Studies on a growth factor in lettuce, Lactuca sativa var. romana Hort., required by the grasshoppers Melanoplus bilituratus (Wlk.) and M. bivittatus (Say)
by Joseph Bernard Kreasky

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Entomology at Montana State College
Montana State University

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The nutritional needs of the two species of grasshoppers used in this study apparently differ from those of their relative, the desert locust, Schistocerca gregaria.

STUDIES ON A GROWTH FACTOR IN LETTUCE, LACTUCA SATIVA VAR. ROMANA HORT., REQUIRED BY THE GRASSHOPPERS MELANOPLUS BILITURATUS (WLK.) AND M. BIVITTATUS (SAY)

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ABSTRACT

The grasshoppers *Melanoplus bilituratus* (Wlk.) and *M. bivittatus* (Say) require a water soluble factor in lettuce, *Lactuca sativa* var. *romana* Hort., for nymphal growth. Manipulation of water extracts of dried lettuce showed that the active factor is heat stable, dialyzable, adsorbed on Norite A, and is cationic. The acid soluble nucleotides of lettuce, synthetic nucleic acid derivatives, and the ninhydrin-positive components of a fractionated, aqueous lettuce extract all failed to satisfy the need for the lettuce factor. The active component in lettuce is probably different from a factor in corn leaves which is required for growth of the European corn borer, *Pyrausta nubilalis*. The nutritional needs of the two species of grasshoppers used in this study apparently differ from those of their relative, the desert locust, *Schistocerca gregaria*.

INTRODUCTION

In its broadest usage the term "growth factor" might be considered as any exogenous substance(s), identified or unidentified, which is necessary for, or has a favorable effect upon, growth of an organism under specified experimental conditions. In addition, a growth factor is usually thought of as an accessory substance to the so-called main components of food, i. e., fats, proteins, carbohydrates, mineral salts, and water. While investigating the nutritional requirements of two species of grasshoppers, Melanoplus bilituratus (Walker) and M. bivittatus (Say), a factor which is present in an aqueous lettuce extract was found necessary for growth of nymphs fed an artificial diet. The purpose of this study is to provide information on the properties of this growth factor.

Woods (1953) divides growth factors into two groups: substances of ultimate catalytic function, and substances that are incorporated into cell constituents. A consideration of the catalytic function of a growth factor would include the possibility of its role as a coenzyme or as a component of a coenzyme.

Neilands and Stumpf (1958) have arbitrarily separated the coenzymes into the hydrogen-carrying and the group-carrying types. Examples of the hydrogen-carrying coenzymes and their vitamin moieties are the pyridine nucleotides, flavin nucleotides, and lipoic acid. Diphosphopyridine nucleotide and triphosphopyridine nucleotide function as hydrogen carriers by means of their nicotinamide moieties. Pullman (1953) has presented evidence to show that a reversible reduction occurs at the <u>para</u> position of the pyridine nucleus and Loewus <u>et al</u>. (1956) have shown that this reduction is stereospecific. The flavin nucleotides, of which riboflavin (vitamin B_2) is a component, are believed to transport hydrogen by means of a reversible reduction of the isoalloxazine nucleus. Singer and Kearney (1950) demonstrated that several isoalloxazine derivatives are capable of catalyzing the transfer of hydrogen from the reduced pyridine nucleotides to cytochrome c. A more recent addition to the list of hydrogen-carrying coenzymes is lipoic acid. Reduction of the sulfur atoms after opening of the cyclic disulfide ring may account for the activity of this vitamin. Its participation in the oxidation of pyruvate in <u>Escherichia coli</u> has been reported (Hager and Gunsalus, 1953).

Among the group-carrying coenzymes the role of the adenosine phosphates and the transphosphorylases is recognized in the synthesis of high energy phosphate bonds. The adenosine pyrophosphate compounds may then be used in phosphorylation reactions as well as in many other biological processes. Besides the adenosine pyrophosphates, research during the past decade has implicated other nucleic acid derivatives as participants in enzymatic reactions. Uridine diphosphate glucose has been shown to participate as a coenzyme in the conversion of galactose-1-phosphate to glucose-1-phosphate (Caputto <u>et al</u>., 1950); cytidine diphosphate choline functions in the process of incorporating phosphorylcholine into lecithin (Kennedy and Weiss, 1956); and guanosine triphosphate is required to phosphorylate adenosine diphosphate (Sanadi and Ayengar, 1954; Sanadi <u>et al</u>., 1956).

A number of vitamins B are components of group-carrying coenzymes. Thiamine (vitamin B_1) is known to act as a coenzyme in decarboxylation reactions such as those mediated by pyruvic acid oxidase, a-ketoglutaric oxidase, and transketolase. Lohman and Schuster (1937) showed that this coenzyme is thiamine pyrophosphate (cocarboxylase). The discovery that pantothenic acid (vitamin B_3) is a component of coenzyme A led to the elucidation of the function of this vitamin. Coenzyme A is concerned with the transfer of acetyl groups by means of the alternate acetylation and deacetylation of the terminal sulfhydryl groups (Lynen, 1953). Pyridoxal-5-phosphate, a relative of pyridoxine (vitamin B_6), is required as a coenzyme in decarboxylation, transamination, and racemization of amino acids (Snell, 1952). Folic acid, known also as pteroylglutamic acid, participates in the transfer of the formimino group (Sager et al., 1956; Miller and Waelsch, 1956).

Other organic compounds such as biotin, glutathione, vitamin B_{12}, ascorbic acid and the fat soluble vitamins have important physiological manifestations, but their status as coenzymes is yet to be made clear. It is probable that many of these compounds will be identified as coenzymes or components of coenzymes in the future.

Besides the role of organic compounds in catalytic processes, trace elements are known to be concerned with many enzymatic reactions. Iron, copper, zinc, magnesium, manganese, molybdenum, cobalt, potassium, and calcium are contained in various enzymes or function as enzyme activators.

A review of the metal enzymes has been presented by Williams (1953).

The second group of growth factors, those which ultimately become part of the main cell constituents, could conceivably include a whole host of various compounds. A few examples might include amino acids, peptides, fatty acids, and choline. Chattaway *et al.* (1944) and Chattaway *et al.* (1949) found that a serine-glycine-glutamic acid peptide had growth promoting activity for *Corynebacterium diphtheriae*, *Streptococcus faecalis* R., and *Lactobacillus casei*. It has been suggested by Cheldelin (1954) that the need for peptides may be the result of poor assimilation of the component amino acids or the inability on the part of the organism to conjugate specific amino acids. The essential fatty acids, linoleic acid, linolenic acid, and arachidonic acid, are at least in part concerned with structural tissues. Alkaline hydrolysis of tissue gives a greater yield of these acids than does exhaustive extraction with solvents (Rieckehoff *et al.*, 1949; Reiser, 1950). The nutritionally important compound choline is known to be incorporated into structural tissues and to provide a source of labile methyl groups for transmethylation reactions (Wendell and Nyc, 1954).

Because of the many different compounds that could be active biologically, it is evident that a growth stimulus imparted by an unknown substance could be due to any of a large array of biochemicals. However, from the outline just presented, the vitamins, nucleic acid derivatives, trace metals, amino acids, peptides, fatty acids, and so forth are likely groups of which a growth factor could be a member.

Investigations of the basic nutritional requirements of insects have produced data concerning the need for growth factors by several insects. The beetle Tenebrio molitor requires a substance for larval growth that is water soluble, insoluble in fat solvents, heat stable, acid resistant, and not adsorbed on charcoal (Fraenkel, 1951a; 1951b). This factor was shown to be carnitine, a relative of choline (Carter et al., 1952). The European corn borer, Pyrausta nubilalis, grows best when a dialyzable and heat stable factor in corn is added to the diet (Beck, 1953). An unknown factor or factors affect emergence of the Asiatic rice borer, Chilo suppressalis, from its pupal case (Ishii, 1956) and, recently, it was found that inositol would satisfy the need for a growth factor for the boll weevil, Anthonomous grandis (Vanderzant, 1959).

Most of the literature that deals with grasshopper nutrition is concerned with the effect of host plants on growth and egg production (Hodge, 1933a; Chauvin, 1939; Sanderson, 1939; Tauber et al., 1945; Brett, 1947; Pfadt, 1949; Smith et al., 1952; Barnes and Nerney, 1953; Pickford, 1958; Smith, 1959). Only a few workers have conducted studies designed to provide information on the chemical nature of those substances present in host plants which are related to the nutritional needs of the grasshopper. Hodge (1933b) reported that when wheat leaves are boiled for 10 minutes some substance necessary for growth of M. differentialis is destroyed. M. bivittatus is unable to absorb carbohydrate polymers from its food but readily assimilates monosaccharides (Brown, 1937). Smith and Northcott (1951) maintain that there is a positive correlation between an increase in nitrogen content of a wheat host and greater vitality of M. mexicanus (bilituratus) as measured by survival, growth rate, and egg production, while wheat having a high phosphorous content lessens vitality of this species (Smith, 1960). One worker has succeeded in rearing Schistocerca gregaria on a semi-synthetic diet and has indicated that vitamin A (or a derivative) and ascorbic acid are required for growth (Dadd, 1957).

For general information concerning the nutritional needs of the phytophagous insects the reader is referred to the review by Friend (1958). Other important reviews are those by Levinson (1955), Lipke and Fraenkel (1956), Trager (1953), and Wigglesworth (1950).

Preliminary work in this study consisted of testing three artificial diets as possible starting points in an investigation of the basic nutritional needs of the grasshopper species previously mentioned. One of the diets used was chemically defined and has been used successfully for rearing the onion maggot, Hylemya antiqua (Friend and Patton, 1956). The second was a semi-synthetic diet which is adequate for growth of the pink bollworm, Pectinophora gossypiella (Vanderzant and Reiser, 1956b). The third, also a semi-synthetic diet, was designed to support growth of a wide variety of organisms (Luckey, 1954). All of these diets (see Appendix   for compositions) failed to support growth of the grasshopper nymphs beyond the second molt. It was learned, however, that growth to adulthood can be obtained if the pink bollworm diet is supplemented with a water soluble fraction of dried lettuce. Ether and 95 percent ethanol extracts of lettuce proved ineffective. As a result of these preliminary findings, the problem of elucidating some properties of the active lettuce component evolved.

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METHOD

Rearing the test insects.

The egg pods of two species of grasshoppers, *Melanoplus bilituratus* (Walker) and *M. bivittatus* (Say), were collected at sites in Lake and Teton counties, Montana, and western Oklahoma in the autumn of 1955, 1956, 1957, and 1958. The pods were mixed with moist soil, brought into the laboratory, and stored in loosely-capped Mason jars at 4°C. When needed for use the desired number of pods was removed from the refrigerator and placed on moist blotting paper in a petri dish. The pods were incubated at a constant temperature of 30°C. until hatching occurred, which usually required a week to 10 days. Each newly hatched nymph was removed from the petri dish as soon as possible after hatching and placed in a clean glass tube (6" X 1"). The rearing tubes were stoppered at each end with gauze-covered cotton plugs. These nymphs were reared at a constant temperature of 30°C. and in continuous light.

Experimental diets were prepared as agar gels and consisted of a basic diet (Table I) to which was added aqueous lettuce fractions or synthetic compounds. Portions of the autoclaved diet to be fed were transferred from a 300 ml. Erlenmyer flask to a petri dish by sterile technique. Individual feedings were made by transferring small portions of the diet from the petri dish to the rearing tubes once each 24 hours. Care was taken to maintain sterility in the main reservoir of food.

The criterion used for the effectiveness of a diet in promoting growth was the number of molts achieved by the nymphs fed exclusively on the test diet. Either five or six molts occur from egg to adult in these species (Shotwell, 1941). The duration of the stadia between molts varied widely and depended on the composition of the medium being fed. Approximately seven days were required for each stadium when the nymphs were fed a diet capable of producing adults. The length of any one test ranged from three to six weeks and depended on the number of molts achieved by the nymphs of a particular test group.

<u>The basic diet.</u>

The diet shown in Table I will be referred to throughout this study as the basic diet and was used as the base to which various additions were made. This diet was patterned after that used by Vanderzant and Reiser (1956b) with certain modifications. Ascorbic acid was added as an anti-oxidant and brewer's yeast was substituted for the vitamin solution used in the original diet. Vanderzant and Reiser used 2 M potassium hydroxide to dissolve the casein, but in these experiments this was omitted because the dissolved protein tended to precipitate after autoclaving. Likewise, sodium alginate, a component of the original diet, was omitted because it caused the agar medium to boil out of the flasks during autoclaving. The final water content was adjusted to 83 percent.

The basic diet was prepared by dissolving the sucrose in a portion of the water and adding this solution to the rest of the ingredients. Agar was excluded at this stage. The mixture was then thoroughly blended in a Waring Blendor. After placing up to 190 grams of the blended mixture in 300 ml. Erlenmyer flasks, agar and experimental additives were introduced into the individual flasks. The mixtures were then autoclaved at 20 pounds pressure for 20 minutes. An even distribution of ingredients was insured by swirling the flasks during the cooling period. Sterile media were refrigerated until needed.

Preparation of boiled water extracts of dried lettuce.

Boiled water extracts of dried lettuce were prepared by adding one liter of distilled water to 12, 24, or 36 grams of lettuce powder and boiling gently for one hour. Lettuce powder was obtained by pulverizing dried romaine lettuce, *Lactuca sativa* var. *romana* Hort., in a Waring

Table I. Composition of the Basic Diet

Ingredient	Gms.	Ingredient	Gms.
Casein	5.0	Wesson's salts	0.7
l-Cystine	0.1	Choline chloride	0.1
Glycine	0.15	Cellulose (powdered)	4.0
Sucrose	8.0	Brewer's yeast	1.5
Corn oil	1.0	Ascorbic acid	0.5
Cholesterol	0.3	Agar	4.0
		Water	125.0

Blendor. The mixture was boiled and then filtered through several layers of cheese cloth. The pulp was discarded. A second filtration through a double layer of muslin was used to remove the more finely divided particles, and the filtrate was concentrated by gentle boiling in the open air. Precipitates that formed during the period of boiling were removed by frequent filtration. The final filtrate was evaporated to 25 ml. before addition to the basic diet.

Treatment with dialysis.

The water extract was concentrated to 75 ml. by heating and placed in a tube made of cellulose casing (Visking Co., Chicago, Ill.). The dialysis tube and a magnetic stirring rod were placed in two liters of distilled water in an Erlenmyer flask. After autoclaving at 20 lbs. pressure for 20 minutes, the flask was placed on a Cole-Parmer "Magne-stir" and the water was agitated for 15 hours. Both dialyzable and nondialyzable fractions were reduced to 25 ml. by gentle boiling in the open air before inclusion in the basic diet.

Treatment with Norite A.

The dialyzable fraction of the boiled water extract was stirred with Norite A at pH 6.5 until completely decolorized. This usually required from 8 to 10 grams of Norite A to treat an extract derived from 24 grams of dried lettuce. After filtration, the charcoal was stirred with an eluant consisting of 300 ml. of either 50 percent ethanol or 50 percent ethanol plus 2 percent concentrated ammonium hydroxide. A filtrate, an ethanol eluate, and an ammoniacal ethanol eluate of Norite A were prepared using two separate boiled water extracts, each derived from 24 grams of dried lettuce. The resulting filtrate and eluates were concentrated to 25 ml. over an open flame before inclusion in the basic diet.

<u>Treatment with Dowex 50 and Dowex 1.</u>

The ammoniacal ethanol eluate of Norite A was treated with Dowex 1 (an anion exchanger in the chloride form) and Dowex 50 (a cation exchanger in the hydrogen form). Glass tubing (diameter: 9/16 in.) was packed with the resins to provide a bed six inches long. The ethanol and ammonia were removed from three ammoniacal ethanol eluates by heating. One eluate was passed through a column of Dowex 1, the second eluate was passed through a column of Dowex 50, and the third eluate was treated with both exchange resins. Because the Dowex 1 became exhausted during passage of the eluate, it was replaced as often as needed. Exhaustion of this resin was noted when the entire resin bed became discolored. Replacement of the Dowex 50 resin was unnecessary as indicated by the continued displacement of hydrogen ions by the eluate. Flow rates were maintained as rapid as the physical nature of the resins would permit. The Dowex 1 effluent was concentrated over an open flame before addition to the basic medium. The Dowex 50 effluent, which contained displaced hydrogen ions, was concentrated under vacuum and then adjusted to pH 6.5 with potassium hydroxide.

<u>The semi-purified extract.</u>

The Dowex 1 effluent, just described, was designated as the semi-purified extract and will be referred to by that term in subsequent experiments. A recapitulation of the events leading to the semi-purified extract is as follows:

1. Extraction of 24 grams of dried lettuce with boiling water.
2. Dialysis of the water filtrate.
3. Decolorization of the dialyzable fraction of the filtrate with Norite A.
4. Elution of the Norite A with 50 percent ethanol plus 2 percent concentrated ammonium hydroxide.
5. Treatment of the ammoniacal ethanol eluate of Norite A with Dowex 1.

Chromatographic determination of purine and pyrimidine bases.

Attempts were made to determine the presence of free purine and pyrimidine bases in the semi-purified extract by using the chromatographic method of Vischer and Chargaff (1948). A small amount of the extract was placed on a paper strip with a micropipette and the chromatogram was run with butanol: ethanol:water (50:15:35) as the solvent system. For purines: the paper strip was dipped in 0.25 M mercuric nitrate in 0.5 N nitric acid, bathed in 0.5 N nitric acid, and sprayed with ammonium sulfide. For pyrimidines: the paper strip was placed in buffered 0.01 M mercuric acetate solution of pH 6.2 for 30 seconds (1 part 0.1 M mercuric acetate, 3 parts 1 M sodium acetate, and 6 parts water), bathed for exactly 20 seconds in slowly renewed water, and dipped in ammonium sulfide solution. The presence of the mercuric salts of the bases is indicated by a black color following the ammonium sulfide treatment.

Chromatographic separation of ninhydrin-positive compounds.

Gross separations of the ninhydrin-positive compounds were carried out by means of paper chromatography. Three boiled water extracts, each derived from 24 grams of dried lettuce were prepared. One of the extracts was modified in the manner specified for the semi-purified extract (Norite A step omitted). The dialyzable fractions of the other two extracts were passed through separate columns of Dowex 50. The resin columns were eluted with 500 ml. of 4 N ammonium hydroxide and the resulting eluates were concentrated under vacuum. One of the eluates was added to the basic diet directly. The semi-purified extract and the other Dowex 50 eluate were each applied to four sheets of Whatman 3 MM chromatography paper (22½" X 18¼") by streaking with a pipette. The solvent system used was butanol:acetic acid: water which was prepared by saturating butanol with a solution of 1 part glacial acetic acid and 5 parts water. The direction of the solvent flow was descending. Thin strips cut from the center and sides of each paper sheet were developed with a 0.2 percent solution of ninhydrin in acetone. Bands of ninhydrin-positive compounds were located on the undeveloped areas by using the developed strips as reference points. The sheets containing the semi-purified extract were cut in half which provided low and high R_f fractions. The bands on the sheets containing the Dowex 50 eluate were separated by placing a prominent band having an R_f of .50

into one fraction and combining the remaining bands into a second fraction. The portions of the sheets were eluted with distilled water, concentrated to 25 ml. by heating, and added to the basic diet.

Synthetic nucleic acid derivatives.

Synthetic nucleic acid derivatives were included in the basic diet either in water solution (facilitated by heating) or by direct addition. These were obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

Acid soluble nucleotides of fresh lettuce.

A maximum of the acid soluble nucleotides was obtained by the method of Bergvist (1956). A homogenate of 350 grams of fresh lettuce and 1½ liters of 10 percent perchloric acid was prepared in a Waring Blendor. After filtration, the pulp was re-extracted with ½ liter of 5 percent perchloric acid. Both extracts were combined and treated with 10 grams of Norite A. The charcoal was then eluted with a solution of 25 percent ethanol and 0.5 percent concentrated ammonium hydroxide. The eluate was concentrated under vacuum, filtered through a Seitz filter, and added to the sterile basic diet.

Treatment with partially deactivated charcoal.

Further purification of the semi-purified extract was attempted by using the method of Asatoor and Dalgliesh (1956). This method is based on the selective adsorption of aromatic substances by partially deactivated charcoals. Accordingly, charcoal was deactivated to varying degrees by stirring it with a 1.5 percent solution (w/v) of stearic acid in ethanol for one hour. The mixture was then diluted with water at a rate of 9 liters of water per liter of ethanol. The charcoal was collected in a Buchner funnel, washed with water, and air dried. A series of 6 10-gram batches of charcoal was treated in the above manner. An amount of stearic acid solution was added to each batch of charcoal so that the stearic acid constituted 15, 10, 7, 4, 2, and 0 percent of the weight of the charcoal. A semi-purified extract, derived from 24 grams of dried lettuce, was prepared and treated first with the 15 percent deactivated charcoal. The resulting filtrate was then treated with the 10 percent deactivated charcoal. This process was repeated by treating each filtrate with the next lower deactivated charcoal. Each of the charcoals was eluted with 300 ml. of a 7.2 percent aqueous phenol eluant. Phenol was removed by evaporating the eluates to dryness in a drying oven. The temperature in the oven was not permitted to exceed 70° C. The dried eluates were dissolved in 25 ml. of distilled water before inclusion in the basic diet.

Treatment of the boiled water extract with acid.

To test the effect of low pH on the activity of the growth factor, hydrochloric acid was used to adjust an unfractionated, water extract to pH 2. The acidified extract was permitted to stand overnight in a refrigerator after which it was reduced to 25 ml. under vacuum. The concentrated extract was then adjusted to pH 6 with sodium hydroxide before being added to the basic diet.

RESULTS

GROWTH RESPONSES TO UNMODIFIED AND MODIFIED BOILED WATER EXTRACTS OF DRIED LETTUCE.

Unmodified boiled water extracts and ashed lettuce.

The results of feeding the ash of dried lettuce and various concentrations of boiled water extracts of dried lettuce are shown in Table II. Grasshopper nymphs fed diets lacking lettuce extracts failed to develop beyond the second molt in most instances. There was no apparent difference between the stimuli imparted by the extracts derived from 12 or 24 grams of lettuce, but the extract derived from 36 grams of lettuce imparted a weaker stimulus than did the smaller amounts. One possibility for this inhibition of growth is the toxic effect of large concentrations of the required substance or of extraneous materials.

Trace elements are not the cause of the growth stimulus, since nymphs fed ashed lettuce failed to elicit a growth response.

Nymphs fed diets lacking lettuce extracts appeared to be normal until several days after the second molt. At this time signs of starvation appeared and the nymphs wasted away slowly until they died. Diets containing the extracts resulted in adults that were slightly smaller than those reared on natural diets. Some had deformed wings but appeared normal otherwise.

The growth factor is heat stable and resists air oxidation, since concentrating the extract over an open flame and autoclaving does not inactivate it.

Table II. Growth Responses by M. biliturατus to Unmodified Boiled Water Extracts of Dried Lettuce and Ashed, Dried Lettuce.

Added to 150 gms. basic diet[a]	No. insects in test	No. insects completing 1 - 6 molts					
		1	2	3	4	5	6[b]
Distilled water	15	15	12	1	0	0	0
Water extract 12 gms. dried lettuce	15	15	15	15	15	14	13
do. 24 gms. do.	15	14	14	14	14	13	13
do. 36 gms. do.	15	15	14	14	7	4	0
Ash 24 gms. dried lettuce	15	15	14	0	0	0	0

[a]All additions reduced or diluted to 25 ml.

[b]The sixth molt marked the appearance of the adult in this species.

Dialyzable and nondialyzable fractions.

The data in Table III were intended to compare growth responses to the low and high molecular weight fractions of the boiled water extract. Dialysis through cellulose casing was used to effect a gross separation of the small and large molecules.

Heavy mortality and poor vitality of the newly hatched nymphs occurred during this test. Such difficulties appeared from time to time during the course of the experiments. It was not uncommon for egg pods collected at the same site and the same time to give rise to vigorous nymphs at one hatching and to weak nymphs at another. This could be the result of the innate condition of the eggs or of storage conditions in the laboratory.

The fact that the growth response by those insects fed the unfractionated water extract was far weaker than that previously obtained (Table II) provides additional evidence that the condition of the nymphs would have resulted in poor growth regardless of dietary inclusions. Therefore, the assumption was made that the dialyzable fraction was the active one, since the response to this fraction more nearly paralleled that of the whole extract. That this assumption was correct is shown in the next experiment.

Norite A filtrate and eluates.

Table IV shows that data obtained from feeding the filtrate of the Norite A treated dialyzable fraction of the water extract and two Norite eluates. Also, the effect on growth by the nondialyzable fraction was

Table III. Growth Responses by M. biliturtus to Dialyzed, Boiled Water Extracts of Dried Lettuce.

Added to 150 gms. basic diet[a]	No. insects in test	No. insects completing 1 - 6 molts					
		1	2	3	4	5	6
Water extract 12 gms. dried lettuce	10	3	3	2	0	0	0
Dialyzable fraction water extract	10	4	4	3	0	0	0
Nondialyzable fraction water extract	10	2	1	0	0	0	0

[a]All additions reduced to 25 ml.

repeated.

It is evident from Table IV that the Norite A treatment renders a water extract ineffective in stimulating growth of the nymphs. The 50 percent ethanol eluant fails to dislodge the active factor from the charcoal, but the ammoniacal ethanol eluant apparently accomplishes this. That the growth promoting factor is dialyzable is also confirmed by the data. The nondialyzable fraction imparted a slight stimulus to the nymphs which might have been the result of incomplete removal of the active substance during dialysis.

Dowex 1 and Dowex 50 effluents.

The effect of Dowex 1 (Cl^-) and Dowex 50 (H^+) exchange resins on the active factor in lettuce is shown in Table V. Growth was slightly inferior on the diet containing the ammoniacal eluate of Norite A compared with the same experiment in Table IV. Reduced growth activity was observed in the eluate treated with Dowex 1, but growth was far superior than with those eluates treated with Dowex 50. The ammoniacal ethanol eluate of Norite A treated with Dowex 1 will be referred to, hereafter, as the semi-purified extract.

In those extracts which were passed through Dowex 50 columns, the high acidity of the effluents prevented the agar media from solidifying after sterilization in the autoclave. These diets were adjusted to pH 6 with sodium hydroxide. Addition of more agar then resulted in solidification. Because excess acid might have caused chemical changes in the diet, this part of the experiment was repeated with the following

Table IV. Growth Responses by M. biliturutus to the Dialyzable Fraction of Water Extracts Treated with Norite A, to Norite A Eluates, and to the Nondialyzable Fraction.

Added to 150 gms. basic diet	No. insects in test	No. insects completing 1 - 6 molts 1	2	3	4	5	6
D + N[a]	10	8	4	0	0	0	0
D + N + E[b]	10	6	5	2	0	0	0
D + N + EA[c]	10	8	7	7	7	7	5
ND[d]	10	7	6	4	2	0	0
Extract 24 gms. dried lettuce	10	8	8	8	7	6	5
25 ml. distilled water	10	7	3	0	0	0	0

[a]Dialyzable fraction of boiled water extract derived from 24 gms. dried lettuce treated with 10 gms. Norite A at pH 6.5.

[b]Same as (a) with Norite A eluted with 300 ml. 50 percent ethanol.

[c]Same as (a) with Norite A eluted with 300 ml. 50 percent ethanol plus 2 percent concentrated ammonium hydroxide.

[d]Nondialyzable fraction of boiled water extract derived from 24 gms. dried lettuce.

Table V. Growth Responses by M. biliturutus to Boiled Water Extracts of Dried Lettuce Treated with Dialysis, Norite A, Dowex 1 and Dowex 50.

Added to 150 gms. basic diet	No. insects in test	No. insects completing 1 - 6 molts 1	2	3	4	5	6
D + N + EA[a]	10	6	5	5	5	4	2
(D + N + EA) + Dowex 1	10	6	5	5	5	3	0
(D + N + EA) + Dowex 50	10	5	3	0	0	0	0
(D + N + EA) + Dowex 1 & 50	10	5	1	0	0	0	0

[a]Dialyzed fraction of water extract derived from 24 gms. dried lettuce treated with 10 gms. Norite A at pH 6.5; Norite A eluted with 300 ml. 50 percent ethanol plus 2 percent concentrated ammonium hydroxide.

changes. The effluent from the Dowex 50 column was concentrated under vaccuum using a minimum of heat and neutralized with sodium hydroxide before addition to the basic diet. The results of this retest were essentially the same as with the acid media. The active factor is, therefore, either retained on the Dowex 50 resin or inactivated by it.

Ninhydrin-positive components.

Efforts to elicit a growth response by incorporating some of the ninhydrin-positive components of the semi-purified extract into the basic diet failed.. Paper chromatography was used with two different solvent systems, phenol:water (80:20) and butanol saturated with a solution of 1 part glacial acetic acid and 5 parts water.

Since the data in Table V showed that Dowex 50 rendered a water extract inactive, it was hoped that elution of this resin would result in recovery of the activity. In order to test the possibility that a ninhydrin-positive compound is responsible for growth stimulation and is retained on the Dowex 50 column, the dialyzable fractions of two boiled water extracts, each derived from 24 grams of dried lettuce, were passed through separate columns of Dowex 50. After each of the columns was eluted with 500 ml. of 4 N ammonium hydroxide, the eluates were concentrated under vacuum. One of the eluates was chromatogrammed with butanol:acetic acid: water as the solvent system; the other was added to the basic diet intact. The results are shown in Table VI.

The data in Table VI show that the active material was absent from the Dowex 50 eluates, since the intact eluate failed to provide a growth

Table VI. Growth Responses by *M. bilituratus* to some Chromatogrammed Ninhydrin-Positive Components of Lettuce.

Added to 190 gms. basic diet	No. insects in test	No insects completing 1 - 6 molts 1	2	3	4	5	6
Low R_f bands Dowex 50 eluate[a]	10	8	7	0	0	0	0
High R_f bands Dowex 50 eluate	10	10	9	0	0	0	0
Intact Dowex 50 eluate	10	10	10	1	0	0	0
25 ml. distilled water	10	9	6	0	0	0	0

[a]The area covered by the ninhydrin-positive bands on the paper sheets was cut approximately in half, thus providing two fractions--low and high R_f compounds.

response. An adverse effect on the growth factor due to the nature of the eluant does not seem likely because ammonium hydroxide in ethanol was used with success to elute Norite A. It is possible that the eluant was ineffective in displacing the growth factor from the resin, assuming that inactivation did not occur.

Since the supply of M. bilituratus eggs became depleted during the winter of 1957-1958, it was necessary to use those of M. bivittatus. It was determined that this species had the same requirements for the water soluble lettuce factor as M. bilituratus (Table VII). At this time the effect of the ninhydrin-positive compounds in the semi-purified extract was repeated. The Norite A step in the preparation of the semi-purified extract was omitted because the elution of the charcoal might be a significant cause of loss of the active factor. The development of reference strips from the chromatograms revealed an unusually heavy concentration of a substance having an R_f of .50. This band was eluted separately; the remaining bands were combined.

The ninhydrin-positive compounds again failed to impart a stimulus to the nymphs when M. bivittatus was used as the test insect.

Effect of acid on the boiled water extract.

In view of the inability to obtain a stimulus with fractions from the paper chromatograms, the effect of acid on the extract was tested because of the acid nature of the solvent systems. Also, the growth factor was subjected to acid conditions during treatment with Dowex 50. In addition to tests with acid, the activity of the semi-purified extract

Table VII. Growth Responses by M. bivittatus to Chromatogrammed Ninhydrin-Positive Components of the Semi-Purifed Extract.

Added to 190 gms. basic diet	No. insects in test	No. insects completing 1 - 6 molts 1	2	3	4	5	6
.50 R_f band	10	9	8	0	0	0	0
Remaining bands	10	9	7	0	0	0	0
Water extract 24 gms. dried lettuce	10	9	9	9	9	8 (4A*)	4
25 ml. distilled water	10	10	8	0	0	0	0

*A equals adult. The number of molts required to produce the adult in this species varied between 5 and 6. Those nymphs that achieved adulthood after the fifth molt are so designated.

was re-checked in order to be certain that previous results were reproducible. These data are shown in Table VIII.

The data support the previous finding that a reduced but obvious stimulus is elicited by the semi-purified extract and the active factor must, therefore, be present prior to chromatography. Low pH has little effect on the activity of the water extract. The possibility that the growth factor is labile to adsorption on paper would be difficult to reconcile with its stability on charcoal.

Partially deactivated charcoal eluates.

The partially deactivated charcoal method of Asatoor and Dalgliesh (1956) for purification of aromatic substances was also unsuccessful in effecting a further purification of the active substance in the semi-purified extract.

Difficulties in effecting further purifications might be explained by the progressive loss of the active component after each treatment until the lower threshold of the growth stimulus is reached. It would seem appropriate to increase many times the amount of starting material, but this would necessitate the handling of large amounts of extraneous materials. Other methods would have to be devised rather than the ones used herein.

GROWTH RESPONSES TO NUCLEIC ACID DERIVATIVES.

Synthetic nucleic acid derivatives.

Figure 1 shows the results of scanning the semi-purified extract at pH 7 and pH 9.5 in the ultraviolet range. Two peaks are clearly evident

Table VIII. Growth Responses by M. bivittatus to the Semi-Purified Extract and an Unfractionated, Acidified Water Extract of Lettuce.

Added to 190 gms. basic diet	No. insects in test	No. insects completing 1 - 6 molts 1	2	3	4	5	6
Acidified extract[a]	10	9	9	8	8	6 (4A)	2
Semi-purified extract	10	9	9	9	7	1 (1A)	0
25 ml. distilled water	10	10	5	0	0	0	0

[a]Water extract of 24 gms. dried lettuce reduced to 500 ml., acidified to pH 2 with hydrochloric acid, allowed to stand overnight, reduced to 30 ml. under vacuum, adjusted to pH 6.5 with sodium hydroxide.

at pH 7; one occurs at 258 mu. and the other at 263 mu. Both peaks are lost at pH 9.5.

Since the nucleic acid derivatives absorb, generally, in the region of 260 mu. and have been implicated in the nutrition of Drosophila (Schultz et al., 1946) and the onion maggot (Friend and Patton, 1956), the possibility was considered that these compounds are present in the semi-purified extract. The chromatographic method of Vischer and Chargaff (1948) for the determination of free purine and pyrimidine bases revealed the absence of purines but was inconclusive for pyrimidines. The test for pyrimidines appeared to be positive at first but attempts to duplicate the test were inconsistent. Nevertheless, a test was conducted in which various pyrimidine bases, purine and pyrimidine nucleosides, and deoxyribonucleic acid were fed in the basic diet.

All the compounds tested failed to impart a growth stimulus as shown in Table IX. Another pyrimidine, 5-hydroxymethylcytosine, was also found to be ineffective.

Nucleic acid derivatives of lettuce.

When the semi-purified extract was chromatogrammed on paper strips, two distinct fluorescent areas appeared on the strips when they were scanned with a short wave ultraviolet lamp. In order to determine the effect of these materials on growth, the semi-purified extract derived from 24 grams of dried lettuce was reduced to approximately 10 ml. and applied to large sheets of Whatman 3 MM chromatography paper. The ascending solvent system consisted of butanol:ethanol:water (50:15:35). The

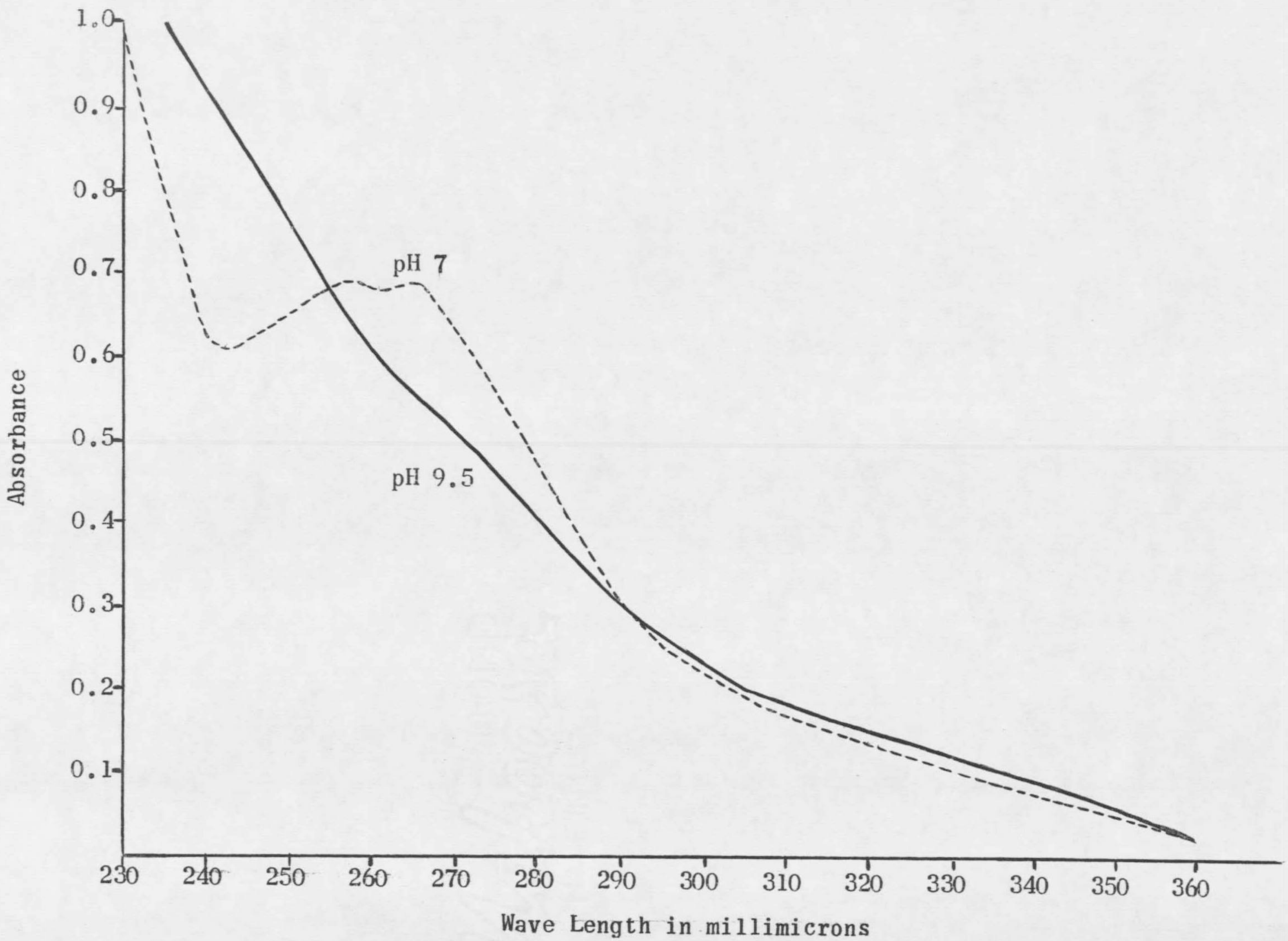

Fig. 1 Ultraviolet Spectrum of the Semi-Purified Lettuce Extract. (G. Baker, Dept. of Chemistry, Montana State College)

Table IX. Growth Responses by M. biliturtatus* to Pyrimidines, Nucleosides, and Deoxyribonucleic Acid.

Added to 190 gms. basic diet	No. insects in test	No. insects completing 1 - 6 molts					
		1	2	3	4	5	6
1 cc. pyrimidine solution[a]	10	8	1	0	0	0	0
2 cc. do.	10	9	2	0	0	0	0
10 cc. do.	10	10	3	0	0	0	0
DNA plus nucleosides[b]	10	10	8	0	0	0	0
Water extract 24 gms. lettuce	10	10	10	10	10	10	9

[a]Each cc. pyrimidine solution contained in water: 4 mg. each of uracil, thymine, cytosine, 5-methylcytosine HCl, and orotic acid.

[b]200 mg. DNA plus 10 mg. each of inosine, adenosine, guanosine, uridine, and cytidine.

*Collected in Oklahoma.

fluorescent bands were cut out of the paper, eluted with distilled water, and added to the basic diet. No growth stimulation was observed.

The foregoing data cast doubt on the role of nucleic acid derivatives in stimulating the growth of the nymphs. It would be helpful if this entire class of compounds could be eliminated from suspicion with some degree of certainty. Since synthetic nucleotides were omitted from the test diets, the method of Bergvist (1956) was used to isolate the acid soluble nucleotides of fresh lettuce. The results of incorporating this fraction into the basic diet are shown in Table X.

A slight stimulus occurred in the diet containing the nucleotides compared with the negative control. While it cannot be said that the acid soluble nucleotides of lettuce are ineffective in stimulating growth, it seems doubtful that they play a very significant role. Furthermore, there is no evidence that the growth factor is a nucleic acid derivative.

A diagrammatic representation of the results of the experiments is given in Figure 2.

Table X. Growth Responses by M. biliturutus to Acid Soluble Nucleotides of Lettuce.

Added to 190 gms. basic diet	No. insects in test	No. insects completing 1 - 6 molts					
		1	2	3	4	5	6
Acid soluble nucleotides	10	9	8	4	1	0	0
25 ml. distilled water	10	10	5	0	0	0	0

Inactive or Activity Much Reduced		Active
95% ETHANOL EXTRACT ←	DRIED LETTUCE	→ BOILED WATER EXTRACT
ETHER EXTRACT		HCl to pH2 NaOH to pH 6.5
		BOILED WATER EXTRACT

NONDIALYZABLE FRACTION ← (dialysis) — BOILED WATER EXTRACT — (dialysis) → DIALYZABLE FRACTION

FILTRATE ← (Norite A) — DIALYZABLE FRACTION

ELUATE ← (50% ethanol) — NORITE A — (50% ethanol, 2% conc. NH_4OH) → ELUATE

EFFLUENT ← (Dowex 50 (H^+)) — ELUATE — (Dowex 1 (Cl^-)) → EFFLUENT

ELUATE ← 4 N NH_4OH — DOWEX 50 (H+)

NINHYDRIN-POS COMPOUNDS ← (Paper chromatography) — EFFLUENT

ELUATES ← (partially deactivated charcoals, aq. phenol)

- -

ACID SOLUBLE NUCLEOTIDES ← perchloric acid — FRESH LETTUCE

- -

SYNTHETIC DNA AND NUCLEOSIDES

- -

Figure 2. Diagrammatic Representation of Activity of Various Lettuce Fractions and Some Synthetic Compounds When Added to the Basic Diet.

DISCUSSION AND CONCLUSIONS

It is tempting to conclude from the results that *M. bilituratus* and *M. bivittatus* nymphs have an absolute requirement for the growth factor in question, in view of the rather consistent growth responses demonstrated by wild populations from both Montana and Oklahoma. While it is possible that this is the case, it is conceivable that populations of these species might be encountered which vary widely in their responses to aqueous lettuce extracts.

The effects of the genetic constitution on the metabolism of organisms is treated extensively by Wagner and Mitchell (1955), however, these authors ask: "But the question arises: in the absence of mutation and in a constant environment, is it to be expected that the metabolic processes of a cell will remain constant?"[1] Evidence is accumulating in support of a negative answer to this question. The effects of crowding on the physiology of populations of small mammals has been of interest to several investigators. Chitty (1957) reported that hypertrophy of the spleen occurs in dense breeding populations of voles and Christian (1955) found a direct relationship between adrenal hypertrophy and increasing population density of mice. Clarke (1953) has suggested that intraspecific strife is responsible for a reduction in fertility and a decreased breeding season in high populations of voles. In other experiments with mice, Christian and Lemunyan (1958) were not only able to show that crowding produced adverse effects on lactating females, but also that these effects persisted in the progeny for at least two generations.

Few experiments such as those just mentioned have been performed using insects as test animals, but Wellington (1957) made observations

[1]Wagner, R.P. and Mitchell, H.K. 1955. Genetics and Metabolism. John Wiley and Sons, New York, p. 390.

on populations of the tent caterpillar, *Malacosoma pluviale*, and proposed that changes in the vigor of populations are reflected in a variation of the range of individual requirements and responses with respect to environmental factors. Studies on the migratory locust, *Locusta migratoria migratorioides*, have revealed that crowding adult females can reduce their fertility (Albrecht *et al.*, 1958).

The hypothesis that the physiological state of animal populations varies due to their own numbers and that this variability, in turn, produces changes in responses of the populations to the environment is currently receiving the attention of certain groups who are interested in the dynamics of grasshopper populations in the western United States.[2] If this is found to be true, changes in the nutritional requirements of grasshopper populations could be manifestations of a dynamic physiology.

During the course of this study, field collected eggs gave rise to nymphs of different vitality (see page 23 and Table III). Whether these changes in vitality of the nymphs can be attributed to mixtures of physiologically different eggs is open to speculation. The variables to which the eggs were subjected during storage make an answer difficult. Unfortunately, little is known about the effects of temperature,

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[2]Regional Grasshopper Research (Cooperative Regional Project W-37, Revised, January 1, 1960). Cooperating Agencies: Experiment stations of Colorado, Montana, Wyoming, California, Idaho, Arizona, and the U. S. Department of Agriculture.

moisture, and gas tensions of the atmosphere on stored eggs.

The similarity of the lettuce factor to a corn leaf factor which Beck (1953) found to be necessary for optimum growth of the European corn borer is of particular interest. Table XI shows a comparison of the two factors.

Examination of Table XI will reveal the similarity of characteristics between the two growth factors. Only in the case of the charcoal filtrate fraction do they differ but this provides evidence that the two factors do not have the same structure, assuming that both charcoal treatments were carried out under the same conditions. In the isolation of carnitine, an essential nutrient for the mealworm, Tenebrio molitor, Fraenkel (1951) remarked that the inability of the charcoal to adsorb this compound provided a significant step in its purification.

The success of Dadd (1957) with his semi-synthetic diet for the desert locust, Schistocerca gregaria, is significant because of the taxonomic relationship between the genera Schistocerca and Melanoplus. The diet, with which this author was able to rear from 20 to 40 percent of the nymphs to adulthood, is given in Table XII.

Dadd's diet contains three ingredients that are not present in the basic diet used for M. bilituratus and M. bivittatus, namely, glucose, wheat germ oil, and carotene, but it is doubtful that these substances could supply the missing factor required by the grasshoppers used in these tests. Glucose is a component of the onion maggot diet (see Appendix for composition) which was found to be ineffective in promoting

Table XI. A comparison of Growth Factors Required in the Nutrition of the European Corn Borer, Pyrausta nubilalis, and the Grasshoppers M. bilituratus and M. bivittatus.

Fraction tested[a]	Growth activity corn borer[b]	Growth activity grasshoppers
Unmodified water concentrate	Active	Active
Ashed concentrate	Inactive	Inactive
Heat coagulable fraction	Inactive	Inactive
Heat uncoagulable fraction	Active	Active
Ether soluble do.	Inactive	Inactive
Ether insoluble do.	Active	Active
50% acetone insoluble do.	Inactive	?
50% acetone soluble do.	Active	?
Charcoal filtrate	Active	Inactive
Dialyzable fraction	Active	Active
Nondialyzable do.	Inactive	Inactive

[a]Sources: corn leaves for corn borer and lettuce for grasshoppers

[b]Data from Beck (1953)

Table XII. Composition of a Semi-Synthetic Diet for *Schistocerca gregaria* Formulated by Dadd (1957).

Ingredient	Gms.	Ingredient	Gms.
Cellulose powder	2.0	Cholesterol	0.5
Glucose	5.0	Carotene (88 percent B-carotene)	0.025
Sucrose	5.0	Ascorbic acid	0.1
Steenbock's salt mixture no. 40	0.5	Choline chloride	0.1
Casein	3.0	Wheat germ oil	1.0 ml.
Dried, debittered yeast	3.0	Water	2.5 ml.

nymphal growth. Carotene and related compounds are water insoluble and would not be expected to be present in water extracts of lettuce. They would be present in ether extracts and, since these extracts produced no stimuli when added to the basic diet, it must be concluded that the carotenes play no role in the nutrition of _M. bilituratus_ and _M. bivittatus_ when nymphal growth is used as the criterion. Again, on the basis of solubility, it is unlikely that wheat germ oil could be a source of the water soluble lettuce factor. It seems reasonable to assume, therefore, that there are important differences between the nutritional requirements for the grasshoppers used in this study and _S. gregaria_, despite their taxonomic relationship.

The present findings cast some doubt on the validity of the conclusion by Hodge (1933b) that boiling wheat for 10 minutes caused the destruction of some necessary nutrient for the grasshopper _M. differentialis_. If the factor required for this species is the same as that encountered in this study, it would be expected that Hodge merely extracted it from the plant tissue rather than destroyed it.

That the stadium between the second and third molts is critical with respect to the growth factor is evidenced by the usual failure of the nymphs to achieve the third molt without the inclusion of a lettuce preparation in the diet. It might be inferred from this that a specific dietary need is manifest at this time. However, it is the writer's opinion that the newly hatched nymphs possess an endogenous supply of the required matabolite which is sufficient to support growth until after the second molt occurs, since starvation symptoms are not exhibited immediately following the second molt. The progressive wasting of the body, which finally results in death, begins several days after molting.

It remains to be shown whether different plant species contain varying amounts of the growth factor encountered in this study. A requirement for an adequate amount of the nutrient for maintenance and egg production as well as growth in grasshoppers might also be worthy of investigation. The reasons for different effects of host plants on egg production (see Introduction page 9) could be associated with varying amounts of the ingested factor. Pepper and Hastings (1943) were able to show that the fecundity of the sugar beet webworm, Loxostege sticticalis, was positively correlated with the linoleic acid content of the host plant tissue and Grison (1952) reported that the fecundity and longevity of the Colorado potato beetle, Leptinotarsa decemlineata, depended on the lecithin content of its host plant.

The problem of how the basic nutritional needs of insects fit into the mechanisms involved in insect-host plant relationships has been the subject of considerable discussion by investigators. Fraenkel (1953) is of the opinion that host plant specificity is determined by the presence or absence of chemical substances which act as stimuli for the sense organs of smell and taste. He states: "...leaves from different plants differ relatively little in chemical composition as far as the nutritional needs of insects for food substances proper are concerned, i. e. protein, carbohydrates, fat, minerals, sterols, and vitamins, and that the specificity of food plants for various insects is based on the presence of chemical compounds which serve as chemical stimuli for the sensory organs of smell and taste."[3] He goes on to say: "The results of numerous investigations have shown conclusively that the basic food requirements of all insects which have so far been examined are essentially similar."[4] While this may be true for the main nutritional requirements of insects, differences in need for accessory factors can hardly be ignored when their omission from the diet results in death or poor growth of the test organisms. On the other hand, it seems reasonable that repellents, for instance, could make a plant undesirable as a host despite its suitability otherwise. For a discussion in opposition to Fraenkel's view, see Beck (1956).

As the basic nutritional needs of more phytophagous insects are investigated, it appears that the need for new growth factors will be discovered. This information will be vital in helping to unravel the puzzle of insect-host plant relationships as well as in assessing the direct nutritional effects of the environment on insect populations.

The results of the experiments conducted in this study have provided information on some general characteristics of "lettuce factor".

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[3]Fraenkel, G. 1953. The Nutritional Value of Green Plants for Insects. Trans. IXth Interntl. Congr. Ent. 2:90-100, p. 90.

[4]Ibidem.

This organic substance in water solution exhibits extreme stability to prolonged periods of heating and is resistant to air oxidation. It is dialyzable through a cellulose membrane. Lettuce factor is strongly adsorbed on charcoal from which it can be eluted with ammoniacal ethanol. An aqueous solution containing lettuce factor is rendered inactive after treatment with a cation exchange resin (Dowex 50 H^+), but is largely unaffected after treatment with an anion exchange resin (Dowex 1 Cl^-). The active material is resistant to acids and to mild alkaline conditions. Lettuce factor is not a nucleic acid derivative.

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APPENDIX

Composition of Experimental Diets Used in Preliminary Tests.

1. Pink bollworm diet, Vanderzant and Reiser, (1956b)

Ingredient	Gms./100 Gms. Medium
Casein	5.0
L-cystine	0.1
Glycine	0.15
Sucrose	8.0
Corn oil	1.0
Cholesterol	0.3
Wesson's salts	0.7
Choline chloride	0.1
Cellulose (powdered)	4.0
Agar	3.0
Sodium alginate	0.5
Vitamin solution	1.0 ml.
2 M potassium hydroxide to pH 6.5	
Water to 100 grams	

The composition of the vitamin solution was such that 1 ml. contained the following amounts of vitamins, Vanderzant and Reiser, (1956a):

Thiamin	0.60 mg.
Riboflavin	0.90
Calcium pantothenate	2.00

Niacin	6.25
Pyridoxine	0.80
Folic acid	0.125
Biotin	0.018
p-Aminobenzoic acid	1.50
Vitamin B_{12}	0.005

A substitute vitamin preparation ("Homicebrin", Eli Lilly & Co.), used in place of the above vitamin solution, was specified as having the following concentrations of vitamins in 1 cc:

Thiamin chloride	0.20 mg.
Riboflavin	0.24
Pyridoxine HCl	0.20
Nicotinamide	2.00
Vitamin B_{12}	0.0006
Ascorbic acid	12.00
Vitamin A	600 U.S.P. Units
Vitamin D	200 U.S.P. Units

2. "Universal Diet No. 1", Luckey (1954):

	Gms./Kilo. of Diet
Purified casein	300
Corn oil	80
Corn starch	300
Cellulose (powdered)	120
Sucrose	120

Vitamin A	10,000 IU
Vitamin D	2,000 IU
a-Tocopherol	0.1
Vitamin K	0.01
Ascorbic acid	10.0
Thiamin Cl	0.02
Riboflavin	0.02
Nicotinamide	0.10
Calcium pantothenate	0.05
Inositol	2.0
Choline chloride	2.0
Pyridoxine Cl	0.02
Biotin	0.001
Folic acid	0.02
Vitamin B_{12}	0.0005
K acetate	20.0
$CaCO_3$	18.0
$CaHPO_4$	13.5
Na_2HPO_4	12.0
NaCl	3.0
KI	0.045
$MgSO_4 \cdot 7H_2O$	4.5
MgO	4.0
$MnSO_4 \cdot 4H_2O$	0.75

$Fe(C_6H_5O_7)_2$	4.5
$CuSO_4.5H_2O$	0.23
$CoCl.6H_2O$	0.03
$ZnSO_4.7H_2O$	0.06
$Na_2B_4O_7.10H_2O$	0.03
$AlK(SO_4)_2.10H_2O$	0.045

3. Onion Maggot Diet, Friend and Patton (1956).

	Gms.
Agar	25.0
Ribonucleic acid	1.0
Inosine	0.03
Thymine	0.004
Cholesterol	0.1
Dextrose	15.0
Wesson's salts	2.0
Casein	24.21
L-cystine	0.50
Glycine	0.70
Distilled water	1000.0
	uGms.
Biotin	20.0
Vitamin B_{12}	40.0
Calcium pantothenate	6,000.0
Choline chloride	20,000.0

Coenzyme A	1,000.0
Folic acid	6,000.0
Nicotinic acid	10,000.0
Pyridoxine HCl	30,000.0
Riboflavin	2,400.0
p-Aminobenzoic acid	1,500.0
Thiamin	1,500.0
Thioctic acid	500.0
Inositol	20,000.0