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Histochemistry of selected tissues from vitamin-deficient Tribolium confusum

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HISTOCHEMISTRY OF SELECTED TISSUES FROM
VITAMIN-DEFICIENT TRIBOLIUM CONFUSUM

by

Albert LeRoy Broseghini

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Zoology

Approved:
Signatures have been redacted for privacy.

Iowa State University
Of Science and Technology
Ames, Iowa

1959
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INTRODUCTION AND REVIEW OF LITERATURE

_Tribolium confusum_ Duval, the confused flour beetle, has been extensively used in laboratories for experimental purposes. Because of its size, ease of rearing and relatively short developmental period, _Tribolium_ has been the subject of a great many investigations. A considerable amount of literature has been published on various aspects of the nutrition of this species. Because of the very excellent reviews dealing with the nutrition and biology of _Tribolium_ (Uvarov, 1928; Hoskins and Craig, 1935; Craig and Hoskins, 1940; Trager, 1941; Fraenkel, 1943; Trager, 1947; Lipke and Fraenkel, 1956), no attempt will be made to repeat these reviews. Only those papers dealing with the vitamin requirements of _Tribolium_ are included here.

As early as 1928 Sweetman and Palmer attempted to ascertain the vitamin requirements of _Tribolium_. Their work was closely followed by that of Street and Palmer (1935) and Chiu and McCay (1939). Not until Fraenkel and Blewett (1943a) began their series of experiments on _Tribolium_ was much learned of _Tribolium_ nutrition. These two workers were the first to establish definitely the fact that _Tribolium_ required thiamin, riboflavin, nicotinic acid and pantothenic acid for normal larval development (1943a). Frobrich and Offhaus (1953) and Magis (1954) confirmed Fraenkel and Blewett's (1943a, b) assertion that _Tribolium_ also required folic acid and biotin. Choline was also

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shown to aid growth of Tribolium larvae when added to the diet of this insect (Fraenkel and Blewett, 1943a).

Carnitine (vitamin B<sup>7</sup>) has often been demonstrated as a compound necessary for normal pupal development. Fraenkel and Printny (1954) were the first to demonstrate this need. Frobrich (1954) showed that Tribolium required a "Tribolium-Imago-Faktor" which is now considered identical with carnitine (French and Fraenkel, 1954). Offhaus (1957-1958) has also demonstrated the need of Tribolium for carnitine. More recently, Fraenkel (1958) has examined the interrelationships between zinc, potassium and carnitine in the nutrition of Tenebrio molitor L. This study is of interest inasmuch as it suggests that such a relationship might exist in Tribolium.

A major concern in nutritional studies of Tribolium has been the presence of several unknown factors in yeast. Fraenkel (1949) first showed the need by this insect for such factors. French (1954) and Offhaus (1952) have also shown experimentally that Tribolium requires some as yet uncharacterized factor in yeast for normal development. More recently Chiragos (1958) mentions such factors in his work on the protein requirements of Tribolium.

Dick (1937) made the first observations on ovarian development of Tribolium as affected by diet. Broseghini (1956) attempted to determine more specifically the nutritional requirements of Tribolium for
normal ovarian development. With the exception of these two experiments nothing more has been attempted along this line with Tribolium.

Ovarian development of adult Tribolium confusum is strongly influenced not only by the quantity of food available, but also the quality (Broseghini, 1956). Such observations have been made on other insects (Rasso and Fraenkel, 1954) but no study has been undertaken to determine what cellular or cytochemical changes occur in gonadal tissues during periods of nutritional restrictions and deficiencies. One study on the effect of avitaminosis on the gut of the rice moth, Corcyra cephalonica has been reported by Swamy and Sreenivasay (1942). It was found that after 60 days on a thiamin deficient diet the nuclei of the gut epithelium are Feulgen negative. Since this report, Chang and Fraenkel (1954) have presented observations dealing with the effect of carnitine deficiency on the gut epithelium of Tenebrio molitor larvae. No attempt was made by these authors to investigate cytochemical changes.

The present study is an outgrowth of earlier work and was made to determine, if possible, some cytological and cytochemical changes which occur in certain tissues of Tribolium during periods of nutritional inadequacy.

The choice of ovaries as test organs for cytochemical changes during nutritional deficiencies was prompted by a number of factors:
(1) As mentioned previously, the gross changes of the ovaries of Tribolium fed various dietary mixtures have already been investigated (Broseghini, 1956). Results obtained from that study suggested that a more detailed investigation would be profitable.

(2) Tribolium has been investigated extensively from the nutritional standpoint and an adequate diet of known chemical composition is available. Although previous studies were concerned primarily with the larvae and utilized growth rate as a criterion for nutritional adequacy of various synthetic diets, it was felt, in this species, that information obtained from larval studies is, to a great extent, applicable to the adults because both larval and adult stages normally exist on the same diet under identical environmental conditions. Preliminary experiments indicated that this assumption is valid.

(3) The histochemical relationships of various insect ovaries has been studied by a number of authors. Recently, Bonhag (1958) has summarized present knowledge concerning insect vitellogenesis. Because of these studies detailed histochemical techniques adapted to insect tissues are available, and the necessity of trial and error is reduced.

A few observations were also made on the mid-gut because of its obvious role in digestion and absorption. It was realized that any disruption of these functions probably would adversely affect other
tissues. A special effort was made, therefore, to determine what changes in the ovaries were due primarily to vitamin deficiencies and what changes were brought about as a result of the inability of the mid-gut to perform its normal physiological tasks.
METHODS AND MATERIALS

General Procedures

Female pupae of the confused flour beetle, *Tribolium confusum* Duval, were isolated from stock cultures, maintained on a stock diet of rolled oats, fish meal, and whole brewers yeast, by the method of Chapman (1918), and placed in individual vials (35 x 12 mm.) to complete their development. On emergence, the virgin females were transferred to a control or experimental diet and allowed to feed for 30 or 60 days.

The composition of the control diet used in this study is listed in Tables 1 and 2. In preparing this diet, amino acids, sodium bicarbonate, cholesterol, and wheat germ oil were weighed out in quantities 20 times those shown in Table 1, mixed in a ball mill for 5 hours, and then passed through a sieve having 50 meshes to the linear inch. All experimental diets were based on this concentrated mixture. To fabricate a diet, 26 grams of the concentrate were added to 69 grams of dextrin. Vitamins were added in aqueous solution by means of an atomizer. Depending on the solubility of the vitamins, this involved the addition of about 8 ml of fluid to a 100 gram sample of dry mixture. The moistened diet was desiccated overnight at 60°C., and then ground in a mortar until the largest particles would readily pass through a 20
Table 1. Composition of control diet for study of effects of vitamin deficiencies on ovarian tissue of *Tribolium confusum* Duval

<table>
<thead>
<tr>
<th>Principal ingredients</th>
<th>%</th>
<th>Vitamins</th>
<th>μgm/gm of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid mixture</td>
<td>20.7</td>
<td>Thiamin</td>
<td>1</td>
</tr>
<tr>
<td>(Table 2)</td>
<td></td>
<td>Riboflavin</td>
<td>2</td>
</tr>
<tr>
<td>Salts (No. 2, U.S.P. XIII)</td>
<td>2.0</td>
<td>Pyridoxine</td>
<td>1</td>
</tr>
<tr>
<td>Wheat germ oil</td>
<td>1.5</td>
<td>Nicotinic acid</td>
<td>8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td>Calcium pantothenate</td>
<td>4</td>
</tr>
<tr>
<td>Brewers yeast</td>
<td></td>
<td>Choline</td>
<td>1500</td>
</tr>
<tr>
<td>Dextrin</td>
<td></td>
<td>Biotin</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Folic acid</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carnitine</td>
<td>0.01</td>
</tr>
</tbody>
</table>


\textsuperscript{b}Nutritional Biochemical Corporation.

\textsuperscript{c}General Biochemicals, Inc.

\textsuperscript{d}National Biochemical Corp.: 2 units Vitamin E per gram.

\textsuperscript{e}National Biochemical Corp." Vitamins in micrograms, per gram: Thiamin, 150; Riboflavin, 65; Niacin, 475; Pantothenic Acid, 125; Pyridoxine, 30; Folic Acid, 22; Biotin, 2.2; Choline, 3600; Inositol, 4500.

\textsuperscript{f}B and A, General Chemical Company.

\textsuperscript{g}International Minerals and Chemical Corporation.
Table 2. Composition of amino acid mixture of control diet\textsuperscript{a, b}

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Gms /100 gms of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-alpha alanine</td>
<td>0.49</td>
</tr>
<tr>
<td>L-arginine HCl</td>
<td>0.59</td>
</tr>
<tr>
<td>DL-aspartic acid</td>
<td>0.49</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.24</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>2.44</td>
</tr>
<tr>
<td>glycine</td>
<td>0.12</td>
</tr>
<tr>
<td>L-histidine HCl</td>
<td>1.05</td>
</tr>
<tr>
<td>L-hydroxyproline</td>
<td>0.12</td>
</tr>
<tr>
<td>DL-isoleucine</td>
<td>1.95</td>
</tr>
<tr>
<td>L-leucine</td>
<td>1.47</td>
</tr>
<tr>
<td>L-lysine HCl</td>
<td>1.83</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.98</td>
</tr>
<tr>
<td>DL-phenylalanine</td>
<td>1.47</td>
</tr>
<tr>
<td>L-proline</td>
<td>0.24</td>
</tr>
<tr>
<td>DL-serine</td>
<td>0.24</td>
</tr>
<tr>
<td>DL-threonine</td>
<td>1.71</td>
</tr>
<tr>
<td>L-tryptophane</td>
<td>0.49</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>0.73</td>
</tr>
<tr>
<td>DL-valine</td>
<td>2.44</td>
</tr>
</tbody>
</table>


\textsuperscript{b}1.59% sodium bicarbonate added to reduce acidity due to presence of HCl.
mesh sieve. By compounding a concentrated stock mixture, the same basic medium could be used for all test diets. Also, potential errors inherent in weighing individual minute amounts of ingredients for small volumes of diets were eliminated.

Yeast was incorporated in the control diet since the presence of yeast has been shown to have a beneficial effect on ovarian development (Broseghini, 1956). However, brewers yeast contains vitamins which would prevent the evaluation of the effect of a B-vitamin deficiency. But, a complete absence of yeast from the control diet seriously impairs ovarian development. This was determined in a preliminary experiment.

Since yeast cannot be omitted entirely, but at the same time cannot be used in experiments involving vitamin deficiencies, a substitute must be found. The most obvious substitute is the insoluble portion of yeast. French (1954) has pointed out that the addition of the insoluble portion of yeast to a diet fed to Tribolium larvae, while not as good as whole yeast, is still a desirable addition. Further, Broseghini (1956) has shown that the insoluble residue of brewers yeast alone will support normal ovarian development. Therefore, hot water extracted yeast was substituted for whole yeast in the control diet. This was accomplished by boiling for 15 minutes approximately 10 grams of whole brewers yeast in 100 ml of distilled water and then centrifuging. The supernatant was discarded. Boiling and centrifuging were done at least six times on each sample of
yeast used. After extraction, the yeast was dried overnight at 60° C. and ground to a powder fine enough to pass through a sieve having 50 meshes to the linear inch.

All experimental diets in which a vitamin had been omitted contained 5 per cent yeast residue rather than whole yeast. Differences observed as a result of this substitution will be discussed in a later section.

To check the adequacy of extraction, the yeast residue was assayed microbiologically for its vitamin content according to methods suggested in the Difco Manual (1953). Results of this assay are shown in Table 3.

Samples of diets were held in shell vials (50 x 25 mm.), each containing approximately 5 grams of food. Ten beetles were placed in each vial. A total of 200 beetles was used in each trial. One hundred of these beetles were dissected 30 days after feeding commenced. At this time the remaining 100 were transferred to a fresh diet of identical composition as originally started, and dissected after they had been feeding for a total of 60 days. Beetles were kept at 32° C. with 70 per cent relative humidity for the entire feeding period.

Dissections were accomplished by immobilizing the beetles, ventral side down, in paraffin-bottomed watch glasses and covering with saline. Ovaries were exposed by removing the elytra and tergal plates. The gut, tracheoles and as much of the fat bodies as possible were removed.
Table 3. Vitamin content of water extracted whole brewers yeast as determined by microbiological assay\(^a\)

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>(\mu\text{gm/gm. of yeast residue} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamin</td>
<td>0.6</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>less than 0.8</td>
</tr>
<tr>
<td>Niacin</td>
<td>less than 0.8</td>
</tr>
<tr>
<td>Ca Pantothenate</td>
<td>0.38</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.106</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.2</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>1.12</td>
</tr>
</tbody>
</table>

\(^a\)Ames Assay Laboratories, Inc., Box 422, Station A, Ames, Iowa

The saline was poured off and fixative added. As each gut was removed it was placed in a depression in a spot plate containing fixative.

Preliminary work indicated that Serra's fixative was compatible with all histological techniques contemplated, so this solution was used exclusively. Cellular fixation was excellent and the yolk material was preserved better with Serra's than with any other fixative tried. Ovaries were fixed in situ for 45 minutes, washed in 95 per cent ethanol, then removed from the insects and placed in depression plates containing
absolute alcohol. All further changes of fluids as well as infiltration in equal parts of benzene and paraffin were carried out in these micro-spot plates. After infiltration, which was carried out overnight, embedding was accomplished in an hour with three changes of paraffin (m.p. 56°-58°C.) containing 10 per cent bayberry wax. Sections were cut at 5 micra.

Histochemical Techniques

**Desoxyribonucleic acid**

The Feulgen reaction was used to localize DNA. Hydrolysis took place in N HCl at 60°C. for 8-10 minutes. Fast green was used as a counterstain. A 5 per cent solution of trichloroacetic acid heated to 70°C. was used to remove DNA (Gomori, 1952).

**Ribonucleic acid**

The toluidine blue-ribonuclease method of Brachet (1953) was used to visualize ribonucleic acid. A 0.05 per cent solution of toluidine blue (pH 4) in 5.0 per cent ethanol was found to be satisfactory (Bonhag, 1955). Ribonuclease was used as a 0.1 per cent solution in glass distilled water. Tissues were stained for 15 minutes in the toluidine blue solution; control sections were incubated in ribonuclease for 3-5 hours at 60°C. All slides were differentiated and dehydrated overnight in tertiary butyl alcohol. A solution of 10 per cent perchloric acid was also used to remove RNA (Ogur and Rosen, 1950). Slides treated with perchloric acid were

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placed in this solution overnight in a refrigerator. After removal from the perchloric acid the tissues were stained and treated as usual. Both perchloric acid and RNAse effectively removed all cytoplasmic basophilia and this basophilia was presumed to be due to RNA.

**Polysaccharides**

Polysaccharides were localized by use of the periodic acid-Schiff (PAS) reaction as outlined by Lillie (1954). No counterstain was used. Glycogen was detected by the lead tetraacetate method of Glegg, Cleremont and LeBlond (1952). Confirmation was obtained by treating control sections with a 0.5 per cent malt diastase solution for 30 minutes at 37°C. Hyaluronidase was used in an attempt to detect mucopolysaccharides. Sections were treated with a hyaluronidase solution (1 mg/1 ml) in distilled water for 3 and 24 hours at room temperature (Bonhag, 1955). The reverse acetylation technique for carbohydrates was carried out exactly as outlined by McManus and Cason (1950). Pearse’s method for metachromasia utilizing aqueous toluidine blue was also used (1953).

**Proteins**

Proteins were localized by means of the mercuric chloride-bromphenol blue technique (HgBPB) of Mazia, Brewer and Alfert (1953). After staining in a bromphenol blue and mercuric chloride solution in
95 per cent alcohol for 15 minutes, the sections were washed for 20 minutes in 0.5 per cent acetic acid. This was followed by either washing in tap water for 2 minutes, to develop color, then dehydrating and mounting, or washing in several changes of tertiary butyl alcohol to remove excess dye and to dehydrate the tissue. The latter method gave the better results as it prevented excessive loss of color whereas this was not always the case when water was used as a wash.

**Alkaline phosphatase**

A modification of the Gomori method (1952) for alkaline phosphatase was used. Tissues were fixed in a solution consisting of 95 ml absolute alcohol and 5 ml of glacial acetic acid. The substrate consisted of 20 ml sodium barbital, 20 ml sodium glycerophosphate, 8 ml calcium chloride, 2 ml magnesium sulfate and 50 ml distilled water. All solutions were 2 per cent. In the control substrate, water replaced the glycerophosphate. Incubation time varied from 45 minutes to 5 hours. Cobalt nitrate and ammonium polysulfide were used as described by Gomori (1952) to demonstrate sites of activity.

**Additional techniques**

For general histological observations a few slides were stained with Heidenhain's iron haematoxylin. Differentiation was accomplished in a saturated solution of picric acid in 70 per cent alcohol.
RESULTS AND DISCUSSION

Histologically, the ovaries of Tribolium confusum are quite similar to those of Tenebrio molitor, described by Schlottman and Bonhag (1956). Both species possess ovaries of the teletrophic type. Each ovary of Tribolium is composed of four ovarioles. Individual ovarioles normally contain three or four follicles. Because of the small size of the ovaries and the fact that they tend to curl when fixed, complete sagittal sections were difficult to obtain. The present conclusions have, therefore, been based upon a composite picture obtained by viewing large numbers of sections.

Results after 30 Days on Control Diet

Each ovariole can be divided into two portions—the apical germarium and the basal vitellarium. Both are invested by two sheaths, the outer and inner ovariole sheath (Fig. 10). The inner sheath ends at the base of the terminal filament but the outer sheath continues apically to envelop the terminal filament. A transverse septum separates the terminal filament from the germarium proper. Cells of both sheaths are stretched extremely thin because of pressure of the egg follicles. Nuclei of both sheaths contain sparse chromatin granules, and the cytoplasm is only slightly basophilic. Mitotic figures were never observed in either sheath although this does not preclude their presence.
Germarium

The germarium can be further subdivided into two parts, the apical trophic tissue which includes approximately the anterior three-fourths of the germarium, and the prefollicular tissue which occupies the basal one-fourth of the germarium. In the apical trophic tissue the predominant cell is the trophocyte or nurse cell; interstitial nuclei are scattered throughout. The most noticeable characteristic of the nurse cells is the presence of extremely large nuclei (Fig. 1). Many of the nurse cells contain two and sometimes three nuclei. Differences existing between trophocytes in various areas of the germarium of Tribolium are not great enough to warrant division of the germarium into various zones, as Bonhag and Wick (1953) were able to do with Oncopeltus fasciatus.

Toluidine blue preparations reveal extremely dark basophilic bodies in the trophocyte nucleus (Fig. 1). These basophilic bodies are absent after treatment with either RNase or perchloric acid and hence are assumed to consist of RNA. Because of this it is felt that these bodies are nucleoli although Schlottman and Bonhag (1956) found no discernible nucleoli in the trophocyte of Tenebrio molitor. The trophocytic nucleoli, as compared with nucleoli found in the oocytes, are irregular in outline and of variable size. Their distribution is somewhat reminiscent of the large chromatin masses of the trophocytes displayed in Feulgen preparations. Therefore, an attempt was made to determine if any heterochro-
matin associated with nucleoli could be demonstrated. A mixture of safranin and fast green FCF was used according to the directions given by Bryan (1955). No relationship between heterochromatin and nucleoli could be shown by this technique.

The cytoplasm of the nurse cells is very basophilic, especially near the nuclear membrane (Fig. 1). This basophilia is completely removed upon treatment with RNAse.

The anterior prefollicular tissue at the base of the germarium of Tribolium does not present the same picture as that observed in Tenebrio molitor by Schlottman and Bonhag (1956). These authors found only a slight basophilia in this tissue. In Tribolium there are no striking differences in the staining qualities of this tissue as compared with the results seen in the apical trophic tissue. Cellular dissolution was also commonly found by the above authors in Tenebrio. Some of this degeneration was due to parasites but was also observed in non-parasitized ovaries at the apical end of the nutritive cords. Cytological examination of the germarium of Tribolium, especially the posterior regions, failed to disclose any signs of extensive degeneration. In this respect Tribolium closely resembles the condition described for Popillus disjunctus (Bryan, 1954). It is at once obvious, however, that there seems to be some definite change in the nuclei of this region, but there is no evidence to indicate that whatever changes are occurring are brought about by
normal pycnosis. The absence of pycnosis is quite noticeable when the situation in Oncopeltus fasciatus is taken into account (Bonhag, 1955). In this organism it was shown that numerous DNA droplets are found in the posterior part of the germarium and this condition is a consequence of normal degeneration. The same condition has been reported in Acanthocephala bicloripes (Schrader and Leuchtenberger, 1952).

At the base of the germarium of Tribolium are found numerous nutritive cords containing an extremely high concentration of RNA (Fig. 2). These cords extend from the nurse cells to the oocytes and are the means by which the developing oocytes obtain nutritive material. Treatment of slides with RNAse and subsequent staining with toluidine blue does not reveal any of these plasmatic strands. None of the tissues stained by the Feulgen method showed any discernible DNA in the nutritive cords.

Periodic-acid Schiff positive material was found in all areas of the germarium. In the trophocytes a non-uniform reaction was obtained in the cytoplasm; the nuclei were entirely negative (Fig. 3). It was repeatedly observed that the cytoplasm of the nurse cells nearest the center of the germarium was much more reactive than other areas of the cell, and the cells on the periphery of the germarium gave a more intense reaction than trophocytes nearer the center of the germarium (Fig. 3). This uneven distribution of PAS material is possibly due to the type of fixation used and probably represents a diffusion artefact. When
sections were tested with lead tetraacetate according to Glegg et al. (1952) these same sites yielded a positive reaction. Pretreatment of such sections with malt diastase indicates that these reactive areas are due to glycogen. The epithelial sheaths surrounding the germarium also gave a positive reaction when tested by the PAS technique, but when tested for glycogen no reaction was elicited. The reverse acetylation technique of McManus and Cason (1950) was performed to check the validity of the PAS localizations. Results indicate that the reactive sites mark the presence of compounds with 1, 2 glycol groups.

An attempt was made to identify mucopolysaccharides, notably hyaluronic acid, by the use of hyaluronidase. Pretreatment of slides with a solution of this enzyme failed to alter the PAS reaction.

In sections stained with the mercuric chloride-bromphenol blue technique a relatively uniform reaction was obtained. This stain is not specific for any one type of protein. There can be noted, however, slight variations in the staining intensity within the cells themselves, indicating a higher concentration of total protein in one part of the cell than in another. In the trophocytes all parts of the cell stained but, with careful illumination, it was noted that the nucleoli were more intensely stained than the surrounding nucleoplasm; also, a very narrow border surrounding the nuclear membrane was shown to stain somewhat heavier. This could be interpreted to mean that these areas have a
higher concentration of protein than other areas. These same areas also
gave a distinctly more intense reaction when tested for RNA (Fig. 1).

Alkaline phosphatase was not demonstrated in the germarium or in
the vitellarium. In view of the work by Mulnard (1950, 1954) on
Acanthocephala and Yao on Drosophila (1950) this result is not too sur­
prising. Neither of these authors was able to demonstrate alkaline
phosphatase although Yao did find a very high concentration of acid
phosphatase.

The prefollicular tissue lies just anterior to the vitellarium and is
characterized by the presence of oocytes and the fact that cell boundaries
in this region are rather indistinct. The prefollicular tissue gradually
becomes oriented around the developing oocytes thereby forming the
definitive follicular epithelium (Schlottman and Bonhag, 1956). Nucleic
acid studies of the prefollicular tissue reveal that the chromatin material
is randomly distributed in the nuclei, and the cytoplasm is intensely
basophilic. Numerous small nuclei are present which are quite similar
to the interstitial nuclei found in more anterior regions of the germarium.

Scattered among the cells comprising the anterior prefollicular
tissue are to be found small cells with almost perfectly spherical nuclei
(Fig. 4). The chromatin of these cells, in contrast with that of the
trophocytes, appears to be more diffuse. These cells are the oocytes.
Vitellarium

As the oocytes develop they become oriented one behind the other. Each oocyte acquires a single layer of epithelial cells called the follicular epithelium which is generally considered to be derived from the anterior prefollicular tissue (Schlottman and Bonhag, 1956).

The oocytes themselves present a picture similar to that given for other insects. Young oocytes possess a strong basophilic cytoplasm when stained with toluidine blue (Fig. 5). This basophilia is completely removed upon treatment with either RNAse or perchloric acid. As the oocyte enlarges the basophilia diminishes in intensity, but this decrease in staining intensity is probably a dilution effect from the accumulation of yolk material which is being gradually laid down. In sections stained with Feulgen's the chromatin material in all oocytes is seen to be in the form of long, fine filaments (Fig. 6). Upon closer examination the chromomeres become evident. Nucleoplasm surrounding the chromosomes contains small granules of Feulgen-positive material. Variable numbers of nucleoli are present in the oocyte nucleus, the largest number found in any one nucleus being five (Fig. 5). Each group of nucleoli appears to be surrounded by a distinct "membrane". These nucleoli are absent after treatment with RNAse; they are not Feulgen-positive.

As the oocyte enlarges the nucleus also increases in size and
eventually becomes what is termed the germinal vesicle. In *Tribolium* the chromosomes remain distinctly filamentous throughout the successive stages of oocyte growth. Nucleoli are also prominent structures in the germinal vesicle and have been observed in all follicles except the largest. The chromosomes and nucleoli occupy a definitely circumscribed area in the germinal vesicle. Surrounding both nucleoli and chromosomes is a limiting "membrane" (Figs. 5, 7, and 8). This "membrane" can be seen in toluidine blue sections, as well as in haematoxylin stained sections, but is absent in Feulgen preparations. Some of the larger granules seen in the germinal vesicle may be the "emission bodies" spoken of by Mulnard (1954), but it is felt that the largest are nucleoli. The entire area enclosed by the "membrane" in the germinal vesicle is probably homologous to the caryonucleolus of Mulnard (1954) and the karyosphere of Schlottman and Bonhag (1956).

In the largest oocyte the germinal vesicle loses most of the above characteristics and proceeds to break down. The fate of the karyosphere was not investigated.

Surrounding each oocyte is the follicular epithelium. The origin of this tissue and a detailed description of it are presented in Schlottman and Bonhag's paper (1956). In *Tribolium* this tissue gives a negative reaction when stained for carbohydrates by means of the PAS technique. After treatment with lead tetraacetate a negative reaction is obtained.
The epithelial sheaths surrounding the follicular epithelium also gave negative reactions when tested for glycogen but were intensely stained by the PAS technique (Fig. 9).

In very young oocytes only a slight PAS reaction was noted. However, as the oocyte enlarges, more and more of the oocyte becomes reactive. At first PAS-positive granules are deposited adjacent to the follicular epithelium. This is exactly the same process observed in the milkweed bug by Bonhag (1955). As oocyte development progresses there is an increase in both size and number of PAS-positive deutoplasmic particles (Figs. 9, 10, 11).

When staining for protein, the follicular epithelium and epithelial sheaths react positively because of the ubiquitous distribution of proteins. In young oocytes the entire cytoplasm yields a uniform, intense protein reaction. This is undoubtedly due to the association of a high concentration of RNA and protein found in oocytes at this stage. However, after the deposition of yolk material commences the distribution of protein is seen to resemble the pattern of the PAS-positive bodies (Fig. 12). In fact, the arrangement of the protein and carbohydrate inclusions found in the yolk are identical, suggesting that they are glyco-protein. Both are laid down in the same progressive manner.

Glycogen was found distributed throughout the yolk. When the lead tetraacetate method for glycogen was used, the large PAS-positive bodies
(which are also protein positive) remained unstained but surrounding each of these bodies were very fine glycogen granules (Fig. 13). This is the same distribution of glycogen in the yolk that Bonhag observed in Anisolabis maritima (1956).

As with the germarium, the vitellarium did not give a positive reaction when tested for alkaline phosphatase and mucopolysaccharides.

**Results after 60 Days on Control Diet**

When beetles were fed the control diet for 60 days, some differences in the ovaries were noted. Grossly, the ovaries tended to be slightly smaller than those in the 30 day controls, and the surrounding fat bodies were not as extensive. Nevertheless, the ovaries were judged to be fully developed according to the criteria established by Broseghini (1956).

Microscopic examination also revealed some changes. In approximately 50 per cent of the ovaries examined the trophocyte nucleoli were fragmented (Fig. 14). A few of the ovaries showed degeneration of the follicular epithelium. There was no reduction in Feulgen stained tissues. Distribution and development of proteins and carbohydrates were normal both in the germarium and vitellarium.

Although the diet used as a control in this study is considered adequate for larval growth and pupation (Lemonde and Bernard, 1951a, b) it appears that it is not entirely satisfactory for adults over an extended...
period of time. Even so, ovarian development, when viewed grossly, is not affected to any large degree even after 60 days.

The question now arises whether the cellular changes observed after 60 days are due to some inadequacy of the diet or to some non-dietary factor. Two possibilities in the latter category immediately come to mind--aging, and the stage of sexual activity of the beetles. The effect of age possibly can be dismissed when one considers the fact that egg-laying in Tribolium may continue for as long as 14 months (Good, 1933). Thus, a period as short as 60 days would not be expected to produce any marked changes in the ovaries or their ability to produce eggs. To support this assumption, a group of 100 virgin female beetles were fed a stock diet consisting of rolled oats, ground fishmeal and whole brewers yeast. This diet has definitely proven to be adequate in all respects (Broseghini, 1956). When the beetles had been feeding for 30 and 60 days, the ovaries were dissected out, sectioned and examined histo-chemically. No cellular changes were noted, even in beetles fed such a diet for 60 days.

Since only virgin females were used for the above test, another experiment was designed. In this experiment 100 newly emerged females were placed on the control diet with 100 newly emerged males. They were allowed to feed for 30 and 60 days after which the females were dissected and the ovaries sectioned and stained. Again, it was found that after 30
days no cellular changes were evident, but, after 60 days, some cellular
degeneration had occurred. This consisted of pycnosis, cellular swelling
and a reduction in cytoplasmic basophilia (Fig. 15).

Thus, the experimental evidence indicates that the diet used as a
control in this study can only be considered adequate for a period of
approximately 30 days. For longer periods of time the diet fails to
prevent cellular changes which, although slight, are a constant feature
of beetles fed such a diet. The reason for this is not clear.

Results with Extracted Yeast Substituted
for Whole Yeast

As mentioned previously, whole yeast contains more than adequate
amounts of those vitamins needed by Tribolium. Nothing would be gained
if whole yeast were to be retained in diets intended to test the effect of a
specific vitamin deficiency. Therefore, hot water extraction was carried
out to remove the known water soluble vitamins from the yeast. The
resulting water insoluble portion of yeast was used in all vitamin-
deficient experimental diets in place of the whole yeast. This was
necessary because a preliminary experiment showed that the complete
absence of yeast from the control diet resulted in retarded ovarian
growth.

However, in addition to recognized vitamins, yeast is known to
contain at least two factors necessary for normal larval growth and
development of Tribolium. One is water soluble, and one water insoluble (French, 1954). Obviously, treatment of whole yeast with water will remove the soluble fractions plus any other as yet undetermined water soluble factor which may be necessary for Tribolium. Also, if there are any moderately heat labile factors present in yeast, these, too, might be altered in such a way as to prevent their availability to Tribolium. Thus, the resulting residue of hot water extracted yeast is of unknown composition and any results obtained with diets containing this portion of yeast must be interpreted with care.

When water extracted yeast is substituted for whole yeast in the control diet, gross ovarian development is slightly inferior, but complete, nevertheless, after 30 days. Microscopically, the tissues were, for the most part, normal. However, signs of pycnosis were apparent in the germarium and simple necrosis of the follicular epithelium was noted after 30 days (Fig. 16). Nucleoli were found in the trophocytes after 60 days on this diet. The significance of this will be discussed in a later section.

Why the substitution of extracted yeast for whole yeast in the diet of Tribolium should result in inferior ovarian development after 30 days remains an enigma. One could, of course, explain it on the basis of the unknown factors mentioned previously. However, the need for these factors by the larval form does not necessarily mean that the adults will
also require the same factors. Furthermore, Fraenkel and Printny (1954) have managed to raise Tribolium larvae in the absence of yeast altogether. However, as Magis (1954) has pointed out, the best synthetic diet (larval) devised so far is considerably better when whole yeast is added. Its effect cannot be explained on the basis of presently known ingredients. Thus, the entire role yeast plays in larval Tribolium nutrition is still not wholly known, even after considerable investigation. Its function in adult Tribolium nutrition is equally obscure.

Results after 30 Days on a Thiamin-Deficient Diet

The most striking cytological and cytochemical changes were observed in tissues from Tribolium maintained on a thiamin-deficient diet. The effect of a vitamin B₁ deficiency was so pronounced that the experiment could not be carried beyond 30 days. Of the 200 beetles started on this diet, less than 100 remained alive at the end of 30 days. Upon dissection, the surviving beetles were found to be in an emaciated condition. Fat bodies were much reduced and in many cases were virtually non-existent. The fat that was present was brittle, difficult to remove, and obviously considerably altered from the type found in beetles on a better diet. The ovaries were either undeveloped or, at best, partially developed.

Histologically, the cells of the germarium seem to be most
affected. Pycnosis was a common finding (Fig. 17). Most trophocyte nuclei stained significantly darker with toluidine blue than the surrounding cytoplasm (Fig. 18). Treatment with RNAse indicated that this material was RNA. Swelling of the nurse cells and loss of cell boundaries were observed in most instances (Fig. 17). The Feulgen reaction was not affected except in those cells which had begun to cytolyze. Nucleoli were never observed in any of the nurse cells in thiamin-deficient beetles.

The vitellarium also showed rather widespread changes. The follicular epithelium was uniformly affected. Cells of this tissue were vacuolated; nuclei were pycnotic; and there was a diminished cytoplasmic basophilia (Fig. 20).

Yolk material was not materially affected except in follicles showing degenerative changes in the epithelium. In these instances the follicle was filled with an amorphous material (Fig. 16).

In light of these findings, it is interesting to speculate as to the relationship between the follicular epithelium and the production of yolk material. Bonhag (1955, 1956) has presented evidence that carbohydrates, lipids and proteins are contributed to the oocytes by the follicular epithelium. In Tribolium the first PAS positive granules are formed adjacent to the follicular epithelium. This suggests that the follicular epithelium may have some connection with their formation. Although this is only indirect evidence, the fact that yolk production appears to be affected
when the follicular epithelium becomes degenerative strongly suggests that there is indeed a relationship between the two observations.

Probably the most notable change was seen in some of the young oocytes. In the normal condition the nucleoplasm has little, if any, basophilic material, while the cytoplasm contains a heavy concentration of such material (Fig. 5). In thiamin-deficient beetles an occasional oocyte showed an absence of basophilia but a very intensely stained nucleus (Figs. 19 and 20). Since there was no way of determining beforehand the oocytes which would show this it was impossible to test the effect of RNAse on this basophilic material. Therefore, it can only be assumed that the basophilic material found in nuclei of some of the oocytes of thiamin-deficient beetles is RNA.

The results obtained with a thiamin deficiency are difficult to assess. First of all, it must be remembered that the diet contained water-extracted yeast. It was noted previously that substitution of extracted yeast for whole yeast in the control diet with synthetic vitamins added, produced swelling of the nurse cells, pycnosis, and degeneration of the follicular epithelium. Obviously, any changes in ovarian tissues caused by a thiamin deficiency will be superimposed on the tissue alterations in beetles kept on a diet containing extracted yeast. Thus, what is being dealt with here is more than a simple vitamin deficiency. However, a careful evaluation of the results may shed some light on those changes.
caused primarily, or possibly entirely, by a thiamin deficiency.

Degeneration of the follicular epithelium and pycnosis certainly cannot be attributed solely to a thiamin deficiency inasmuch as these conditions were present in diets containing all the vitamins and extracted yeast. Whereas these changes were encountered infrequently in the yeast residue-all vitamin diet, they were a constant feature of the yeast residue-thiamin-deficient diet. One might conclude from this that thiamin lack played a major, but not exclusive role in the development of pycnotic nuclei and follicular epithelium degeneration.

The situation is equally complex with respect to the other cellular disturbances encountered when thiamin is omitted from the diet. Absence of nucleoli in the nurse cells, for example, was encountered in thiamin-deficient beetles, but nucleoli were present in animals fed diets containing extracted yeast. Nucleoli were also absent in the nurse cells in about half the beetles fed the control diet (containing whole yeast) for 60 days. This latter finding most certainly was not caused by a vitamin lack. On the other hand, absence of nucleoli in the trophocytes of beetles on a thiamin-deficient diet would appear to be associated with a deficiency of this dietary factor.

The increased basophilia of nuclei in the nurse cells and oocytes observed in thiamin-deficient animals is also difficult to explain. Fabiny (1957), working with embryonic chick skin, found that when such tissue
is treated with 2-thienylalanine ($\beta$−2-T) the epidermal cells lose a considerable amount of their cytoplasmic RNA. However, these cells did retain a nucleolus although it was abnormally large and stained exceedingly dark with toluidine blue. He offers several explanations for this. The $\beta$−2-T may have affected an enzyme system associated with the release of RNA by the nucleolus. It might also have affected the synthesis of peptides and proteins. Since such synthesis is thought to occur in both the nucleolus (Stich, 1956) and cytoplasm (Mazia and Prescott, 1955), the addition of $\beta$−2-T might inhibit the formation of such compounds with a concomitant increase in their precursors, in this case, RNA. A thiamin deficiency might also disrupt, either directly or indirectly, the normal nucleocytoplastic relation existing in Tribolium. However, current knowledge of the activity of thiamin in intermediate metabolism does not support such an assumption.

Observations on the Mid-Gut

The above conclusions remain valid only until the role of the mid-gut is considered. As mentioned previously, the mid-gut was also examined in an attempt to ascertain whether the changes observed in the ovary were due to a specific deficiency or whether they resulted from an inability of the mid-gut to function properly in its absorptive processes.

Detailed studies of the mid-gut were not considered to be a part of
this study. Hence, only those observations bearing on the problem at
hand are presented.

Cells of the mid-gut of beetles fed the control diet containing whole
yeast for either 30 or 60 days are intensely basophilic (Fig. 21). It will
be noticed that the proximal end of the cells stain distinctly more
intensely than the distal portion. This heavier concentration of RNA
would indicate that the proximal end of the cells is the site of the greater
protein concentration. All basophilia is completely abolished by pre-
treatment with either ribonuclease or perchloric acid.

The Feulgen reaction revealed small, oval nuclei with chromatin
granules scattered indiscriminately within the nucleoplasm. No definite
conclusions could be reached by using the PAS or Hg-BPB techniques.
Glycogen could not be demonstrated in any of the mid-gut cells.

When the beetles had been fed a thiamin-deficient diet for 30 days
the mid-gut cells were found to have lost their basophilic properties to a
very large extent (Fig. 22). The proximal portion of the cells continued
to stain more intensely than the other portions of the cell. No evidences
of cellular degeneration were noted.

Since the basophilia exhibited by the gut cells has been shown to be
due to RNA, it follows that a loss of basophilia would also indicate a loss
of RNA. The significance of this is not entirely clear, but an attempt at
explanation can be made based upon present concepts of the function of
RNA.

Caspersson (1947, 1950) has shown that a relationship exists between RNA and protein synthesis. Correlated with a high rate of cell activity is the presence of large, conspicuous RNA-positive nucleoli. There is a considerable literature showing the relations among RNA content of cells, nucleoli and protein synthesis (Brachet, 1947; Caspersson, 1947; Davidson, 1949; Crook, 1957). In almost all tissues studied to date this relationship has been shown to exist. Tribolium ovaries from specimens on adequate diets show this correlation quite clearly.

If the relationship between RNA and protein synthesis is accepted, then the loss of RNA in the gut epithelium could be taken to mean that the ability of the epithelial cells to synthesize protein would be correspondingly reduced. The mid-gut has long been assigned the role of digestive enzyme formation (Wigglesworth, 1950; Day and Waterhouse, 1953; Waterhouse, 1957). Thus, if the production of proteinaceous digestive enzymes were to be hindered in any way, through a decrease of RNA, for example, it could be reasonably assumed that the process of digestion would also be impaired. If this were the case then the amount of digested food materials available for absorption from the gut would either be greatly reduced or non-existent, depending upon the amount of digestion which had taken place. Therefore, the lesions previously described in the ovaries of Tribolium may well have been caused by an inability of the gut to function properly due to a loss of its synthesizing
ability, and only secondarily to any influence of a thiamin deficiency on the ovaries. It should be pointed out that food material was observed in the lumen of the mid-gut of thiamin-deficient beetles, although the amount may have been quantitatively reduced. Thus, the effect cannot be attributed to an inability of the beetles to ingest food substances.

However, although the lesions observed in ovaries of thiamin-deficient beetles appear to be the result of a loss of function of the mid-gut, the loss of cytoplasmic basophilia in the nurse cells and oocytes may also be attributed directly to a thiamin deficiency. Support for this assumption is to be found in the work of Guggenheim and Halevy (1957) and Halevy and Guggenheim (1958). They showed that liver RNA was uniformly decreased in rats suffering from a thiamin deficiency. However, as will be pointed out later, the above assumption is open to serious question.

Since the question had been raised that proper digestion and subsequent absorption by the gut were impaired in thiamin-deficient specimens, it was deemed advisable to test the effect of starvation on both the ovaries and gut. Therefore, a series of experiments was devised whereby a group of 50 newly-emerged virgin females was starved for a period of 10 days (Petersen, 1959). Starvation for more than 10 days resulted in beetles in such poor condition, with markedly atrophied

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viscera, as to make dissection impractical. After 10 days of starvation beetles were dissected and the ovaries and guts were prepared for observation as before. Grossly, the ovaries appeared to be in the same condition as those obtained from beetles fed a thiamin-deficient diet for 30 days. Histologically, the results were no different from those obtained from thiamin-deficient beetles (Fig. 23).

When the mid-gut from starved beetles was stained with toluidine blue, it showed a noticeably lighter staining reaction, although not as light as in the thiamin-deficient beetles (Fig. 24).

A study by Lagerstedt (1949) might also be mentioned at this point. Working with rats, he states that "In starving animals . . . the nucleolar apparatus could be shown to diminish rapidly . . . , and after this the basophil cytoplasmal inclusions (BC1) disappear from the cytoplasm."

These results would seem to lend support to the idea that starvation, brought about by the effect of a thiamin deficiency on the mid-gut epithelium, plays a leading role in the development of the lesions observed histologically in the ovaries.

Results with Diets Deficient in Riboflavin, Nicotinic Acid, Calcium Pantothenate or Pyridoxine

The results obtained with diets deficient in riboflavin, niacin, calcium pantothenate or pyridoxine are so similar that they can be discussed collectively.
Tissues from beetles fed for 30 days on a diet deficient in any one of the above vitamins appeared to be normal, both grossly and microscopically. Occasional areas of cellular degeneration were observed in the germarium but they were not widespread enough to suggest a vitamin deficiency as a causative factor. Such areas of degeneration are observed in tissues from beetles on what is considered to be an adequate diet. After 60 days on diets deficient in any of the above vitamins the incidence of cellular degeneration in the germarium and vitellarium was increased. However, here again, such changes were seen in 60 day control beetles. Nucleoli were, in a great many instances, absent in the trophocytes. No changes were noted in the distribution of ribonucleic acid. Neither were there any alterations in the distribution of glycogen, polysaccharides or protein.

To ascribe the lesions observed to a deficiency of any of the above mentioned vitamins is impossible. The cellular changes found were no different from those described in Tribolium fed the control diet for 60 days. Thus, it would appear that the absence of a vitamin other than thiamin from an otherwise complete diet has no visible cytochemical effect on the tissues studied, beyond the changes noted in normal tissues.

In contrast with a thiamin deficiency, removal of any of the above vitamins did not affect the mid-gut, either histochemically or histologically.
SUMMARY AND CONCLUSIONS

1. A series of experiments was performed in an attempt to determine what cytochemical and cytological changes occurred in the ovaries of adult Tribolium confusum Duval fed various vitamin-deficient diets. Over 2,000 beetles were dissected and prepared for histological study.

2. Newly emerged virgin females of Tribolium confusum were fed a control diet consisting of 20.7 percent amino acid mixture (DL-alpha alanine, L-arginine HCl, DL-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine HCl, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, L-tryptophane, L-tyrosine, DL-valine), 2.0 per cent salts (No. 2, U.S.P. XIII), 1.5 per cent wheat germ oil, 1.0 per cent cholesterol, 5.0 per cent whole brewers yeast, 69.3 per cent dextrin and a mixture of nine vitamins (thiamin, riboflavin, pyridoxine, nicotinic acid, calcium pantothenate, choline, biotin, folic acid, carnitine).

3. After 30 days on such a diet, gross ovarian development is both normal and complete as judged by the appearance of mature follicles. After 60 days on this diet gross ovarian development is somewhat inferior in that the ovaries are slightly smaller and the surrounding fat bodies of the insects are not as extensive; even so, follicular maturation seemed unaffected.
4. Ovaries were examined histochemically for DNA, RNA, polysaccharides, glycogen, protein, and alkaline phosphatase. Comparisons of the presence and distribution of these cellular components with those reported for other insects are made. In general, Tribolium ovaries are identical, or very similar, to the ovaries of other insects studied in a like manner. A particular similarity was found between Tribolium and Tenebrio molitor L.

5. When ovaries of Tribolium fed the control diet for 60 days were examined histochemically, trophocyte nucleoli were fragmented, nurse cell nuclei were pycnotic and the follicular epithelium showed evidence of simple necrosis.

6. It was concluded that the diet used as a control is not entirely adequate for more than approximately 30 days.

7. It was found necessary to substitute hot water-extracted yeast for whole yeast in the control diet in order to assess the effect of a deficiency of a water-soluble, yeast-contained vitamin. When such a substitution is made, gross ovarian development is slightly inferior, but complete, nevertheless. Microscopically, the ovaries exhibit signs of pycnosis in the germarium and degeneration of the follicular epithelium.

8. When beetles are fed a diet deficient in thiamin, less than 50 percent of such beetles survive for 30 days. Grossly, the ovaries are in an undeveloped state, and fat bodies are either much reduced or absent
entirely.

9. Trophocyte nuclei of thiamin deficient beetles were found to be packed with RNA. Nucleoli were never observed in these cells. It was suggested that the absence of thiamin might be disrupting the normal nucleocytoplasmic relation existing in Tribolium.

10. The cells of the follicular epithelium of thiamin deficient beetles were found to be vacuolated to a large extent. In such instances the yolk material lacked any definite form. Evidence was presented suggesting that the follicular epithelium plays a role in the production of yolk material.

11. Some oocytes of thiamin deficient beetles showed a reversal in the distribution of basophilic material. Nuclei of such oocytes were found to be intensely basophilic, while the cytoplasm exhibited no such basophilia. This is directly opposite to the distribution found in normal oocytes. This basophilic material was assumed to be RNA.

12. The mid-gut of Tribolium was examined in both normal and thiamin deficient animals. In beetles fed a complete diet the gut epithelium was found to be intensely basophilic. This basophilia was demonstrated to be RNA.

13. In thiamin deficient beetles the mid-gut epithelium was found to have lost much of its basophilia. It was suggested that the loss of RNA of the mid-gut cells resulted in a decrease in their production of
proteinaceous enzymes. This in turn impaired the process of digestion. With impaired digestion the amount of digested food materials available for absorption would be greatly reduced.

14. It was further suggested that the lesions observed in the ovaries of thiamin-deficient beetles were due primarily to a decrease in amounts of circulating end-products resulting from reduced enzymatic activity in the mid-gut. Consequently, ovarian cells would not be able to synthesize their normal products.

15. An absence of riboflavin, nicotinic acid, calcium pantothenate or pyridoxine in the diet produced no changes in either the mid-gut or ovary of Tribolium that could be attributed to such a deficiency.


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PLATES
Plate I

Fig. 1. Trophocytes from germarium of *Tribolium confusum* fed control diet, containing whole brewers yeast, for 30 days. Note the intense cytoplasmic basophilia, especially adjacent to the nuclear membrane. The darkly stained bodies within the nuclei are nucleoli. Serra's, 5⁒, toluidine blue, X 885.

Fig. 2. Longitudinal section near base of the germarium of control insect showing the nutritive cord (nc). Serra's, toluidine blue, 5⁒, X 885.

Fig. 3. Trophocytes of germarium from control insect showing distribution of PAS-positive material. Note that the nuclei are completely negative and the rather uneven distribution of material in the cytoplasm. Serra's, 5⁒, periodic acid-Schiff technique, X 485.

Fig. 4. Longitudinal section near base of germarium of a control insect showing developing oocytes (arrows). Serra's, Feulgen technique, 5⁒, X 190.

Fig. 5. Young oocyte of beetle fed control diet for 30 days. Note the intense basophilia of the ooplasm and the relatively unstained nucleus. Within the nucleus are a number of nucleoli surrounded by a "membrane". This latter structure is thought to be homologous to the caryonucleolus of Mulnard (1954) and the karyosphere of Schlottman and Bonhag (1956). Serra's, 5⁒, toluidine blue, X 885.

Fig. 6. Oocyte of control beetle showing filamentous condition of the chromosomes. Serra's, Feulgen technique, 5⁒, X 885.

Figs. 7 and 8. Young oocytes showing germinal vesicle with nucleoli and surrounding "membrane." Serra's, 5⁒, iron haematoxylin, oil immersion, X 995.
Plate II

Fig. 9. Section of developing oocyte from a normal insect showing early formation of PAS material. Note the relationship of the follicular epithelium (fe) to the stained material and the negative reaction of the nucleus. Note also that the follicular epithelium is practically negative but the epithelial sheaths (es) are strongly positive for PAS material. The shape of the oocyte is an artefact due to stretching during dissection. Serra's, 5/, periodic acid-Schiff technique, X 430.

Fig. 10. Sagittal section of oocyte showing progressive development of PAS-positive material near the follicular epithelium. Serra's, 5/, periodic acid-Schiff technique, X 430.

Fig. 11. Same section as Fig. 10 but at a lower magnification. Notice the distribution of PAS positive material in the fully formed oocyte. Note also that the upper, youngest oocyte is almost entirely lacking in PAS positive material. Serra's, 5/, periodic acid-Schiff technique, X 190.

Fig. 12. Mature oocyte of control beetle showing distribution of protein. PAS material is distributed in a similar manner suggesting that the material is actually a glycoprotein; compare with Fig. 11. Serra's, 5/, mercuric chloride-bromphenol blue technique, X 485.
Plate III

Fig. 13. Mature follicle from control beetle showing distribution of glycogen. Clear areas represent the glycoprotein yolk bodies; compare with Fig. 11, showing PAS reaction and Fig. 12 showing protein distribution. Serra's, 5/, lead tetraacetate technique, X 485.

Fig. 14. Section of germarium from beetle fed control diet for 60 days. Note the lack of nucleoli in the trophocytes. The dark bodies in the nuclei are chromatin granules. Serra's, 5/, toluidine blue, X 485.

Fig. 15. Section of germarium from mated female fed control diet for 60 days. The trophocytes are swollen, nuclei are pycnotic (arrow) and there is a reduction in cytoplasmic basophilia. Serra's, 5/, toluidine blue, X 485.

Fig. 16. Cross section of oocyte from a beetle fed a thiamine-deficient diet for 30 days. The follicular epithelium is necrotic and the yolk material lacks definite form. This same condition was observed occasionally in beetles fed a diet in which whole yeast was replaced by hot water-extracted yeast. Serra's, 5/, toluidine blue, X 485.

Fig. 17. Germarium from a beetle fed a thiamin deficient diet for 30 days. Note the widespread cellular degeneration. This section was chosen because it demonstrates the extreme condition found in thiamin-deficient beetles. Serra's, 5/, toluidine blue, X 720.

Fig. 18. Section of germarium from a beetle fed a thiamin-deficient diet for 30 days. Nuclei of the trophocytes stain significantly darker than normal with a corresponding decrease in cytoplasmic basophilia. Treatment with RNase indicated the nuclear material to be RNA. Serra's, 5/, toluidine blue, X 485.
Figs. 19-20. Oocytes of beetles fed a thiamin-deficient diet for 30 days. The nucleus is strongly basophilic and the ooplasm is not. This is the reverse of what is observed in beetles fed a complete diet for the same period of time (see Fig. 5). Serra's, 5/N, toluidine blue, X 190.

Fig. 21. Section of mid-gut epithelium from beetle fed the control diet for 30 days. The cells are strongly basophilic. Note that the proximal portion of the cell stains more intensely than the distal portion. Serra's, 5/N, toluidine blue, X 885.

Fig. 22. Section of mid-gut from a beetle fed a thiamin-deficient diet for 30 days. Note the lack of basophilic material within the cells. The proximal portion of the cells continues to stain darker than the distal portion. Serra's, 5/N, toluidine blue, X 885.

Fig. 23. Section of germarium from beetle starved for 10 days. Serra's, 5/N, toluidine blue, X 485.

Fig. 24. Section of mid-gut epithelium from beetle starved for 10 days; compare with Fig. 21. Serra's, 5/N, toluidine blue, X 485.