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The Distribution of Hydrolytic Enzymes and Ribonucleic Acid in Subcellular Fractions from Eggs and Adult Tissue of Arbacia punctulata

Charlotte Jackson
College of William & Mary - Arts & Sciences

THE DISTRIBUTION OF HYDROLYTIC ENZYMES AND RIBONUCLEIC ACID IN SUBCELLULAR FRACTIONS FROM EGGS AND ADULT TISSUE OF ARBACIA PUNCTULATA

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Charlotte Jackson
Author

Approved, January 1965:

Robert E. L. Black
Robert E. L. Black, Ph.D.

Mitchell A. Byrd
Mitchell A. Byrd, Ph.D.

Charlotte P. Mangum
Charlotte P. Mangum, Ph.D.

Bruce L. Welch
Bruce L. Welch, Ph.D.

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ABSTRACT

Homogenates of adult gut tissue and unfertilized eggs of *Arbacia punctulata* have been fractionated by centrifugation to determine the distribution of several hydrolytic enzymes and of nucleic acid among the subcellular fractions. The isolation of a large granule fraction containing an appreciable concentration of acid hydrolases within unfertilized eggs has been attempted.

Differential centrifugation was applied to both adult gut tissue and unfertilized eggs suspended in sucrose homogenates to determine the distribution of acid hydrolases among the various subcellular fractions. Isolation of specific types of granules containing acid hydrolases and ribonucleic acid was attempted on the large visible granule fraction of the egg homogenate by centrifugation on sucrose density gradients.

The results obtained suggest that three types of large granules exist in the egg. The first type contains high specific activities of acid phosphatase, esterase, and lipase. The second type contains the highest specific activity of proteolytic activity at pH 5; this fraction also contains the highest specific activity of succinic dehydrogenase, a mitochondrial enzyme. The third type contains the highest specific activity of RNAase and the greatest amount of ribonucleic acid.

Differential centrifugation of both gut and egg homogenates indicates the existence of submicroscopic granules containing high concentrations of acid phosphatase and esterase; these may represent either fragments of endoplasmic reticulum or lysosomes.

The possible roles of the visible granules at fertilization and during embryonic development are discussed.

THE DISTRIBUTION OF HYDROLYTIC ENZYMES AND RIBONUCLEIC ACID IN SUBCELLULAR FRACTIONS FROM EGGS AND ADULT TISSUE OF *ARBACIA PUNCTULATA*

INTRODUCTION

Specific cellular granules containing hydrolytic enzymes were first identified by de Duve and co-workers (1955) by means of centrifugal fractionation of rat-liver homogenates. The name lysosome was proposed for these granules because five distinct acid hydrolases, apparently located within them, seemed to be released in a parallel manner in preparations subjected to disruptive treatments such as freezing and thawing. Prior to de Duve's isolation of a fraction rich in hydrolytic enzymes however, Berthet et al (1951) had studied the nature of the linkage between acid phosphatase and mitochondria in rat-liver tissue. Their data indicated that the particles containing acid phosphatase behave as osmotic systems. The simplest schematic model conforming to their description was that of a sac consisting of fluid enclosed in a semi-permeable membrane. Laird et al (1953) had also discovered two "types" of mitochondria in the rat liver, the smaller of which had a ratio of pentose nucleic acid to phosphate/nitrogen that greatly exceeded even the microsomes. The same year, de Duve et al, (1953), while examining further the enzymic content of the mitochondrial fraction, also mentioned the possibility of a separate set of granules containing the hydrolytic enzymes. From the first, quantitative purification has not been

necessary to characterize the lysosomes as a separate group of particles. Most of the information has been obtained by studying the distribution curves of the particle-bound acid hydrolases in a variety of centrifugal systems.

The problems of identifying the granules indicated by the distribution curves of hydrolase activity stimulated the development of techniques to describe the morphology of the granules. Although correlated biochemical and morphological studies have only been carried out on a few tissues, electron micrographs demonstrate the existence of particles thought to be lysosomes. Novikoff, Beaufay, and de Duve (1956) made a preliminary investigation of lysosome-rich fractions obtained from rat liver by differential centrifugation. This investigation revealed the presence of hitherto undescribed cytoplasmic particles which they designated "dense bodies." The dense bodies were tentatively identified by them as hepatic lysosomes and later investigations proved them to be correct. Essner and Novikoff (1961) then attempted to locate the acid phosphatase activity and thus the lysosomes by staining frozen sections of rat and human liver for acid phosphatase by the Gomori lead-glycerophosphate procedure. The electron micrographs revealed that the acid phosphatase was located in the structures believed to be lysosomes. Holt (1959) by using a modification of the Gomori acid phosphatase procedure, obtained even more accurate results on the cytological distribution of acid phosphatase. Holt and Hicks (1961) then correlated the results obtained by anatomical and biochemical data, and definitely concluded that the vacuolated dense

bodies seen in the electron microscope are the morphological counterparts of the lysosomes defined biochemically by de Duve. Rahman (1962a) later made similar correlations of biochemical and cytological data in the rat thymus. Bauhuin and Beaufay (1963) also found that the specific granules found by Holt and Hicks (1961) to be lysosomes corresponded to suspected lysosomal "dense bodies" in similar electron micrographs. In addition, biochemical and staining analyses have recently revealed that the Golgi apparatus is involved in the formation of the acid-phosphatase rich secretory vacuoles and dense bodies of neurons and hepatic parenchymal cells (Novikoff, 1963). A summary of the morphological details revealed by electron microscopy has been given by Rhodin (1963), who has described lysosomes as bodies having a highly pleomorphic appearance with large or small, dense granules and short, irregular or long, concentrically arranged membranes. He has also noted that structurally, this type of inclusion body can be identified in almost any kind of cell. A more detailed morphological concept seems to be limited at the moment since the fine structure of lysosomes appears to vary considerably from one cell to another. Although reliable cytochemical techniques exist only for acid phosphatase at the present, the development of similar techniques for the localization of other hydrolytic enzymes is being explored (Novikoff, 1963).

Although the present concept of the lysosome is based on both its biochemical and morphological properties, the biochemical properties have been more extensively

investigated. The accepted schematic models of the lysosome at the present are therefore derived primarily from biochemical data. Novikoff (1961) and de Duve (1963) have presented the most recent model based on a summary of data obtained with rat-liver lysosomes. The model suggests that the lysosome contains at least twelve separate hydrolytic enzymes showing an acid pH optimum. These include ribonuclease, acid-deoxyribonuclease, acid phosphatase, phosphoprotein phosphatase, cathepsin, collagenase, alpha-glucosidase, beta-N-acetylglucosaminidase, beta-glucuronidase, alpha-mannosidase, and aryl-sulfetase. The accessibility of these hydrolases to substrates in the surrounding cytoplasm is presumed to be restricted by a lipoprotein membrane (Beaufay and de Duve, 1959). The enzymes within the lysosome therefore become soluble and accessible only when this membrane is injured or destroyed (Shibko, et al, 1963). However, since this model is based primarily on rat-liver lysosomes, it will be necessary to determine to what extent the results with this tissue are applicable to other biological materials before the biochemical concept of the lysosome can be generally accepted. An alternative model to the membrane hypothesis of de Duve and Novikoff has been presented by Koenig (1962). On the basis of high concentrations of brain gangliosides found in grenules having some of the properties of lysosomes, he proposed that lysosomes are composed of macromolecules of enzyme and non-enzyme protein conjugated to acidic glycolipids by ionic, covalent, and/or other bonds. He stated that acid hydrolases within the lysosome are inert because they are bound to glyco-

lipoproteins and that these enzymes are released or activated by cleavage of the glycolipoprotein-enzyme bonds instead of membrane rupture. Koenig (1963) added support to this hypothesis by demonstrating the vital staining of lysosomes by acridine orange and a wide variety of other cationic molecules which liberate acid phosphatase from lysosomes in vitro without disrupting the lysosomal matrix. This also indicated, according to his interpretation, that the lysosomal enzymes are bound to anionic sites of the matrix by electrostatic bonds. Most investigators at the present, however, support the sac-like model of de Duve. Recently, Sawant, Desai, and Tappel (1964) added additional support to de Duve's model by carefully studying factors such as osmotic swelling, temperature, pH, and permeability changes that affect the lysosomal membrane. Their results on the availability of lysosomal enzymes over a wide range of pH values did indicate binding of these enzymes at different sites in the lysosome. The differential release of bound hydrolases from rat-liver lysosomes treated with a non-ionic surface active substance Triton X-100 (Ugazio and Pani, 1963) also indicates that some of the hydrolases have different binding sites.

Both biochemical and morphological concepts of the lysosome must be considered in determining the function of the lysosome particles within a cell. The possible roles of the acid hydrolases within cells have been investigated by several workers. However, the variety of cell structures which demonstrate hydrolase activity has made it very

difficult to formulate a concise functional concept (see below). The presence of acid hydrolases characteristic of lysosomes indicates that, regardless of the cell type or location, acid digestion is the principal role of the lysosome. de Duve (1959) has stated that the lysosomes appear to be powerful digestive systems, capable of breaking down nucleic acids, phosphate esters and nucleotides, proteins and phosphoproteins, as well as the various components of mucopolysaccharides. The relatively impermeable lipoprotein membrane enclosing the enzymes presumably protects the cell contents against the destructive action of its own hydrolases (Beaufay and de Duve, 1959). Cell injury and destruction however can both be caused by the lytic action of the lysosome (Quie and Hirsch, 1964). Recent studies of leucocytes and macrophages exposed to streptolysins indicate that the lytic effect of the streptolysins on the lysosomes causes rapid and massive degranulation of the living phagocytic cells, soon followed by extensive alterations of the cytoplasm and nucleus and by the death of the cell (Keiser, et al, 1964). The lysosome, regardless of location, is considered to be involved in the digestion of foreign material engulfed by phagocytosis or pinocytosis and in the breakdown of the cell's own constituents, both under many normal conditions, and in necrosis and other degenerative conditions (de Duve, 1959).

A diversity of cell structures in tissues other than rat liver has been shown to exhibit acid hydrolase activity. Those cell structures include the protein droplets of the

mammalian kidney (Straus, 1954); the phagocytic vacuoles of the polymorphonuclear leucocytes and the macrophages (Quie and Hirsch, 1964; Rahman, 1962b); the dense bodies found especially in neurons and in most mammalian livers (Novikoff, et al, 1956; Holt and Hicks, 1961); the cytolysosomes of the kidney and neurons (Novikoff, 1961, 1963); the early secretory granules found in exocrine and endocrine cells as well as functional hepatoma cells (Novikoff, 1961, 1963; Essner and Novikoff, 1962); and alpha and beta metachromatic granules observed in embryonic development (Dalcq, 1963; Marsland, et al, 1960; Rebhun, 1959). The latter structures are described in detail below. Investigation of acid hydrolases has also been extended to some invertebrates, although for most only brief surveys have been made of characteristic enzymes, and no attempts to isolate any specific granules have been reported. Dodgson, Spencer and Lewis (1953) examined aryl-sulfatase and beta-glucuronidase activity in various marine mollusks. Müller, Tóth, and Töró (1960) have investigated the histophagous ciliate, Tetrahymena corlissi, as well as other protozoans, in an attempt to correlate the protozoan food vacuoles with the lysosome concept. The most extensive survey of invertebrates has been done by Shibko, Caldwell, Sawant, and Tappel (1963) who found measureable acid hydrolases in the tissues of the Coelenterata, Mollusca, Echiurida and Arthropoda, as well as in those of birds and amphibians. Janoff and Haurylko (1963) examined the amoeboid leucocytes of Mercenaria mercenaria and Asterias forbesi for acid hydrolases known to occur in rabbit phagocytic cells. Slight

acid phosphatase activity and considerable acid-RNAase activity were found in both species. Differential centrifugation of the cell lysates revealed that the activity of each enzyme was as high or higher in the supernatant fraction as in the lysosome-mitochondria fraction. Zeidenberg and Janoff (1964) have recently demonstrated acid hydrolase properties in homogenates of the lobster kidney, as well as in the excretory organs of three species of marine fish.

Granules having the characteristics of lysosomes have not been studied extensively in embryonic tissues. Lysosomal activity has been suspected in a few instances involving regression and resorption of embryonic cells, particularly those of the Müllerian duct rudiments of male chick embryos (Scheib-Pfleger and Wattiaux, 1962) and in the tails of amphibians undergoing metamorphosis (Weber, 1963). The latter investigator fractionated the tail tissue of growing amphibian embryos and demonstrated that cathepsin and acid phosphatase are concentrated in cytoplasmic particles of relatively low stability. Dalcq (1963) demonstrated cytochemically that high acid phosphatase activity is associated in the rat egg with granules which also exhibit metachromatic properties. Such granules exist in oocytes and undivided fertilized eggs in minute size and gradually increase in size during cleavage. Dalcq concluded that their primary role was in such energy consuming activities as cleavage. Large granules exhibiting metachromatic properties have also been observed in developing eggs of various invertebrates. Staining with suitable vital dyes has made

it possible to study the distribution of granules during successive cleavage stages more successfully than in mammalian eggs. Pasteels and Mulnard (1957, 1963), and Dalcq (1963) have followed the fate of such granules in bivalved mollusks, sea urchins, and tunicates. The metachromatic granules observed in invertebrate eggs appear to be of two types, designated as alpha and beta granules by Pasteels (1958). The smaller, more rapidly staining granules are the alpha granules, and the more conspicuous granules, which stain more slowly are the beta granules. Pasteels concluded that the beta granules are not stained directly but receive dye from the smaller alpha granules. Pasteels and Mulnard (1957) found that after centrifugation the alpha granules are found near the centrifugal extremity of the egg or blastomeres, while the beta granules are sedimented in the hyaline part of the cytoplasm. Dalcq (1963) has recently concluded from comparative electron microscopic and cytochemical investigations of early developmental stages that the yolk platelets are a source of definite organelles with phosphatase activity. Pasteels and de Harven (1963) confirmed this by a series of electron microscope findings which demonstrated the transformation of yolk platelets into microvesicular bodies similar to the metachromatic granules observed in the living eggs, and the rupture of the microvesicular bodies to release minute phosphohydrolase granules. The only resemblances these granules have to lysosomes, however, are their apparent ability to rupture and their possession of acid phosphatase.

The identification of acid hydrolase granules in embryonic tissue has been based only on electron microscopy and cytochemistry. Dalcq (1963) proposed at a recent symposium that the most direct approach to ascertaining the presence or absence of particles containing specific hydrolases would be the application of homogenization and density gradient centrifugation to appropriate eggs at various stages to determine whether a layer of particles containing an array of lytic enzymes can be isolated. This type of investigation has not previously been reported for eggs of any animal. The present investigation is therefore concerned with the problem of isolating a fraction containing an appreciable concentration of acid hydrolases from homogenates of eggs and adult gut tissue of the sea urchin, Arbacia punctulata. To determine the distribution within the major subcellular fractions, differential centrifugation has been applied to sucrose homogenates of both eggs and adult gut tissue. Isolation of specific sets of granules containing acid hydrolase activity has been attempted only on the large visible granule fraction of the egg homogenate by centrifugation on sucrose density gradients. The results obtained by the differential and density gradient centrifugation clearly indicate the existence in the egg of several types of large granules possessing different hydrolytic enzymes.

MATERIALS AND METHODS

Biological Procedures

The animals used in this study, Arbacia punctulata, were collected along the Eastern Shore region of the Chesapeake Bay. They were stored in the laboratory at 23° C in aerated, polyethylene aquaria filled with artificial sea water at 33 0/00 made from Utility Seven-Seas Marine Mix (Utility Chemical Company, Patterson, New Jersey). In order to obtain adult tissue, the entire gut was removed by dissection, placed in cold, artificial sea water, blotted carefully, and weighed. Eggs were obtained by electrically inducing shedding in the females, using alternating current at 30 volts (Harvey, 1954). The eggs were washed three times by settling in artificial sea water prepared from reagent grade salts and distilled water. To remove the jelly coat, the eggs were treated with acid sea water at pH 4.6-4.8 and allowed to settle (Allen, 1957). The eggs were washed an additional time in sea water buffered at pH 8.0 with 0.02 M tris-(hydroxymethyl) aminomethane (hereafter referred to as "tris") and packed in a hand centrifuge to prepare them for homogenization.

Adult gut and eggs were prepared for fractionation by the same methods. A 10% homogenate of each tissue was made, based on a weight/volume ratio for the gut tissues and

on a volume/volume ratio in the eggs. The tissues were homogenized at 0° C in a hand-operated Tenbroeck glass homogenizer in 0.98 M sucrose containing 10^{-3} M ethylenediamine tetraacetic acid (EDTA) and buffered at pH 7.5 with 0.05 M tris. This buffered sucrose was used for suspending all of the cell fractions obtained by later centrifugation, as indicated below. The nuclei-free starting material was prepared by the procedure of Berthet and de Duve (1951) with modifications to allow for the differences in sucrose density. The homogenate was centrifuged for ten minutes at 2000 rpm for the gut tissue and at 500 rpm for the eggs in rotor No. 253 in an International PR-2 centrifuge at 0° C. The egg homogenate was centrifuged at the much lower speed to remove debris and whole cells without removing the majority of heavy granules. The supernatant fluid was then decanted and saved. The nuclear fraction from each tissue was rehomogenized in an additional 3 to 5 ml of buffered 0.98 M sucrose-EDTA and recentrifuged at the same speed as before. The combined supernatant fluids were used for the subsequent isolation of granules. The final precipitate containing a few clumped nuclei, cell fragments, and whole cells was discarded.

The various subcellular fractions were obtained by a modification of the technique used by Applemans, Wattiaux, and de Duve (1955). The procedure is outlined in the flow sheet given below. All of the operations described were carried out at or near 0° C. The granule fractions were obtained by centrifuging the homogenates in a swinging-

bucket type SW rotor in a Beckman Model L preparative ultracentrifuge. In the procedure outlined below, each granule fraction was washed once by resuspension in buffered sucrose-EDTA and recentrifugation at the forces and times indicated. Each supernatant fluid obtained by washing the granules of a particular fraction was combined with the original supernatant fluid before centrifugal isolation of the subsequent fraction. All granule fractions obtained were suspended in 2 to 5 ml of 0.98 M sucrose, containing 0.05 M tris and 10^{-3} M EDTA, at pH 7.5. The preparations were either used immediately or frozen at -18° C for later use. The times and forces of centrifugation for each fraction were as follows. Fraction I, consisting of heavy granules, was obtained by centrifugation at 17,500 times gravity for 15 minutes. Fraction II, light granules, was obtained by centrifuging the supernatant fluid after removal and washing of Fraction I at 63,000 times gravity for 28 minutes. The supernatant fluid from Fraction II was centrifuged at 90,000 times gravity for 150 minutes to obtain a microsomal fraction, designated Fraction III. The final supernatant fluid remaining after removal and washing of the above granule fractions was designated Fraction IV.

Fraction I from eggs was centrifuged on a layered sucrose gradient to separate particles of different densities (de Duve, Berthet, and Beaufay, 1959). Cold sucrose layers of densities 1.1513, 1.1663, 1.1868, 1.1972, and 1.2092 were placed in the order given over a cushion of 2.5 M sucrose,

by use of a syringe and blunted 22 gauge needle. The tubes used for the gradients were kept cold to eliminate convection currents during the layering process. The granule fractions were layered on the top of the gradients, and the tubes were centrifuged for one hour at 90,000 times gravity. The isolated granule layers were removed by carefully pipetting the layers from the top, or by puncturing the bottom of the tubes and allowing the sucrose to drip out slowly. The granule fractions were then suspended in 0.98 M sucrose-EDTA and either used immediately or stored at -18° C.

The staining properties of the granules obtained on the density gradient were examined by dividing Fraction I into three equal portions. One tube served as a control, the other two contained 10 to 15 drops of 0.1% toluidine blue or methyl red. After an initial centrifugation at 17,500 times gravity to remove the excess stain, the stained preparations were placed over the same density gradients as above, and centrifuged for one hour at 90,000 times gravity.

Chemical Procedures

Determinations were made for protein, nucleic acid, succinic dehydrogenase, acid phosphatase, proteolytic activity, esterase, lipase, aryl-sulfatase, ribonuclease, and beta-galactosidase according to the methods outlined below.

The fractionation method of Schmidt and Thannhauser (1945) was used to obtain nucleic acid and protein. Two ml

Outline of Fractionation Procedure

Whole homogenate in 0.98M sucrose, 10^{-3} EDTA, 0.05 tris, pH 7.5.

Centrifuged at 500-2000 rpm for 10 minutes to remove nuclei, whole cells, and debris. Washed once.

Precipitate discarded.

Supernatant fluids combined and centrifuged at 17,500 times gravity for 15 minutes. Washed once.

Precipitate suspended in buffered 0.98 M sucrose. Fraction I.

In eggs only, Fraction I recentrifuged on gradient of densities 1.1513, 1.1663, 1.1868, 1.1972, 1.2092, and 2.5 M for one hour at 90,000 times gravity. Granule fractions A. B. C. D. and E.

Combined supernatant fluids centrifuged at 63,000 times gravity for 28 minutes. Washed once.

Precipitate suspended in buffered 0.98 M sucrose. Fraction II.

Combined supernatant fluids centrifuged at 90,000 times gravity for 150 minutes. Washed once.

Precipitate suspended in buffered 0.98 M sucrose. Fraction III.

Combined supernatant fluids. Fraction IV.

of cold 10% W/V trichloroacetic acid (TCA) were added to 0.5 to 1.0 ml of each fraction. The precipitate was washed in additional cold TCA until the pigment was completely removed from each fraction. All supernatant fluids were discarded. The precipitate was resuspended in 2 ml of boiling ethanol-ether (3:1 V/V). The precipitate was cooled, centrifuged, and washed once in additional ethanol-ether. The supernatant fluids were discarded. The precipitate was then suspended in 1 to 2 ml of 5% TCA and heated for 15 minutes at 90° C on a water bath. This process was repeated with an additional 1 to 2 ml of 5% TCA. The hot TCA extracts were combined and used for the nucleic acid determinations. The protein precipitate was suspended in 0.5 to 1.0 ml of 1 N sodium hydroxide. The method of Lowry et al (1951) was used for the protein determinations. The incubation reagent was prepared by mixing 50 ml of 2% W/V sodium carbonate with 1 ml of 0.5% W/V cupric sulfate in 1% W/V sodium-potassium tartrate immediately before use. An aliquot of 0.2 to 0.4 ml of the suspended protein solution was mixed with 5 ml of the incubation reagent and allowed to stand for ten minutes at room temperature. Five-tenths ml of 1 N Folin-Ciocalteau reagent (#So-F-24 Fisher Scientific Company) were then added rapidly with a syringe, and the solution was mixed thoroughly and allowed to stand for 30 minutes at room temperature. The readings were made at room temperature on a Klett-Summerson colorimeter using green filter #54. The values were converted to micrograms of protein by comparison with a standard curve obtained from 0.5% W/V crystalline bovine serum albumin

(Nutritional Biochemicals Corporation) in 1 N sodium hydroxide.

The extraction procedure used for the nucleic acid was that of Schmidt and Thannhauser (1945) as described above. The ultra-violet procedure mentioned by Schneider (1957) was used to determine total nucleic acid. Samples were diluted appropriately and read at 260 millimicrons on the Beckman DU spectrophotometer. Several concentrations of ribonucleic acid were prepared from a stock solution of 1 mg per ml of commercial reagent grade ribonucleic acid (Nutritional Biochemicals Corporation #1576) dissolved in 5% TCA, and the absorbencies of these were determined at 260 millimicrons, in order to obtain a standard curve.

The method of Bonner (1955) was used for the determination of succinic dehydrogenase. A reaction mixture was prepared by adding a total of 2.6 ml of 0.07 M phosphate buffer (pH 7.4), 0.01 M neutralized potassium cyanide, 0.001 M potassium ferricyanide, and 0.015 M sodium-succinate and incubating the mixture at 25° C for 30 minutes. Readings were made on a Beckman DB recording spectrophotometer against water blanks containing the same amount of enzyme. Calculations were based on that part of the curve obeying zero order kinetics. Activity was expressed in micromoles of ferricyanide reduced per minute per microgram of protein.

Acid phosphatase was determined by a method based on that of Burch (1957). The substrate used, p-nitrophenyl phosphate (Sigma 104 Phosphatase Substrate, Sigma Chemical Company), was prepared by dissolving 40 mg in 10 ml of glass distilled water. The substrate, 0.5 ml p-nitrophenyl

phosphate, was added to 0.5 ml of 0.1 M sodium acetate (pH 5.0) and 0.1 ml of enzyme and incubated at 25° C for 15 minutes. To stop the reaction, 0.04 M sodium hydroxide was added to make a total volume of 5.0 ml. Determinations of p-nitrophenol were then made with blue filter #42 on a Klett-Summerson colorimeter. The enzyme activity was expressed as micromoles of p-nitrophenol liberated per minute per microgram of protein, based on a p-nitrophenol standard curve. The p-nitrophenol was obtained from Sigma Chemical Company.

Proteolytic activity at pH 5 was determined by the method of Herriott (1955). The substrate contained 2% W/V denatured hemoglobin (#8938 Nutritional Biochemicals Corporation) in sodium-acetate buffer pH 5.0. One-tenth ml of enzyme and 0.5 ml of substrate were incubated for 15 minutes at 25° C. One ml of 5% TCA was added, and the solution was centrifuged to settle the precipitate. To 0.7 ml of the digestive supernatant were added 1.4 ml 0.5 M sodium hydroxide and 0.4 ml 1N Folin-Ciocalteau phenol reagent (#So-P-24 Fisher Scientific Company). This mixture was allowed to stand 15 minutes before reading at 660 millimicrons on the Beckman DU spectrophotometer. Blanks were prepared in the same manner except the 5% TCA was added to the tubes before the hemoglobin and enzyme. A stock tyrosine solution was prepared by dissolving 289.7 mg dry tyrosine in one liter of 0.02 M hydrochloric acid and diluting this solution ten-fold in 0.2 M hydrochloric acid. A standard tyrosine curve was

prepared by using different concentrations of the diluted stock tyrosine in a total of 0.7 ml. These concentrations of the diluted stock tyrosine were treated in the same manner as the digestive supernatant. The enzyme activity was expressed in micromoles of tyrosine liberated per minute from hemoglobin per microgram of homogenate protein.

The esterase method was based on the procedure outlined by Huggins and Laprides (1947). P-nitrophenyl acetate (Nutritional Biochemicals Corporation) was prepared as a substrate by dissolving 32 mg in 5 ml of methanol. One ml of this solution was then diluted to 50 ml in water for immediate use. For each reaction, 0.6 ml of 0.06 M phosphate buffer (pH 6.8), 0.4 ml p-nitrophenyl acetate, 1.5 ml glass distilled water, and 0.3 ml of enzyme were added to each cuvette and incubated for 15 minutes at 25° C. The reaction rate was determined by the change in readings at 0 and 15 minutes at 400 millimicrons on the Beckman DU or DB spectrophotometer. Blanks were prepared by omitting the substrate. Enzyme activity was expressed in micromoles of p-nitrophenol liberated per minute per microgram of protein based on a p-nitrophenol standard curve.

The method of determining lipase was modified from the esterase procedure of Huggins and Laprides (1947). P-nitrophenyl stearate (Nutritional Biochemicals Corporation) was prepared as a substrate by dissolving 32 mg in 5 ml of methanol and diluting one ml of this mixture to 50 ml in glass distilled water. The p-nitrophenyl stearate is extremely hard to dissolve and precipitates easily when diluted in

water unless the solution is added slowly with constant swirling. The reaction cuvettes, containing 0.6 ml of 0.06 M phosphate buffer (pH 6.8), 0.4 ml of p-nitrophenyl stearate, 1.5 ml of glass distilled water, and 0.3 ml of enzyme, were incubated for 15 minutes at 25° C. Readings were made at 0 and 15 minutes at 400 millimicrons on the Beckman DU or DB spectrophotometer against blanks prepared without substrate. Enzyme activity was expressed in micromoles of p-nitrophenol liberated per minute per microgram protein, based on a p-nitrophenol standard curve.

The procedure for aryl-sulfatase was similar to the esterase technique (Huggins and Laprides, 1947) except for the substrate and pH. Potassium p-nitrophenyl sulfate (Nutritional Biochemicals Corporation) was prepared by dissolving 40 mg in 10 ml of glass distilled water. The reaction cuvettes, containing 0.1 ml of enzyme, 0.5 ml of sodium acetate buffer (pH 5.0), 0.5 ml of p-nitrophenyl sulfate, and 1.9 ml of glass distilled water, were incubated 30 minutes at 25° C. Determinations of p-nitrophenol were then made at 0 and 30 minutes on the Beckman DU spectrophotometer against blanks prepared without substrate. The enzyme activity was expressed in micromoles of p-nitrophenol liberated per minute per microgram protein based on a p-nitrophenol standard curve.

Ribonuclease was determined by the method of MacDonald (1955). Commercial ribose nucleic acid (reagent grade, #1576 Nutritional Biochemicals Corporation) was prepared as a

substrate by dissolving it in 0.1 M acetate buffer (pH 5.0) to a concentration of 3 mg per ml. This solution was freshly prepared and the pH was carefully adjusted each time before use. Two-tenths ml of enzyme and 0.5 ml of substrate were incubated for 10 minutes at 25° C. To stop the reaction, 1 ml of MacFayden's reagent (0.25% W/V uranium acetate in 2.5% TCA) was added. After standing for 30 minutes at room temperature, the solution was centrifuged to settle the precipitate. The supernatant was then decanted and its absorbence at 260 millimicrons was determined on the Beckman DU spectrophotometer after it had been diluted 2.5 times. The blank cuvettes contained 2.8 ml of MacFayden's reagent diluted 5 times and 0.2 ml of enzyme. The enzyme activity was expressed as micrograms of ribonucleic acid solubilized per minute per microgram of protein.

Three techniques were used in an attempt to measure beta-galactosidase activity. The same substrate, p-nitrophenyl-B-D-galactopyranoside (Sigma Chemical Company), was used for each technique in the concentrations indicated below. In the method of Wallenfels (1962), 50 mg of substrate was dissolved in 10 ml of 0.05 M tris buffer, pH 7.6. Five-tenths ml of the substrate were incubated with 0.125 ml of 1 N sodium chloride, 1.675 ml of 0.05 M tris buffer, pH 7.6, and 0.2 ml of enzyme. Determinations were made at 405 millimicrons at 25° C. The change in optical density was noted every 30 seconds for 5 minutes, zeroed against blanks containing appropriate amounts of enzyme and distilled water.

In a similar method to that used for the determination of acid phosphatase (Burch, 1957), 40 mg of substrate were dissolved in 10 ml of glass distilled water. To 0.5 ml of the substrate was added 0.5 ml of 0.1 M sodium acetate pH 5.0, and 0.1 ml of enzyme. The mixture was incubated at 25° C for 5-15 minutes, and the reaction then stopped by adding enough 0.04 M sodium hydroxide to make a total volume of 5.0 ml. Determinations of p-nitrophenol were then made with blue filter #42 on a Klett-Summerson colorimeter. Blanks were obtained by adding the sodium hydroxide to the tubes prior to incubation. The third attempt was based on the esterase technique of Huggins and Laprides (1947). The substrate was prepared in the same manner as the previous acid phosphatase technique. The reaction cuvettes, containing 0.1 ml of enzyme, 0.5 ml of sodium acetate buffer pH 5.0, 0.5 ml of substrate, and 1.9 ml of glass distilled water, were incubated 1-15 minutes at 25° C. Determinations were made on the Beckman DU spectrophotometer against blanks prepared without substrate. None of the above techniques measured any beta-galactosidase activity in the egg homogenates.

RESULTS

Distribution of Enzymes and Nucleic Acid in Subcellular Fractions

The distributions of acid phosphatase, esterase, and nucleic acid in the various fractions obtained by differential centrifugation from the gut are shown in Table I. The highest total acid phosphatase and esterase activities were found in the soluble fraction , while the large granules contained only 10 to 15 percent of the total activity. The highest specific activity of esterase was found in the microsomal fraction (III), while in most experiments the specific activity of acid phosphatase was rather uniformly higher in all the granule fractions than in the whole homogenate. The highest percent of total nucleic acid, as determined from the ultraviolet method, was found in the supernatant fraction, probably representing mostly soluble ribonucleic acid. The percentage in the microsomal fraction, although slightly higher than in the larger granules, is still quite low, possibly indicating that the cells are poor in ribosomal RNA. These findings may be a consequence of the starvation of the animals prior to the fractionation of the tissue.

The distribution of enzyme activities within the subcellular fractions of the egg is presented in Table II. In addition to the enzymes examined in the gut subcellular fractions, determinations were made of proteolytic activity at pH 5, aryl-sulfatase, and RNAase. The highest total acid phosphatase and esterase activities were found in the soluble fraction , but the large granules contained from 25 to 50 percent of the total acid phosphatase activity and from 15 to 40 percent of the esterase activity. This difference in percentage of activity present in the large granules of the eggs is probably due to the presence of granules which would not be present in the gut. The variation in percent activity in the individual experiments was due to the removal of many of these granules in earlier experiments while removing whole cells and debris. The highest specific activity of esterase was found in the microsomal fraction (III); in this respect the distribution is similar to that in the adult intestine. The acid phosphatase in the egg also resembled that of the gut in being rather consistently more concentrated in all granule fractions than in the whole homogenate. Lipase activity, which was not detected in the gut, was prevalent in Fractions I and II as well as in the supernatant fraction (IV). The concentration of activity in these fractions and the absence of activity in the gut indicates that this activity is associated with the granules prevalent in the heavier fractions derived from the egg and absent from the adult tissues. Activity in the supernatant fraction could be due

to the release of enzyme from granules which rupture during homogenization. Proteolytic activity at pH 5 is about evenly distributed between Fraction I, the largest granules, and Fraction IV, the supernatant fluid; little or no activity is found in the other granule fractions. The activity in the supernatant in this case, as with lipase, could be due to the rupture of granules during homogenization. The distribution of aryl-sulfatase is similar to that of lipase and protease at pH 5, ranging from 20 to 30 percent in Fraction I and from 20 to 40 percent in Fraction IV. The highest specific activity of RNAase, as well as the greatest nucleic acid: protein ratio were found in the microsomal fraction, Fraction III. The highest percentages of both RNAase activity and total nucleic acid were found in the soluble fraction; however the recoveries of both these substances were quite high, when the combined amounts in the separate fractions are compared to those in the whole homogenates. It is interesting to note that considerable percentages of the recovered nucleic acid and RNAase were found in the visible granule fraction (I), and in the intermediate granules (II), indicating the probable association of both substances with non-microsomal particles. This association is examined in more detail below.

Density gradient Centrifugation of Fraction I

In order to investigate the possible heterogeneity of the visible granules with respect to their contents of

hydrolytic enzymes, Fraction I was further centrifuged in tubes containing several layers of sucrose solutions having different densities. The separation of granules into layers of different densities as a result of this centrifugation is depicted in Figure 1. In most experiments four separable layers were obtained; in one case a fifth, denser layer was also found. Because the centrifugation was performed for only one hour, it seems unlikely that complete separation of granules of different densities was achieved. This incompleteness of separation, as well as a certain amount of mixing which occurred upon removal of the different fractions, undoubtedly contributed to the variations in distribution and activities reported below.

Assays of the hydrolytic enzymes in question, and of ribonucleic acid, were performed on the subfractions obtained by the gradient centrifugation. Tests for DNA were made by the diphenylamine procedure of Dische (1930) as modified by Seibert (1940). These indicated that only trace amounts were present in each fraction. The absorption of the hot trichloroacetic acid extracts at 260 millimicrons was therefore taken as a measure of ribonucleic acid content. In addition to the hydrolytic enzymes, succinic dehydrogenase was assayed as an indicator of the presence of mitochondria in the subfractions. The results of these determinations are presented in Table III and the average specific activities of enzymes and amounts of ribonucleic acid in each granule fraction are shown in a series of histograms in Figure 2.

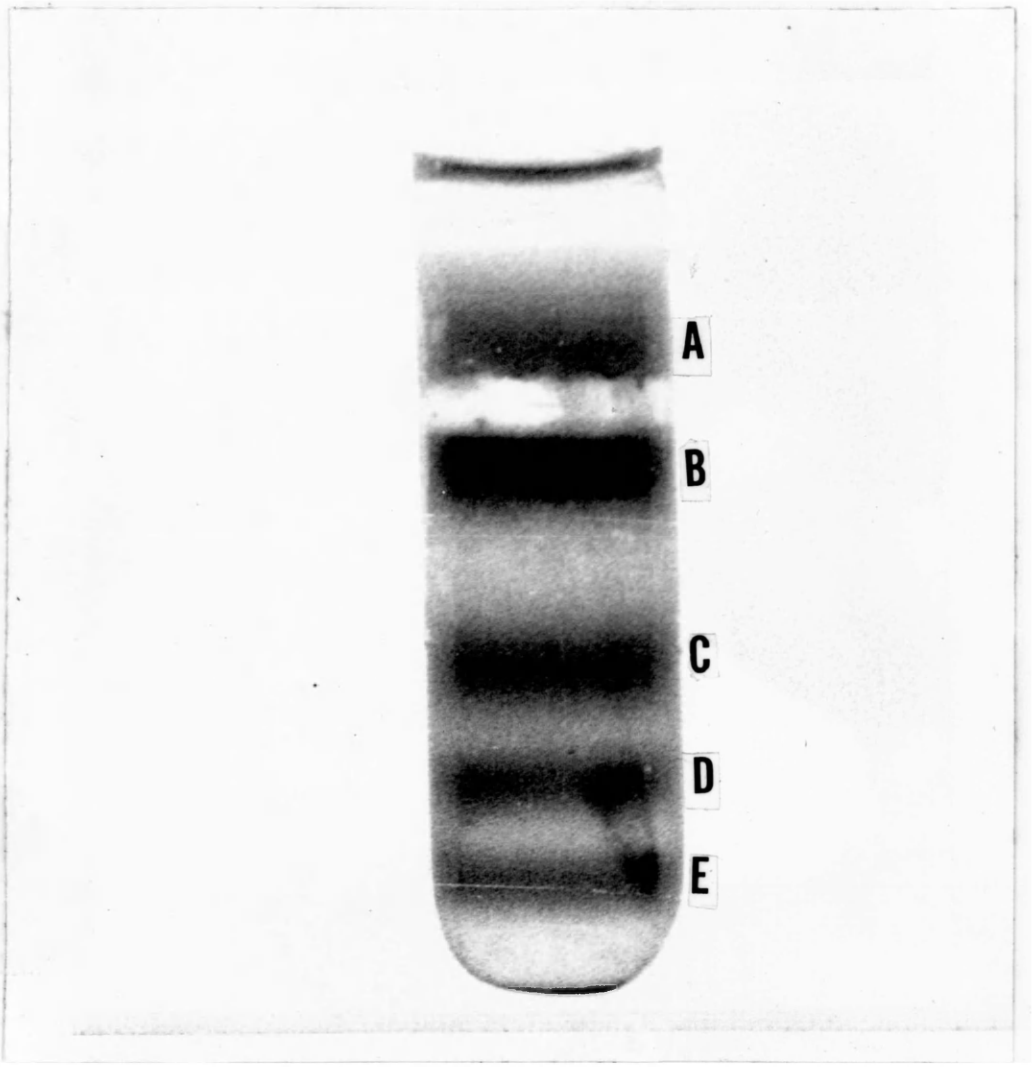
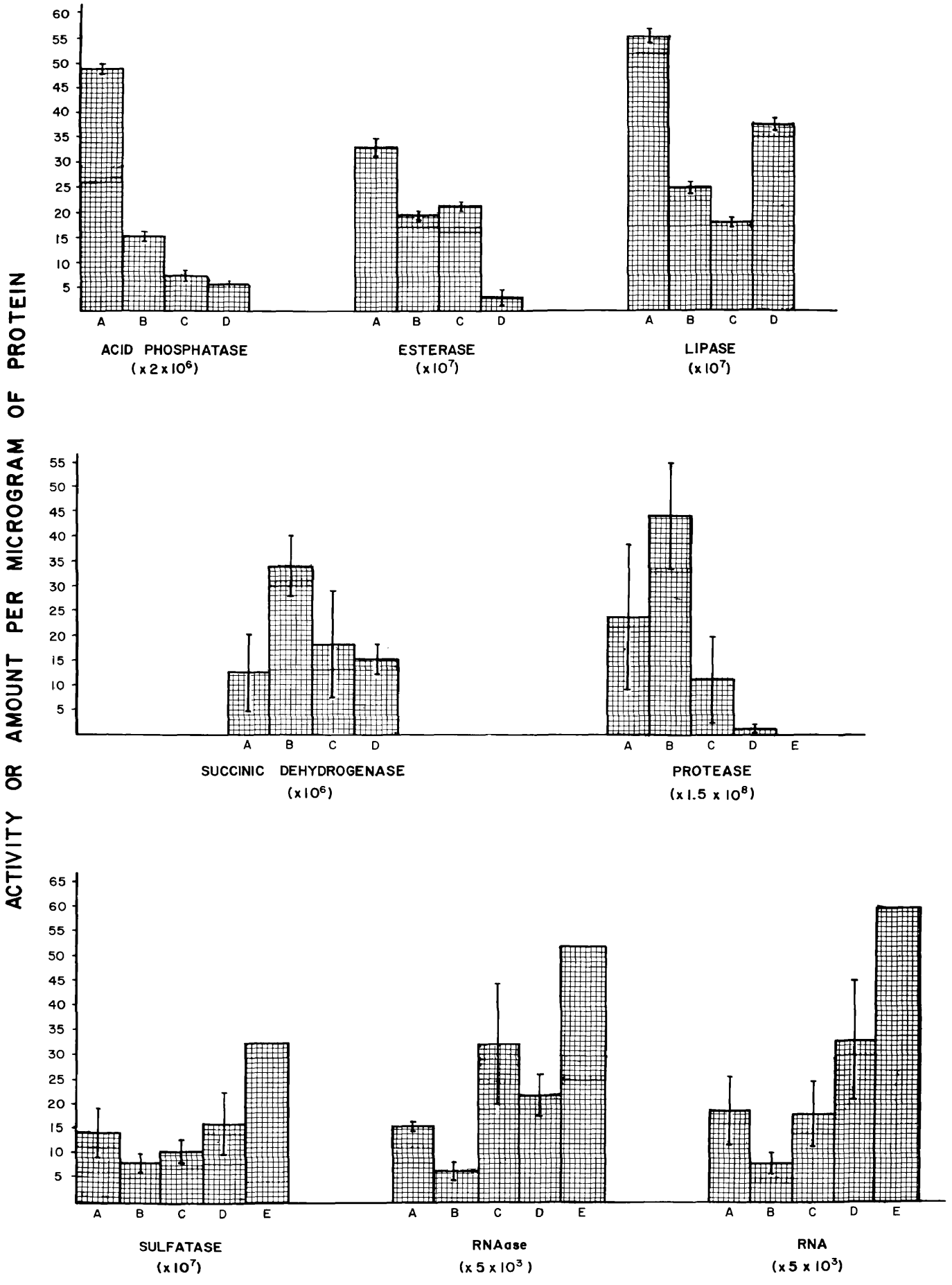


Figure 1. Subfractions obtained from Fraction I by density gradient centrifugation. The subfractions were obtained by centrifuging granules on the following densities of sucrose for one hour at 90,000 times gravity: 1.1513, 1.1663, 1.1868, 1.1972, 1.2092, and 1.3163.

The results indicate considerable heterogeneity in granule types. Acid phosphatase, lipase, and esterase have the highest specific activities in the granules of lowest density, Subfraction A. Acid phosphatase in these granules has a relative specific activity as much as nine times that of the whole homogenate, while esterase and lipase are about four times as concentrated as in the whole homogenate. Subfraction B, which contains the highest specific activity of succinic dehydrogenase, is presumed to be the chief mitochondrial fraction; however it also contains the largest percentages of all other enzymes assayed as well as the highest percentage of total protein. This is assumed to be a result of incomplete separation of granules as noted above. The echinochrome pigment granules are also concentrated in this subfraction. Pigment presumably derived from these granules was always found in the fluid at the top of the density gradient. Proteolytic activity at pH 5 is also most concentrated in Subfraction B. Aryl-sulfatase, a characteristic lysosomal enzyme, has somewhat higher specific activities in Subfraction A and in the denser granules D and E; however, its distribution is fairly uniform throughout all the subfractions isolated. Ribonuclease activity and ribonucleic acid are low in the mitochondria fraction and are only moderately high in Subfraction A. The denser granules, especially D and E, contain unusually high concentrations of both RNAase and ribonucleic acid.

Figure 2. Average specific activities of enzymes and amounts of ribonucleic acid in the subfraction granules isolated from Fraction I. The values are multiplied by the factors indicated. The lines represent one standard deviation below and above the mean.


ACTIVITY OR AMOUNT PER MICROGRAM OF PROTEIN
ACID PHOSPHATASE
(x2x10⁶)
ESTERASE
(x10⁷)
LIPASE
(x10⁷)
SUCCINIC DEHYDROGENASE
(x10⁶)
PROTEASE
(x1.5 x 10⁸)
SULFATASE
(x10⁷)
RNAase
(x5 x 10³)
RNA
(x5 x 10³)

Microscopical Observation and Vital Staining of Granules

The granules in Subfraction A were observed under oil immersion in order to estimate their size range. The spherical granules varied in size from about one to three microns, with 60-70 percent being in the 1-1½ micron range, and about 20 percent in the 2-2½ micron range. Only a very few granules were evident in the 3-3½ micron range, probably less than one or two percent.

The staining of granules from Fraction I prior to isolation on the sucrose layers gave conclusive results only with toluidine blue. With this stain Subfractions A and E stained slightly with a very pale green color. Subfractions B, C, and D appeared to exhibit metachromasia, with most of the red color concentrated in Subfraction B. Neutral red and methyl red were predominantly taken up by Subfraction B, but this may have resulted from the presence of most of the granules of all types in this subfraction. The presence of red, echinochrome pigment granules in this fraction interfered with the detection of its staining properties with all dyes. It seems clear that the granules of Subfraction A, which possess the highest specific activity of acid phosphatase, do not stain metachromatically in vitro. A direct analysis of the nature and content of polysaccharides in the different granules would appear to be desirable from the standpoint of correlating this finding with that of Dalcq (1963), who reported that granules possessing acid phosphatase activity also exhibited metachromatic properties.

DISCUSSION

From the results illustrated in Figure 2, it may be tentatively concluded that at least three types of visible granules, differing in their content of hydrolytic enzymes, exist in the egg. The first type, exemplified by Subfraction A, exhibits acid phosphatase, esterase, and lipase activities. The second type, most abundant in Subfraction B, contains proteolytic activity at pH 5. This fraction also probably includes the mitochondria, since succinic dehydrogenase is most concentrated here. The third type, found in Subfractions D and E, contains ribonuclease and ribonucleic acid, as well as a fairly high content of aryl-sulfatase.

The granules of Subfraction A contain at least three of the hydrolytic enzymes believed by de Duve (1963), Novikoff (1961), and others to be located within the lysosomes. The absence of metachromasia in these granules after *in vitro* staining suggests that they may differ from the *alpha* and *beta* granules of Dalcq (1963) and Pasteels and Mulnard (1957) in their polysaccharide content; however, these investigators worked only with fertilized eggs stained *in vivo*. Rebhun (1959) demonstrated that in *Spisula solidissima* staining of the *alpha* and *beta* granules appeared only

after fertilization. In stratifying eggs of various species of sea urchins, Immers (1960) expressed doubt that regions of mucopolysaccharide concentration evident after *in vivo* staining corresponded to the metachromatic *alpha* and *beta* granules of Dalcq and Pasteels because his staining was performed only on unfertilized eggs.

In spite of their high content of hydrolytic enzymes, it is not possible to identify the granules of Subfraction A as lysosomes. De Duve (1963) has warned that the present definition of the lysosome, although based primarily on his rat liver tissue work, must not include any incidental details such as size and other physical characters, osmotic properties, centrifugal behaviour, mechanism of structure-linked latency, or sensitivity to individual disrupting treatments. If these factors are therefore omitted in defining the lysosome, the essential characteristic remaining is the association within a special group of cytoplasmic particles of a number of soluble acid hydrolases of widely differing specificity. The accessibility of these enzymes to the surrounding substrate must be restricted, making the latency of the enzymes dependent on the structural complexes of the particles. Such a definition would be broad enough to include the hydrolytic granules in Subfraction A isolated from *Arbacia* eggs, if it were shown that the accessibility or activity of the enzymes in question is restricted by the granular structure.

The second type of granule recovered from egg

homogenates exhibits a high concentration of proteolytic activity at pH 5. This enzyme activity was most concentrated in Subfraction B; however, this Subfraction also contained considerable percentages of the total activities of all the other enzymes studied, as well as the highest percentage of total protein in the visible granule fraction. Subfraction B probably contains most of the mitochondria, since succinic dehydrogenase is most concentrated here; however, the presence of mitochondria can only be confirmed after a study of the ultrastructure with the electron microscope. From its high content of protein, it is assumed that most of the proteinaceous yolk is also concentrated in this subfraction. This subfraction appeared to show some metachromasia after in vitro staining with toluidine blue, indicating that the metachromatic granules of Dalcq (1963) may be included in this layer. Because of the apparent heterogeneity of this group of granules, it is not possible to make any reasonable hypothesis regarding the identity of the protease-containing granules.

The most dense granules in the visible granule fraction, recovered in Subfractions D and E after gradient centrifugation, contain much higher specific concentrations of ribonucleic acid and ribonuclease than do the other visible granules. The existence of similar dense RNA bodies in eggs has been reported by other workers. Raven (1945) demonstrated the presence of heavy RNA particles in the centrifugal pole of stratified Limnaea eggs. Pasteels (1958), by centrifuging

Paracentrotus eggs, discovered "heavy bodies" of RNA, ranging from 1-3 microns, in the centrifugal cap region. This region, which also contained the mitochondria, was intensely stained with pyronine. Pasteels postulated that, in addition to being found in the ribosomes and in annulate membranes within the egg, RNA could also be found in undefined structures that could be linked to the mitochondria but which contained the most dense material in the egg. Balinsky and Devis (1963) observed electron dense granules in the young oocytes of Xenopus laevis which presumably accumulated between adjacent mitochondria. Afzelius (1956) has also described "heavy bodies" which stain vitally with toluidine blue in the sea urchin egg. Immers (1960) described dense RNA granules which were separate from the mitochondria in the most centrifugal zone of stratified eggs of Paracentrotus lividus. A few workers have claimed that the heavy yolk granules, especially in the Amphibia, contain an appreciable amount of RNA (Grant, 1953; Rounds and Flickinger, 1958), but others have shown by histochemical and cytological studies that there is little or no RNA within the yolk granules of most species examined. Collier (1960) found no evidence of either RNA or proteolytic enzymes in the yolk granules of Ilyanassa obsoleta. The recent work by Karasaki (1963) and Ohno et al (1963) revealed no evidence for the presence of RNA in the yolk granules of Triturus pyrrhogaster and Rana pipiens embryos.

It may be tentatively concluded that the heavy granules in Subfractions D and E correspond to those described

by Immers and Pasteels. These bodies are probably not polyribosomes, since Wilt (1964) has shown that these appear only after fertilization in the eggs of Strongylocentrotus purpuratus. It is possible that the annulate lamellae described by Pasteels (1958) are sufficiently dense to be included in this fraction; these structures consist of membranes to which bodies similar in size and density to ribosomes are attached. No previous report has been made concerning the association of ribonuclease with any granule fraction in the egg; however, a comparison of the present finding with that of Reid and Node (1959) for granules of rat liver is of particular interest. These authors provided evidence that acid ribonuclease was present in particles which were more rapidly sedimented from homogenates than the lysosomes, indicating the possible existence of a separate set of granules which contain this enzyme.

The distribution of activities of the hydrolytic enzymes in Fraction II and III of the egg homogenates indicates the presence of these enzymes in submicroscopic structures (see Table 2). The possibility therefore exists that granules resembling rat liver lysosomes in size may also be present in the egg. If such granules are easily ruptured during preparation, as are liver lysosomes, this may account for the high enzyme activities found in the soluble fraction. It is of interest that Fractions II and III of the gut tissue of adult Arbacia (Table I) contain approximately the same specific activities of acid phosphatase and esterase as do

the corresponding fractions of the egg.

It is not possible to speculate in detail about the relationship of the granules described above to developmental processes; however, a number of studies may be mentioned which tend to implicate hydrolytic granules in fertilization and cleavage. Immers (1960) reported that the fertilization membrane is more highly elevated and more refractive in the heavy pole of stratified Arbacia lixula which contains the yolk layer exhibiting strong periodic acid-Schiff staining and acid phosphatase activity. In Psammechinus miliaris, the yolk layer with similar staining is located near the light pole, and a similar elevation and refraction of the fertilization membrane occurs in this region. After fertilization however, the yolk granules become more evenly distributed throughout the cytoplasm and stain more intensely. Immers postulated that the results indicate that one or several factors active in the organization of the fertilization membrane are bound to particles which follow the displacement of the yolk granules in centrifuged eggs. He was undecided as to whether the activity stems from unmodified yolk globules or from more specialized particles. By centrifuging fertilized eggs of Temnopleurus and Mesophilia into light and heavy halves, Kojima (1959) demonstrated that only the halves receiving the metachromatic granules as well as most of the other visible granules during centrifugation are able to cleave. Marsland et al (1960), in pressure centrifugation work on premature furrowing in Arbacia eggs, found that

nuclear rupture is a necessary prelude to furrow induction, but that the induced furrowing does not occur unless the metachromatic granules in the cytoplasm are also ruptured. Maggio (1957) reported that proteolytic activity at pH 5.4 to 5.9 is a characteristic mitochondrial enzyme in Paracentrotus lividus; however, his preparations of mitochondria undoubtedly contained other types of granules as well. Maggio found that the protease activity in the granular fraction decreases immediately after fertilization, increases slightly during the sperm-aster stage, and then decreases again. The cytoplasmic protease undergoes a temporary increase immediately after fertilization. These changes in activity could be due to the differential release of enzyme during preparation in different stages or to changes in activity of the enzyme itself.

No work has been reported which directly implicates the dense RNA-containing granules in any phase of early development. These may represent a storage form of RNAase and ribonucleic acid in the unfertilized egg. Further studies should be made of the fertilized egg to determine if the granules contribute to the rapid disappearance of ribosomal RNA during cleavage and early blastulation (Comb and Brown, 1964), or to the release of bound messenger RNA which has been postulated to occur at fertilization (Tyler, 1963).

The heterogeneity of the populations of granules which contain hydrolytic enzymes in the egg may be generally related to the timing with which different enzymes become active

during development. It is postulated that such a separation of enzymes in different granules could result in the specific release or activation of some hydrolases, but not others, at particular developmental stages. Furthermore, partial segregation of the granules into different cells during cleavage may confer different developmental potentialities on the daughter cells. Segregation of granules and certain enzymes have been observed in numerous eggs exhibiting "mosaic" cleavage (cf. Brachet, 1950 for review); however, similar differentiation has not been observed in the sea urchin. Experimental testing of the latter hypothesis must await the development of techniques for visual identification of the granules in question.

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APPENDIX

TABLE I

DISTRIBUTION OF ENZYME ACTIVITIES, NUCLEIC ACID AND PROTEIN IN SUBCELLULAR FRACTIONS OF THE GUT

Specific activities of acid phosphatase and esterase are expressed in micromoles of substrate converted per minute per microgram of protein, multiplied by the factors indicated.

	Exp. #	Whole	I	II	III	IV
Acid Phosphatase						
Total	G1*	100.0	12.6	5.4	4.5	47.2
activity as	G2*	100.0	15.3	5.6	4.9	42.4
percent of whole	G3	100.0	17.4	11.0	7.5	37.7
homogenate	G4	100.0	16.9	13.8	15.7	36.1
Average		100.0	15.5	8.9	8.2	40.8
Specific acti-	G1*	4.7	7.4	10.7	13.4	4.7
vity ($\times 10^6$)	G2*	5.4	12.1	10.7	13.4	5.4
	G3	6.7	15.4	15.4	11.4	2.7
	G4	7.4	12.7	10.7	10.7	2.7
Average		6.0	11.9	11.9	12.3	3.9
Specific acti-	G1*	1.0	1.6	2.3	2.8	1.0
vityrelative to	G2*	1.0	2.2	2.0	3.0	1.0
that of whole	G3	1.0	2.3	2.3	1.7	0.4
homogenate	G4	1.0	1.7	1.4	1.4	0.4
Average		1.0	2.0	2.0	2.2	0.7

TABLE I (cont.)

	Exp. #	Whole	I	II	III	IV
Esterase						
Total activity as per cent of whole homogenate	G3	100.0	8.0	8.7	14.7	98.0
	G4	100.0	10.5	8.6	30.4	97.2
Specific activity ($\times 10^6$)	G3	3.0	3.4	6.0	11.0	3.8
	G4	3.2	3.4	3.0	9.2	3.6
Specific activity relative to whole homogenate	G3	1.0	1.1	2.0	3.6	1.2
	G4	1.0	1.1	0.9	2.9	1.1
Total Nucleic Acid						
Total amount as percent of whole homogenate	G3	100.0	7.1	4.3	8.8	74.0
	G4	100.0	10.2	6.1	11.6	66.0
Micrograms per microgram of protein ($\times 10^3$)	G3	22.4	20.6	14.6	29.4	19.8
	G4	20.6	21.4	13.0	22.2	15.2
Concentration relative to whole homogenate	G3	1.0	1.1	0.7	1.3	0.8
	G4	1.0	1.0	0.5	1.1	0.7

TABLE I (cont.)

Protein						
Total amount as	G1*	100.0	8.0	2.4	1.6	45.0
percent of whole	G2*	100.0	7.0	2.7	1.6	57.0
homogenate	G3	100.0	8.1	4.9	4.7	89.0
	G4	100.0	9.7	9.6	11.0	89.0

* Entire gut of animal not removed.

TABLE II

DISTRIBUTION OF ENZYME ACTIVITIES, NUCLEIC ACID AND PROTEIN IN SUBCELLULAR FRACTIONS OF THE EGG

Specific activities for lipase and aryl-sulfatase are expressed in micromoles of substrate converted per minute per microgram of protein, multiplied by the factors indicated. RNAase is expressed in micrograms of RNA solubilized per minute per microgram of protein, and proteolytic activity at pH 5 in micromoles of tyrosine liberated from hemoglobin per minute per microgram of protein, all multiplied by the indicated factors. The other enzymes are expressed in the same terms as in Table I.

	Exp. #	Whole	I	II	III	IV
Acid Phosphatase						
Total activity	E1*	100.0	32.9	19.7	3.8	39.1
as per cent of	E2**	100.0	26.8	7.1	7.9	77.5
whole homogenate	E3**	100.0	51.1	10.4	5.5	64.4
	E4	100.0	33.6	8.0	6.0	68.0
Specific activity ($\times 10^6$)	E1*	4.7	7.4	12.7	8.7	2.1
	E2**	3.0	7.4	5.4	23.4	3.6
	E3**	2.6	8.0	4.7	14.1	3.0
	E4	10.7	16.1	12.7	20.1	4.0
Specific activity relative	E1*	1.0	1.6	2.7	1.8	0.4
	E2**	1.0	2.4	1.8	7.7	1.2
to that of	E3**	1.0	3.1	1.7	5.4	1.2
whole homogenate	E4	1.0	1.5	1.2	1.9	0.4

TABLE II (cont.)

	Exp. #	Whole	I	II	III	IV
Lipase						
Total activity	E1*	100.0	36.6	18.8	1.7	69.8
as per cent of	E2**	100.0	25.0	15.0	0	55.0
whole homogenate	E3**	100.0	40.0	13.0	0	58.0
	E4	100.0	42.8	15.0	0	56.0
Specific activity ($\times 10^7$)	E1*	4.5	8.3	12.0	3.6	3.4
	E2**	5.0	12.0	20.0	0	4.4
	E3**	3.0	8.0	8.0	0	3.0
	E4	7.2	14.0	22.0	0	5.8
Specific activity relative	E1*	1.0	1.8	2.7	0.8	0.8
	E2**	1.0	2.4	3.3	0	0.9
to that of	E3**	1.0	2.7	2.7	0	0.9
whole homogenate	E4	1.0	2.0	3.0	0	0.9
Esterase						
Total acti	E1*	100.0	15.3	19.6	7.8	58.4
vity as per	E2**	100.0	8.2	11.6	5.5	72.6
cent of whole	E3**	100.0	26.5	8.2	5.0	74.7
homogenate	E4	100.0	42.9	9.8	5.1	71.0
Specific activity ($\times 10^7$)	E1*	5.0	4.0	15.0	20.0	3.0
	E2**	10.0	7.3	29.0	54.0	11.3
	E3**	9.5	16.0	14.0	47.0	12.4
	E4	8.9	17.6	15.0	48.0	11.0

TABLE II (cont.)

	Exp. #	Whole	I	II	III	IV
Esterase						
Specific activity relative to whole homogenate	E1*	1.0	0.8	3.0	4.2	0.6
	E2**	1.0	0.7	2.9	5.4	1.3
	E3**	1.0	1.7	1.5	4.9	1.3
	E4	1.0	2.0	1.7	5.3	1.2
Proteolytic activity at pH 5						
Total activity as per cent of whole homogenate	E7	100.0	55.1	0	0	64.4
	E8	100.0	46.0	0***	0***	82.3
	E9	100.0	54.9	2.2	0	82.1
Average		100.0	52.0	0.7	0	76.3
Specific activity ($\times 10^8$)	E7	18.0	47.3	0	0	44.0
	E8	22.4	26.0	0***	0***	31.6
	E9	17.8	34.2	2.8	0	17.0
Average		19.4	36.0	0.9	0	30.9
Specific activity relative to whole homogenate	E7	1.0	2.6	0	0	2.5
	E8	1.0	1.2	0***	0***	1.4
	E9	1.0	1.9	0.2	0	1.0
Average		1.0	1.9	0.1	0	1.6

TABLE II (cont.)

	Exp. #	Whole	I	II	III	IV
Aryl-sulfatase						
Total activity	E7	100.0	24.0	1.9	1.4	18.8
as per cent of	E8	100.0	33.0	1.1***	0***	43.0
whole homo-	E9	100.0	22.3	3.2	1.2	38.7
genate						
Average		100.0	26.4	2.1	0.9	33.5
Specific acti-	E7	8.4	6.2	1.5	4.5	6.1
vity (x 10^7)	E8	7.9	6.4	1.3***	0***	6.1
	E9	16.0	12.7	3.6	2.7	7.2
Average		10.8	8.4	2.1	2.4	6.5
Specific acti-	E7	1.0	0.7	0.2	0.5	0.7
vity relative	E8	1.0	0.9	0.2***	0***	0.8
to whole homo-	E9	1.0	0.8	0.2	0.2	0.4
genate						
Average		1.0	0.8	0.2	0.2	0.7
RNAase						
Total activity	E7	100.0	27.8	18.0	16.4	69.2
as per cent of	E8	100.0	33.3	17.3***	12.8***	92.9
whole homogenate	E9	100.0	35.2	17.7	17.3	88.6
Average		100.0	32.1	17.7	15.5	83.6

TABLE II (cont.)

	Exp. #	Whole	I	II	III	IV
RNAase						
Specific activity ($\times 10^3$)	E7	37.0	29.0	59.0	136.0	89.0
	E8	56.0	48.0	140.***	56.0***	126.0
	E9	48.0	57.0	53.0	102.0	48.0
Average		47.0	44.6	84.0	98.0	87.6
Specific activity relative to whole homogenate	E7	1.0	0.8	1.7	4.7	2.7
	E8	1.0	0.8	2.7***	1.2***	2.3
	E9	1.0	1.2	1.2	2.4	1.0
Average		1.0	1.0	1.9	2.8	2.0
Total Nucleic Acid						
Total amount as per cent of whole homogenate	E7	100.0	22.4	11.6	18.9	41.6
	E8	100.0	17.8	10.5***	37.1***	77.8
	E9	100.0	20.7	20.4	42.3	77.5
Micrograms per microgram of protein ($\times 10^3$)	E7	79.9	54.1	89.1	435.5	128.3
	E8	73.5	33.3	117.3***	260.0***	140.0
	E9	74.6	54.0	106.0	336.0	67.5
Concentration relative to that of whole homogenate	E7	1.0	0.7	1.1	5.4	1.6
	E8	1.0	0.4	1.5	3.5	1.9
	E9	1.0	0.7	1.4	4.5	0.9

TABLE II (cont.)

	Exp. #	Whole	I	II	III	IV
Protein						
Total amount	E1*	100.0	20.3	7.2	2.1	72.2
as per cent of	E2**	100.0	11.0	4.0	1.0	64.0
whole homogenate	E3**	100.0	17.0	6.0	1.0	57.0
	E4	100.0	22.0	6.0	4.0	60.0
	E7	100.0	33.0	10.0	4.0	26.0
	E8	100.0	39.0	6.5***	10.0***	58.0
	E9	100.0	28.0	14.0	7.0	85.0

* Protein determinations made without extracting with TCA etc., and the original homogenate was centrifuged at 2000 rpm to remove debris instead of 500 rpm.

** Original homogenate was centrifuged at 2000 rpm to remove debris instead of 500 rpm.

*** Fractions II and III were not washed after the initial centrifugation.

TABLE III

DISTRIBUTION OF ENZYME ACTIVITIES, RNA, AND PROTEIN IN SUBFRACTIONS DERIVED FROM FRACTION I

Succinic dehydrogenase activity is expressed in micromoles of substrate converted per minute per microgram of protein. The other enzymes are expressed in the same terms as in Tables I and II, multiplied by the factors indicated. The letters A through E represent subfractions of different densities, obtained by centrifuging Fraction I on sucrose layers. The granules in Subfraction A are those of the lowest density.

	Exp. #	A	B	C	D	E
Acid phosphatase						
Specific activity ($\times 10^6$)	E4	95.8	32.6	14.9	13.4	-
	E5	100.5	26.9	13.5	14.7	-
	E6	95.1	29.6	14.1	12.1	-
Average		97.1	29.7	14.1	13.4	-
Total activity as per cent of Fraction I	E4	27.9	54.6	12.5	6.5	-
	E5	28.2	50.2	13.4	6.8	-
	E6	28.0	52.1	12.2	5.0	-
Average		28.0	52.3	12.7	6.1	-

TABLE III (cont.)

	Exp. #	A	B	C	D	E
Acid phosphatase						
Specific activity relative to whole homogenate	E4	8.9	3.0	1.4	1.2	-
	E5	9.0	3.1	1.5	1.3	-
	E6	9.0	2.9	1.3	1.2	-
Average		9.0	3.0	1.4	1.2	-
Esterase						
Total activity as per cent of Fraction I	E4	33.0	19.6	21.3	0	-
	E5	36.0	18.2	22.1	3.1	-
	E6	31.0	19.0	20.1	4.2	-
Average		33.3	18.9	21.2	2.4	-
Specific activity ($\times 10^7$)	E4	8.6	31.2	16.9	0	-
	E5	8.9	32.0	17.0	1.5	-
	E6	8.4	31.0	17.1	2.0	-
Average		8.6	31.4	17.0	1.2	-
Specific activity relative to whole homogenate	E4	3.7	2.2	2.4	0	-
	E5	4.0	2.1	2.5	0.2	-
	E6	3.5	2.0	2.3	0.2	-
Average		3.7	2.1	2.4	0.1	-

TABLE III (cont.)

	Exp. #	A	B	C	D	E
Lipase						
Total activity as percent of Fraction I	E4	18.8	56.9	17.8	21.1	-
	E5	24.2	50.1	16.1	20.2	-
	E6	19.1	57.2	18.1	20.9	-
Average		20.7	54.7	17.3	20.7	-
Specific activity ($\times 10^7$)	E4	55.6	29.0	17.8	37.2	-
	E5	58.1	26.3	16.5	36.2	-
	E6	54.2	29.5	17.9	37.0	-
Average		56.0	28.3	17.4	36.8	-
Specific activity relative to whole homogenate	E4	4.0	2.1	1.3	2.6	-
	E5	4.2	1.9	1.1	2.5	-
	E6	3.9	2.2	1.3	2.6	-
Average		4.1	2.1	1.2	2.6	-
Proteolytic activity at pH 5						
Total activity as percent of Fraction I	E7*	10.5	75.8	8.4	0	0
	E8	19.1	74.1	0	0	-
	E9	22.5	30.1	0	0	-
	E11	5.0	50.4	4.2	0.3	-
	E12	6.2	81.8	6.1	0.4	-
Average		12.7	62.4	3.8	0.1	0

TABLE III (cont.)

	Exp. #	A	B	C	D	E
Proteolytic activity at pH 5						
Specific activity ($\times 10^8$)	E7*	7.8	39.3	21.1	0	0
	E8	22.2	19.0	0	0	-
	E9	36.6	22.6	0	0	-
	E11	11.1	30.6	4.6	1.6	-
	E12	7.0	32.0	10.3	0.9	-
Average		15.7	28.9	7.3	0.5	0
Specific activity relative to whole homogenate	E7*	0.4	2.2	1.2	0	0
	E8	1.0	0.8	0	0	-
	E9	1.7	1.2	0	0	-
	E11	0.7	1.8	0.3	0.1	-
	E12	0.4	1.9	0.6	0.05	-
Average		0.8	1.6	0.4	0.03	0
Succinic dehydrogenase						
Total activity as percent of Fraction I	E4	13.9	82.3	0	3.9	-
	E7*	0	95.1	18.0	7.4	3.0
	E8	20.7	60.9	11.1	8.9	-
	E9	27.9	95.6	13.7	13.7	-
Average		15.6	83.5	10.7	8.5	3.0

TABLE III (cont.)

	Exp. #	A	B	C	D	E
Succinic dehydrogenase						
Specific activity ($\times 10^6$)	E4	12.5	24.0	0	15.0	-
	E7*	0	34.0	25.0	12.5	5.0
	E8	17.1	39.2	27.5	20.0	-
	E9	20.5	38.5	20.0	12.5	-
Average		12.5	33.9	17.9	15.0	5.0
Specific activity relative to whole homogenate	E4	0.5	0.5	0	0.3	-
	E7*	0	1.8	1.3	0.7	0.3
	E8	0.5	1.3	1.0	0.6	-
	E9	0.7	1.3	0.7	0.4	-
Average		0.4	1.2	0.7	0.5	0.3
Aryl-sulfatase						
Total activity as per cent of Fraction I	E7*	40.0	43.9	22.4	12.9	25.1
	E9	20.3	46.6	13.5	17.0	-
	E11	17.8	22.2	24.6	6.0	-
	E12	20.2	46.5	10.9	27.8	-
Average		24.6	39.8	17.8	15.9	25.1
Specific activity ($\times 10^7$)	E7*	20.4	6.7	9.5	8.2	32.0
	E9	10.2	8.6	13.5	11.0	-
	E11	15.4	10.2	10.1	23.5	-
	E12	8.0	6.0	7.2	20.3	-
Average		13.5	7.9	10.1	15.7	32.0

TABLE XXI (cont.)

	Exp. #	A	B	C	D	E
Aryl-sulfatase						
Specific activity relative to whole homogenate	E7*	2.4	0.8	1.1	1.0	3.8
	E9	0.6	0.5	0.8	0.7	-
	E11	1.4	0.9	0.9	2.1	-
	E12	0.7	0.5	0.6	1.7	-
Average		1.3	0.7	0.9	1.4	3.8
RNAase						
Total activity as per cent of Fraction I	E7*	33.7	32.9	29.1	35.4	41.8
	E8	45.5	33.9	32.9	28.2	-
	E9	37.3	52.6	43.1	37.6	-
Average		38.8	39.8	35.0	33.7	41.8
Specific activity ($\times 10^3$)	E7*	80.0	23.0	74.0	106.0	260.0
	E8	96.0	26.0	224.0	147.0	-
	E9	85.0	48.0	187.0	108.0	-
Average		87.0	32.3	161.7	120.3	260.0
Specific activity relative to whole homogenate	E7*	2.4	0.7	2.1	3.1	4.9
	E8	1.9	0.6	4.9	3.4	-
	E9	1.8	1.1	4.3	2.3	-
Average		2.1	0.8	3.1	2.9	4.9

TABLE III (cont.)

	Exp. #	A	B	C	D	E
Ribonucleic Acid						
Total amount	E4	15.6	36.5	22.2	24.5	-
as per cent of	E7*	11.7	18.1	9.6	10.1	15.9
Fraction I	E11	10.1	19.9	27.7	16.0	-
	E12	15.3	20.2	12.6	9.0	-
Average		13.2	23.7	18.0	14.9	15.9
Micrograms per	E4	126.7	52.5	64.0	124.0	-
microgram of	E7*	88.0	38.5	60.0	95.0	300.0
protein	E11	66.7	35.4	88.6	242.0	-
($\times 10^3$)	E12	61.8	26.5	140.0	200.0	-
Average		90.8	38.2	88.2	165.2	300.0
Concentration	E4	2.3	0.8	1.1	2.0	-
relative to	E7*	1.1	0.5	0.8	1.2	3.7
whole homo-	E11	0.9	0.5	1.2	3.3	-
genate	E12	1.3	0.5	2.9	4.1	-
Average		1.4	0.6	1.5	2.6	3.7
Protein						
Total amount	E4	1.1	6.1	3.5	1.7	-
as percent of	E5	2.1	10.5	6.0	2.2	-
whole homo-	E6	3.0	15.0	6.0	3.0	-
genate	E7*	4.0	13.0	4.7	3.1	1.6
	E8	9.2	24.0	2.7	3.2	-
	E9	7.1	12.8	3.6	5.9	-
	E11	3.7	14.1	7.9	1.6	-
	E12	4.2	13.0	3.1	2.3	-

TABLE III (cont.)

* Fraction I was first centrifuged on gradient of the following densities: 1.1513, 1.1868, 1.2092, and 2.5 M. Five sets of granules were isolated, the top two sets being very close together. The top two sets of granules were recentrifuged on the following gradient to further separate the granules: 1.1513, 1.1663, 1.1868, 1.1972, 2.5 M.

VITA

Charlotte Jackson

Born in Bowie, Texas, August 21, 1941. Graduated from Bowie High School, May, 1959. Received Bachelor of Science in Biology degree from North Texas State University, Denton, Texas, May, 1963. Associated with the Department of Biology at the College of William and Mary in Virginia as a Research Assistant from September, 1963 until January, 1965.