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Part I. A study of Mosquito Larvae Nutrition in the Relations to Microorganisms, the Vitamins or Accessory Growth Factors, and the Utilization of Solutes by the Larvae.

Part II. The Survival of the Larvae in Media of Different Concentrations of the Hydrogen Ion.

Thesis and abstract approved: Professor in Charge of Thesis

May 11, 1940

# SOME STUDIES IN THE NUTRITION AND METABOLISM OF THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI* L. AND THE COMMON HOUSE MOSQUITO, *CULEX PIPIENS* L.

PART I. A STUDY OF MOSQUITO LARVAE NUTRITION IN THE RELATIONS TO MICROORGANISMS, THE VITAMINS OR ACCESSORY GROWTH FACTORS, AND THE UTILIZATION OF SOLUTES BY THE LARVAE.

PART II. THE SURVIVAL OF THE LARVAE IN MEDIA OF DIFFERENT CONCENTRATIONS OF THE HYDROGEN ION.

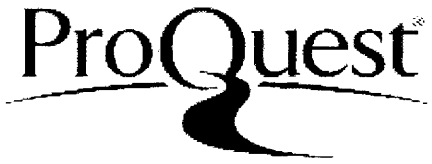
by

Arthur R. Buddington

Thesis submitted to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June, 1940.

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# A C K N O W L E D G M E N T S

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For supplying some of the salts, expensive vitamins, and concentrates, the author is indebted to Dr. Hugo Neilson of the United States Bureau of Fisheries here at College Park.

The author also wishes to thank Mr. Raymond Young of the Department of Bacteriology for his aid in the isolation of microorganisms of various pond waters and supplying pure cultures of various bacteria and yeasts.

Mosquito cultures were obtained at various times from the Army Medical Center, Walter Reed Hospital.

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PART I

# A STUDY OF MOSQUITO LARVAE NUTRITION IN THE RELATION TO MICROORGANISMS, THE VITAMINS OR ACCESSARY GROWTH FACTORS, AND THE UTILIZATION OF SOLUTES BY THE LARVAE.

## INTRODUCTION

In the life of any organism the role of growth factors or growth substances can by no means be overlooked. It has been known for a long time that the larvae of mosquitoes are associated in their development with microorganisms; namely, bacteria, yeasts, molds, algae, desmids, and protozoa. Biologists have known that these low forms of life probably supply the necessary food for the mosquito larvae and also the growth substances or factors. However, in recent years it has been the tendency of research workers to remove the microorganisms from the diets of the larval stages of mosquitoes and other flies, supply the various foods, salts, proteins, carbohydrates, and possibly fats, and attempt to add the accessory food factors or vitamins that seem to be supplied by microorganisms in nature.

According to R. A. Gortner in his textbook on Biochemistry, "Vitamins may be defined as organic substances which must be supplied in the diet of animals or may be synthesized in animals from essential dietary or metabolic precursors, and which exert a hormone-like or enzyme action in the animal body." Gortner further points out that the vitamins are definite nutritive substances and the nutritional quantitative requirements for them are relatively very small just as are the quantitative needs for inorganic salts in the animal body.

In part I of this report the author has approached the problem of mosquito larvae nutrition along three lines of reasoning; First, a consideration of the growth of the larvae of the yellow fever mosquito Aedes aegypti and the common house mosquito Culex pipiens in pure cultures of living bacteria and yeast; Second, a consideration of the effects on larval development of pond water which has been autoclaved and Berkefeld filtered; Third, the effects of various accessory growth factors or vitamins under sterile conditions upon larval development. In this last phase of the nutritional problem one experiment was performed to ascertain the effects of substances in pure solution of the growth of mosquito larvae. Thus the general outline of the author's intentions has been presented.

With aid rendered by the Department of Bacteriology at the University of Maryland some isolation analyses were made on pond water in which Culex pipiens was found breeding. Members of the genera Aedes and Anopheles were also present but in much smaller numbers. The most common forms of microorganisms present were: bacteria: Escherichia (Bacillus) coli, Aerobacter aerogenes, Bacillus subtilis, Bacillus cereus, and other spore forming bacilli; chromogens of the genus Flavobacterium; unknown white molds; Actinomycetes; common yeast.

It was thought advisable to obtain pure cultures of the common intestinal fecal bacterium, Escherichia (Bacillus) coli, the spore forming rod-shaped bacterium, Bacillus subtilis, and the common brewer's yeast, Saccharomycetes cerevisae, and study the growth of mosquito larvae in each of these organisms. These were

obtained from Mr. Raymond Young of the Department of Bacteriology and sub-cultured on an autoclaved medium composed of oyster meal, dried yeast, glucose, and Osborne-Mendel salt mixture. This medium is one described by the author in his thesis, "Some Studies in the Biology and Metabolism of Culex pipiens L.", 1939, University of Maryland (unpublished). Hence the role of three microorganisms very abundant in the natural life of mosquito larvae could be studied.

The effects of autoclaving and Berkefeld W filtering of pond water on Culex pipiens have been previously reported by the author. These same experiments have been repeated using Aedes aegypti. The survival of mosquito larvae in autoclaved oyster meal, dried yeast, glucose and Osborne-Mendel salt mixture was likewise studied and the results will be presented later in this report. In this phase of the problem, another experiment was carried on which seems unique in insect nutritional studies. An apparatus was designed wherein this synthetic medium was autoclaved in an Erlenmeyer flask connected to another flask by means of a sterilized Seitz filter. In this other flask the same synthetic medium was not sterilized and it contained many bacteria. Hence by raising and lowering one of the flasks on a ringstand the soluble products of bacterial metabolism could flow throught the sterile filter into the autoclaved (sterile) medium. It was thought that bacteria and materials not in true solution could be kept from the sterile cultural medium. If the growth factors that microorganisms produce are solutes they should pass through the filter under pressure

(one flask lower than other) to the other flask. Sterile eggs or sterile larvae were inoculated into the sterile flask.

In the third phase of Part I of this report the author studied the growth of the larval stage of Aedes aegypti on diets of known chemical composition free from microorganisms. A basal diet of 0.1% oyster meal, 0.1% dried yeast, 0.1% glucose, and 0.1% Osborne-Mendel salt mixture that had been autoclaved was used in these experiments. Since autoclaved yeast supplied a heat-stable growth factor it was replaced in some tests with pure chemicals to determine if any of them was the growth factor. The vitamins employed in this problem are water soluble and were added to the autoclaved media by means of the sterile Berkefeld.

The composition of the Osborne-Mendel salt mixture used throughout these experiments is as follows:

| | | |
|---|---|---|
| Calcium carbonate | 134.8 | grams |
| Magnesium carbonate | 28.9 | " |
| Sodium carbonate (Anhydrous) | 34.2 | " |
| Potassium carbonate (dried at 180°C) | 141.3 | " |
| Phosphoric acid | 119.3 | " |
| Hydrochloric acid | 148.3 | " |
| Sulphuric acid | 9.6 | " |
| Citric acid | 111.1 | " |
| Ferric citrate | 7.44 | " |
| Potassium iodide | 0.020 | " |
| Magnesium sulphate | 0.117 | " |
| Sodium fluoride | 0.062 | " |
| Potassium alum | 0.044 | " |

These chemicals were mixed thoroughly in a "ball mill" to a very fine powder which was uniform in composition throughout.

The fat soluble vitamins of higher animals, vitamins A, D, E, have not been considered by the writer in his experiments. Vitamin A which chemically is closely related to the carotenes of plants is

apparently synthesized by animals alone. This factor is not found in plants although its forerunners or parent compounds are present in no small quantities. About 90% of this vitamin present in the body of animals is stored in the liver. Cod liver oil and other fish oils are good sources of this substance. Lack of vitamin A causes in rats and man an atrophy of epithelium cells and a keratinization of the epithelium, particularly the eyes. Heat in the presence of oxygen destroys vitamin A.

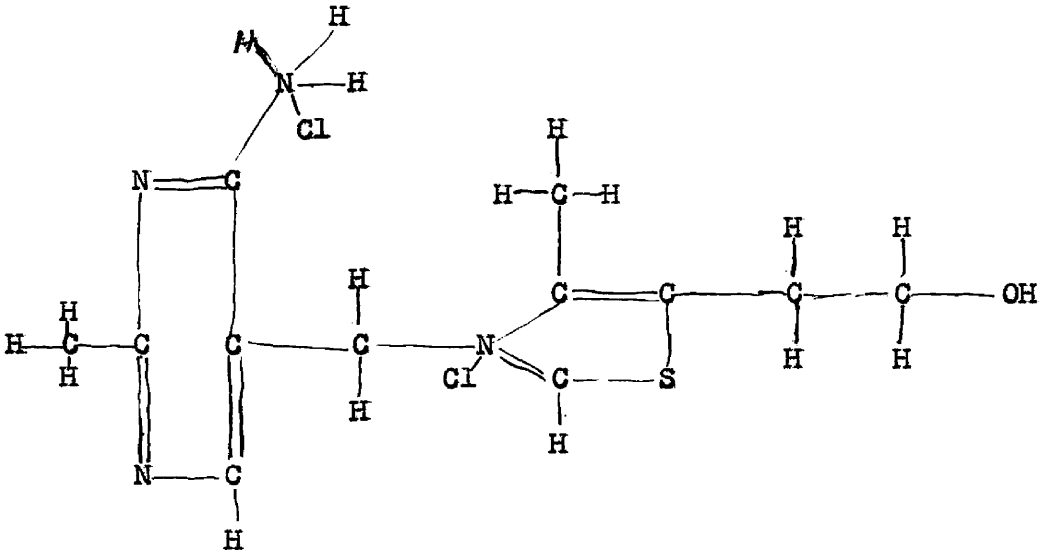
According to Gortner (1938) "ergosterol and other sterols, whether in plants or animal tissue, are transformed into vitamin D under the influence of the proper light rays and other forms of energy". It is not clear in the mind of the present writer just exactly what vitamin D is chemically. Gortner presents evidence from the chemical literature that possible nine forms of the D vitamin exist. Ergosterol and calciferol are sterols very closely related to cholesterol which can readily be detected in animal tissue. Calciferol is derived from ergosterol by various forms of irradiation, has been isolated in pure crystalline form and is now known as vitamin $D_2$. In mammals the D vitamins are associated with the metabolism of the bone, and the utilization of calcium and phosphorus, and prevent rickets, hence the name antirachatic vitamin. Heat in the presence of oxygen is generally considered destructive to these vitamins of the D group.

Vitamin E is the sterility vitamin of mammals and is known as A-tocopherol. Both the male and female animal are unable to produce

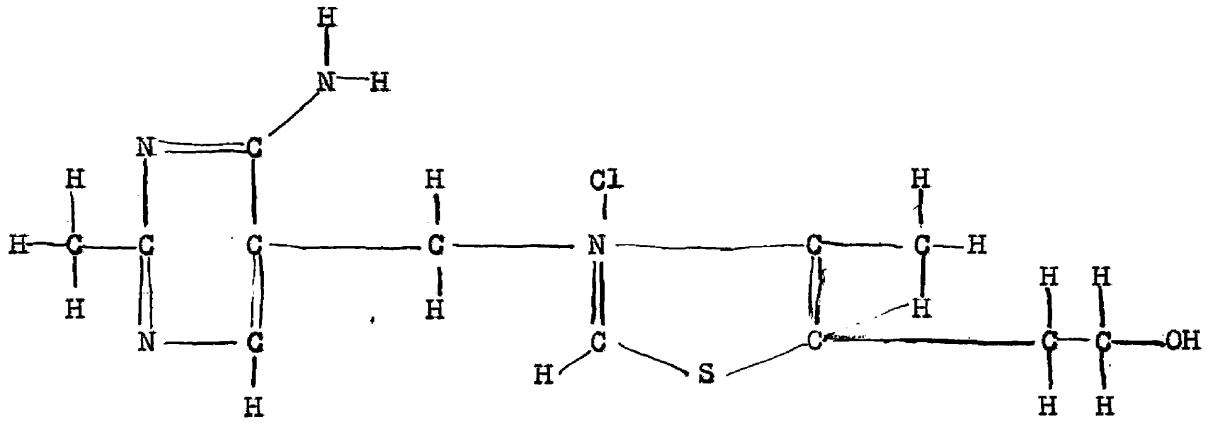
fertile reproductive cells in the absence of this vitamin. Vitamin E is present in nature in the germ oils of barley, oats, rice, wheat, corn, and many other plants. This factor is very sensitive to oxidation and is destroyed by autoclaving in the presence of oxygen.

The insect requirements of vitamins A, D, and E are not thoroughly understood by the present writer. As he will show in the review of literature the majority of the evidence tends to support his belief that insects in general do not require any of these three substances unless they themselves synthesize them from various forerunners. The experimental evidence presented by this worker seems to show any need by mosquito larvae for A, D, and E is so small that measurement of it cannot be made from our present knowledge of vitamins.

Vitamin B, known to the chemist as thiamin or its derivative thiamin chloride hydrochloride, is a water soluble substance present in yeast, wheat germ, rice polishing and other products. In nature its primary source is microorganisms but it is now readily synthesized by the chemist. In the presence of alkali this vitamin is readily destroyed by heat but in very acid medium (pH. 3.5) it resists destruction by heat. For a long time the effect of vitamin B on the nervous system has been known. It rapidly cures polyneuritis in pigeons and rats and is the anti-beriberi factor of man. Thiamin is very definitely associated with the metabolism of carbohydrates. The structures of thiamin and thiamin chloride hydrochloride are given below. The author used pure crystalline thiamin chloride hydrochloride in his experiments.



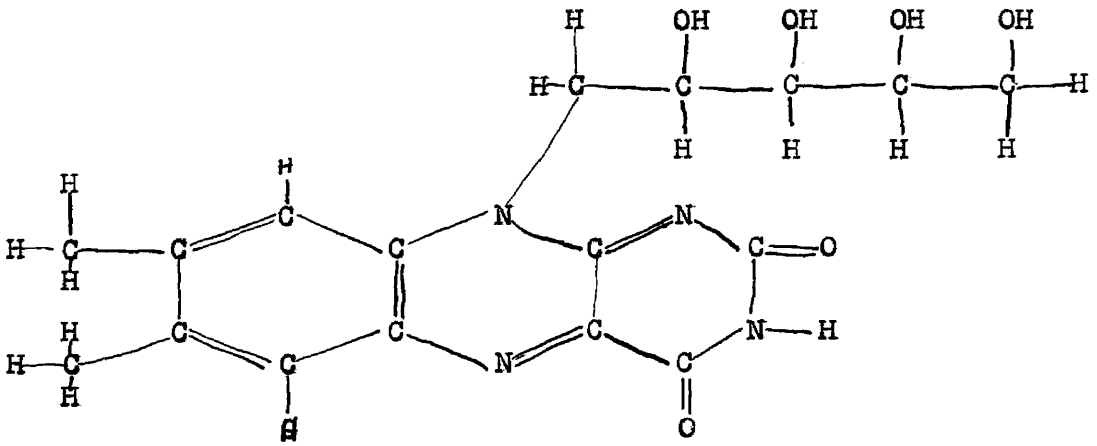
Thiamin Hcl.



Thiamin

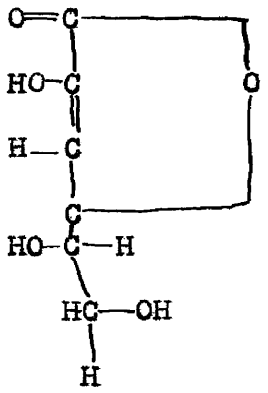
Vitamin $B_2$ or G, known to the biochemist as d. riboflavin, is water soluble. It is chemically related to the yellow-green fluorescent pigment known as the "yellow enzyme". This substance has commanded attention of chemists and biologists from 1879 to the present time. In the presence of blood the yellow pigment turns white but returns to its original color on exposure to oxygen. Riboflavin is relatively

heat-stable but autoclaving for a long time in the presence of oxygen will destroy it. It was first discovered in yeast but is now known to be widely distributed in both the plant and animal worlds. It has been isolated from egg white, milk, liver, kidney, urine, barley malt, dandelion blossoms, grasses, egg yolk, and retinas of fish eyes. It is readily soluble in water, insoluble in the fat solvents, stable in the presence of strong acids and heat but readily destroyed by alkali. Vitamin $B_2$ is supposed to be required by all living cells. This is almost undoubtedly true because of its relation to the oxidation-reduction enzyme and its widespread occurrence in nature. The lack of riboflavin in the diet of rats results in the appearance of pellagra-like skin lesions, hence the name "anti-pellagra"vitamin. Chickens require the vitamin to prevent "chick pellagra". If it is removed from the diet of dogs a condition of blacktongue results. The present worker used pure crystalline riboflavin in his experiments and the influence of it on the life of mosquito larvae will be shown. The formula for d. riboflavin is given below:



d. riboflavin

Vitamin C or l. ascorbic acid (also cevitamic acid) is a water-soluble, highly unstable substance, present in all citrus fruit (lemons, oranges, grapefruit in particular), spinach, turnip greens, pepper, cabbage, and many other green vegetables. Milk is another rich source of ascorbic acid but it is often destroyed in pasteurization. In mammals it is important in calcium metabolism and is associated with the development of bone, teeth, and muscles. Vitamin C prevents scurvy and is known as the antiscorbutic factor of higher vertebrates. The role of ascorbic acid in insect nutrition will be discussed later. This worker used pure crystalline l. ascorbic acid in his experiments. The formula for this very heat-labile vitamin is shown below:

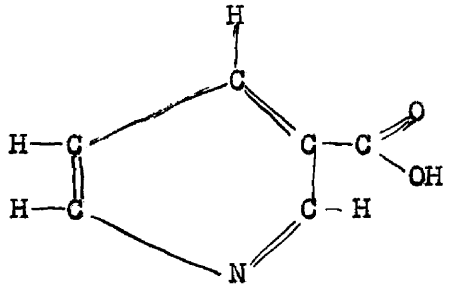


l. ascorbic acid

The vitamins $B_1$ (thiamin) and $B_2$ (riboflavin) have been isolated from the B complex and their structures and properties determined, as has been described in the preceding pages. However, there are still others in the water-soluble B complex whose chemistry and physiology

are not definitely known. The author would like to mention three of those substances. One of them was carefully studied in the experiments on mosquito larvae. Another was obtained in a very small quantity too late for the author to obtain significant results from its use. The third has been synthesized only in the last three months and is not yet available.

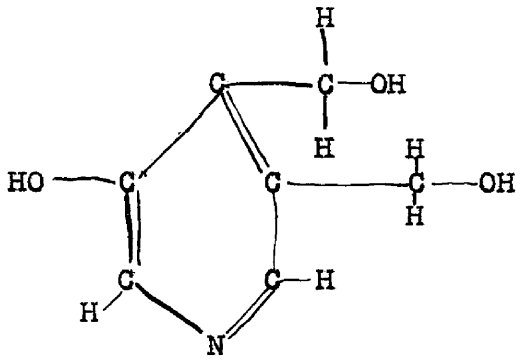
In the past few years it has been claimed by several workers that a pellagra-preventing factor (P.P.) exists which is thought to be nicotinic acid or a derivative, nicotinic acid amide. This substance is thought to be required in addition to riboflavin in the prevention of pellagra. Crystalline nicotinic acid was obtained and its effects on the growth of mosquito larvae determined. The formula for nicotinic acid is shown below.




Nicotinic Acid

The second of these none-too-well-understood B vitamins is $B_6$ or adermin. It is the rat antipellagra or anti-dermatitis factor of Gyorgy (1935). The work of Chick, Copping and Edgar (1930-35) and of Copping (1936) presents further evidence to chemists and physiologists

that this factor does exist. It is very heat and alkali stable but is destroyed by light on long exposure. So little of this chemical was available that the author was unable to complete his study of the effect of $B_6$. The formula for adermin is given below.



Adermin

A third factor of the unknown B vitamins appears to be the factor of Williams (1934038), who calls it "pantothenic acid" and claims it is the bios factor required in the development of yeast. In the past few months Williams and his associates have synthesized this substance. It is present in large quantities in yeast and liver. "Pantothenic acid" according to Williams withstood autoclaving for nineteen hours in acid media but was destroyed by autoclaving in alkaline media. The author has been unable to secure "pantothenic acid" and has not seen its chemical formula.

At some time in the near future some experiments will be performed on the effects of $B_6$ and the Y factor on larval growth of mosquitoes. As the author's results definitely will show, a

third heat stable factor exists in yeast and liver very vital in the growth and development of mosquito larvae.

In the last few weeks of this study in mosquito larvae nutrition the author was able to secure in pure crystalline form the following amino acids; leucine, glycine, histidine, trytophane, aspargine, d. cystine, and l. tryosine. Concentrated solutions of these amino acids were made and combined with 0.1% liver extract, 0.1% glucose, 0.1% Osborne-Mendel salt mixture, plus Vitamins $B_1$, $B_2$, $B_6$, (each 1 mg./500cc.) and sterilized by filtering through Berkefeld W. Time permitted only a study of this diet in the presence of microorganisms. However, the author feels that his results are significant enough to report and will be presented in the chapter devoted to results.

## REVIEW OF LITERATURE

The entomological literature on the subjects of insect digestion, nutrition, metabolism, and growth substances is voluminous. Dr. Wigglesworth in his new textbook, The Principles of Insect Physiology, lists and partially reviews two hundred and sixty-five scientific publications in his chapter on digestion and nutrition and two hundred and three references are found in the chapter devoted to metabolism. It is beyond the ability of this worker to review by any means all of these publications. At the same time, the factors of time and space are also prohibitive. However, many of the research papers directly and indirectly related to this problem will be considered in this review.

Boyce and Lewis (1910) noticed that mosquito larvae in ordinary tap water caused an increase in the numbers of bacteria present.

Loeb, J. (1915), in some of his early work, studied the role of inorganic salts and microorganisms in the development of Drosophila. According to this great scientist, the larvae of the fruit fly require only potassium acid phosphate and magnesium sulphate as the inorganic salt components of their diets. In addition to these, grape sugar, cane sugar, ammonium tartrate, and bacteria were present. These substances mixed with water in chopped up bits of ashless filter paper were used as a diet and normal growth resulted. In another diet, Loeb obtained normal growth with grape sugar and the two inorganic salts with the addition of the amino acids glutamic acid and alanine. These two amino acids were the only nitrogen compounds required, and

either one of them, provided the other was present, could be replaced with ammonium tartrate or succinate. Of course, bacteria and yeast were present in the diets. Sodium chloride and likewise calcium chloride were unable to replace either of the two salts.

Atkin and Bacot (1917) studied the effects of bacteria and yeasts on the development of the larvae of Aedes aegypti. They concluded that the larvae of this mosquito grow only exceptionally on dead foods, and that living bacteria and yeasts are a much more suitable food than dead forms.

Northrup (1917,26) did extensive research on the role of yeast in the nutrition of Drosophila larvae. He found that the total numbers of emerging flies were greater on definite quantities of yeast when banana, casein, or sugar was added. Growth of the larvae was more rapid on a mixture of banana and yeast than on yeast alone, provided that at least thirty-three percent of the diet was yeast. Northrup found that kidney, liver, and pancreas were also adequate sources of food for the larvae. In his later paper this worker reared aseptic Drosophila larvae for two hundred and thirty generations inbred in the dark on heat-killed yeast and banana. Hence, he concludes that the absence of microorganisms has no effect on the duration of life and growth of the fruit fly.

Baumberger (1919) studied the former problem of Loeb on the relation of microorganisms and their substrata and their role in the nutrition of Drosophila larvae. He used the sugars, inorganic salts (potassium acid phosphate and magnesium sulphate), ammonium tartrate, and agar-agar as a rearing medium. He observed that this medium when sterilized by autoclaving supported the growth of larvae from sterile

eggs, but they lived for only five days and showed no increase in size. Baumberger then inoculated the medium with living yeasts and the larvae grew at a normal rate, reaching their maximum size in ten days and pupating normally. In the presence of living yeast, the only source of nitrogen was ammonium tartrate. This worker concludes that sugars, salts and the nucleoprotein of yeast are an adequate food for Drosophila larvae. However, as the present author would like to point out, during the era of Loeb, Northrup (his early work), and Baumberger, most of the research workers thought along the lines of protein requirements and overlooked in many instances the importance of the accessory growth factors or vitamins, many of which are synthesized by the microscopic forms of life.

Bacot and Harden (1922) performed some very simple experiments on the vitamin requirements of Drosophila. They are possibly the earliest workers to consider vitamins and insect nutrition. Using butterfat as a source of vitamin A, yeast as a source of B, and lemon juice for C, they studied the needs of these three factors by Drosophila larvae under sterile conditions. It was reported by them that development of aseptic larvae occurred in the presence of vitamins A and B. The larvae did not require vitamin C in lemon juice for their development under sterile conditions. The basal diet used by these workers, which consisted of caseinogen, starch, and salts, was autoclaved along with the various sources of the vitamins. As will be pointed out later by the present writer, it seems odd that growth could be obtained with the autoclaved yeast since the important factor thiamin ($B_1$) is certainly destroyed by the heat of autoclaving.

Glaser (1924) determined the relation of microorganisms to the development and longevity of flies. Working with Drosophila melanogaster, Musca domestica, Stomoxys calcitrans, and Lyperosia irritans he found that the completion of the larval stage of flies was dependent upon certain accessory growth factors which must be ingested with the food. The neccessary growth substances are obtained according to Dr. Glaser from bacteria and yeasts, as well as from higher plants and animal tissue. He sterilized media at high temperatures and observed that these vital substances were destroyed. On the other hand, contaminating the media with living bacteria or yeasts brought about normal growth when inoculated with sterile eggs. These substances could also be returned to cultural media by adding plant juices or large quantities of dead bacteria or yeast killed at low heat. Glaser then concludes that "microorganisms and their activities are not absolutely essential to normal growth, development, and longevity of the flies investigated." However the microorganic flora "may be one of the principal sources for the accessory growth factors of some larval flies found breeding in certain types of media in a state of nature but cannot be regarded as a proved fact." The present author will later show that all the necessary growth factors (accessory) required by mosquito larvae are present in yeast.

Boyd (1926) reports that yeast serves as an adequate food for the larvae of Anopheles quadrimaculatus and Anopheles crucians.

Wollman (1926) reared the roach Blatella germanica for twelve generations free from microorganisms and free from vitamins.

He also claims that vitamins A and B fed to other individuals had no effect in increasing the growth rate. Seldom does one read in the literature such a remarkable article as this one. None of the American research workers using Blatella as a test animal have come to these conclusions.

Richardson (1926) studied the growth of the Mediterranean flour moth (Ephestia kuehniella Z.) in wheat flour. He found that highly milled wheat flour was not a good rearing medium for the larvae of this moth unless an alcoholic extract of yeast was added. He claims that vitamin B present in yeast, also in the embryo of the wheat kernel, is required for normal development. However, the addition of egg yolk and butter also caused an improved growth rate. Hence, Dr. Richardson believes that vitamin A is likewise needed by this insect. Ether and chloroform extracted this substance from egg yolk and butter.

Barber (1927) reports on the food of Anopheline larvae and in 1928 on the food of Culicine larvae. Autoclaved pond water supported the growth of Anopheline larvae for one month, when they died at the "half-grown" stage. Either autoclaved pond water or broth failed to yield pupae under sterile conditions. Culicine larvae could live for twelve days in autoclaved broth but could not pupate. However, when the broth was contaminated, normal growth was restored in both groups of mosquito larvae. Continuing his experiments with pure cultures of living yeast plus the protozoa, Colpidium or a pure culture of the alga, Scenedesmus, Barber was able to rear to maturity Anopheles crucians, A. quadrimaculatus, Aedes argenteus (aegypti), and Culex fatigans. He definitely shows that microorganisms are important in the normal life of mosquito larvae.

Sweetman and Palmer (1928) investigated the vitamin requirements of the flour beetle, Tribolium confusum, Duval. They used as a basal diet or ration purified casein, dextrin, and salts. Removal of the extract of these materials prolonged growth but the occurrence of pupation showed that Tribolium did not absolutely need a fat-soluble vitamin. However, prolonged extraction with alcohol prevented pupation which demonstrated a need for one or more vitamins of the B complex. Highly purified vitamin B from yeast when added to the alcohol extracted ration resulted in the production of pupae in one-half the period normally required. Hence the need for one or more of the B vitamin is shown. However fat of some nature although free from vitamin A did shorten the larval period but this may have been a food factor.

Cleveland (1928) describes his additional observations and experiments on the symbiosis between termites and their intestinal protozoa. When termites of the genera Reticulitermes, Calcaritermes, Neotermes, and Kalotermes were treated with oxygen at sixty pounds pressure for one hour all of their intestinal protozoa were removed. The termites which were uninjured by this process were then fed on wood, cellulose, cotton, and other products. In no case could they survive and they died of starvation. Spirochaetes were removed by feeding termites 5% aqueous acid fuchsin plus cellulose leaving the protozoa inside the digestive tract. The termites were still able to digest wood and cellulose; hence, spirochaetes play no role in the digestion of wood and cellulose. On the other hand protozoa are indispensable in the life of the termites.

Rudolfs and Lackey (1929) reported some unusual observations of the effect of food upon the phototrophic behavior of mosquito larvae. Working with Culex pipiens, Aedes sylvestris, and Aedes canadensis they observed

that so long as the larvae of these three mosquitoes fed on a mixed diet they were positively phototropic. However, on a diet of pure ciliates the sign of their phototropism was changed within two days from positive to negative. On a diet of dinoflagellates, Chlamydomonas or Euglena their positive sign was greatly intensified and remained so until the pupation period.

MacGregor (1929) reared the larvae of Aedes argenteus (aegypti) to maturity in 10-15 days at 30°C on a sterile diet on one-tenth percent "standard bread" that had been autoclaved for 15-20 minutes at 120°C. This experiment has been repeated by several workers including the present author (unpublished Master's Thesis) and no one has been able to duplicate MacGregor's results. He claims that "bacteria themselves furnish the larvae with some important accessory food factors (vitamins?) from their own bodies or produce this factor by conversion of the materials of the bread". He further points out that possibly in the baking of ordinary bread the yeast vitamins are not destroyed. Likewise the autoclaving from MacGregor's viewpoint may have no effect on the accessory food factor or factors. He argues that "it is questionable whether vitamins can effect the life of some insects at least, since many exhibit a successful existence on such materials as paper, starch paste, spun cotton, and silk, etc. However, in the light of Dr. Cleveland's work on the role of protozoa in the digestive tracts of termites, it does seem that wood and pure cellulose should fall into the category outlined by MacGregor. He fails to consider that bacteria, yeasts, molds, and protozoa are certainly present "on such materials as paper, starch paste, spun cotton, and silk, etc."

Matheson and Hinman (1929) observed that Chara fragilis was toxic to

the larvae of Culex pipiens, Aedes vexans, and Anopheles punctipennis. Chara is a siliceous, unicellular alga, rarely eaten as food by animals. In aquaria containing Chara these workers found that larvae of the above mosquito species were killed in almost 100 percent of their observations. Watery solutions containing one half to one ounce of dried Chara to every twelve and one half pints of water were also lethal. These notations are very interesting in that an algal form of a detrimental nature is reported since most algae are important in mosquito larvae nutrition.

Howland (1930) studied the relation of algal food to the nutrition of mosquito larvae. She dissected the larvae of Aedes argenteus (aegypti) and found many forms of algae present and concluded that they formed an important part of the larval diet. On the other hand she was able to rear them on other diets containing no algae. Thus this worker believes that other organic forms assume the role played by algae in nature. These other forms were undoubtedly bacteria and yeasts since Howland made no attempt to sterilize either media or eggs.

The Russian worker Beklemishev (1930) by means of microdissection technique investigated the food of Anopheline larvae. He reports that the larvae of Anopheles maculipennis utilize particles smaller than *5 mu and develop as far as the fourth instar on colloid fractions of organic substances present in water. These small particles were insufficient to bring about maturity. Larvae of the fourth instar utilized coarser particles and albuminous substances. Beklemishev concludes that colloidal particles are very important in the nutrition of young Anopheline larvae which feed chiefly by filtering. Bacteria were present in the pond water

*1 mu = 1 micron, 0.00025 inch, 0.001 millimeter.
1 m. mu = .001 micron, 0.0000025 inch, 0.0001 millimeter.

and mosquito larvae analyzed by this worker.

Shipitzina (1930,35) likewise studied the nutrition problem of mosquito larvae as affected by organic colloids and used feeding experiments and microdissection technique. She fed first instar larvae of Anopheles maculipennis solutions of Chinese ink (particle size, 1 mu to 0.1 mu in diameter), carmine, and medical preparation of colloidal silver (diameter = 20.m. mu). In her later paper she reports the use of quartz sand of varying degrees of fineness and colloidal gold and haemoglobin. Shipitzina's observations revealed that the filtering apparatus of the larvae was capable of catching colloidal particles, as the concentration of the substances tested was much greater in their guts than in the water. Particles of soluble starch (5 m. mu.) were not filtered from the water by the first instar larvae. When water was passed through filter paper the growth of larvae in it was retarded showing that coarser particles in suspensions are important as food. First instar larvae filtered out sand particles from 22.8 mu. to 34.2 mu. (diameter of particles = 20% head width). Larvae of the fourth instar were able to filter particles of sand as large as 68 mu. to 165.3 mu. (31.2% diameter of head width). Colloidal gold particles (26-31.5 m. mu.) and haemoglobin particles (2-4 m. mu.) were recovered from the guts of all instars.

The work of these two Russians will be discussed later as it might aid in the explanation for the facts that autoclaving and Berkefeld filtering of pond water renders it incapable of supporting growth of mosquito larvae beyond the fourth instar.

Hinman (1930, 32(a), 32(b), 33) did extensive research on the food of mosquito larvae. He studied the roles of microorganisms, solutes, and the utilization of water colloids. From dissections of the larvae of eight

species of Aedes, six species of Culex, Anopheles punctipennis, Theobaldia dyari, Th. inornata, and Wycomyia smithii he found that "different species of mosquito larvae (within the same genera) from the same habitat normally digest about the same material". He further observed that the larvae are not selective in their feeding but sweep a constant stream of water containing organisms and other materials into the intestinal tract. Prominent among the biota were Vorticella, ciliates, Trachelmonas, Euglena, rotifers, many filamentous green algae, unicillular green algae, desmids, diatoms, blue green algae, and crustacea. Hence Hinman is of the opinion that mosquitoes cannot be controlled by the elimination of larval food. He then tried to rear larvae of Aedes aegypti on sterile media. Autoclaved pond water if uncontaminated failed to support the growth of the larvae of this species beyond the third instar. On the other hand many of the contaminated cultures supported growth through pupation and emergence. Similar results were obtained with Culex pipiens as the test animal. As Hinman states "autoclavings of water from breeding pools destroyed certain elements necessary for larvae transformation". Likewise bacterial cells killed chemically failed to support growth under sterile conditions. With Berkefeld W or N filtered pond water he was able to rear to maturity small numbers of Aedes aegypti under aspptic conditions. Repeated experiments with species of Culex or Anopheles resulted in failure to obtain adults in sterile Berkefeld filtered pond water. In some later experiments (1932) Hinman studied dialyzed water that was sterilized by filtration. During dialysis most of the colloids are removed from the medium and the dialyzed pond water failed to support the growth of Culicid larvae. Anopheline larvae lived for eleven days in

Seitz-Werke filtered pond water whereas in pure solution the larvae could survive for only three days. Colloids of small molecules supported growth for six days. The complete absence of solutes caused death although on a solute diet alone the larvae lived for the shortest periods in all of the experiments. The present writer at this time would like to bring out the fact that accessory growth factors are often water soluble and hence solutes. At the same time there is the possibility that Hinman may have removed all of his salts in the dialysis operations.

White (1931) reports that sterile maggots for surgical use can be reared on autoclaved glucose, peptonized agar, and meat.

Hobson (1931, $32_{(a)}$, $32_{(b)}$, $32_{(c)}$, 33, $35_{(a)}$, $35_{(b)}$, $35_{(c)}$) has contributed much to the field of insect nutrition and metabolism in a series of papers on the physiology of blowfly larvae. In an early paper (32) he reported that blowfly (Lucilia) larvae could be reared on sterile brain (guinea pig) mush and that the rate of growth was the same as on infective tissue. Therefore he concluded that microorganisms played no part in intestinal digestion. Hobson also noticed that when sterile unheated muscle was used as a rearing medium for aseptic larvae their growth rate was decreased. When this muscle was infected with Bacillus coli normal growth was restored. The addition of yeast extract to the muscle did the same thing. This worker then concluded that some growth promoting factor was lacking in sterile muscle. He then studied the growth of blowfly larvae on blood which also was deficient in growth substance. Sterile horse blood was absorbed on sterile cotton and allowed to coagulate. Hobson found that for normal growth to occur under asepsis with this blood diet, at least three growth factors must be added: a substance absent from aqueous or alcoholic extracts of yeasts, but present in a soluble form in

yeast autolysate, a heat-labile factor which could be supplied by Peters "antineuritic concentrate", a heat stable factor present in autoclaved yeast extract. In the analysis of the results of experiments on mosquito larvae that are to be presented later, the present writer will demonstrate at least three factors present in yeast similar to those described by Hobson. According to Hobson "tests with autoclaved yeast have shown that blowfly larvae require a heat-labile factor which is probably analogous to members of the vitamin B complex and they may also be identical with vitamins required by higher animals". One of the factors present in yeast is very resistant and stable in the presence of both heat and alkali; the other sensitive to alkali and somewhat labile to long periods of heating. Hobson discovered another factor required by blowfly larvae, a fat soluble substance of which in an earlier paper he states that "the distribution of the blowfly factor shows that it is not vitamin A, D, or E". However he later shows that this fat soluble factor is cholesterol which is chemically related to vitamin D. The extensive work of this English insect physiologist has been a great aid to the present writer in his experiments on the vitamin requirements of mosquito larvae.

Michelbacher, Hoskins, and Herms (1932) investigated the adequacy of sterile synthetic diets in the nutrition of the flesh fly larva, Lucilia sericata. These workers reared blowfly larvae under aseptic conditions on purified casein, brewers yeast powder, Fleishmann's yeast (bakers) butter fat, or dod-liver oil, salt mixture, cystine, and agar. The addition of the amino acid cystine had a remarkable effect in increasing the numbers of pupae. It can be noted that casein is a protein very low in cystine content. Cystine or related sulphur-containing amino acids is a very important substance in the nutrition of higher animals.

Cook and Scott (1933) reported that the termite Zootermopsis augusticollis must have in its diet vitamins A, B, D, and G. They fed this termite on a diet consisting of sucrose, casein, Crisco, salts, and agar gel. Vitamins A and D were given as the non-saponifiable portions of cod liver oil and B and G in rice polishings. The food and containers were changed daily to inhibit the growth of bacteria and molds. It must be remembered that these workers did not completely control the effects of microorganisms (complete sterility) nor did they use chemically pure materials (vitamins).

Hoog, van't (1935,36) studied the effect of vitamins on the aseptic culture of Drosophila melanogaster. He claims that the life history of this species can be completed in the absence of vitamins A, D, and E. However Drosophila larvae need the non-saponifiable fraction of fats in their food. The active principle according to this worker was cholesterol for which the following could be substituted: sitosterol, phytosterol, stigmasterol, or ergosterol; but it could not be replaced in the food by calciferol or lumisterol. A dialysate of yeast extract contains all of the necessary water soluble factors. Irradiation of the dialysate by means of visible light rendered it inactive and the addition of lactoflavin partially restored the activity. Van't Hoog infers that the vitamins $B_1$, $B_2$, and other water soluble factors of yeast are required by Drosophila larvae.

Nelson and Palmer (1935) determined the phosphorus content of the eggs, larvae, pupae, and adults of Tribolium confusum and studied the need for vitamin D. Analysis showed that phosphorus content of the eggs, larvae, pupae, and adults was 0.445%, 0.456%, 0.522%, 0.598% of the dried weight respectively. They also observed that vitamin D was not needed for any physiological function that could be detected by length of time to pupation, also that the phosphorus content of the pupae was in no way effected by

vitamin D.

Street and Palmer (1935) studied the requirements of Tribolium confusum for vitamins of B group. They used a basal diet consisting of 15 parts of casein, 3 parts of Crisco, 4 parts Osborne-mendel salt mixture, and 10 parts of dextrin. A 5 gram portion of the basal diet was mixed with the various vitamin requirement supplements and placed in a vial with twenty two-hour old larvae. All vials were incubated at 32°C and 70% humidity and average time of pupation recorded. In the presence of a vitamin $B_1$ concentrate no pupation resulted. Likewise in the presence of 5% autoclaved yeast no pupation resulted. On the other hand when these workers mixed a vitamin $B_1$ concentrate (2% rice polish) and 1% autoclaved yeast the average pupation time was 192 days. Thus it can be seen that a heat-labile factor ($B_1$) and a heat stable factor are required by the larvae of the confused flour beetle. Street and Palmer also observed that at a pH. of 13 the heat stable factor was destroyed by four hours of autoclaving.

Rozeboom (1935) studied the relation of bacteria and bacterial filtrates to the development of mosquito larvae. He found that the filtering of pond water by means of a Berkefeld filter failed to support the growth of Aedes aegypti, Culex pipiens, C. territans, and C. salinarius under aseptic conditions. When the same filtered pond water was inoculated with a mixed culture of Escherichia coli, Bacillus subtilis, and B. megatherium it was noticed that 144 larvae of A. aegypti yielded 60 fourth instar water larvae and 4 adults. With this same mixture 230 first instar larvae of Culex spp. failed to enter the second instar. On the other hand a contaminated bread mixture produced 90 adults, 102 fourth instar larvae, out of 147 inoculated first instar larvae of Aedes aegypti. Both extra-cellular and intra-cellular extracts of E. coli and B. subtilis were prepared and added by means of the

sterile Berkefeld filter to a medium composed of autoclaved dove feces in water. Growth could not proceed on either of these diets. Hence Rozeboom concludes that the growth factors were not intra or extra-cellular products of bacterial metabolism. Pond water when autoclaved gave negative results but when contaminated produced good growth.

Trager ($1935_a$, $35_b$, 36, 37) and Trager, Miller, and Rhoads (1938) have investigated the growth substances required by the larvae of Aedes aegypti under sterile conditions. Trager in his earlier work demonstrated that the growth of this mosquito to maturity could occur on a sterile medium composed of vitamin-free casein, salts, and extracts from Eli Lilly liver extracts No. 343 and Harris yeast extract. It was concluded by Trager that two accessory growth factors, one in liver, and another in yeast were required by mosquito larvae for normal development. Factor A, in liver, was found to be soluble in water and dilute alcohol, heat stable, but was destroyed by alkali and absorbed by fuller's earth. This factor was later shown to be present in small quantities in yeast. Factor B, found in yeast and yeast extract, also in egg white and wheat, was both heat and alkali stable and was not absorbed by fuller's earth. Later Trager reared mosquito larvae on the yeast extract material without the liver extract. However, the concentration of the yeast had to be greatly increased in order to support growth without the liver extract. When Merck's crystalline vitamin $B_1$ was autoclaved and used to replace the yeast extract the larvae died in the first or second instar. However, when sterilized by filtration through a Seitz filter the larvae could reach the third instar, but none reached the fourth. At the same time relatively large amounts of $B_1$ were required. Trager definitely states that vitamin $B_1$ was not the liver growth factor required by mosquito larvae. The ability of larvae to utilize solutes

was also investigated by this worker. He found that larvae of Aedes aegypti could exist on solutes alone as far as the fourth instar. Calcium ions had to be present in the solute diet, in order for the larvae to reach the fourth instar. Trager, Miller, Rhoads, (38) observed that extracts prepared from the urine of normal individuals or patients with aplastic anemia contained a substance which "enhances the growth of the larvae of the mosquito, Aedes aegypti". This substance according to them, is possibly a flavine or a flavine compound. It is "lacking or is present in smaller quantities or extracts from the urine of pernicious anemia patients showing symptons of the disease". However, when these same patients were given adequate treatment, the urine then contained as much of the substance as normal urine extracts.

Frost, Herms, and Hoskins (1936) have studied the nutritional requirements of the larvae of the mosquito, Theobaldia incidens Thom. These workers found that yeast (Fleischmann's) was an adequate food for the larvae. The most favorable concentration was 2-2.5 milligrams per cc. Brewers yeast in a concentration of 1 milligram per cc. was also satisfactory. Theobaldia incidens was reared to maturity under aseptic conditions, but most of the experiments were made under non sterile conditions by a daily transfer of the larvae to fresh foods. Technical and purified casein alone proved to be inadequate as rearing media but were improved by the addition of certain inorganic salts. The addition of dried yeast to vitamin free casein supported the growth of larvae to maturity. The essential growth factor contributed by yeast according to these workers was thermostable and not fat soluble and was thought to be vitamin G or a member of the G complex. "Proteins are by far the most important class of food stuffs, and carbohydrates and fats are required,if at all, in small amounts", according

to these workers. They also claim that vitamins A, B, C, D, and E did not appear to be needed for the development of this insect.

The technique of Frost, Herms, and Hoskins was adopted for two experiments since it occasions much less loss of time because of contamination and consequent necessity of repeating experiments. However, it is likely that its results are not so accurate as those obtained by the use of sterile media, since microorganisms may in 24 hours produce sufficient vitamins to vitiate the results.

Jacobs and Raichoudhury (1937) reared to maturity Ephestia kuehniella Z. (Mediterranean flour moth) under aseptic conditons. They used wholemeal flour that had been kept at 180°C for two hours and no bacterial growth was observed. Eggs of this moth were immersed in 0.1% mercuric chloride solution for ten minutes and inoculated into the medium. These workers noted that growth was slower and that emergence of the first moths occurred four days later on the sterilized media, than on the same unheated media. It was claimed by them that the slowing of the growth rate was probably due to the destruction of the A and B vitamins.

Rakhmanova (1937) investigated the role of bacteria in the growth of the larvae of Anopheles maculipennis. In suspensions of Bacillus fluorescens the larvae gradually died off in the course of their development. However, in pure cultures of B. coli in sterilized tap water, normal growth of larvae occurred, and adults were obtained in high concentrations of the bacteria. He thought that the medium was not sufficiently nourishing to allow the bacteria to increase in appreciable numbers and to become "toxic". In too low a bacterial concentration, the larvae died of hunger.

Melampy and Maynard (1937) studied the nutrition of the cockroach, Blatella germanica. They found diets of milk and milk products inadequate for the growing roach. Ground wheat and dried skim milk or ground wheat and

meat were very suitable diets. An ether extract of milk which withstood saponification produced a growth stimulating substance. Lard or butter oil could not be substituted for the oil (fat) from wheat. A vitamin of the B complex which was heat and alkali stable was also required.

Crowell and McCay (1937) report that the webbing clothes moth, Tineola bisselliella requires part or all of the vitamin B complex. Fat and the fat soluble vitamins were not needed by this insect. They also report that purified casein was not an adequate protein for optimum development, but when purified lactalbumin was added, normal growth of Tineola resulted. Cystine could not replace lactalbumin in the diet.

Busnel (1937) in an anatomical and physiological study of the larvae of Ephestia kuehniella reports that the larvae could not mature on flour which had its vitamins removed by treatment with X-rays or heat.

McCay (1938) studied the nutritional requirements of Blatella germanica. He states that "the combined evidence from all of these experiments indicated that this species has little if any need for vitamins A and D, but that there is a requirement for some of the factors of yeast". The yeast factors were in part soluble in water and in part in the fat solvents.

Bushnell (1938) reports that the larvae of Tribolium confusum require the addition of yeast to their cornmeal rearing media for a high percentage of emergence. At the same time the developmental period was reduced by thirteen days when yeast was added to the cornmeal diet.

Ludwig and Fox (1938) studied the growth and survival of Japanese beetle larvae in different media. They observed that wheat or yeast, when added to soils containing beetle larvae and plant materials, increased the numbers of surviving beetles, shortened the metamorphic period, and produced

individuals of larger size. The facts were attributed by Ludwig and Fox to the presence of accessory food factors belonging to the vitamin B complex in wheat and yeast.

Glaser (1938) revealed that the common housefly, Musca domestica, could be reared under sterile conditons on a medium composed of autoclaved yeast, liver, water, and sawdust.

Chiu, Shin Foon and McCay (1939) have continued the study of the nutritional requirements of the confused flour beetle, Tribolium confusum Duv. These workers also studied the food requirements of the bean beetle, Acanthoscelides obtectus Say. A high protein diet was needed for the development of Tribolium. A purified diet composed of casein, corn starch, yeast, cotton seed oil, and Osborne-Mendel Salt mixture was sufficient for normal development. Vitamins $B_1$ and G (lactoflavin) alone did not fulfill the requirements for the growth of Tribolium. They confirmed the previous work of McCay, that the fat soluble vitamins A and D were not needed by the confused flour beetle. One or more additional factors present in yeast were essential for the growth of this beetle. Tribolium larvae grew normally when fed dextrose as their only source of carbohydrate.

## METHODS

The role of microorganisms in the nutrition and metabolism of insects has been considered in the portion of this thesis devoted to the review of literature. It can be readily seen that for significant results experiments must be conducted on sterile media inoculated with either sterile eggs or sterile larvae.

Preliminary experiments were conducted by the present writer to determine a successful technique for sterilizing the eggs of mosquitoes. White (1931) and Trager (1935) recommended a solution composed of mercuric chloride 0.25 gram, sodium chloride 6.5 grams, hydrochloric acid 1.25 cc., ethyl alcohol (95%) 250 cc., and distilled water 750 cc. They exposed the eggs for periods of five to ten minutes and transferred them by means of a sterile loop or sterile pipette to the various media. Other workers have used 80% alcohol, 0.1% aqueous mercuric chloride, and 5% formalin. The author adopted the technique described by Hinman (1932) and later used by Rozeboom (1935). Eggs were submerged in a dilute soap solution for five minutes, rinsed in sterile water, and then immersed in a 1/1000 dilution of hexylresorcinol (commercial S.T.37) for a period of two minutes.

All methods have two weaknesses, the difficulty of obtaining sterile cultures and of obtaining a reasonable percentage of hatch. Many tubes had to be discarded because of contamination. The mosquito egg presents a relatively large surface and penetration of the disinfectant through the chorion kills the embryo. The

author considered 60-80% hatch of the treated eggs free from micro-organisms very fortunate. Sterility was checked either by plating a portion of the rearing media on Petri-dishes on standard bacteriological nutrient agar or by making streaks on agar plants.

Another problem in technique was the matter of egg manipulation. The method of MacGregor (1929) of using glass "boats" proved to be the most feasible. Glass cover slips were curled in the flame of a Bunsen burner and eggs were pipetted into these "boats" where they adhered to the inner surfaces. The "boats" were easily manipulated with sterile forceps. The raft-like egg masses of Culex pipiens had to be teased apart and the eggs separated. Eggs of Aedes are laid singly and are more readily handled. In many experiments sterile eggs were inoculated directly into test tubes or Erlenmeyer flasks containing the desired diets. However, if excess of the materials to make up one of the diets was on hand the eggs were transferred to sterile water and after the larvae had hatched they were transferred to the diet. This latter method enhances the possibilities of contamination but has an advantage when a low percentage of hatching occurs.

Another problem encountered in nutritional studies in insect physiology is in the preparation of diets. Microorganisms can be easily destroyed in food stuffs by means of heat (usually boiling or autoclaving), chemicals, or ultraviolet light. Since biochemists have presented data showing that the use of heat, ultraviolet or even visible light destroys several of the vitamins, it was fortunate that the vitamins used by this author are soluble in water and could be sterilized by being passed through a sterile Berkfeld W

filter. The oyster meal protein used in these experiments was vitamin-free material and was autoclaved. Glucose and the Osborne-Mendel salt mixture and dried yeast were autoclaved.

Two rearing cages were maintained in a large incubator (28°C. ± 1°). In one cage Aedes aegypti cultures were maintained throughout the year. Culex pipiens was bred in the other cage. Males of both species were fed on moist raisins and the females on living plucked pigeons. Either species oviposits readily on filter paper floating on large finger bowls of water. Oviposition also occurs on the top of water. Eggs of Aedes aegypti are laid singly whereas eggs of Culex pipiens are laid in rafts of 200-300 or more.

# RESULTS

## Growth of Mosquito Larvae in Pure Culture of Microorganisms.

In these experiments it was thought advisable to alter the basal diet of 0.1% oyster meal, dried yeast, glucose, and Osborne-Mendel salt mixture. At this concentration of oyster meal, dried yeast, and glucose the bacterial growth is too great for the well-being of mosquito larvae. Therefore a diet of 0.05% oyster meal, 0.05% dried yeast, 0.05% glucose, and 0.1% Osborne-Mendel salt mixture was made in distilled water and autoclaved. Portions 20-50 cc. in volume were transferred to sterile test tubes or flasks and inoculated with the desired organisms. Twenty-four hours later, sterile day-old larvae of the mosquitoes were inoculated into the various tubes and flasks.

Table I shows the relation of E. coli to the growth of the larvae, Table II, the relation of B. subtilis, and Table III, the relation of S. cerevisae.

Thus it can be seen that of the three microorganisms, only two of them, Bacillus subtilis, and Saccharomycetes cerevisae (yeast) could support the growth of mosquito larvae in pure culture. The results of the tests with Escherichia coli reveal that some of the fourth instar larvae were able to pupate but none of them emerged. This may be considered unusual in that when an insect species pupates it is generally able to emerge. Many diets will support the growth of mosquito larvae to the fourth instar before death occurs. On inadequate or incomplete diets the pupal stage is almost never reached.

Table I. Survival of Sterile Mosquito Larvae on Autoclaved 0.05% Oyster Meal, 0.05% Dried Yeast, 0.05% Glucose, and 0.1% Osb.-Mendel Salt Mixture. Inoculated with Escherichia coli. p.H. 6.8-7.0 - NaOH - $KH_2PO_4$

Aedes aegypti

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 20 | 14 | 2 | 0 |
| 2 | 20 | 11 | 0 | 0 |
| 3 | 20 | 16 | 3 | 0 |
| 4 | 20 | 12 | 1 | 0 |
| 5 | 20 | 14 | 2 | 0 |
| 6 | 20 | 10 | 0 | 0 |
| 7 | 20 | 11 | 0 | 0 |
| 8 | 20 | 8 | 2 | 0 |
| 9 | 20 | 12 | 4 | 0 |
| 10 | 20 | 15 | 1 | 0 |
| TOTAL | 200 | 123 | 15 | 0 |

Culex pipiens

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 10 | 2 | 0 | 0 |
| 2 | 10 | 4 | 1 | 0 |
| 3 | 10 | 2 | 0 | 0 |
| 4 | 10 | 3 | 0 | 0 |
| 5 | 10 | 5 | 2 | 0 |
| 6 | 10 | 2 | 1 | 0 |
| 7 | 10 | 3 | 0 | 0 |
| 8 | 10 | 1 | 0 | 0 |
| 9 | 10 | 3 | 1 | 0 |
| 10 | 10 | 2 | 1 | 0 |
| TOTAL | 100 | 27 | 6 | 0 |

Table II. Survival of Sterile Mosquito Larvae on Autoclaved 0.05% Oyster Meal, 0.05% Dried Yeast, 0.05% Glucose, and 0.1% Osb.-Mendel Salt Mixture. Inoculated with Bacillus subtilis. p.H. 6.8-7.0

Aedes aegypti

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 20 | 15 | 14 | 14 |
| 2 | 20 | 12 | 12 | 12 |
| 3 | 20 | 16 | 15 | 14 |
| 4 | 20 | 14 | 14 | 12 |
| 5 | 20 | 12 | 12 | 12 |
| 6 | 20 | 13 | 10 | 9 |
| 7 | 20 | 16 | 13 | 13 |
| 8 | 20 | 15 | 12 | 11 |
| 9 | 20 | 12 | 12 | 12 |
| 10 | 20 | 15 | 14 | 14 |
| TOTAL | 200 | 140 | 128 | 123 |

Culex pipiens

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 10 | 7 | 4 | 4 |
| 2 | 10 | 8 | 4 | 3 |
| 3 | 10 | 7 | 3 | 3 |
| 4 | 10 | 5 | 2 | 2 |
| 5 | 10 | 7 | 5 | 4 |
| 6 | 10 | 6 | 2 | 2 |
| 7 | 10 | 5 | 2 | 2 |
| 8 | 10 | 8 | 4 | 3 |
| 9 | 10 | 7 | 3 | 3 |
| 10 | 10 | 8 | 3 | 3 |
| TOTAL | 100 | 68 | 32 | 29 |

Table III. Survival of Sterile Mosquito Larvae on Autoclaved 0.05% Oyster Meal, 0.05% Dried Yeast, 0.05% Glucose, and 0.1% Osb.-Mendel Salt Mixture. Inoculated with *Saccharomycetes cerevisae*. p.H. 6.8-7.0

*Aedes aegypti*

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 20 | 18 | 18 | 16 |
| 2 | 20 | 16 | 15 | 15 |
| 3 | 20 | 16 | 16 | 16 |
| 4 | 20 | 17 | 15 | 14 |
| 5 | 20 | 15 | 14 | 14 |
| 6 | 20 | 18 | 16 | 14 |
| 7 | 20 | 17 | 17 | 16 |
| 8 | 20 | 16 | 15 | 15 |
| 9 | 20 | 14 | 12 | 12 |
| 10 | 20 | 15 | 12 | 12 |
| TOTAL | 200 | 162 | 150 | 144 |

*Culex pipiens*

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 10 | 9 | 9 | 9 |
| 2 | 10 | 8 | 8 | 8 |
| 3 | 10 | 7 | 6 | 6 |
| 4 | 10 | 6 | 6 | 6 |
| 5 | 10 | 8 | 7 | 6 |
| 6 | 10 | 8 | 8 | 8 |
| 7 | 10 | 9 | 8 | 8 |
| 8 | 10 | 6 | 6 | 6 |
| 9 | 10 | 8 | 8 | 7 |
| 10 | 10 | 9 | 8 | 8 |
| TOTAL | 100 | 78 | 74 | 72 |

Of Aedes ageypti, 62.5% of the larvae reached the fourth instar and 7.5% pupated when reared on a pure culture of E. coli; 27% of the larvae of Culex pipiens reached the fourth instar and 6% pupated on this same culture.

When reared on a pure culture of B. subtilis 70% of the larvae of Aedes aegypti reached the fourth instar, 64% pupated, and 62.5% appeared as adults; 68% of the larvae of Culex pipiens reached the fourth instar, 32% were able to pupate, and 29% appeared as adults on a culture of this same bacterial organism.

On a pure culture of yeast 81% of the larvae of Aedes aegypti reached the fourth instar, 75% survived to the pupal stage and 72% appeared as adults; 78% of the larvae of Culex pipiens went into the fourth instar, 75% pupated, and 72% of them emerged as adults.

Growing on pure cultures of the first two microorganisms, A. aegypti seemed to be a more vigorous type than C. pipiens.

Growth of Mosquito Larvae in Autoclaved and Berkefeld Filtered Pond Water

Some of the natural pond water in the vicinity of the campus at College Park, in which numerous mosquito larvae were found, was brought into the laboratory and filtered through cotton to remove all of the debris. Part of the pond water was autoclaved in large test tubes at 15 pounds pressure (120°C.), for thirty minutes. Eggs sterilized by the described hexylresorcinol method were inoculated into the tubes. Results are presented in Table IV.

Some of the same pond water described above was filtered through cotton to remove the debris and filtered through the Berkefeld W to remove the microorganisms. After filtration the sterile water was transferred to test tubes or small Erlenmeyer flasks and sterile eggs were inoculated into them. The results are shown in Table V.

Thus it can be readily seen that the autoclaving of pond water destroys its chemical, physical, or biotic factor or factors necessary for development of mosquito larvae since only 12.9% of the larvae of Aedes aegypti and 7.6% of the larvae of Culex pipiens reached the fourth instar before death occurred. Those larvae reaching the fourth instar were small and very inactive.

The Berkefeld filtration of pond water likewise renders it useless for the development of mosquito larvae. Only 8.3% of the larvae of A. aegypti and 4.7% of the larvae of Culex survived to the fourth instar.

Growth of Mosquito Larvae on Alcohol and Heated Sterilized Yeast

From the results presented in the author's Master thesis (ibid) and the analysis of the results of other workers, it can be seen that yeast plays an important role in the development of mosquito larvae. It was decided to study the relation of dried yeast sterilized by means of 70% ethyl alcohol to the nutrition of mosquito larvae. The alcohol was placed over a relatively large amount of yeast (dried) and allowed to remain for periods of 4-6 weeks. From time to time the mixture was agitated in order that the alcohol could penetrate all parts of the yeast and flask. The alcohol was removed by vacuum distillation. Bacteriological

technique was employed in order to prevent contamination. A heavy suspension of this chemically sterilized yeast was mixed with 0.1% glucose and 0.1% Osborne-Mendel salt mixture and portions of it were transferred to sterile test tubes or flasks. Sterile day-old larvae were added to the tubes and flasks. The results are shown in Table VI.

The effects of autoclaved yeast (0.1%) in addition to autoclaved 0.1% oyster meal, 0.1% Osborne-Mendel salt mixture, and 0.1% glucose were then studied. These substances after autoclaving were transferred to sterile test tubes and small sterile flasks and sterile larvae of Aedes aegypti. These results are presented in Table VII.

Since it was thought that soluble products of bacterial metabolism might contain growth factors required by mosquito larvae, an attempt was made to rear larvae in a sterile solution consisting of autoclaved 0.1% dried yeast, 0.1% oyster meal, 0.1% glucose, and 0.1% Osborne-Mendel salt mixture that was connected to a contaminated solution of these same substances by means of a sterile Seitz filter. However, in every instance bacteria were able to grow through the asbestoes filter and contamination occurred.

It can be easily seen by analysis of Table VI that alcohol sterilized yeast besides being a suitable food for the larvae of mosquitoes, provides all of the accessory food (growth) factors. As the results in Table VI show, 75% of larvae of Aedes aegypti reached the fourth instar, 71% pupated, and 69.5% were able to emerge as adults. In the case of Culex pipiens 74% of the larvae reached the fourth instar, 71% pupated, and 65% emerged.

On the autoclaved yeast diet none of the larvae of either species was able to leave the fourth instar. Of the day old larvae of Aedes aegypti 17.9% reached the fourth instar before death occurred, whereas only 10.6% of the larvae of Culex pipiens reached the fourth instar stage.

Table IV. Survival of Mosquito Larvae from Sterilized Eggs Inoculated into Autoclaved Pond Water. p.H. 6.8-7.0

Aedes aegypti

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 12 | 1 | 0 | 0 |
| 2 | 15 | 8 | 0 | 0 | 0 |
| 3 | 15 | 11 | 0 | 0 | 0 |
| 4 | 15 | 7 | 2 | 0 | 0 |
| 5 | 15 | 7 | 1 | 0 | 0 |
| 6 | 15 | 12 | 3 | 0 | 0 |
| 7 | 15 | 8 | 0 | 0 | 0 |
| 8 | 15 | 9 | 2 | 0 | 0 |
| 9 | 15 | 10 | 3 | 0 | 0 |
| 10 | 15 | 11 | 0 | 0 | 0 |
| TOTAL | 150 | 95 | 12 | 0 | 0 |

*Culex pipiens

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 5 | 2 | 0 | 0 | 0 |
| 2 | 10 | 6 | 0 | 0 | 0 |
| 3 | 30 | 18 | 2 | 0 | 0 |
| 4 | 15 | 9 | 2 | 0 | 0 |
| 5 | 5 | 3 | 0 | 0 | 0 |
| 6 | 5 | 2 | 0 | 0 | 0 |
| 7 | 12 | 10 | 1 | 0 | 0 |
| 8 | 22 | 16 | 0 | 0 | 0 |
| 9 | 14 | 9 | 0 | 0 | 0 |
| 10 | 5 | 3 | 1 | 0 | 0 |
| TOTAL | 123 | 78 | 6 | 0 | 0 |

*Results on Culex pipiens in this table taken from author's Master's Thesis (Non-Buffered Media).

Table V. Survival of Mosquito Larvae from Sterilized Eggs Inoculated into Pond Water Filtered Through Berkefeld W.

*Aedes aegypti*

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 7 | 0 | 0 | 0 |
| 2 | 15 | 8 | 0 | 0 | 0 |
| 3 | 15 | 8 | 1 | 0 | 0 |
| 4 | 15 | 9 | 2 | 0 | 0 |
| 5 | 15 | 7 | 0 | 0 | 0 |
| 6 | 15 | 10 | 1 | 0 | 0 |
| 7 | 15 | 6 | 1 | 0 | 0 |
| 8 | 15 | 11 | 0 | 0 | 0 |
| 9 | 15 | 11 | 2 | 0 | 0 |
| 10 | 15 | 7 | 0 | 0 | 0 |
| TOTAL | 150 | 84 | 7 | 0 | 0 |

**Culex pipiens*

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 6 | 2 | 0 | 0 | 0 |
| 2 | 12 | 8 | 0 | 0 | 0 |
| 3 | 8 | 3 | 0 | 0 | 0 |
| 4 | 13 | 10 | 1 | 0 | 0 |
| 5 | 9 | 6 | 0 | 0 | 0 |
| 6 | 15 | 8 | 1 | 0 | 0 |
| 7 | 12 | 5 | 0 | 0 | 0 |
| TOTAL | 75 | 42 | 2 | 0 | 0 |

*Results on *Culex pipiens* in this table taken from author's Master's Thesis (Non-Buffered Media.)

Table VI. Survival of Sterile Mosquito Larvae on Heavy Suspension of Alcohol Killed Yeast plus Autoclaved 0.1% Glucose and 0.1% Osb-Mendel Salt Mixture. p.H. 6.8-7.0

Aedes aegypti

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 20 | 17 | 17 | 16 |
| 2 | 20 | 10 | 10 | 10 |
| 3 | 20 | 16 | 15 | 15 |
| 4 | 20 | 13 | 12 | 11 |
| 5 | 20 | 17 | 15 | 15 |
| 6 | 20 | 18 | 16 | 16 |
| 7 | 20 | 16 | 16 | 16 |
| 8 | 20 | 12 | 12 | 11 |
| 9 | 20 | 15 | 15 | 15 |
| 10 | 20 | 16 | 14 | 14 |
| TOTAL | 200 | 150 | 142 | 139 |

Culex pipiens

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 10 | 8 | 8 | 6 |
| 2 | 10 | 10 | 9 | 9 |
| 3 | 10 | 7 | 7 | 7 |
| 4 | 10 | 8 | 8 | 8 |
| 5 | 10 | 9 | 9 | 9 |
| 6 | 10 | 7 | 7 | 7 |
| 7 | 10 | 6 | 5 | 5 |
| 8 | 10 | 7 | 6 | 4 |
| 9 | 10 | 6 | 6 | 4 |
| 10 | 10 | 6 | 6 | 6 |
| TOTAL | 100 | 74 | 71 | 65 |

The results of Table VI and Table VII show that at least one or more factors present in the yeast are destroyed by heat (autoclaving).

## Growth of Mosquito Larvae on Sterile Media with the Addition of Vitamins

Since heating destroyed the growth promoting qualities of yeast, the author then studied the effects of adding pure crystalline vitamins. Since the vitamins of yeast (B complex) are water soluble, this could be done under aseptic conditons by means of the sterile Berkefeld filter. Autoclaved vitamin-free oyster meal, glucose, and Osborne-Mendel salt mixture was used as a basal diet. Yeast was added to this basal diet previous to autoclaving in some of the tests and it was omitted in others. Thiamin($B_1$), a vitamin found in yeast, was tested alone in the presence of yeast. Riboflavin ($B_2$), also found in yeast, was likewise tested. A combination of autoclaved yeast, thiamin, and riboflavin was next tried to determine the effects of the three substances on larval growth and development. Table VIII gives the results of growth under aseptic conditons on the basal diet plus thiamin hydrochloride and autoclaved yeast; Table IX plus riboflavin and autoclaved yeast; Table X plus thiamin hydrochloride, riboflavin, and autoclaved yeast; Table XI plus thiamin hydrochloride and riboflavin (no yeast).

Analysis of Tables VIII, IX, X, and XI reveals that three substances are required for the growth and development of the larvae of Aedes aegypti and Culex pipiens. The vitamins thiamin hydrochloride ($B_1$) and riboflavin ($B_2$) are required in addition to at least one heat stable factor present in autoclaved yeast. If any one of the three factors, $B_1$, $B_2$, or autoclaved yeast were removed, growth could not proceed beyond the fourth instar.

Table VII. Survival of Mosquito Larvae from Sterilized Eggs Inoculated into Autoclaved 0.1% Oyster Meal, 0.1% Dr. Yeast, 0.1% Glucose, and 0.1% Osb-Mendel Salt Mixture. p.H. 6.8-7.0

Aedes aegypti

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 12 | 2 | 0 | 0 |
| 2 | 15 | 11 | 0 | 0 | 0 |
| 3 | 15 | 9 | 0 | 0 | 0 |
| 4 | 15 | 9 | 3 | 0 | 0 |
| 5 | 15 | 13 | 6 | 0 | 0 |
| 6 | 15 | 11 | 1 | 0 | 0 |
| 7 | 15 | 15 | 2 | 0 | 0 |
| 8 | 15 | 10 | 1 | 0 | 0 |
| 9 | 15 | 12 | 2 | 0 | 0 |
| 10 | 15 | 15 | 4 | 0 | 0 |
| TOTAL | 150 | 117 | 21 | 0 | 0 |

Culex pipiens

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 10 | 6 | 0 | 0 | 0 |
| 2 | 10 | 7 | 2 | 0 | 0 |
| 3 | 10 | 4 | 0 | 0 | 0 |
| 4 | 10 | 8 | 1 | 0 | 0 |
| 5 | 10 | 5 | 0 | 0 | 0 |
| 6 | 10 | 4 | 0 | 0 | 0 |
| 7 | 10 | 4 | 0 | 0 | 0 |
| 8 | 10 | 6 | 2 | 0 | 0 |
| 9 | 10 | 10 | 2 | 0 | 0 |
| 10 | 10 | 8 | 0 | 0 | 0 |
| 11 | 10 | 8 | 0 | 0 | 0 |
| 12 | 10 | 9 | 3 | 0 | 0 |
| 13 | 10 | 4 | 0 | 0 | 0 |
| 14 | 10 | 9 | 0 | 0 | 0 |
| 15 | 10 | 4 | 0 | 0 | 0 |
| 16 | 10 | 5 | 1 | 0 | 0 |
| 17 | 10 | 10 | 1 | 0 | 0 |
| 18 | 10 | 8 | 0 | 0 | 0 |
| 19 | 10 | 7 | 1 | 0 | 0 |
| 20 | 10 | 6 | 1 | 0 | 0 |
| TOTAL | 200 | 132 | 14 | 0 | 0 |

Table VIII. Survival of Mosquito Larvae from Sterilized Eggs Inoculated into Autoclaved 0.1% Oyster Meal, 0.1% Dr. Yeast, 0.1% Glucose, and 0.1% Osb-Mendel Salt Mixture plus Thiamin HCL (1 mg./ 500cc.) p.H. 6.8-7.0 Thiamin added by Berkefeld W

Aedes aegypti

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 12 | 0 | 0 | 0 |
| 2 | 15 | 9 | 2 | 0 | 0 |
| 3 | 15 | 8 | 0 | 0 | 0 |
| 4 | 15 | 8 | 0 | 0 | 0 |
| 5 | 15 | 10 | 2 | 0 | 0 |
| 6 | 15 | 11 | 1 | 0 | 0 |
| 7 | 15 | 11 | 1 | 0 | 0 |
| 8 | 15 | 7 | 0 | 0 | 0 |
| 9 | 15 | 9 | 3 | 0 | 0 |
| 10 | 15 | 10 | 0 | 0 | 0 |
| 11 | 15 | 13 | 0 | 0 | 0 |
| 12 | 15 | 12 | 1 | 0 | 0 |
| 13 | 15 | 12 | 1 | 0 | 0 |
| 14 | 15 | 10 | 2 | 0 | 0 |
| 15 | 15 | 13 | 0 | 0 | 0 |
| TOTAL | 225 | 155 | 13 | 0 | 0 |

Culex pipiens

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 9 | 0 | 0 | 0 |
| 2 | 15 | 8 | 0 | 0 | 0 |
| 3 | 15 | 12 | 2 | 0 | 0 |
| 4 | 15 | 10 | 1 | 0 | 0 |
| 5 | 15 | 6 | 0 | 0 | 0 |
| 6 | 15 | 9 | 3 | 0 | 0 |
| 7 | 15 | 9 | 2 | 0 | 0 |
| 8 | 15 | 10 | 2 | 0 | 0 |
| 9 | 15 | 7 | 2 | 0 | 0 |
| 10 | 15 | 9 | 3 | 0 | 0 |
| TOTAL | 150 | 89 | 15 | 0 | 0 |

Table IX. Survival of Mosquito Larvae from Sterilized Eggs Inoculated into Autoclaved 0.1% Oyster Meal, 0.1% Dried Yeast, 0.1% Glucose, and 0.1% Osb-Mendel Salt Mixture plus Riboflavin (1 mg./ 500cc.) p.H. 6.8-7.0 Riboflavin added Berkefeld W

Aedes aegypti

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 12 | 2 | 0 | 0 |
| 2 | 15 | 9 | 0 | 0 | 0 |
| 3 | 15 | 12 | 3 | 0 | 0 |
| 4 | 15 | 11 | 1 | 0 | 0 |
| 5 | 15 | 8 | 1 | 0 | 0 |
| 6 | 15 | 8 | 0 | 0 | 0 |
| 7 | 15 | 14 | 0 | 0 | 0 |
| 8 | 15 | 8 | 2 | 0 | 0 |
| 9 | 15 | 15 | 4 | 0 | 0 |
| 10 | 15 | 12 | 3 | 0 | 0 |
| 11 | 15 | 12 | 0 | 0 | 0 |
| 12 | 15 | 8 | 1 | 0 | 0 |
| 13 | 15 | 9 | 2 | 0 | 0 |
| 14 | 15 | 14 | 1 | 0 | 0 |
| 15 | 15 | 10 | 0 | 0 | 0 |
| TOTAL | 225 | 162 | 20 | 0 | 0 |

Culex pipiens

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 12 | 1 | 0 | 0 |
| 2 | 15 | 9 | 2 | 0 | 0 |
| 3 | 15 | 10 | 4 | 0 | 0 |
| 4 | 15 | 8 | 1 | 0 | 0 |
| 5 | 15 | 8 | 0 | 0 | 0 |
| 6 | 15 | 14 | 2 | 0 | 0 |
| 7 | 15 | 12 | 4 | 0 | 0 |
| 8 | 15 | 9 | 2 | 0 | 0 |
| 9 | 15 | 5 | 1 | 0 | 0 |
| 10 | 15 | 8 | 2 | 0 | 0 |
| TOTAL | 150 | 95 | 19 | 0 | 0 |

Table X. Survival of Mosquito Larvae from Sterilized Eggs Inoculated into Autoclaved 0.1% Oyster Meal, 0.1% Dried Yeast, 0.1% Glucose, and 0.1% Osb-Mendel Salt Mixture plus Thiamin HCL and Riboflavin (Both vitamins 1 mg./ 500cc. and added thru Berkefeld W.) p.H. 6.8-7.0

Aedes aegypti

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 12 | 12 | 12 | 11 |
| 2 | 15 | 12 | 12 | 11 | 11 |
| 3 | 15 | 11 | 10 | 10 | 9 |
| 4 | 15 | 9 | 9 | 9 | 9 |
| 5 | 15 | 13 | 12 | 10 | 10 |
| 6 | 15 | 14 | 12 | 12 | 11 |
| 7 | 15 | 14 | 14 | 13 | 13 |
| 8 | 15 | 12 | 10 | 10 | 10 |
| 9 | 15 | 9 | 8 | 7 | 7 |
| 10 | 15 | 13 | 11 | 11 | 10 |
| TOTAL | 150 | 119 | 110 | 105 | 101 |

Culex pipiens

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 12 | 10 | 11 | 11 |
| 2 | 15 | 8 | 8 | 8 | 8 |
| 3 | 15 | 11 | 11 | 11 | 11 |
| 4 | 15 | 9 | 8 | 7 | 7 |
| 5 | 15 | 9 | 7 | 7 | 7 |
| 6 | 15 | 14 | 12 | 12 | 12 |
| 7 | 15 | 6 | 6 | 6 | 6 |
| 8 | 15 | 10 | 8 | 8 | 8 |
| 9 | 15 | 13 | 11 | 10 | 10 |
| 10 | 15 | 8 | 6 | 6 | 6 |
| 11 | 15 | 8 | 8 | 8 | 8 |
| 12 | 15 | 10 | 8 | 8 | 8 |
| 13 | 15 | 12 | 12 | 11 | 11 |
| 14 | 15 | 8 | 7 | 7 | 7 |
| 15 | 15 | 5 | 5 | 5 | 5 |
| TOTAL | 225 | 143 | 127 | 125 | 125 |

In the presence of vitamin $B_1$ and autoclaved yeast 8.3% of the larvae of Aedes aegypti reached the fourth instar and 16.8% of the larvae of Culex pipiens reached this same stage.

In the presence of vitamin $B_2$ and autoclaved yeast, similar results were obtained. Of the Aedes aegypti larvae, only 12.3% of them reached the fourth instar before death occurred. In the case of Culex pipiens 19.8% of the larvae survived to the fourth instar.

It can be seen that when a combination of thiamin hydrochloride ($B_1$), riboflavin ($B_2$), and autoclaved yeast is used as a rearing medium, both species of mosquitoes reached maturity. Aedes aegypti 91.6% of the larvae reached the fourth instar, 88.2% pupated, and 84% appeared as adults on this medium; 88.8% of the larvae of Culex pipiens reached the fourth instar, 87.4% pupated, and 87.4% emerged.

In the presence of thiamin hydrochloride and riboflavin but without the addition of autoclaved yeast growth beyond the fourth instar was impossible. The results show that 6.3% of the A. aegypti larvae and 16.3% of the C. pipiens larvae reached the fourth instar.

It was then decided to remove the autoclaved yeast and add other vitamins to the $B_1$ and $B_2$ diet. Vitamin C (ascorbic acid) and nicotinic acid replaced the yeast.

It can be seen that nicotinic acid and ascorbic acid could not replace the autoclaved yeast in the diet of mosquito larvae as growth beyond the fourth instar was impossible. The results show that 14.7% of the larvae of Aedes aegypti and 23.4% of Culex pipiens reached the fourth instar.

At a very late date a small quantity of adermin ($B_6$) was obtained through the courtesy of Dr. Hugo Neilson of the United States Bureau of Fisheries at College Park. This vitamin was substituted for the autoclaved

yeast and combined with thiamin hydrochloride and riboflavin in a prepared diet. However, because of contamination, caused possibly by a minute hole in the Berkefeld filter, the author was unable to secure significant results. An attempt was made to employ the daily transfer of larvae, technique described by Frost, Herms, and Hoskins (1936) but by that time the supply of vitamin $B_6$ was exhausted and the experiment must be repeated at a later date.

Another source of water soluble vitamins, the exact nature of which is unknown, is liver. The author substituted the 0.1% autoclaved yeast with a 0.1% autoclaved concentrate of liver. Thiamin hydrochloride and riboflavin were added by means of the Berkefeld filter to the liver concentrate (0.1%) which had been autoclaved with the oyster meal, glucose, and Osborne-Mendel salt mixture. The results are presented in Table XIII.

Thus it can be seen that autoclaved liver, as does autoclaved yeast, contains one or more heat stable substances required by mosquito larvae. The results show that 91.2% of the larvae of Aedes aegypti reached the fourth instar, 81.8% pupated and 79.3% emerged as adults; whereas 90.2% of the larvae of Culex pipiens reached the fourth instar, 85.8% pupated, and 81.6% appeared as adults.

In order to study the effects of material in true solution upon the growth and development of mosquito larvae, the amino acids, leucine, glycine, histidine, tryptophane, aspargine, cystine, and tyrosine were obtained and a strong solution of them was made. Thiamin hydrochloride, riboflavin, adermin, 0.1% liver concentrate, glucose, and Osborne-Mendel salt mixture were added to the amino acid solution and filtered through the sterile Berkefeld. A shortage of time forced the author to inoculate non-sterile day old larvae into this diet. The results can be seen in Table XIV.

The results show that no larvae were able to develop beyond the fourth instar on this diet. In the case of Aedes aegypti 71% of the larvae reached the fourth instar, whereas 63.5% of the larvae of Culex pipiens reached this stage.

Table XI. Survival of Mosquito Larvae from Sterilized Eggs Inoculated into Autoclaved 0.1% Oyster Meal, 0.1% Glucose, and 0.1% Osb-Mendel Salt Mixture plus Thiamin HCL and Riboflavin (Both vitamins 1 mg./ 500cc. and added thru Berkefeld W.) p.H. 6.8-7.0

Aedes aegypti

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 6 | 0 | 0 | 0 |
| 2 | 15 | 8 | 0 | 0 | 0 |
| 3 | 15 | 10 | 1 | 0 | 0 |
| 4 | 15 | 5 | 0 | 0 | 0 |
| 5 | 15 | 7 | 2 | 0 | 0 |
| 6 | 15 | 8 | 0 | 0 | 0 |
| 7 | 15 | 8 | 0 | 0 | 0 |
| 8 | 15 | 11 | 1 | 0 | 0 |
| 9 | 15 | 6 | 0 | 0 | 0 |
| 10 | 15 | 10 | 1 | 0 | 0 |
| TOTAL | 150 | 79 | 5 | 0 | 0 |

Culex pipiens

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 12 | 2 | 0 | 0 |
| 2 | 15 | 9 | 0 | 0 | 0 |
| 3 | 15 | 9 | 0 | 0 | 0 |
| 4 | 15 | 8 | 0 | 0 | 0 |
| 5 | 15 | 10 | 1 | 0 | 0 |
| 6 | 15 | 15 | 4 | 0 | 0 |
| 7 | 15 | 8 | 2 | 0 | 0 |
| 8 | 15 | 8 | 3 | 0 | 0 |
| 9 | 15 | 11 | 2 | 0 | 0 |
| 10 | 15 | 8 | 3 | 0 | 0 |
| TOTAL | 150 | 98 | 17 | 0 | 0 |

Table XII. Survival of Mosquito Larvae from Sterilized Eggs Inoculated into Autoclaved 0.1% Oyster Meal, 0.1% Glucose, 0.1% Osb-Mendel Salt Mixture plus Thiamin HCL, Riboflavin, Ascorbic Acid, Nicotinic Acid (All vitamin 1 mg./ 500cc. added thru Berkefeld W). pH 6.8-7.0 - NaOH - $KH_2PO_4$

Aedes aegypti

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 10 | 2 | 0 | 0 |
| 2 | 15 | 12 | 2 | 0 | 0 |
| 3 | 15 | 8 | 0 | 0 | 0 |
| 4 | 15 | 11 | 1 | 0 | 0 |
| 5 | 15 | 10 | 4 | 0 | 0 |
| 6 | 15 | 10 | 2 | 0 | 0 |
| 7 | 15 | 9 | 1 | 0 | 0 |
| 8 | 15 | 12 | 1 | 0 | 0 |
| 9 | 15 | 8 | 0 | 0 | 0 |
| 10 | 15 | 12 | 2 | 0 | 0 |
| TOTAL | 150 | 102 | 15 | 0 | 0 |

Culex pipiens

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 8 | 2 | 0 | 0 |
| 2 | 15 | 10 | 6 | 0 | 0 |
| 3 | 15 | 12 | 4 | 0 | 0 |
| 4 | 15 | 10 | 0 | 0 | 0 |
| 5 | 15 | 9 | 0 | 0 | 0 |
| 6 | 15 | 8 | 3 | 0 | 0 |
| 7 | 15 | 6 | 1 | 0 | 0 |
| 8 | 15 | 8 | 2 | 0 | 0 |
| 9 | 15 | 7 | 1 | 0 | 0 |
| 10 | 15 | 7 | 1 | 0 | 0 |
| TOTAL | 150 | 85 | 20 | 0 | 0 |

Table XIII. Survival of Mosquito Larvae from Sterilized Eggs Inoculated into Autoclaved 0.1% Oyster Meal, 0.1% Liver Conc., and 0.1% Osb-Mendel Salt Mixture plus Thiamin HCL and Riboflavin (Both vitamins 1 mg./ 500cc. and added thru Berkefeld W.) p.H. 6.8-7.0

Aedes aegypti

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 10 | 8 | 8 | 8 |
| 2 | 15 | 12 | 12 | 10 | 10 |
| 3 | 15 | 14 | 12 | 12 | 11 |
| 4 | 15 | 11 | 11 | 9 | 9 |
| 5 | 15 | 11 | 10 | 9 | 8 |
| 6 | 15 | 12 | 12 | 10 | 10 |
| 7 | 15 | 13 | 12 | 12 | 12 |
| 8 | 15 | 10 | 8 | 8 | 8 |
| 9 | 15 | 12 | 11 | 9 | 8 |
| 10 | 15 | 11 | 10 | 8 | 8 |
| TOTAL | 150 | 116 | 106 | 95 | 92 |

Culex pipiens

| Test No. | No. Eggs | No. Larvae Hatch. | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|---|
| 1 | 15 | 12 | 10 | 10 | 9 |
| 2 | 15 | 8 | 7 | 7 | 7 |
| 3 | 15 | 8 | 8 | 8 | 8 |
| 4 | 15 | 9 | 7 | 6 | 6 |
| 5 | 15 | 7 | 6 | 6 | 5 |
| 6 | 15 | 7 | 7 | 7 | 7 |
| 7 | 15 | 12 | 11 | 10 | 9 |
| 8 | 15 | 10 | 9 | 8 | 8 |
| 9 | 15 | 12 | 11 | 11 | 10 |
| 10 | 15 | 7 | 7 | 6 | 6 |
| TOTAL | 150 | 92 | 83 | 79 | 75 |

Table XIV. Survival of Mosquito Larvae (Non-Sterile) Inocubated into a Sterile Berkefeld Filtered Solute Diet, Consisting of: 0.1% Glucose, 0.1% Obs-Mendel Salt Mixture, 0.1% Liver Conc., Thiamin, Riboflavin, Adermin (Each vitamin 1 mg./ 500cc.) and Seven * Amino Acids. pH 6.8-7.0

Aedes aegypti

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | Nol Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 20 | 14 | 0 | 0 |
| 2 | 20 | 15 | 0 | 0 |
| 3 | 20 | 11 | 0 | 0 |
| 4 | 20 | 9 | 0 | 0 |
| 5 | 20 | 10 | 0 | 0 |
| 6 | 20 | 16 | 0 | 0 |
| 7 | 20 | 16 | 0 | 0 |
| 8 | 20 | 18 | 0 | 0 |
| 9 | 20 | 15 | 0 | 0 |
| 10 | 20 | 18 | 0 | 0 |
| TOTAL | 200 | 142 | 0 | 0 |

Culex pipiens

| Test No. | No. Day Old Larvae | No. 4th Instar Larvae | No. Pupae | No. Adults |
|---|---|---|---|---|
| 1 | 20 | 14 | 0 | 0 |
| 2 | 20 | 16 | 0 | 0 |
| 3 | 20 | 9 | 0 | 0 |
| 4 | 20 | 11 | 0 | 0 |
| 5 | 20 | 13 | 0 | 0 |
| 6 | 20 | 8 | 0 | 0 |
| 7 | 20 | 12 | 0 | 0 |
| 8 | 20 | 14 | 0 | 0 |
| 9 | 20 | 15 | 0 | 0 |
| 10 | 20 | 15 | 0 | 0 |
| TOTAL | 200 | 127 | 0 | 0 |

* Concentrated Solution of : Leucine, Glycine, Histidine, Tryptophane, Aspargine, Cystine, and Tyrosine.

# DISCUSSION.

It has been shown by the writer that the larvae of Aedes aegypti and Culex pipiens in the presence of certain types of living bacteria and yeasts grew and developed in the normal manner. Bacillus sublitis and Saccaromycetes cerevisae were very useful organisms in the diets of mosquito larvae. However, if Escherichis coli produced suitable growth substances, they were in quantities too small to be effective. On the other hand, E. coli may have produced substances unfavorable or toxic in the medium. Oddly enough, Hobson (1932) was able to restore the growth of blowfly larvae on sterile muscle by the addition of a culture of this bacterium. Rakhmanova (1937) concluded that pure cultures of the colon bacillus in autoclaved tap water was not "toxic" to the larvae of Anopheles maculipennis. However, he observed that Bacillus fluorescens would not support the growth of Anopheles larvae.

The autoclaving and Berkefeld filtering of pond water destroyed its properties as a rearing medium for the larvae of both species. Shipitzina (1930, 1935) has shown the value of colloids in mosquito larvae nutrition as has Beklimshen (1930). Many of the colloids are removed by both the autoclaving and Berkefeld filtering technique. Shipitzina observed that the small larvae (1st through 3rd instars) utilized very small colloids, but that the fourth instar larvae required the coarser particles in suspensions for food. Her observations are analagous to those of Beklemishev who noted that the larvae of Anopheles maculipennis could develop only as far as the fourth instars on particles 5 mm. or less in diameter. Hinman (1932) found that upon dialysis of pond water Anopheline larvae could live for only three days, whereas in Seitz-Werke filtered pond water larvae of this same

group survived for eleven days. The dialyzed water consisted of substances in true solution, whereas the Seitz-Werke filtrate contained small colloids in addition to solutes.

The present author would like to point out that autoclaving had other important effects on pond water, and on the oyster meal, dried yeast substrate. The primary effect was the destruction of the microorganisms present in these media. Should the growth factors be produced by living organisms, and the author's results just presented have shown this to be true, the removal of the microscopic flora removed the principal source of the accessory food factors. Heat also breaks down many chemicals and the destruction of the heat-labile growth factors by autoclaving is inevitable. However, the explanation of the inactivation of pond water by the Berkefeld filtering treatment is somewhat hard to explain. If gorwth substances are water soluble, hence solutes, they should readily pass through the filter and appear in the filtrate. This may be true, but in the absence of larger colloids, the apparent need for which the author has discussed, growth was likewise retarded. (Trager (1935) claimed that the growth factors required by the larvae of Aedes aegypti were solutes and that the larvae on solutes alone survived till the fourth instar. Rozeboom (1935), as mentioned before, was able to rear the larvae of various mosquitoes on a contaminated medium of bread mixture or one of dove feces. However, when the dove feces were autoclaved and intracellular and extracellular extracts of E. coli and B. subtilis added, growth to maturity was impossible. The work of this latter author confuses the nutritional requirement of mosquito larvae problem along the experimental reasonings of Beklimshev, Shipitzana, Hinman, Trager, and the present author in that he had colloidal materials and the

factors of both types of bacterial metabolism present in his diet. However, it is the opinion of the present worker that amounts of growth substances extracted from bacterial cells by no means compare with the amount and nature of the growth substances produced by living bacteria on a nutrient medium. The use of bacterial filtrates in insect nutritional studies has produced no positive results, whereas a careful examination of the physiological literature reveals numerous cases of negative data. For instance, the work of Hobson noted above on the nutritional requirements of blowfly larvae shows that blowfly larvae required in addition to two aqueous or alcoholic extractable factors present in yeast, a third substance "absent from aqueous or alcoholic extracts of yeasts." It is unfortunate that apparatus has not as yet been perfected wherein mosquito larvae could grow in a sterile medium connected by means of a sterile semi-permeable membrane fo some nature with a contaminated medium of the same composition. If this could be done, then any growth substances produced by the cellular activities of microorganisms could be better investigated. The author's experiments with the Seitz filter failed because bacteria were able to grow through the filter.

A careful consideration must be given the vitamins as they influence the normal growth, metabolism, and development of the larvae of Aedes aegypti and Culex pipiens. The results definitely show that thiamin (thiaminhydrochloride), riboflavin, and a heat stable substance present in yeast or a water concentrate of liver were required by mosquito larvae. Nicotinic acid or ascorbic acid failed to supplement the heat-stable factor of yeast or liver. The present author would like to compare these substances with the factors that Hobson (1932) has shown to be required by Lucilia larvae. Hobson's results illustrate

the presence of three factors in yeast. One was heat-labile factor which could be supplied by Peter's "antineuritic concentrate," a heat-stable factor in autoclaved yeast, and a soluble factor in yeast autolysate. It is felt by the present author that since Peter's "antineuritic concentrate" is a rich source of the heat-labile vitimin $B_1$, this factor probably was thiamin or its derriviative. The same stable yeast factor of blowfly nutrition may have been the same heat-stable yeast factor of mosquito larval nutrition. Riboflavin may possibly have been the substance in Hobson's yeast autolysate.

Gyorgy (1935) investigated the G complex requirement of rats. In addition to lactoflavine (riboflavin) a second antidermatitis factor was needed. This latter factor was heat-stable even in the presence of strong alkali. Chick, Copping, and Edgar (1935) studied the B complex and confirmed the results of Gyorgy that very heat- and alkali-stable factor was present in the complex. This factor has been called vitamin $B_6$. Unfortunately, the present writter cannot in this thesis report the effect of this vitamin on mosquito larvae. Williams (1934, 1938) and co-workers have studied the B vitamins and their effect on the growth of yeast. These workers claim that a filtrate factor or "pantothenic" acid which is heat-stable in the presence of acid is quired for the normal growth of yeast cells. Williams has called this substance the Y factor. Dann and Subbarow (1938) in a study of rat dermatitis factors and chick dermatitis factors conclude that "the vitamin $B_2$ complex contains the following four different sharply characterized entities; riboflavin, nicotinic acid, vitamin

$B_6$, and the filtrate factor." Interesting results can be expected when these substances are studied under controlled conditions in insect nutrition especially in the diets of mosquito larvae.

Trager (1935) has determined two factors required by mosquito larvae, both of which are solutes. However, Trager was unable to rear the larvae beyond the fourth instar. It is unusual that Trager got mosquito larvae to grow under sterile conditions in the presence of autoclaved liver and yeast extracts in that heat destroys vitamin $B_1$ very readily and has a marked effect on riboflavin. Since all three growth factors required by mosquito larvae are present in yeast, it is only natural that Frost, Herms, and Hoskins (1938) could rear the larvae of Theobaldia incidens to maturity on yeast alone. The work of Ludwing and Fox (1938) on Japanese beetle larvae, and the work of Chiu and McCay (1939) with the confused flour beetle tends to further show the value of yeast in providing growth substances that are essential for larval growth.

## SUMMARY.

1. Mosquito larvae can develop on pure culture of some bacteria and cannot develop on others. Escherichia coli fails to support the growth of the larvae of Aedes aegypti and Culex pipiens. Bacillus subtilis and Saccharomycetes cerevisae support the gorwth of larvae to maturity.

2. Autoclaved and Berkefeld W filtered pond water supports the growth of mosquito larvae only as far as the fourth instar under sterile conditions.

3. Mosquito larvae develop to maturity on alcohol sterilized yeast, but cannot develop beyond the fourth instar on autoclaved yeast alone under sterile conditions.

4. Mosquito larvae require for development to maturity at least three factors; thiaminhydrochloride ($B_1$), riboflavin ($B_2$), and a heat-stable factor in yeast under aseptic conditions.

5. Nicotinic acid and ascorbic acid do not replace the heat-stable factor in yeast.

6. The heat-stable factor of yeast is also present in autoclaved liver concentrate.

7. The heat-stable factor has properties similar to those of vitamin $B_6$ and pantothenic acid.

8. Mosquito larvae failed to develop beyond the fourth instar on a solute diet containing amino acids, vitamins, glucose, and Osborne-Mendel salts.

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# THE SURVIVAL OF MOSQUITO LARVAE IN DIFFERENT CONCENRATIONS OF THE HYDROGEN ION.

## INTRODUCTION.

In Part I of this disseration the effects of microorganisms, vitamins, and solutes on the normal growth and development of the larvae of Aedes aegypti and Culex pipiens has been considered. However, before successful experiments on the dietary needs of mosquito larvae could be performed, a knowledge of the optimum pH ranges was greatly needed. As the review of the literature will show, laboratory experimentation on the influence of the hydrogen ion on the larvae of mosquitoes, has never been carefully considered. Hence, the present worker deemed it highly desirable to study this problem before the growth experiments were performed.

Since Part II of this thesis is of a supplementary nature and the author does not think it either necessary or advisable to discuss or explain the physico-chemical phenomena associated with the hydrogen ion and its activity. Briefly stated, electrolytes in aqueous solution are capable of dissociation into their components or ions. This dissociation results in the production of an acid, neutral, or basic (alkaline) condition in water solutions. The measurement of the acidity or alkalinity of a system constitutes what is known as a measurement of the pH (hydrogen ion concentration).

The author has studied the influence of different concentrations of the hydrogen ion on the development of the larvae of the yellow fever mosquito and the common house mosquito. The factors of size of the larvae at a definite age and percentage emergence of the adults have been considered.

## REVIEW OF LITERATURE

MacGregor (1921,29) endeavored to study the influence of the hydrogen ion concentration upon the development of mosquito larvae. In his early work he noticed that an acid condition (pH. 4-4) was lethal to Anopheles maculipennis, a. bifurcatus, and Ochlerotatus nemorosous. On the other hand a pH. range of 8.2-8.4 to 9.6 was favorable. His early paper consisted of a few field observations, but later MacGregor performed some experiments on the effect of pH. under laboratory conditions. He observed that under sterile conditions on his autoclaved "standard bread" diet, which other workers have been unable to duplicate, the larvae of Aedes argenteus (aegypti) developed equally well at hydrogen ion concentrations of 4.0, 6.0, and 8.0. In a 0.5% raisin diet the pH. at first was 4-6.5 but on standing for twenty-four hours it dropped to a 2-3 range; "Nevertheless," according to MacGregor, "the larvae at the end of 24 hours are unaffected and in this medium develop almost as well as they do in the standard bread medium." All of the larvae died on diets whose concentrations of the hydrogen ion were between 8.5 and 9.0, made up with dilute ammonia to increase the alkalinity. But when these same diets wherein larvae had died because of excess alkali were made acid with acetic acid to pH.4.0, inoculated larvae were able to develop and reach maturity. MacGregor unfortunately made the mistake of using unbuffered media. Throughout his paper he constantly explains the sudden changes in hydrogen ion concentration.

Barber and Komp (1922) in their water surveys observed that Anopheles punctipennis, A. quadrimaculatus, and A.crucians were found breeding in waters whose pH. ranged from 5.0 to 8.0.

Morishita (1925) working with Culex fatigaus in Japan noticed that all breeding waters varied in pH. from 6.5 to 7.6. However, between 5.3 and 9.6 under laboratory conditions this mosquito could survive. He concluded the hydrogen ion concentration was of little importance in mosquito development.

Pruthi (1926) investigated the role of pH in the development of the May-fly larva Cloen dipterum L. He observed that in a pH range of 6.0 - 9.5 the larva did not suffer any great harm. Optimum ranges were 7.0 - 9.5. Death was earlier below 6.0 than above 9.5 and at a pH of 3.0 death occurred in 8 hours. Pruthi concludes that "it does not require a long argument to show that hydrogen ion concentration, as such, is also a factor of great importance."

Harold (1926) observed that Aedes maculatus, A. annulipes, A. geniculatus, and A. rusticans were found only in acid waters, but that other members of the same genus, Aedes punctor, A. cinereus along with Theobaldia morsitans were found in waters as alkaline as pH.7.5. Anopheles species were found in waters from 6.0 to 6.8. Culex pipiens was never found in acid waters. Since this worker's observations were made in the field over a small area they contribute but little to the problem. Many other factors, especially hosts for the adult female, govern the breeding places for mosquitoes.

Beklemishev and Mitrofanova (1926) studied the problem of larval distribution of Anopheles maculipennis. They never recorded

the larvae of this mosquito in water whose pH was below 7.0. A pH range from 7.1 - 7.95 always presented the greatest number of larvae.

Senior-White (1926) made an extensive survey of the mosquitoes of Ceylon and studied the physical factors in mosquito populations. This worker observed that natural waters in Ceylon had a pH range from 5.4 to 9.2 but that mosquito larvae in general were found within a pH range of 5.8 to 8.6. He also noticed that mosquitoes living in moving waters had wider tolerances than those in standing waters. Anophelines had wider tolerances than Culicines.

Russel (1927) studied the hydrogen ion concentration of breeding waters in Macedonia. She found that the Anophelines of this area preferred a range of pH 8.0 to 9.5. However the Culicines were not so selective as they were found in a pH range of 6.5 to 10.0.

Rudolfs and Lackey (1929) reported that the concentration of the hydrogen ion had little effect on the development of Aedes canadensis as far as the larvae themselves were concerned, but that it probably determined to a considerable extent the abundance of diatoms, protozoa, fungi, and other food forms.

Sinorodinzen and Adowa (1930) reported that they found Anopheles maculipennis breeding in waters whose pH ranged between 5.0 and 10.0. However a pH of 7.5 was considered to be optimum.

Buchmann (1931) studied the role played by hydrogen ion concentration in the life of Culex pipiens and species of Aedes. According to this worker the larvae of C. pipiens were indifferent to pH values from 4.4 to 8.5-9.0. The larvae of Aedes sticticius A. vexans and A. caspius developed only in water between 6.5 and8.0.

Varashi (1931) noticed that the larvae of Anopheles maculipennis were found in waters whose pH varied between 5.8 and 7.7.

Bradley (1932) has studied the breeding of Anopheline mosquitoes under field conditions. In waters whose pH varied between 6.8 and 9.2 Anopheline larvae were found. However at 6.8 and above 8.3 only very small numbers of larvae occurred. Between a pH of 7.0 and 7.8 the greatest number of larvae were observed.

Beattie (1932) working in Trinidad studied the breeding of Anopheles tarsimaculatus in the field. Her observations revealed that the larvae could be found between a pH of 5.8 and one of 7.5. She states that no definite correlation existed between the hydrogen ion concentration and numbers of mosquito larvae.

Woodhill (1938) reported that variations in the pH from 4.2 to 6.8 up to 9.0 slightly retarded the development of the larvae to the adult stage but had no effect on the total numbers of the adults produced.

## METHODS

In Part II of this report no attempt was made to rear the larvae under sterile conditions since optimum growth occurs in the presence of microorganisms. All media was buffered and checked at the end of 24 hours in case any changes in pH had occurred. Occasionally the pH of a buffered media would suddenly change and the jar had to be discarded. At every pH considered 8 tests were perfored with 25-day old larvae per test. At all concentrations of the hydrogen ion from 5-8 (including thisone) up to 9.0, the media were buffered with NaOH and $KH_2PO_4$. Below 5.8 the author used NaOH and KH phthalate to buffer the media.

Hydrogen ion determinations were made with a slide comparator and series of dyes manufactured by W. A. Taylor and Co. This piece of equipment was loaned by Dr. W. C. Supplee of the Department of Chemistry here at the University. The accuracy of this instrument was checked against a Beckman electrometric pH meter, using buffers of a known hydrogen ion concentration.

All larvae were reared on a 0.1% oyster meal, 0.1% dried yeast, 0.1% glucose, and o.1% Osborne-Mendel salt mixture diet in the presence of microorganisms. However, the author would like to point out that at the hydrogen ion concentrations of 4.0, 5.0, 5.2, 5.4, and 5.6 the phthalate ion was present (in buffer). Although it was given no experimental consideration, the author feels that the phthalate ion was not a toxic factor.

Analyses of results were made on the percentage of adult emergence and size of the larvae at the end of 5 days.

## RESULTS.

The relation of pH to the development of Aedes aegypti are presented in the general table, Table XV, and the composite table, Table XVII; whereas the results for Culex pipiens are shown in the general table, Table XVI, and the composite table, Table XVIII.

At a pH of 4.0 it can be seen that all of the larvae of both species could not survive for a 24 hour period; at 5.0 all larvae were dead at the end of 48 hours; and at 5.2 the larvae could survive for 72 hours before death occurred.

The larvae of Aedes aegypti were able to develop at a pH of 5.4 and 24% of them emerged as adults. On the other hand, the larvae of Culex pipiens lived for 120 hours (5 days) and death occurred.

Between the pH range of 5.6-6.0 larvae of both species responded similarly as shown by the tables. In fact, at a pH of 6.0 both Aedes and Culex yielded 60% emergence.

Concentrations of the hydrogen ion between a pH range of 6.2-7.4 were very favorable for the growth and development of the larvae of both A. aegypti and C. pipiens.

However, at a pH of 7.4 the larvae of Culex pipiens did not survive in as great a number as did the larvae of Aedes aegypti. At this hydrogen ion concentration only 64% emergence was observed for Culex pipiens, whereas 80% emergence was noted for Aedes aegypti.

At a pH range of 7.6 the difference in the emergence percentage between the two species is likewise very wide. Only 4% of the larvae of Culex pipiens emerged. On the other hand, 24% of the larvae of Aedes aegypti emerged. In both cases the fall in the emergence percentage compared to a pH of 7.4 is tremendous.

The larvae of both species could survive for 72 hours at a pH of 7.8; at 8.0 death occurred in 48 hours; at 9.0 in 24 hours.

The larvae of both Aedes aegypti and Culex pipiens at the end of 5 days were very small at pH ranges 5.4-5.8. However, on the higher hydrogen ion concentration side, that is a pH range of 7.4-7.6, no difference could be seen when compared with a 5 day old larvae grown at a pH range of 6.4-7.2.

Table XV. Effect of pH. on the Development of Aedes aegypti.

pH. 4.0 - NaOH - KHphthalate
8 tests with 25 larvae in test
All larvae dead at end of 24 hours.

pH. 5.0 - NaOH - KHphthalate
8 tests with 25 larvae in test
All larvae dead at end of 48 hours.

pH. 5.2 - NaOH - KHphthalate
8 tests with 25 larvae in test
All larvae dead at the end of 72 hours.

pH. 5.4 - NaOH - KHphthalate

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 2 |
| 2 | 25 | 5 |
| 3 | 25 | 7 |
| 4 | 25 | 6 |
| 5 | 25 | 8 |
| 6 | 25 | 7 |
| 7 | 25 | 8 |
| 8 | 25 | 5 |
| 9 | 25 | 4 |
| 10 | 25 | 8 |
| TOTAL | 250 | 60 |
| AVER. | 25 | 6 |

Table XV. - Continued.

pH. 5.6 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 8 |
| 2 | 25 | 12 |
| 3 | 25 | 5 |
| 4 | 25 | 7 |
| 5 | 25 | 3 |
| 6 | 25 | 9 |
| 7 | 25 | 9 |
| 8 | 25 | 7 |
| 9 | 25 | 11 |
| 10 | 25 | 9 |
| TOTAL | 250 | 80 |
| AVER. | 25 | 8 |

pH. 5.8 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 15 |
| 2 | 25 | 9 |
| 3 | 25 | 12 |
| 4 | 25 | 12 |
| 5 | 25 | 8 |
| 6 | 25 | 17 |
| 7 | 25 | 12 |
| 8 | 25 | 10 |
| 9 | 25 | 14 |
| 10 | 25 | 11 |
| TOTAL | 250 | 120 |
| AVER. | 25 | 12 |

pH. 6.0 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 17 |
| 2 | 25 | 13 |
| 3 | 25 | 15 |
| 4 | 25 | 12 |
| 5 | 25 | 10 |
| 6 | 25 | 18 |
| 7 | 25 | 20 |
| 8 | 25 | 17 |
| 9 | 25 | 15 |
| 10 | 25 | 13 |
| TOTAL | 250 | 150 |
| AVER. | 25 | 15 |

pH. 6.2 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 22 |
| 2 | 25 | 19 |
| 3 | 25 | 24 |
| 4 | 25 | 20 |
| 5 | 25 | 17 |
| 6 | 25 | 20 |
| 7 | 25 | 24 |
| 8 | 25 | 22 |
| 9 | 25 | 16 |
| 10 | 25 | 17 |
| TOTAL | 250 | 201 |
| AVER. | 25 | 20 |

Table XV. - Continued.

pH. 6.4 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 24 |
| 2 | 25 | 21 |
| 3 | 25 | 25 |
| 4 | 25 | 25 |
| 5 | 25 | 18 |
| 6 | 25 | 19 |
| 7 | 25 | 24 |
| 8 | 25 | 23 |
| 9 | 25 | 18 |
| 10 | 25 | 23 |
| TOTAL | 250 | 220 |
| AVER. | 25 | 22 |

pH. 6.6 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 25 |
| 2 | 25 | 22 |
| 3 | 25 | 23 |
| 4 | 25 | 25 |
| 5 | 25 | 25 |
| 6 | 25 | 24 |
| 7 | 25 | 22 |
| 8 | 25 | 24 |
| 9 | 25 | 25 |
| 10 | 25 | 25 |
| TOTAL | 250 | 240 |
| AVER. | 25 | 24 |

pH. 6.8 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 21 |
| 2 | 25 | 25 |
| 3 | 25 | 25 |
| 4 | 25 | 25 |
| 5 | 25 | 24 |
| 6 | 25 | 23 |
| 7 | 25 | 25 |
| 8 | 25 | 24 |
| 9 | 25 | 25 |
| 10 | 25 | 23 |
| TOTAL | 250 | 240 |
| AVER. | 25 | 24 |

pH. 7.0 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 25 |
| 2 | 25 | 24 |
| 3 | 25 | 24 |
| 4 | 25 | 24 |
| 5 | 25 | 22 |
| 6 | 25 | 24 |
| 7 | 25 | 23 |
| 8 | 25 | 25 |
| 9 | 25 | 24 |
| 10 | 25 | 25 |
| TOTAL | 250 | 240 |
| AVER. | 25 | 24 |

Table XV. - Continued.

pH. 7.2 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 18 |
| 2 | 25 | 23 |
| 3 | 25 | 19 |
| 4 | 25 | 24 |
| 5 | 25 | 20 |
| 6 | 25 | 24 |
| 7 | 25 | 23 |
| 8 | 25 | 20 |
| 9 | 25 | 24 |
| 10 | 25 | 25 |
| TOTAL | 250 | 220 |
| AVER. | 25 | 22 |

pH. 7.4 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 17 |
| 2 | 25 | 18 |
| 3 | 25 | 22 |
| 4 | 25 | 20 |
| 5 | 25 | 21 |
| 6 | 25 | 24 |
| 7 | 25 | 22 |
| 8 | 25 | 18 |
| 9 | 25 | 20 |
| 10 | 25 | 18 |
| TOTAL | 250 | 200 |
| AVER. | 25 | 20 |

pH. 7.6 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 4 |
| 2 | 25 | 7 |
| 3 | 25 | 7 |
| 4 | 25 | 8 |
| 5 | 25 | 3 |
| 6 | 25 | 5 |
| 7 | 25 | 8 |
| 8 | 25 | 6 |
| 9 | 25 | 5 |
| 10 | 25 | 7 |
| TOTAL | 250 | 60 |
| AVER. | 25 | 6 |

pH. 7.8 NaOH - $KH_2PO_4$
8 tests with 25 larvae in test
All larvae dead at end of 72 hours.

pH. 8.0 NaOH - $KH_2PO_4$
8 tests with 25 larvae in test
All larvae dead at end of 48 hours.

pH. 9.0 NaOH - $KH_2PO_4$
8 tests with 25 larvae in test
All larvae dead at end of 24 hours.

Table XVI. Effect of pH. on the Development of Culex pipiens.

pH. 4.0 - NaOH - KHphthalate
8 tests with 25 larvae in test
All larvae dead at end of 24 hours.

pH. 5.0 - NaOH - KHphthalate
8 tests with 25 larvae in test
All larvae dead at end of 48 hours.

pH. 5.2 - NaOH - KHphthalate
8 tests with 25 larvae in test
All larvae dead at end of 72 hours.

pH. 5.4 - NaOH - KHphthalate
8 tests with 25 larvae in test
All larvae dead at end of 120 hours.

pH. 5.6 NaOH - KHphthalate

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 6 |
| 2 | 25 | 3 |
| 3 | 25 | 5 |
| 4 | 25 | 2 |
| 5 | 25 | 7 |
| 6 | 25 | 10 |
| 7 | 25 | 8 |
| 8 | 25 | 9 |
| 9 | 25 | 7 |
| 10 | 25 | 6 |
| TOTAL | 250 | 63 |
| AVER. | 25 | 7 |

pH. 5.8 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 15 |
| 2 | 25 | 11 |
| 3 | 25 | 13 |
| 4 | 25 | 9 |
| 5 | 25 | 8 |
| 6 | 25 | 8 |
| 7 | 25 | 14 |
| 8 | 25 | 11 |
| 9 | 25 | 12 |
| 10 | 25 | 9 |
| TOTAL | 250 | 110 |
| AVER. | 25 | 12 |

pH. 6.0 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 17 |
| 2 | 25 | 11 |
| 3 | 25 | 12 |
| 4 | 25 | 18 |
| 5 | 25 | 22 |
| 6 | 25 | 18 |
| 7 | 25 | 16 |
| 8 | 25 | 13 |
| 9 | 25 | 16 |
| 10 | 25 | 17 |
| TOTAL | 250 | 160 |
| AVER. | 25 | 15 |

pH. 6.2 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 14 |
| 2 | 25 | 17 |
| 3 | 25 | 21 |
| 4 | 25 | 13 |
| 5 | 25 | 11 |
| 6 | 25 | 16 |
| 7 | 25 | 16 |
| 8 | 25 | 18 |
| 9 | 25 | 10 |
| 10 | 25 | 15 |
| TOTAL | 250 | 151 |
| AVER. | 25 | 15 |

Table XVI. - Continued.

pH. 6.4 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 17 |
| 2 | 25 | 22 |
| 3 | 25 | 23 |
| 4 | 25 | 17 |
| 5 | 25 | 18 |
| 6 | 25 | 15 |
| 7 | 25 | 15 |
| 8 | 25 | 21 |
| 9 | 25 | 17 |
| 10 | 25 | 15 |
| TOTAL | 250 | 180 |
| AVER. | 25 | 18 |

pH. 6.6 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 24 |
| 2 | 25 | 18 |
| 3 | 25 | 25 |
| 4 | 25 | 21 |
| 5 | 25 | 17 |
| 6 | 25 | 23 |
| 7 | 25 | 25 |
| 8 | 25 | 20 |
| 9 | 25 | 24 |
| 10 | 25 | 23 |
| TOTAL | 250 | 220 |
| AVER. | 25 | 22 |

pH. 6.8 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 25 |
| 2 | 25 | 22 |
| 3 | 25 | 25 |
| 4 | 25 | 22 |
| 5 | 25 | 25 |
| 6 | 25 | 24 |
| 7 | 25 | 25 |
| 8 | 25 | 22 |
| 9 | 25 | 25 |
| 10 | 25 | 25 |
| TOTAL | 250 | 240 |
| AVER. | 25 | 24 |

pH. 7.0 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 18 |
| 2 | 25 | 23 |
| 3 | 25 | 21 |
| 4 | 25 | 17 |
| 5 | 25 | 15 |
| 6 | 25 | 22 |
| 7 | 25 | 22 |
| 8 | 25 | 23 |
| 9 | 25 | 18 |
| 10 | 25 | 21 |
| TOTAL | 250 | 200 |
| AVER. | 25 | 20 |

Table XVI. - Continued.

pH. 7.2 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 16 |
| 2 | 25 | 21 |
| 3 | 25 | 14 |
| 4 | 25 | 12 |
| 5 | 25 | 18 |
| 6 | 25 | 22 |
| 7 | 25 | 19 |
| 8 | 25 | 23 |
| 9 | 25 | 16 |
| 10 | 25 | 19 |
| TOTAL | 250 | 180 |
| AVER. | 25 | 18 |

pH. 7.4 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 12 |
| 2 | 25 | 17 |
| 3 | 25 | 19 |
| 4 | 25 | 11 |
| 5 | 25 | 9 |
| 6 | 25 | 20 |
| 7 | 25 | 19 |
| 8 | 25 | 21 |
| 9 | 25 | 16 |
| 10 | 25 | 16 |
| TOTAL | 250 | 160 |
| AVER. | 25 | 16 |

pH. 7.6 NaOH - $KH_2PO_4$

| Test No. | No. Larvae | No. Adults |
|---|---|---|
| 1 | 25 | 0 |
| 2 | 25 | 0 |
| 3 | 25 | 1 |
| 4 | 25 | 2 |
| 5 | 25 | 0 |
| 6 | 25 | 1 |
| 7 | 25 | 1 |
| 8 | 25 | 2 |
| 9 | 25 | 3 |
| 10 | 25 | 0 |
| TOTAL | 250 | 10 |
| AVER. | 25 | 1 |

pH. 7.8 NaOH - $KH_2PO_4$
8 tests with 25 larvae in test
All larvae dead at end of 72 hours.

pH. 8.0 NaOH - $KH_2PO_4$
8 tests with 25 larvae in test
All larvae dead at end of 48 hours.

pH. 9.0 NaOH - $KH_2PO_4$
8 tests with 25 larvae in test
All larvae dead at end of 24 hours.

Table XVII. Composite Table Showing Effect of pH. on Development of the Larvae of *Aedes aegypti*. Average of 8 tests.

| pH. | No. Larvae | End 24 Hrs. | End 48 Hrs. | End 72 Hrs. | No. Adults | % Adults |
|---|---|---|---|---|---|---|
| 4.0 | 25 | All dead | | | | |
| 5.0 | 25 | Living | All dead | | | |
| 5.2 | 25 | " | Living | All dead | | |
| 5.4 | 25 | " | " | Living | 6 | 24 |
| 5.6 | 25 | " | " | " | 8 | 32 |
| 5.8 | 25 | " | " | " | 12 | 48 |
| 6.0 | 25 | " | " | " | 15 | 60 |
| 6.2 | 25 | " | " | " | 20 | 80 |
| 6.4 | 25 | " | " | " | 22 | 88 |
| 6.6 | 25 | " | " | " | 24 | 96 |
| 6.8 | 25 | " | " | " | 24 | 96 |
| 7.0 | 25 | " | " | " | 24 | 96 |
| 7.2 | 25 | " | " | " | 22 | 88 |
| 7.4 | 25 | " | " | " | 20 | 80 |
| 7.6 | 25 | " | " | " | 6 | 24 |
| 7.8 | 25 | " | " | All dead | | |
| 8.0 | 25 | " | All dead | | | |
| 9.0 | 25 | All dead | | | | |

Table XVIII. Composite Table Showing Effect of pH. on Development of the Larvae of *Culex pipiens*. Average of 8 tests.

| pH. | No. Larvae | End 24 Hrs. | End 48 Hrs. | End 72 Hrs. | No. Adults | % Adults |
|---|---|---|---|---|---|---|
| 4.0 | 25 | All dead | | | | |
| 5.0 | 25 | Living | All dead | | | |
| 5.2 | 25 | " | Living | All dead | | |
| 5.4 | 25 | " | " | Living | Larvae dead end 5 days | |
| 5.6 | 25 | " | " | " | 7 | 28 |
| 5.8 | 25 | " | " | " | 12 | 48 |
| 6.0 | 25 | " | " | " | 15 | 60 |
| 6.2 | 25 | " | " | " | 15 | 60 |
| 6.4 | 25 | " | " | " | 18 | 76 |
| 6.6 | 25 | " | " | " | 22 | 88 |
| 6.8 | 25 | " | " | " | 24 | 96 |
| 7.0 | 25 | " | " | " | 20 | 80 |
| 7.2 | 25 | " | " | " | 18 | 76 |
| 7.4 | 25 | " | " | " | 16 | 64 |
| 7.6 | 25 | " | " | " | 1 | 4 |
| 7.8 | 25 | " | " | All dead | | |
| 8.0 | 25 | " | All dead | | | |
| 9.0 | 25 | All dead | | | | |

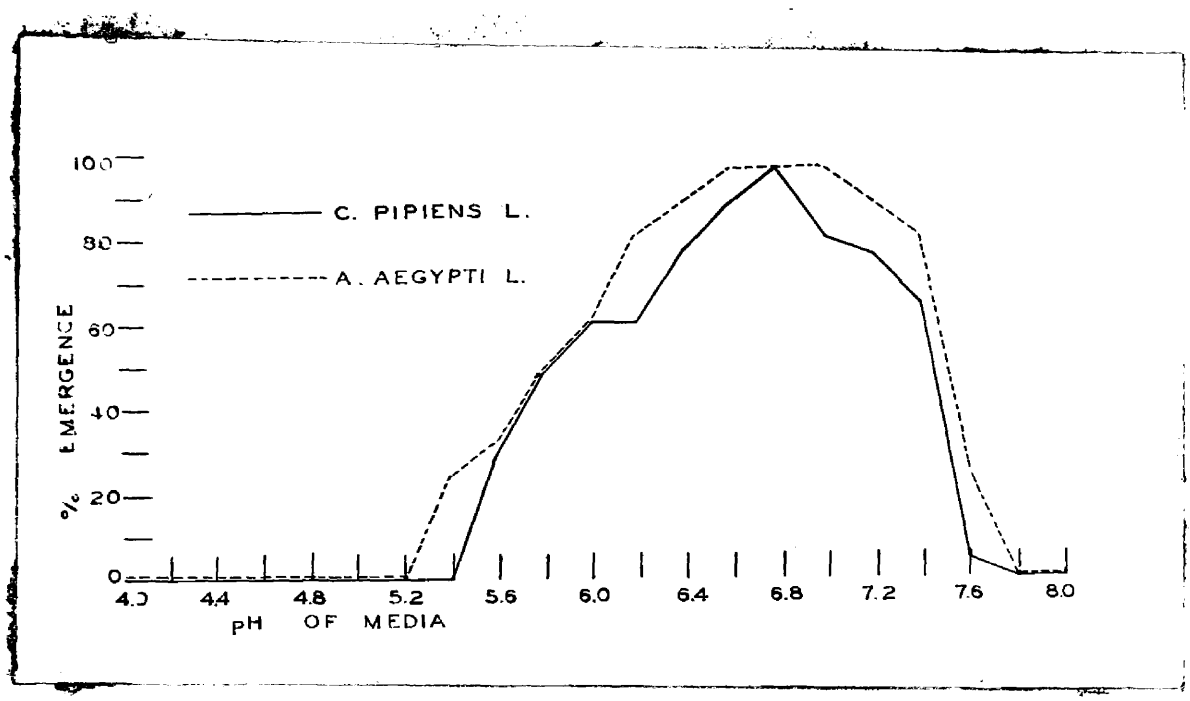


Fig. 1. Effect of pH. on the Development of Aedes aegypti and Culex pipiens

## DISCUSSION

The author has shown that the larvae of Aedes aegypti survive in media whose hydrogen ion concentration varied between a pH. range of 5.4 - 7.6 whereas the larvae of Culex pipiens survived a range of pH. between 5.6 - 7.6. MacGregor (1929) working with Aedes aegypti claimed that the larvae developed equally as well at 4.0, 6.0, and 8.0. However when the experimental technique employed by MacGregor is examined it can be seen that he failed to use buffered media of any type. Under these conditions the present worker cannot feel that this worker (MacGregor) has drawn scientific conclusions. In the present worker's own work he has observed that the oyster meal and dried yeast medium (along with glucose and Osborne Mendel salts) when left unbuffered changed from day to day. This was probably due to action of bacterial metabolism and metabolic activity of the larvae themselves.

The observations of Morishitia (1925) revealed that Culex fatigans larvae was found only in breeding waters whose pH. varied from 6.5 to 7.6 By comparing these observations with the results present in this report close agreement can be seen.

Harold (1926) claimed that Culex pipiens larvae were never found in acid waters. However, the present worker would like to point out that Harold's observations were made over a very small area under field conditions. Other factors often govern the breeding places for mosquitoes and it is inconceivable that adult females can select breeding waters whose hydrogen ion concentration is favorable to larval development.

Barber and Komp (1922) on the other hand did conduct extensive surveys on the breeding places of Anopheline larvae and reported that between a pH. range of 5.0 - 8.0 larvae did occur. These results do closely

approximate the results obtained by the present worker breeding Aedes aegypti and Culex pipiens in the laboratory.

The observations of Senior-White (1926) were in agreement to a certain extent with those presented by the present worker. This worker observed that mosquito larvae in Ceylon were found within a pH. range of 5.8 to 8.6.

It is unfortunate that only a few laboratory experiments have been conducted along these lines of research.

## SUMMARY

1. The larvae of Aedes aegypti survive in media whose hydrogen ion concentration varies between a pH. range of 5.4 - 7.6; whereas the larvae of Culex pipiens survive in a pH. range of 5.6 - 7.6

2. The optimum ranges for both species lie between a pH. range of 6.2 - 7.4.

3. The larvae of Aedes aegypti are able to develop in wider ranges of hydrogen ion concentrations than the larvae of Culex pipiens.

4. Mosquito larvae are smaller when reared in media whose pH. range is between 5.4 - 5.8 than in those closer to neutrality.

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