PROTEIN UPTAKE IN THE OOCYTES
OF THE CECROPIA MOTH

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ABSTRACT
The formation of yolk spheres in the oocyte of the cecropia moth, Hyalophora cecropia (L.), is known immunologically to result largely from uptake of a sex-limited blood protein. Recent electron microscope analyses of insect and other animal oocytes have demonstrated fine structural configurations consistent with uptake of proteins by pinocytosis. An electron microscope analysis of the cecropia ovary confirms the presence of similar structural modifications. With the exception of two apparently amorphous layers, the basement lamella on the outer surface of the follicular epithelium and the vitelline membrane on the inner, there is free access of blood to the oocyte surface between follicle cells. Dense material is found in the interfollicular cell space and adsorbed to the outer surface of the much folded oocyte membrane. Pits in the oocyte membrane and vesicles immediately under it are lined with the same dense material not unlike the yolk spheres in appearance. Introduction of ferritin into the blood of a developing cecropia moth and its localization adsorbed to the surface of the oocyte, and within the vesicles and yolk spheres of the oocyte cortex, is experimental evidence that the structural modifications of the oocyte cortex represent stages in the pinocytosis of blood proteins which arrive at the oocyte surface largely by an intercellular route. Small tubules attached to the yolk spheres are provisionally interpreted as a manifestation of oocyte-synthesized protein being contributed to the yolk spheres.

Both morphological and physiological analyses have established that an important aspect of yolk formation in animal oocytes is the uptake of protein from the blood. Fine structural configurations consistent with intensive pinocytotic activity are seen during periods of rapid yolk formation in oocytes of amphibians (8, 29), chickens (22), and a variety of insects (1, 4, 9, 19, 21), and the sequestering of specific blood proteins in yolk bodies has been demonstrated experimentally in the chicken (11) and in moths (24).

This paper attempts to extend these observations by describing an electron microscope analysis of the ovary of the cecropia moth, Hyalophora cecropia (L.). Immunochemical studies have shown that the mechanism of protein uptake in the cecropia oocyte is particularly adapted for a highly selective removal of a sex-limited lipo-protein from the blood and that this protein attains a substantially higher concentration in the yolk than in the blood (23, 24). Based on these observations it has been proposed that there must be, at some point on the route of protein transfer to the yolk, a site of selective adsorption (25). Fluorescein-labeled antibody studies of the distribution of blood proteins in the ovary indicated that there are large intercellular spaces in the follicular epithelium which surrounds the ovary and that the spaces are filled with blood protein (25). These observations suggested the oocyte surface as the most likely site of adsorption, and free diffusion as the mechanism for penetration of protein through the ovariole wall and the enveloping follicular epithelium (25). This model was convincingly confirmed by Roth and Porter's (19, 21) description of the fine structure of the mosquito ovary.

Published July 1, 1965
With the exception of a basement lamella at the outer surface of the follicular epithelium, the oocyte surface is in communication with the hemocoele by extracellular spaces which should be traversable by freely diffusing proteins. And where pinocytotic vesicles were being formed, the oocyte surface was coated with a dense material not unlike the contents of the yolk spheres in appearance. While not ruling out the possibility of an effect of the basement lamella on protein uptake, Roth and Porter thus agreed that the oocyte surface was a likely site of blood protein adsorption (21).

Aside from the obvious desirability of attempting to confirm these observations on a species in which blood protein uptake has been analyzed physiologically, it seemed desirable to obtain experimental evidence that the material adsorbed to the oocyte surface does in fact include blood protein, and that it is transmitted to the yolk spheres through the agency of pinocytosis.

MATERIALS AND METHODS

The large size and abundant yolk which have made the cecropia oocytes particularly amenable to physiological studies have made the analysis of their fine structure more difficult. Ward (28) found the yolky oocytes of adult frogs also recalcitrant to preparation for fine structure analysis, and therefore studied early formation of yolk in newly metamorphosed frogs. A similar problem, encountered by Hope et al. (8) in preparation of salamander eggs, was dealt with by using one fixing procedure which fixed immature oocytes throughout but fixed only the periphery of more mature oocytes. In cecropia, adequate fixation was obtained by using eggs in early stages of yolk formation and by trimming off the nurse cells, a procedure which presumably enhanced penetration of the fixative.

Oocytes were obtained from pupal cecropia between 13 and 17 days of adult development, a period of active vitellogenesis (27). Ovarioles, dissected from the pupae, were removed briefly to a depression slide containing several drops of pupal blood and a few crystals of phenyl thiourea to inhibit melanin formation. Here follicles between 0.75 and 1.5 mm in length were selected, separated from each other and, in most cases, removed from the ovariole wall and snipped at the anterior ends, removing the nurse cells. The tissue was fixed for 3 to 4 hours at 0 to 4°C in 2 per cent osmium tetroxide buffered at pH 7.4 with 0.15 M phosphate buffer to which was added 0.1 per cent calcium chloride (16). Rapid dehydration in 75 per cent through absolute alcohol was followed by embedding in Araldite 502 or Epon 812. Thin sections were stained with lead (15) and examined with an electron microscope.

The ovariole, follicular epithelium and oocyte cortex are shown in a section cut perpendicular to long axis of the follicle cells (FC). The ovariole wall, bounded on either side by a thin basement lamella, contains granular material (g) in the interstices between tracheal (t) and muscle cells (m). The follicular epithelium is distinguished by a thick basement lamella (BL), and large intercellular spaces containing dense flocculent material (FL). Thin cytoplasmic extensions (EX) maintain the contact of follicle cells across the intercellular spaces, with the basement lamella, and with the oocyte surface. The extensions facing the oocyte are microvillous-like (MV). Lobulated nucleus (N), numerous mitochondria (M) and lipid bodies (L) are seen in follicle cells. The cortex of the oocyte exhibits a brush border (BB) with yolk spheres (Y) beneath it. X 1700.
A section cut tangential to the surface of the oocyte reveals that the brush border is an elaborately folded limiting membrane. Dense flocculent material (FL) covers the membrane. Microvillous extensions (MV) of the follicle cells are seen at the top of the figure. × 19,000.

Part of the oocyte cortex from a median longitudinal section of a follicle. A bit of follicle cells (FC), at upper left, exhibits a microvillus (MV) extending toward oocyte surface. Dense flocculent material (FL) occurs in the interfollicular cell space and on the surface of the oocyte. Separating the microvilli of the follicle cells and the oocyte surface is a layer of less dense and more evenly distributed granular material provisionally identified as a vitelline membrane (VM). Crevices in the oocyte surface are shown in sections cut across (C⊥) and along (C∥) the folds of the cell membrane. Pits (P) which appear to be incipient vesicles occur at the sides and innermost ends of the crevices. Vesicles (V) lined with dense flocculent material and covered with bristles (B) abound in the cortex of the oocyte. Membrane-limited, dense yolk bodies (Y) of various sizes are also numerous. The tiny vesicles (t) near the yolk bodies are the same diameter as tubules (T) which are occasionally found attached to the yolk bodies. Irregularly shaped lipid bodies (L), ribosomes (R) and membranes (E) are also evident, × 23,500.

The inset shows ribosomes (R) and a vesicle with bristled coat (B) on the outside and dense material on the inside of a dark membrane, × 47,500.
Akashi Transcope-TRS-50. Experimental procedures of ferritin injection and ovary transplantation are described along with the observations.

**OBSERVATIONS AND DISCUSSION**

The histology of the cecropia ovary and oocytes has been described previously (25, 26) and is typical of the polytrophic insect ovary (5). Each of the four ovarioles constituting the ovary is a linear array of about 40 follicles enclosed in a thin ovariole wall. A follicle includes a layer of follicle cells surrounding an oocyte and a group of nurse cells at its anterior end. A few follicle cells are interposed between nurse cells and oocytes except for a median pore where the nurse cells maintain cytoplasmic continuity with the oocyte (Fig. 1). A brush border marks the surface of the oocyte and closely associated with its inner side are small yolk spheres just visible with the light microscope. These tiny spheres at the periphery are presumed to coalesce forming the larger spheres which accumulate in the center of the oocyte (25).

**OVARIOLE WALL:** The sieve-like structure of the ovariole wall, which has been demonstrated with the light microscope to consist of fine striated muscle fibers, occasional unoriented tracheoles, and a few fine fibrils in the interstices, would appear to be easily traversed by blood proteins (26).

Electron micrographic analysis of ovariole walls establishes no further evidence of barriers to the passage of blood proteins other than a thin basement lamella on each side of the wall. The granular material, preserved in the large spaces between the meshwork of muscle, and tracheal cells, is tentatively identified as blood protein (Fig. 2).

**FOLLICLE CELLS:** When vitellogenesis begins in the oocyte the most conspicuous feature of the follicular epithelium is the development of large intercellular channels extending from the basement lamella to the oocyte surface (25). In the earlier stages of yolk formation the lateral contact between two follicle cells remains close at some points while at others, where there appears to be no contact in the light microscope, contact is maintained by thin cytoplasmic extensions into the intercellular spaces (Fig. 2). The basal pole of the follicle cells adheres to the basement lamella by irregular cytoplasmic extensions so that intercellular spaces are created here also. At their opposite end, facing the oocyte, the follicle cells develop slender extrusions resembling microvilli. The microvilli are about 1 μ long and 0.1 μ in diameter (Figs. 2 and 4).

A dense flocculent material occupies much but not all of the space surrounding the follicle cells (Figs. 2 and 4). Based on immunohistological localization of blood protein this material is tentatively identified as blood protein (25). The flocculent condition of the blood protein contrasts with the more granular and evenly distributed intercellular material in the ovariole wall. Either the composition of the blood protein is altered in the interfolllicular spaces or fixation produces a different configuration of the material here. The uneven distribution of the flocculent material suggests that fixation causes condensation of blood proteins which were presumably more evenly distributed around the follicle cells.

The conspicuous basement lamella of the follicular epithelium may be 2 or more microns thick and appears amorphous or possibly irregularly fibrillar in structure (Fig. 9). Since there is no alternative route of entry, it has been necessary to presume that the lamella is permeable to blood proteins, and this assertion is confirmed by the results of experiments to be described shortly, in which ferritin introduced into the blood was detected in the basement lamella (Fig. 9).

Under the follicle cells, in the brush border of the oocyte, the presence of a fine line was strikingly demonstrated by non-specific staining with fluorescein antibody; it appeared to divide the brush border so that 1/2 lay on the follicle cell side and 1/2 on the oocyte side (25). Electron micrographs establish this clearly as a layer, about 0.08 μ thick, separating the brush borders of the

Figure 5 A portion of oocyte cortex, from an ovary grown in a male pupa, is shown in median longitudinal section. Note the conspicuous vitelline membrane (VM), irregular crevices (C), flocculent yolk (Y), small vesicles (V). Lipid bodies (L) and annulate lamellae (AL) are conspicuous in the cortex of these abnormal oocytes. X 23,500.
Follicles cells and the oocyte (Fig. 4, VM). It is easily distinguished from the flocculent "blood proteins" by its less dense and more homogeneous granular nature. Its position is that of the vitelline membrane described in vitellogenic dipteran oocytes (10, 14). Although it is of similar density, the dipteran membrane is more discrete, thicker, and, during the period of yolk formation, has perforations through which microvilli extend. Whereas blood proteins could pass through the pores of the dipteran membrane to reach the oocyte surface, in the moth they would necessarily penetrate the vitelline membrane itself. As is the case with the basement lamella, a significant proportion of the materials fixed and stained in the vitelline membrane can be presumed to be blood proteins which have failed to flocculate due to the structure of the membrane itself.

OOCYTE SURFACE: The convolutions which form the brush border of the oocyte surface consist of finger-like projections of the unit membrane but rather of extensive crevices. The crevices are about 0.1 μ in width and extend to a depth of 3 to 4 μ into the cortex of the oocyte. The pattern of crevices is best seen in sections cut tangential to the surface of the oocyte (Fig. 3). From this it is apparent that any section cut perpendicular to the oocyte surface will be parallel to some of the crevices (C₁ in Fig. 4) and will cut across others (C₂ in Fig. 4). Adhering to the outside of the oocyte membrane and more or less filling the crevices is a dense flocculent substance similar to that seen in the interfollicular cell spaces (Figs. 3 and 4, FL).

Pits (Fig. 4, P), reminiscent of those first seen in the mosquito oocyte (19, 21), occur on the sides and inner ends of the crevices. Some of the pits are about the same diameter as the vesicles which abound in the cortical cytoplasm of the oocyte. The pits and vesicles are lined with a layer of dense granular material and are coated on their cytoplasmic surface with fibrils or bristles (Fig. 4, inset). The diameter of the coated vesicles varies from about 0.13 μ to 0.17 μ, the inner lining being about 300 A thick and the outer bristles 200 A long. If it is correct to interpret them as pinocytotic vesicles, then it is clear that the granular lining is derived from material adsorbed to the oocyte surface in the crevices.

Membrane-limited spheres in the oocyte cortex range in diameter from that of the coated vesicles to 2 μ. The granular contents of the larger spheres are homogeneously distributed rather than adsorbed to the inner surface of the membrane. In their position and structure these spheres are indistinguishable from the small yolk spheres seen in the light microscope (25, 26). Based on the absence of any other obvious mechanism for the transfer of extracellular protein to the yolk spheres, one would imagine that the vesicles coalesce to form the yolk spheres.

Vesicles of smaller diameter (420 to 630 A) are also abundant in the cortical cytoplasm particularly around the yolk spheres (Fig. 4, t). Tubules of the same diameter are frequently contiguous with the limiting membrane of the yolk (Fig. 4, T). Similar configurations were described by Roth and Porter (21) who suggest that they are either remnants of coalesced vesicles or membranous cytoplasmic components contributing material to the yolk bodies, and in view of autoradiographic evidence that proteins synthesized in the cortex of the oocyte are contributed to the yolk spheres (13) they may be provisionally interpreted as a manifestation of the transfer of internally synthesized protein to the yolk spheres. However, in the cricket oocyte Favard-Sérène (7) demonstrated a great abundance of similar tubules which, during yolk formation, enclose in their extremities the dense contents of the pinocytotic vesicles, clearly suggesting that the tubules serve as transport channels for extracellular material to the yolk spheres.

These small vesicles and less well defined membranes are likely constituents of the endoplasmic reticulum of the cortical cytoplasm. Ribosomes occur singly, in clusters, and less frequently associated with membranes (Fig. 4). Lipid droplets of various sizes are easily recognized by their multilobular shape. Mitochondria are not abundant in the oocyte cortex in regions of active yolk formation.
OOCYTE SURFACE UNDER NURSE CELLS: Autoradiographic studies have indicated that yolk spheres are not formed at an appreciable rate at the nurse cell borders of the oocyte (3, 13). Electron micrographs of this region (Fig. 6) show that several layers of follicle cells, attenuated laterally and with little intercellular space, are interposed between the nurse cells and the oocyte. In comparison with the vitellogenic surfaces, the microvilli of the follicle cells and the crevices of the oocyte surface are reduced in length, irregularly oriented, and are not clearly separated by a vitelline membrane. The flocculent intercellular material is less dense and less abundant here presumably because of a reduced availability of blood proteins resulting from the greater distance from the hemocoele and the reduced interfollicular cell spaces. Concomitantly coated pits, and vesicles are fewer and smaller (about 0.08 μ in diameter). The small yolk spheres are also less numerous whereas mitochondria are more abundant. Roth and Porter (21) find that even in the previtellogenic mosquito follicle the part of the oocyte facing the nurse cells shows relatively few microvilli, pits, and vesicles. Clearly the abundance of vesicles, their size, and density of contents are directly related to rate of yolk formation.

OOCYTES DEVELOPING IN OVARIES TRANSPLANTED TO MALES: Ovaries transplanted from diapausing female pupae to diapausing male pupae develop during the differentiation of the adult male, producing smaller than normal eggs (27). Since normal size eggs are produced if the males are injected with female blood which is rich in vitellogenic protein, the small size eggs are presumed to result from deficiencies of vitellogenic blood proteins in the male. Thus it was of interest to examine the fine structure of the cortex of these eggs. Ovarian transplants were prepared according to the methods of Telfer and Rutberg (27) and follicles were dissected for fixation when the male was on about the 19th day of adult development. In electron micrographs the cortex of these eggs shows a striking difference from that of the normal egg (Fig. 5). It is similar to the condition found in the cortex under the nurse cells, in that the crevices forming the brush border are irregular and contain little dense flocculent material. The vitelline membrane, however, is wider than in normal eggs where the membrane would be attenuated by the more rapid increase in size of the egg. Coated pits and vesicles are numerous although they are smaller than in normal eggs (0.08 μ to 0.1 μ in diameter). Most yolk bodies in the periphery contain loosely packed flocculent material (Fig. 5, Y); only a few normal ones occur. Lipid droplets and annulate lamellae are conspicuous in the cortex possibly because of the relative paucity of normal yolk bodies. Annulate lamellae are not as frequently seen in normal oocytes and then only well below the cortex.

OOCYTES EXPOSED TO FERRITIN DURING YOLK FORMATION: In order to test the proposal that the material lining the pinocytotic vesicles and the flocculent material observed in the interfollicular cell space and the crevices of the oocyte surface both represent blood proteins destined to be incorporated into the yolk spheres, the follicles of females which had been injected with ferritin were examined. A 20 per cent solution of ferritin (K and K Laboratories, Inc., Jamaica, New York, horse spleen ferritin) was dialyzed through cellophane against 0.15 M NaCl to reduce the possibility of toxic impurities, and 0.7 ml of this prep-
aration plus a few crystals of phenyl thiourea were injected into the hemocoele of a 3.5 gm female on about the 17th day of adult development. Follicles were removed from this animal 21/2 hours later and fixed and sectioned according to the usual procedure, except that the sections were not stained with lead.

Since most foreign proteins introduced into the hemocoele can be recovered from the yolk in minor amounts (24, 25) it was anticipated that a substantial concentration would have to be established in the hemocoele to make it readily detectable in the follicle. This maneuver carried with it the hazard of toxicity effects. Follicles exposed to these levels of ferritin in vitro and those dissected 4 hours or longer after the injection had an abnormally attenuated brush border. Since 21/2 hours' exposure in vivo did not detectably alter the fine structure of the follicle, however, there are grounds for hoping that the distribution of ferritin observed at this time reflected the normal route of entry of vitellogenic blood proteins.

After 21/2 hours' exposure it is clear that the oocytes had incorporated this foreign protein into the yolk spheres (Fig. 8). Within the cortex of the oocyte the ferritin was localized primarily in the vesicles and small yolk spheres (Figs. 7, 8) although there was some ferritin free in the cytoplasm. On the surface of the oocyte an abundance of ferritin was associated with and tended to be on the surface of the flocculent material covering the crevices and their pits (Fig. 7). Ferritin was equally abundant in the vitelline membrane, in the flocculent material between follicle cells, and within the basement lamella of the follicle cells. The cytoplasm of the follicle cells also contained ferritin.

CONCLUSIONS

PROTEIN TRANSPORT TO THE OOCYTE SURFACE: The presence of ferritin in the interfollicular cell spaces and in the crypts of the oocyte surface confirms fluorescein-labeled antibody and autoradiographic studies (25, 26), in indicating that proteins can readily penetrate all the extracellular spaces and membranes of the follicle. The presence of ferritin throughout the matrix of the basement lamella and the vitelline membrane provides additional support for this conclusion. It can be therefore inferred that the flocculent material which occupies the intercellular spaces and the crypts in the oocyte surface is composed, to a significant degree, of blood proteins.

Ferritin within the cytoplasm of the follicle cells might lead one to suppose that the follicle cells also function as a pathway for proteins to the oocyte surface. Ramamurty (17) demonstrated that the follicular epithelium of the scorpion fly oocyte contained detectable amounts of trypan blue only after long exposure to the dye when the oocyte cortex no longer incorporated the dye, suggesting that the transport mechanism through the follicle cells had ceased to function, thus allowing dye to accumulate in the follicle cells. On the other hand, penetration of cells by either dye or ferritin could result from fixation damage or from pathological conditions, and thus these observations must be considered with caution. Ferritin would be expected, under non-pathological conditions, to enter the cells by pinocytosis. The occasional vesicles within the follicle cells which stained immunologically for blood proteins (25) do not indicate intensive pinocytotic activity. Therefore it seems probable that if there is passage of blood proteins through the follicle cells it concerns a much less significant amount than reaches the oocyte surface directly through the intercellular space. Since the follicle cells do not directly contact the oocyte during yolk formation, it is necessary to conclude that even if the follicle cells serve a transport function, they must finally yield the proteins to the intercellular spaces.

PROTEIN UPTAKE BY THE OOCYTE: Structures which suggest pinocytotic activity at the surface of the oocyte include the deeply folded cell membrane with a layer of dense material adhering to it, coated pits along the folds, and coated vesicles in the cortical cytoplasm of about the same diameter as the pits and containing material of similar structure and density.

These features are fundamentally similar to those described in developing oocytes of the mosquito (19, 21), the milkweed bug (9), the American cockroach (1), and the scorpion fly (4). Their interpretation as stages in the process of pinocytosis is confirmed here by their content of ferritin which has reached the oocyte through the blood.

There are a number of additional correlations between pinocytosis and the occurrence of yolk formation. Observations on the preyolk forming oocytes of the mosquito (21) indicate that vesicles appear in the cortical cytoplasm prior to yolk formation but that they are smaller and less numerous than those of a rapidly growing oocyte. In the preyolk forming oocyte of the cockroach,
Anderson (1) notes that the vesicles are fewer and contain less particulate material than during yolk formation; in addition it appears from the illustrations that the vesicles are smaller than those of a vitellogenic oocyte. Even in oocytes which are actively forming yolk, any region of the cell surface to which blood proteins are not readily accessible shows a reduction of pinocytotic configurations. Thus few and smaller vesicles occur at the surface facing the nurse cells in developing oocytes of eecropia and in the previtellogenic oocyte of the mosquito (21). Bier and Ramamurty (4) point out that pits are less numerous under the follicle cells than at the surfaces facing the spaces between them. Finally, in the developing but abnormally small oocytes grown in male cecropia pupae, in which there is a deficiency of vitellogenic blood protein, the vesicles are smaller than normal and contain less internal material. The loosely packed cortical yolk spheres presumably result from the deficient blood protein uptake. These observations are all consistent with the proposal that the formation of vesicles and yolk spheres is profoundly affected by the availability of vitellogenic blood proteins.

Uptake of ferritin at the oocyte surface seems clearly to be by formation of vesicles from the pits in the folded membrane since ferritin is confined to the outer surface of the oocyte and within the vesicles near the surface. That ferritin is taken into cells via plasma membrane invaginations has been demonstrated previously, for example, in the renal glomerulus of the rat (6), in Kupffer cells of rat liver (20), the renal proximal tubules of Necturus (12), and in the spinal ganglion cells of the toad (18). In the latter three cases, as in the insect oocyte, the invaginations are characterized by luminal and cytoplasmic coatings.

It is clear from the distribution of ferritin in the cortical cytoplasm of the oocytes that much of the ferritin introduced into the cell via the vesicles reaches the yolk spheres whereas a lesser amount is free in the cytoplasm. This distinct localization of ferritin suggests that neither fixation damage nor pathological conditions were major factors affecting the distribution of ferritin, and that the transfer of ferritin from the vesicles is more directly to the yolk spheres rather than through the cytoplasm. How this transfer is accomplished in the cecropia oocyte is not resolved. It may be by way of tubes as Favard-Sérêno describes in the cricket oocyte (7). In the mosquito oocyte, Roth and Porter (21) point out that the pieces of membranes and vesicles found attached to yolk spheres may be remnants of vesicles which have coalesced to form yolk spheres or they may be associated with some cytoplasmic contributions to the yolk. The autoradiographic evidence of Melius and Telfer (13) that proteins synthesized in the cortex of the oocyte are contributed to the yolk bodies gives impetus to further investigating the latter proposal.

Intraoocyte synthesis is ascribed as a major source of yolk proteins in the crayfish oocyte, from convincing cytological evidence (2). Granules, apparently precursors of protein yolk, occur within the rough-surfaced endoplasmic reticulum; cisternae of the endoplasmic reticulum are expanded with granules which appear to undergo transformation into finely granular protein yolk bodies (2). It was also noted that pinocytotic vesicles occur at the oocyte periphery (2), thus opening the possibility of uptake of blood proteins as a lesser source of yolk proteins. It is proposed by Wartenberg (29) that yolk platelets of amphibian oocytes are comprised of proteins arising from several sources. Such multiplicity in origin of yolk proteins may prove to be of general occurrence in animal oocytes.

The encouragement and assistance of Dr. W. H. Telfer in the course of this work and in the preparation of the manuscript is gratefully acknowledged, as is the technical assistance of Miss Elizabeth Wallace.

The work was supported by Grants AI-05075 and GM-08-977 from the National Institutes of Health and Grant G-11229 from the National Science Foundation.

Received for publication, August 30, 1964.


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