in the head imparted a deep red color to the sarcosome fraction. Consequently isolated thoraces were used routinely as their use permitted preparation of the sarcosome fraction uncontaminated by the red pigment.

As shown in Figure 6 both substrates were oxidized almost completely to CO_2 and water. This is suggestive

Figure 6



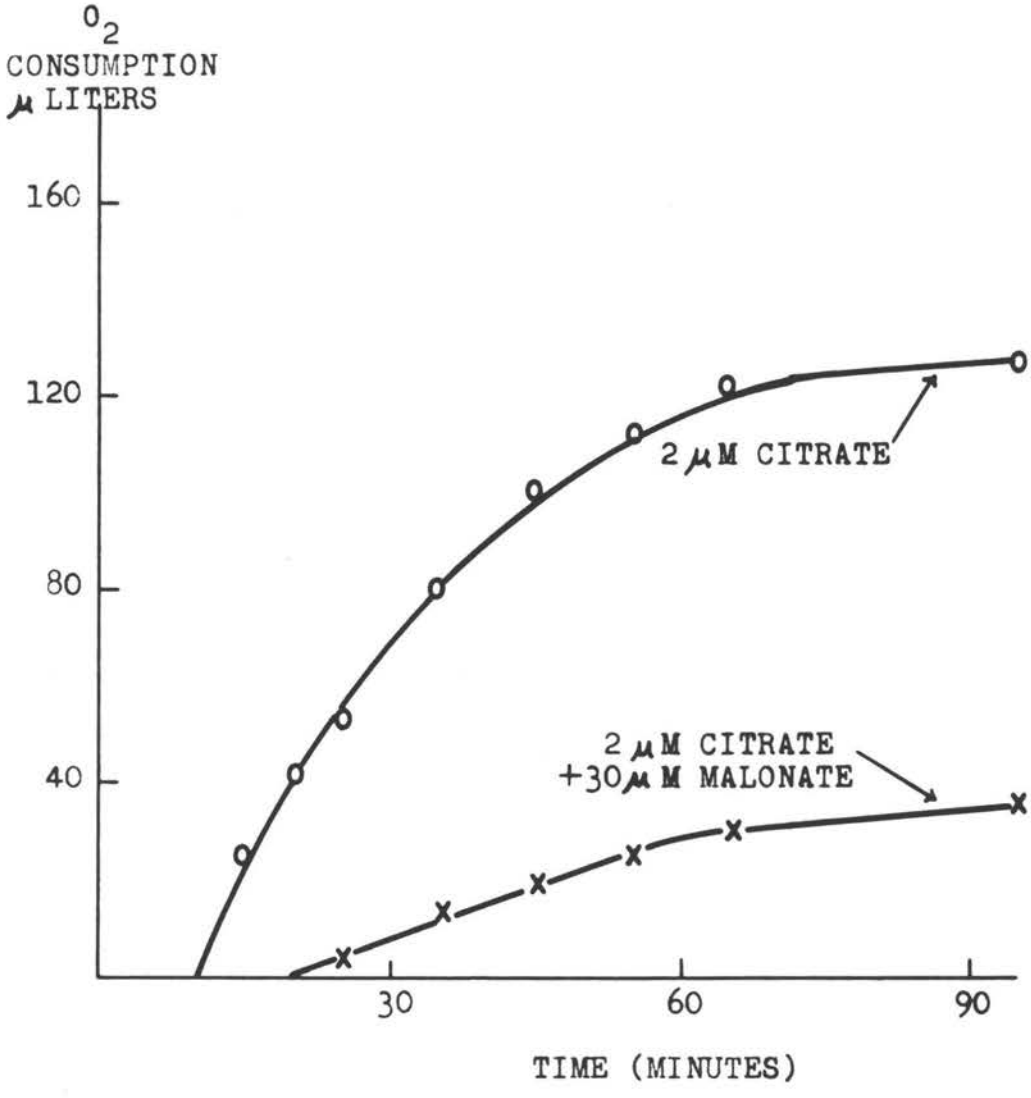
Oxidation of citrate and α-ketoglutarate by adult P. regina sarcosome fraction. To each flask was added 20 μ M phosphate buffer, pH 7.2, 4 μ M $MgCl_2$, 0.35 μ M cytochrome C, 10 μ M AMP[1], 1 mg. Armour liver concentrate, 1 ml. enzyme preparation and substrates as indicated. 0.2 ml. 20% KOH and filter paper was placed in center well. Total volume 2.5 mls.

evidence of an active enzymatic system, possessing tricarboxylic acid cycle activity. To further indicate the presence of this system of terminal oxidation, malonate was introduced into the system. As is shown in Figure 7 oxidation was reduced in the presence of this inhibitor. These results, further support the concept that the enzyme complex involved in the tricarboxylic acid cycle is localized in the sarcosomes.

Experiments were also conducted to compare the oxidizing ability of the whole homogenate, the sarcosomes and the soluble fraction. As shown in Figure 8 the homogenate showed a very high endogenous oxidation compared with either the sarcosomes or the enzymes in the supernatant liquid. When the two fractions were recombined oxidation was again rapid. Citrate was oxidized by the homogenate and the sarcosome fraction. However in the presence of citrate the oxygen consumption by the supernatant fraction was reduced below that of the endogenous.

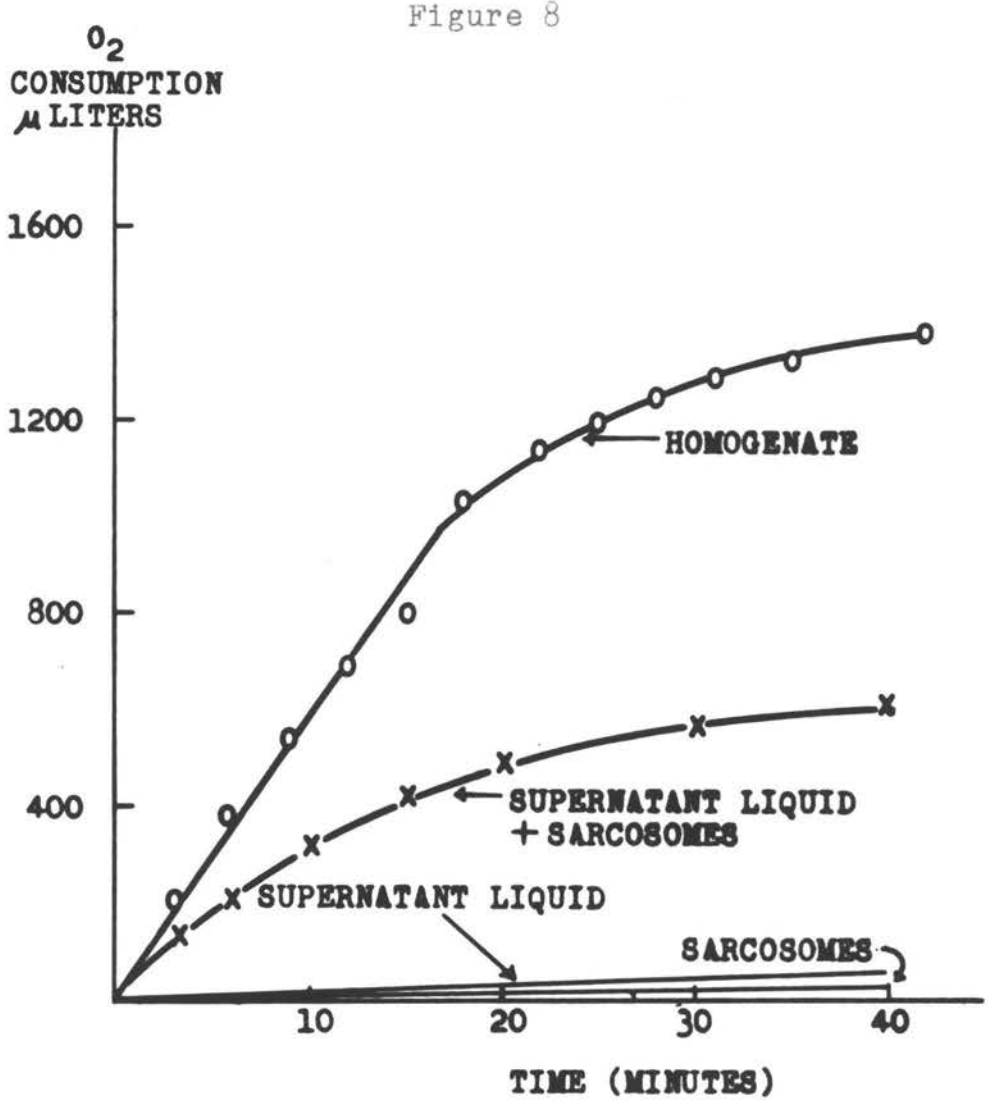
Two points arise from these observations. First, only the sarcosome fraction possesses the ability to oxidize citrate to CO_2 and water. Second there is a high concentration of substrates present in the supernatant liquid which are not oxidized in the absence of the sarcosomes. Since the supernatant fluid was not dialysed part of this endogenous oxidation is likely

Figure 7



Malonate inhibition of citrate oxidation by adult P. regina sarcosome preparation. Additions to flasks the same as Figure 6 except malonate was included as shown.

Figure 8



Endogenous oxidation of homogenate, supernatant and sarcosome fractions of adult P. regina. Additions to flasks the same as Figure 6 except no substrate was added and 1 mg. streptomycin sulfate was included.

owing to the presence of free amino acids. It is probable that glycogen was also an endogenous substrate.

Coenzyme specificity of isocitric dehydrogenase was tested using both acetone powder extracts and fresh sarcosome preparations. TTZ was employed as the electron acceptor and the quantity of dye reduced was a measure of substrate oxidation.

With the acetone powder extract, the reaction mixture consisted of 30 μ moles of phosphate buffer, pH 7.2, 0.5 μ mole $MgCl_2$, 0.1 μ mole TPP[1], 4 μ moles nicotinamide, 10 μ moles TTZ, 1 μ mole citrate and 0.05 μ mole of either DPN or TPN[1]. The total volume was one milliliter. After two hours incubation at 37°C., 0.3 μ moles of TTZ were reduced in the presence of TPN while only 0.15 μ moles were reduced in the presence of DPN.

The reaction mixture described above, with the addition of 60 μ moles KF, 1 mg. streptomycin sulfate and 1 μ mole DL-isocitrate in place of citrate was used with the fresh sarcosome preparations. After incubation at 37°C. for three hours, 0.48 and 0.85 μ mole of TTZ were reduced in the presence of DPN and TPN respectively.

These results suggest that DPN can be used as a coenzyme by isocitric dehydrogenase but that it is less efficient than TPN. However there is the possibility

that sufficient TPN remained in the enzyme preparations to account for the lower rate of oxidation observed.

After having prepared active sarcosome fractions from the adults, attention was directed toward similar oxidative processes in the larvae. Using the same methods of isolation, a fraction from the larvae was obtained which appeared similar to adult sarcosome when examined microscopically.

The oxidative ability of the homogenate was tested manometrically with citrate, α-Ketoglutarate, succinate and malate as substrates. Of these, succinate was oxidized most extensively, but at best only to 30 per cent completion in one hour. Citrate was not oxidized at all. The reduced oxidative activity is reflected in the low endogenous oxidation found. In 60 minutes the larval preparation consumed less than 200 μ liters of oxygen while the adult homogenate consumed in excess of 1000 μ liters in the same time.

Although these particulate preparations were inactive against citrate as measured manometrically, the same preparations were capable of TTZ reduction in the presence of citrate. The soluble fraction also possessed citrate oxidizing ability as evidenced by TTZ reduction.

The identity of isocitric dehydrogenase was established by isolating and identifying the reaction product. For this demonstration the supernatant liquid was dialysed against 0.02 M Tris buffer, pH 8.0, for 11 hours before use. One milliliter of the dialysed solution, containing 13 mg. of protein, constituted the enzyme source. The reaction mixture consisted of 8 μ moles of phosphate buffer, pH 7.4, 75 μ moles potassium bicarbonate, 10 μ moles nicotinamide, 12 μ moles of $MnCl_2$, 5 μ moles of either citrate or isocitrate and 7.5 μ moles of DPN or TPN. The gas phase was 5% CO_2--95% nitrogen and the total liquid volume was 3.0 mls. The reaction was allowed to proceed 80 minutes and was then stopped by addition of 2,4-dinitrophenylhydrazine (0.1%) in 2N HCl. The keto acid derivative was isolated and determined quantitatively by the method of Brummond and Burris (8, pp.754-759). The results are shown in Table VI.

TABLE VI

Formation of α-Ketoglutarate from Citrate and Isocitrate in presence of $MnCl_2$, DPN or TPN and Soluble larval Enzyme Preparation

Substrate	μ Moles	Pyridine nucleotide added	Product	μM	% Conversion
Citrate	5	TPN	α-Ketoglutarate	1.2	24
DL-Isocitrate	5	TPN	α-Ketoglutarate	0.9	18
Citrate	5	DPN	α-Ketoglutarate	0.8	16
DL-Isocitrate	5	DPN	α-Ketoglutarate	0.7	14

From the data in Table VI it may be seen that either DPN or TPN can serve as the coenzyme for isocitric dehydrogenase, but TPN is superior. These results are in agreement with those on the coenzyme specificity of isocitric dehydrogenase from adult blowfly preparations.

Superficially, the data in Table VI suggest that citrate is oxidized more readily than isocitrate. This is the reverse of what is expected if isocitric dehydrogenase is involved. This discrepancy is likely attributable to utilization of only one optical isomer. If only D-isocitrate is oxidized the effective concentration was only one-half of that indicated. Consequently the per cent converted to α-Ketoglutarate is double that shown which is in agreement with the expected action of this enzyme.

The use of eggs as an enzyme source was limited because of the low productivity of the insects. Thus manometric studies with egg enzymes on citric acid cycle members was not possible but the dye method, using TTZ as the elctron acceptor was used. The eggs were homogenized in the KCl-versene solution with a Potter-Elvehjem homogenizer and fractionated by centrifugation. The supernatant was dialysed against 0.02 M Tris buffer, pH 8.0, for three hours and the precipitate was washed three times prior to use.

After incubating the TTZ reaction mixture at 37°C. for three hours, limited oxidation of citrate, isocitrate and malate was noted with the solid fraction. No oxidation occurred with the supernatant enzymes. By way of comparison the adult sarcosome fraction oxidized citrate almost three times as rapidly under similar conditions.

Glycolysis

Glycolysis has been demonstrated in insects by other workers (4, pp.57-76; and 26, pp.353-359). Since it has not been demonstrated in the blowfly an attempt was made to identify and localize the glycolytic system in the adult insect. The system used was that described by LePage for tumor tissue (32, pp.1009-1020). The experiments were conducted in double side arm Warburg flasks in a final volume of 3.6 mls. under a 5% CO_2--95% nitrogen atmosphere. 0.4 mls. of 50% trichloroacetic acid was placed in one side arm and added at the end of the experiment to stop the reaction. Substrate was placed in the other side arm. The system was buffered at pH 7.4 with 75 μ moles of bicarbonate and 7 μ moles of phosphate. In addition to the enzyme preparation, the reaction mixture contained 12 μ moles $MgCl_2$, 1.2 μ moles ATP[1], 0.6 μ mole DPN, 60 μ moles nicotinamide,

30 μ moles KF, 15 μ moles pyruvate and 6 μ moles FDP.

With both the homogenate and the soluble fraction, lactic acid was produced demonstrating that anaerobic oxidation had occurred. The CO_2 evolved by the homogenate far exceeded the lactic acid formed but with the supernatant liquid the CO_2 evolution approximated that expected on the basis of lactic acid formed.

Triply washed adult sarcosomes were also tested for anaerobic oxidation using the same procedure. A very small quantity of lactic acid was detected at the end of the experiment although all the FDP had disappeared from the system. A summary of these findings is given in Table VII.

TABLE VII

Quantities of lactic acid formed, CO_2 evolved, FDP disappearing and Pentose present after anaerobic oxidation with adult P. regina enzyme preparations

Enzyme system	μ M Lactic acid formed	μ MCO_2 evolved	μ M FDP disappearing	μ M Pentose present
Homogenate	3.9	13.5	5.8	1.5
Supernatant	3.0	2.3	6.0	0.5
Sarcosomes	0.5	1.5	6.0	0.5

While these results are inconvlusive, there is evidence for anaerobic oxidation suggesting the presence of the glycolytic system.

Since nearly all the FDP disappeared and only a small amount was accounted for through the glycolytic scheme it is obvious that some other hexose degrading mechanisms was operating. Although some pentose was formed, the quantity was insufficient to account for the missing FDP.

Pentose Cycle

In recent years the hexose monophosphate shunt has been reinvestigated and shown to be a cyclic oxidative mechanism (23, pp.214-220). Since it is a cyclic mechanism it was renamed the pentose cycle (18, pp. 11-26). This has been shown to function in photosynthesis (5, pp.1760-1770) and its presence has been demonstrated in mammals (36) microorganisms (18, pp.11-26) and the pea aphid (35, pp.37-46). Consequently a series of experiments was run on egg, larval and adult stages of the blowfly to determine the presence of this cycle.

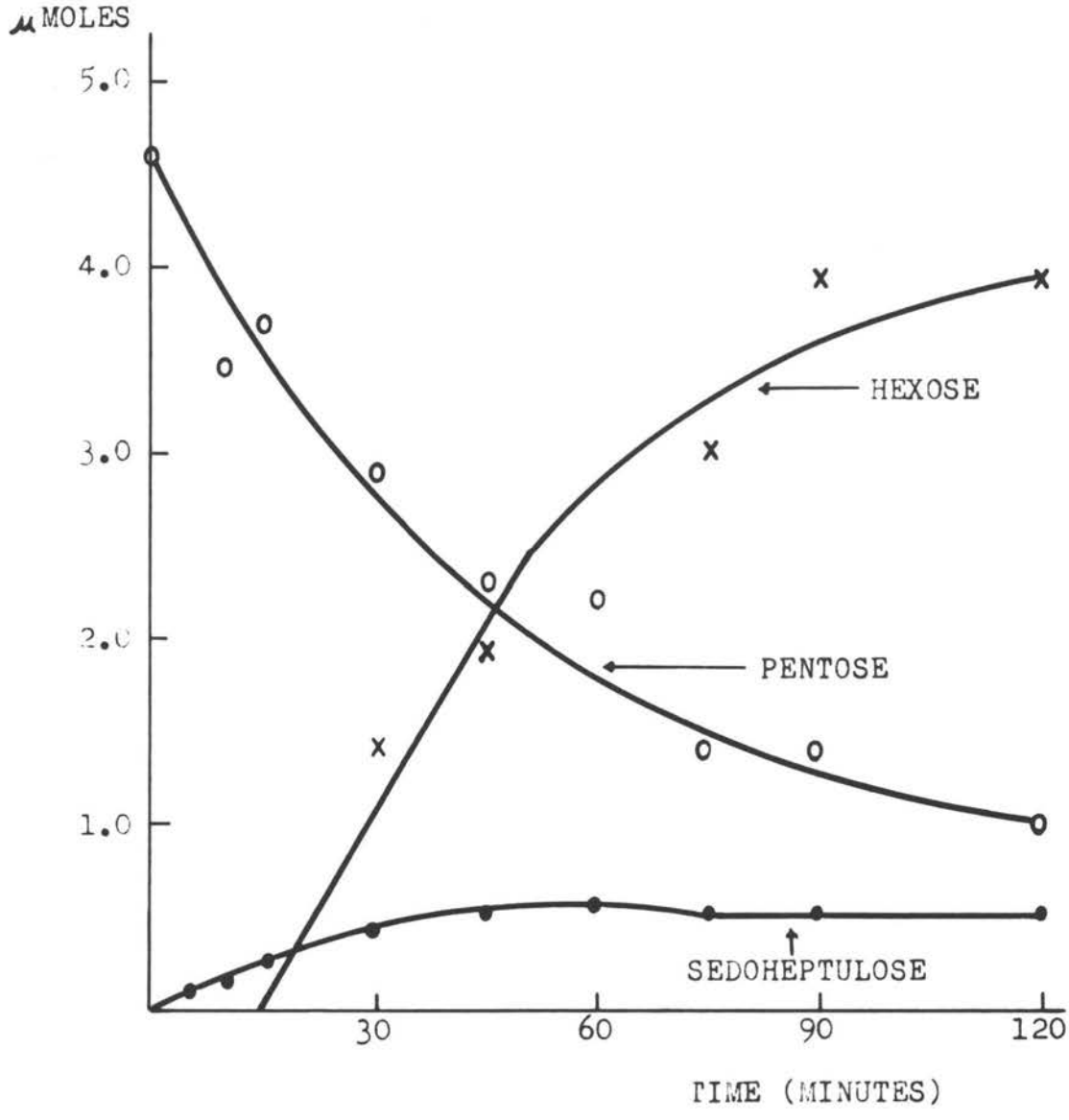
In all experiments the reaction mixture contained 800 μ moles of Tris buffer, pH 8.0, 12 μ moles $MgCl_2$, 0.7 μ mole TPP, 3 mg. streptomycin sulfate, 5 μ moles ribose-5-phosphate and the enzyme preparation. The

total volume was 10.7 mls. The corresponding reaction blank contained no ribose-5-phosphate. The reaction mixtures were incubated at 37°C. Figures 9, 10 and 11 show the changes in concentration of pentose, SH[1] and hexose occurring in the presence of dialysed adult, larval and egg supernatant fractions, respectively.

As the pentose concentration decreases there is a gradual rise in the SH content for the first 30 to 40 minutes, after which it drops slightly and remains constant. Although hexose does not form until after the appearance of SH the concentration rises sharply before plateauing. With the larval preparation there was a high endogenous hexose content which tended to reduce the reliability of the results. However the same general pattern was obtained except that the hexose content decreased after 60 minutes. The significance of this decline has not been determined.

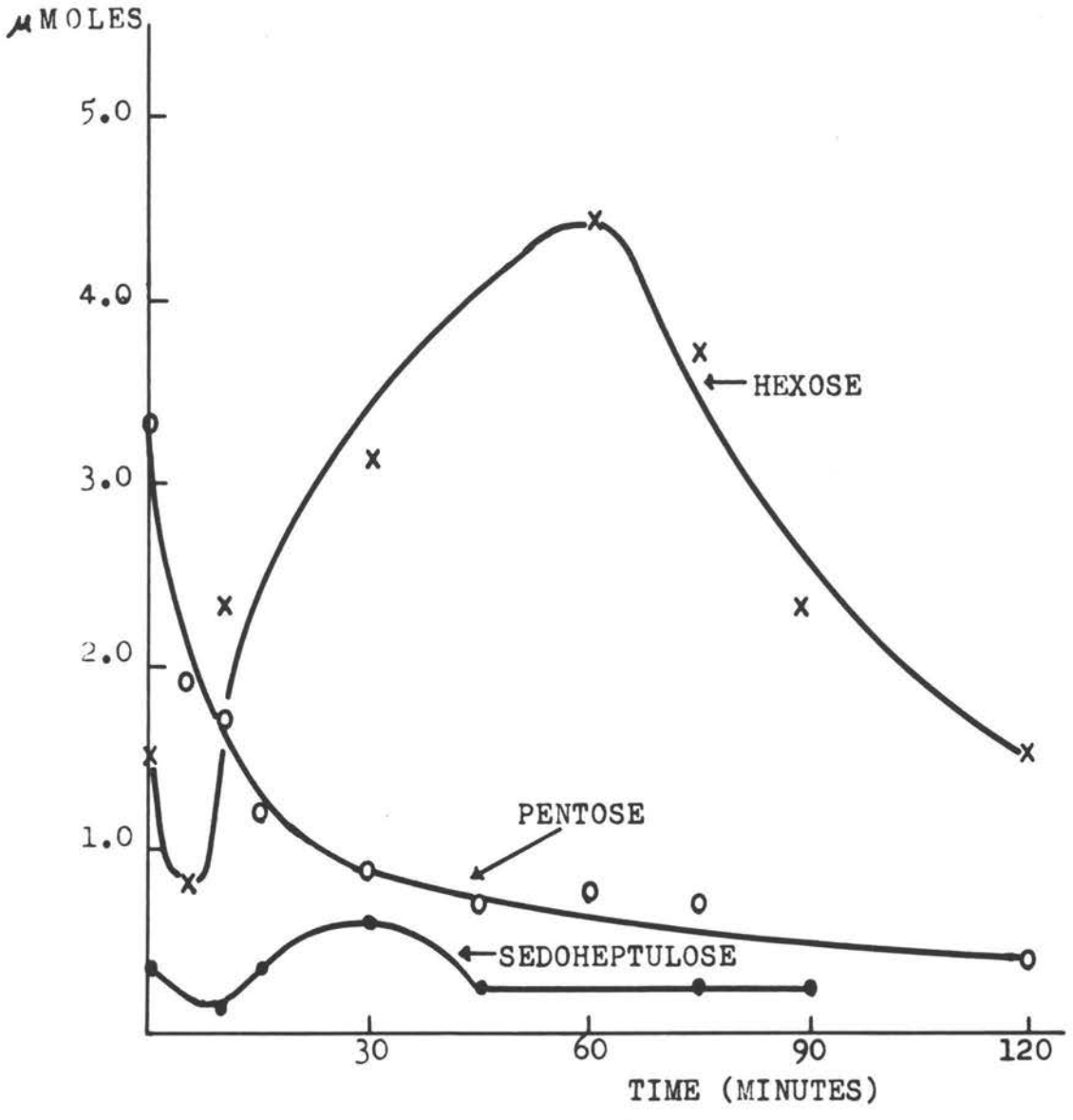
The adult sarcosome fraction was tested for pentose cycle activity also. Although a small amount of pentose disappeared during the two hour incubation period no SH or hexose formation was demonstrated. These results are in agreement with studies on other organisms where pentose cycle activity is localized in the soluble fraction (36; and 35, pp.37-46).

Figure 9



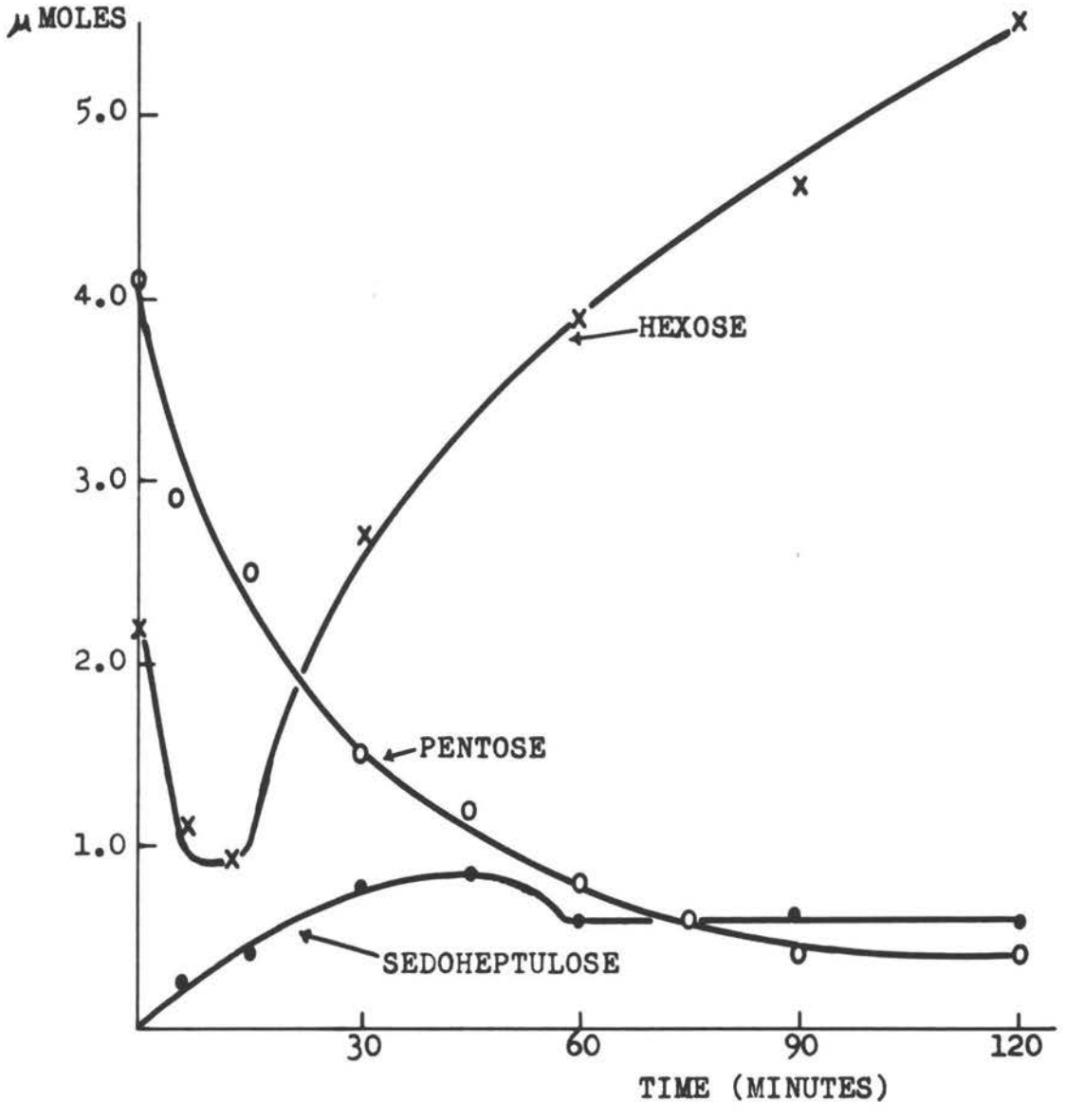
Changes in pentose, SH and hexose concentration in presence of dialysed adult P. regina supernatant fraction.

Figure 10



Changes in pentose, SH and hexose concentration in presence of dialysed larval P. regina supernatant fraction.

Figure 11



Changes in pentose, SH and hexose concentration in presence of dialysed P. regina egg supernatant fraction.

Polyphenol Oxidase

As noted earlier a very active polyphenol oxidase was encountered in studies with late larval and prepupal forms. With prepupal material a noticeable darkening of the homogenate occurred almost immediately after homogenization was begun. When third instar material was used polyphenol oxidase was less striking although still present. Similarly when 2 or 3 day old pupae were used, the activity of this enzyme was much less.

The enzyme is also present in acetone powders as evidenced by the blackening which occurs in the acetone powder extract during dialysis. When catechol was used as substrate with prepupal acetone powder extracts, a very rapid consumption of oxygen occurred. More oxygen was consumed than could be accounted for by the oxidation of the hydroquinone to the quinone. That subsequent reactions occurred was evident, however, because large quantity of black pigment were formed in the reaction flask.

Dennell (10, pp.79-110) studied puparium formation in the fly Sarcophaga falculata and noted a rise in both tyrosine and tyrosinase in the blood immediately prior to pupation. That this system is important in the formation of the puparium seems likely in view of the darkening which occurs following pupation. Whether this is the only

purpose of the enzyme is not known. In view of the apparent differences between larval and adult electron transport systems, it is tempting to speculate that polyphenol oxidase may function in this capacity during the transition period.

SUMMARY

PART I NUTRITION

The diet proposed by Hill (20, pp.213-216) for P. regina has been modified by replacing lanolin with crystalline cholesterol. It was found that replacing 20 per cent of the casein of this diet by glucose reduced growth. When 33 per cent of the casein was replaced by glucose complete inhibition resulted. Part of this inhibition may be the result of the browning reaction between glucose and casein which occurs during autoclaving. Less growth retardation was observed when sucrose was used to replace the casein, and still less when soluble starch was employed.

Neither enzyme hydrolysed casein or acid hydrolysed casein plus added tryptophane was able to replace whole casein in the diet.

It was shown that 0.83 mg. of D-serine per gram of medium caused complete inhibition and resulted in early death of the small immature larvae. Inhibition by this quantity of D-serine could be completely reversed by the addition of 5 mg. of D-alanine. Work with peptides suggests that the free amino group is essential on both amino acids to evoke this response.

A chemically defined medium was prepared on which the blowfly can develop from egg to adult under aseptic conditions. Eight amino acids: arginine, histidine, leucine, lysine, phenylalanine, threonine, tryptophane and valine have been shown to be required in the diet. The essentiallity of the others remains to be determined.

Four vitamins have been shown to be required in the medium. These are thiamin, nicotinic acid, pantothenic acid and choline. From this work it is not possible to specify that these are the only factors necessary.

It seems likely that a high osmotic pressure in the medium is inhibitory to the developing larvae. This concept is supported by three observations on different media:

1. Enzyme hydrolysed casein is inhibitory.
2. Sucrose is more inhibitory than starch when used to replace part of the whole casein in the diet.
3. Concentrations of free amino acids in the chemically defined diet, much in excess of 60 mg. per milliliter of medium are inhibitory.

SUMMARY

PART II METABOLISM

Sarcosomes were isolated by differential centrifugation from the thoracic tissue of adult blowflies in 0.25 M sucrose, 0.001 M versene or 0.9% KCl, 0.001 M versene solutions. The preparations were capable of completely oxidizing citrate and α-Ketoglutarate to CO_2 and water. This oxidation was inhibited by malonate. These results substantiate previous work demonstrating citric acid cycle activity in the adult sarcosomes.

A similar fraction was isolated from mature larvae using identical isolation procedures. With this preparation however, no citrate oxidation could be detected manometrically. When TTZ was used as an artificial electron acceptor, oxidation of citrate was obtained as determined by dye reduction. Isocitric dehydrogenase was detected in the larval supernatant fraction by isolating and identifying α-Ketoglutarate as the reaction product when either citrate or DL-isocitrate was used as the substrate.

Anaerobic oxidation of FDP was demonstrated in the soluble fraction of the adult thoracic preparation. Reduction of pyruvate to lactate and evolution of CO_2 from

a bicarbonate buffer in a fluoride inhibited system suggest that the glycolytic system is present.

Evidence for pentose cycle activity was obtained in the soluble fraction of eggs, larvae and adults by measuring pentose disappearance and SH and hexose formation. No SH or hexose was formed in the presence of adult sarcosome preparations. Thus, as has been shown in other organisms, the pentose cycle activity is localized in the soluble fraction. These findings further support the concept that the pentose cycle is a widely distributed metabolic system.

The presence of an active polyphenol oxidase system in the prepupal form was demonstrated. That it plays a role in pupation is evident but exactly how it functions is at present unknown.

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