EXPERIMENTAL INITIATION OF CELL DIVISION

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Ronald J. Pfohl
1967

THESIS

This is to certify that the

thesis entitled

EXPERIMENTAL INITIATION OF

CELL DIVISION

presented by

Ronald J. Pfohl

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in ZOOLOGY

John R. Shaver

John R. Shaver Major professor

Date July 19, 1967

O-169

ABSTRACT

EXPERIMENTAL INITIATION OF CELL DIVISION

by Ronald J. Pfohl

Previous studies have shown that mature frog eggs can undergo cleavage if parthenogenetically stimulated. The stimulus involves two factors: puncturing the egg and simultaneously inoculating or injecting an appropriate biological material into the egg. The introduction of active material into the egg enables it to undergo normal cleavage. Granules from cells of a variety of amphibian, mammalian,and avian tissues are active in this capacity. A study was undertaken to determine which particulate component in the cell was the active one.

Differential and sucrose density gradient centrifugation techniques were used to fractionate the liver tissue of Rana pipiens. Sucrose-EDTA media, either salt-free or buffered with 0.1M phosphate, pH 7.0-7.2, were used throughout the fractionation procedures. The sucrose concentration varied from 0.25M to 0.7M in the differential centrifugation scheme. The sucrose gradients ranged in steepness from 0.25M to 1.8M sucrose and from 0.8M to 1.8M sucrose. Subcellular fractions obtained by these centrifugal techniques were quantitatively analyzed for the mitochondrial, lysosomal, and peroxisomal marker enzymes. Samples of the fractions were also injected into eggs of Rana pipiens to determine their cleavage-initiating activity.

The results obtained in this study show that cleavage-ini-

tiating activity is associated only with the large granule fraction of cells. This fraction was shown to contain at least mitochondria, lysosomes, and peroxisomes. Microsomes and nuclei were inactive as cleavage-initiating agents.

The structural integrity of the active cell components was shown to be necessary for the components to be effective in initiating cleavage. Subjecting the large granules to freeze-thawing, insonation, storage for days at 0°C, and heating to 52°C resulted in the loss of most or all of their cleavage-initiating activity.

The concentration of granules in the injected material was shown to be of importance in determining the percentage of eggs which undergo cleavage and the quality of the cleavages. If material with a very high concentration of granules is injected, only a few or none of the eggs cleave. Of the eggs which do cleave, most develop only to partial blastulae, with blastomeres covering less than one-half of the egg surface. Progressive dilution of the granule suspension resulted in more of the eggs undergoing total cleavage.

Little correlation was observed between the distribution of cleavage-initiating activity and the distribution of mitochondria, lysosomes, and peroxisomes following gradient centrifugation of untreated and insonated large granule fractions. Upon considering the results of this study, it seems unlikely that any one of the three components is by itself the active factor.

Reports in the literature demonstrate that an egg which is simply activated (but not stimulated to cleave) is capable of

chromosomal replication, protein and RNA synthesis, and aster formation. However, a normally functioning amphiastral system apparently does not form. Whatever the exact nature of the cleavage-initiating material may be, it is proposed that it functions either as a division center or it catalyzes the formation of such a center in the egg.

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Ronald J. Pfohl

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1967

ACKNOWLEDGEMENTS

This investigation was initiated upon the suggestion of Dr. John R. Shaver. I wish to express my sincere gratitude to him for his continued confidence in me, for his encouragement and support when the need arose, and for his endless patience with me during the course of this study.

I am grateful to Dr. Neal Band and Dr. G.B. Wilson for their critical comments and stimulating discussions on various aspects of this investigation; to Dr. Charles Thornton and Dr. John Speck for serving as members on my committee; to Dr. Stephanie Barch for her assistance in obtaining the materials needed in this study; and to Dan Williams for making our office a place to exchange ideas at any time of the day or night.

The ever willing help and encouragement of "Mac" Henderson will always be remembered.

No words of gratitude are adequate to express my appreciation to my wife, Dawn, for her faithful and untiring assistance in preparing this thesis.

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INTRODUCTION

In 1899 Loeb discovered that eggs of the sea urchin, when appropriately treated, follow a course of parthenogenetic development which results in the production of normal larvae. This finding stimulated an abundance of research on the problem of artificial parthenogenesis, particularly on the eggs of marine invertebrates. Discussions of this subject in texts by Wilson (1925), Loeb (1913), and F.R. Lillie (1919), and in a review by Tyler (1941) reveal that a wide variety of agents (physical, chemical, and mechanical) are capable of inciting the eggs of sea urchins to develop. This apparent lack of specificity is in striking contrast with the high degree of specificity in fertilization. A realization of this contradiction by workers in the field led them to abandon the idea that a parthenogenetic agent imitates the sperm or functions in a capacity fulfilled by one or more of the component parts of the sperm.

The frog egg, however, appears to be exceptional in that it has been possible to incite it to develop completely by the use of only one type of procedure. The complete response of the egg to an appropriate stimulus involves two phases described by Bataillon (1911, 1912): 1) an activation phase, and 2) a regulatory phase.

Many of the parthenogenetic agents (puncture, electric shock, heat, cold, ultraviolet radiation, acids, bases, hyper- and hypotonic salt solutions, fat solvents, etc.) which suc-

cessfully incite other eggs to develop are capable only of activating the frog egg. This activation phase is characterized by separation of the vitelline membrane with the subsequent formation of the perivitelline space, rotation of orientation, formation of the grey crescent region, and completion of the second maturation division. Important metabolic changes also occur upon initiation of maturation through the action of hormones. These will be described in the discussion.

These events observed in the activation phase may be followed occasionally by so-called "abortive" cleavages which in effect may involve one or two irregular furrowings or superficial indications of attempts at cytokinesis. Such behavior is not considered to represent bona fide cleavage, although duplication and migration of chromosomes and cyclic sol-gel transformations may occur.

Bataillon (1929) described the behavior of chromosomes and the cyclic formation of monasters in activated eggs of Rana fusca and Bufo vulgaris. He concluded from his cytological studies that eggs simply activated(e.g. by pricking or electric shock) succeed only in accomplishing monocentric mitoses.

The procedure for inciting frog eggs to undergo complete development was first described by Guyer (1907). It involved injecting blood or lymph into unfertilized frog eggs. This author was unaware, however, that he had induced parthenogenetic development. In 1912 Bataillon presented proof that a component must be inoculated into the egg in order for it to proceed along a developmental course involving normal cell divi-

sions (the regulatory phase). The egg of an amphibian can therefore be stimulated to cleave by simply pricking the egg ("first factor") with a fine glass needle in the presence of blood, lymph, or a tissue brei ("second factor"). For a description and analysis of this technique see the review in English by Tchou Su and Chen (1940). Both Herlant (1913) and Bataillon (1929) ascribed to the inoculated material an essential role in positioning or establishing an astral system which is capable of initiating an effective segmentation of the egg.

Normal embryos are less readily and less frequently obtained from eggs of frogs than from eggs of certain marine invertebrates through parthenogenetic development. Nonetheless, the relative ease with which amphibian embryos can be reared through metamorphosis to sexual maturity has made this material a useful tool for the analysis of problems of sex determination and the relative roles of nucleus and cytoplasm in development and heredity (see review by Tyler, 1941).

It soon became apparent to the early workers in the field that the phenomenon of artificial parthenogenesis could be regarded as one aspect of the general problem of cell stimulation. In his chapter "On the Nature of Formative Stimulation (Artificial Parthenogenesis)", Loeb (1912) states: "Cellular physiology has shown that tissues and organs develop only from cells through nuclear and cellular division. The conditions which cause cells to divide and develop into new normal or pathological tissues have, since Virchow, been called formative stimuli. It is the task of modern biology to ascertain first

what is the nature of these stimuli, and second, which change occurs in the cell in the process of formative stimulation."

Much of Loeb's interest in the problem of artificial parthenogenesis was concerned with the nature of the stimulus. The investigations of Shaver (1953) were principally directed toward an elucidation of the nature of the "second factor" in artificial parthenogenesis of frog eggs. Since parthenogenesis generally implies a complete development and since only a few of the eggs which cleave after stimulation by the "second factor" proceed through gastrulation and complete morphogenesis, Shaver preferred to refer to the active factor as the "cleavage-initiating substance."

All the investigations to date have indicated that the active factor can be obtained from a wide variety of biological materials. Rostand (1938) lists some of the sources from which he and other workers have obtained active materials. These include the whole blood of mammals, fish, reptiles, birds, leucocytes of the horse, the contents of earthworm seminal vesicles, and cells from the hermaphroditic glands of snails. Rostand (1924) reported that dried sperm of a frog, when reconstituted with water and inoculated, are still effective in initiating development.

Bogucki, (1923,1926) found the active factor in the brei of the ovary, pancreas, brain, and liver of the frog. Frog embryos at the blastula and gastrula stage also contained active material, while the unfertilized egg and the two blastomere stage did not.

Einsele (1930) first successfully used the injection technique and was able to incite eggs to develop by injecting them with cell-free extracts of testes and blood. Parat (1933) was able to obtain developing embryos by injecting eggs with acrosomes he had dissected from the sperm of Discoglossus pictus. Shaver (1953) obtained embryos which developed to at least the blastula stage by injecting large cytoplasmic granules from the testes, brain, lung, muscle, liver, and blood cells of the adult frog. Activity was also present in the serum of frog blood, in mammalian erythrocytes, and in the cells of frog embryos from late blastula and subsequent stages.

More recently, Huff and Preston (1965) have shown that active extracts could be obtained from frog eggs which had been activated by puncturing or electric shock at least 36 hours previously. A similar development of an active factor was observed in eggs which were activated and then enucleated, indicating the absence of direct nuclear involvement.

Inorganic substances as well as organic and biochemical preparations have, however, thus far proved to be ineffective in initiating development. No improvement in cleavage over mere pricking was obtained when the following materials were inoculated: colloidal platinum, chinese ink, talcum, and finely pulverized carbon (Herlant, 1913); nucleic acid (Voss, 1923; Bogucki, 1921); dextrin, tannin, egg white, and milk (Voss, 1923); pepsin, pancreatin, and rennet (Bogucki, 1922); nucleoproteins and lipids extracted from testes, peptone, tyrosine, thymine, and cysteine (Rostand, 1938).

Voss (1923) suggested that perhaps the materials which he tested were not inoculated in sufficient quantity to incite development. An improvement over the inoculation technique was introduced by Einsele (1930). His injection procedure increased the certainty that sufficient material would be introduced into the egg. Using this technique Einsele (1930) showed that the following solutions at concentrations ranging from 0.002-4.0 molar, when introduced into eggs, were incapable of stimulating them to develop: KSCN, KCl, $MgSO_4$, $MgCl_2$, $CaCl_2$, and mixtures of solutions of acetic acid and sodium acetate, KCl and $MgCl_2$, KCl and $CaCl_2$, and KSCN and $MgSO_4$. Results of injecting peptone solutions were also negative.

Additional materials injected by Shaver (1951, 1953) were shown to be ineffective. He tested 0.005-0.1% solutions of trypsin, chymotrypsin, and pepsin; a nucleohistone preparation from frog testes; a 1 mg/ml solution of denatured lipid-free protein prepared from Ascaris sperm; and 0.1 and 1.0 mg/ml solutions of yeast RNA, adenylic acid, and guanylic acid. To this list of ineffective agents may be added 0.01-0.5% solutions of ATP (Pfohl, 1962).

Negative results were also obtained by inoculating biological materials such as bacteria, flagellates, pollen and ovule extracts, and juices from the meristematic tissue of plants (Rostand, 1928), and by injecting granule fractions and whole cells of brewers yeast and the flagellate Polytomella sp. (Shaver, 1951).

The exact nature of the cleavage-initiating factor is still unknown. Bataillon (1929) strongly adhered to his notion that the inoculated material must include a nucleus or nuclear fragment. This idea was already questioned by his contemporaries (Voss, 1923; Bogucki, 1926; and Herlant, 1913). Positive refutation of this notion has been achieved by the work of Einsele (1930), Shaver (1953), Huff and Preston (1965), and by the results of experiments to be subsequently described in this thesis. These workers have shown that cell- and nuclei-free extracts of a variety of tissues are capable of acting in the regulatory capacity of Bataillon's "second factor". Significant in this regard is Shaver's demonstration of activity in granules from the non-nucleated red blood cell of a mouse.

With the continuing improvement of electron microscopical and biochemical techniques, great advances are being made toward a better understanding of the structure and function of subcellular components. Since the "cleavage-initiating substance" appears to be associated with the large cytoplasmic granules of a cell, consideration was given to the more sophisticated cell fractionation techniques now available as a means of profitably investigating the nature and site of localization of the active factor.

The initiating and motivating spirit of this study was the hope that a knowledge of the nature of this active factor may have significance in relation to the general problem of cell stimulation. A knowledge of the nature of the stimulus could direct investigations on the site and mechanisms of re-

sponse of the cell and hence to a better understanding of the interactions involved.

The following, then, is an account of experiments performed in an attempt to elucidate the nature of the "formative stimulus" of Loeb.

METHODS AND MATERIALS

I. TECHNIQUES OF CELL FRACTIONATION

A. Preparation of Tissue Homogenates: Cell Breakage

Liver Tissue was obtained from fasted adult frogs (Rana pipiens) maintained at 4°C. The frogs were decapitated and bled. The livers were removed, immersed in ice-cold medium, blotted dry and weighed. All subsequent steps in the fractionation were carried out at 0-5°C in order to minimize autodegration of cell components, as for example by hydrolytic enzymes released from damaged lysosomes (Allfrey, 1959, p. 205-207; Sawant, Desai and Tappel, 1964). For each gram of liver, 10 ml of the appropriate sucrose medium were added (1:10 suspension). The tissue was then minced with a scissors into pieces about 0.5 cm in thickness to facilitate homogenization.

Cell breakage was accomplished with a Potter-Elvehjem type coaxial homogenizer modified as described by Pierce and coworkers (1953). It consisted of a smooth-walled glass vessel fitted with a motor driven Teflon pestle (available from Arthur H. Thomas Co., Philadelphia) operated at about 1200 rpm. The homogenate was filtered through several thicknesses of cheesecloth to remove connective tissue fibers. The filtrate was subsequently fractionated by differential centrifugation according to the scheme outlined in a following section.

B. Isolation Media

A medium consisting of 0.25M sucrose and 0.001M disodium ethylenediaminetetraacetate (EDTA) adjusted to pH 7.0-7.2 was used in the initial steps of the cell fractionation scheme (this medium is subsequently designated as 0.25SE). In some experiments the medium was buffered at pH 7.0-7.2 with 0.1M Na_2HPO_4/NaH_2PO_4 (0.25 SEP). Prior to the final centrifugation the pellet was sometimes resuspended in 0.7M sucrose medium (0.7 SE or 0.7 SEP).

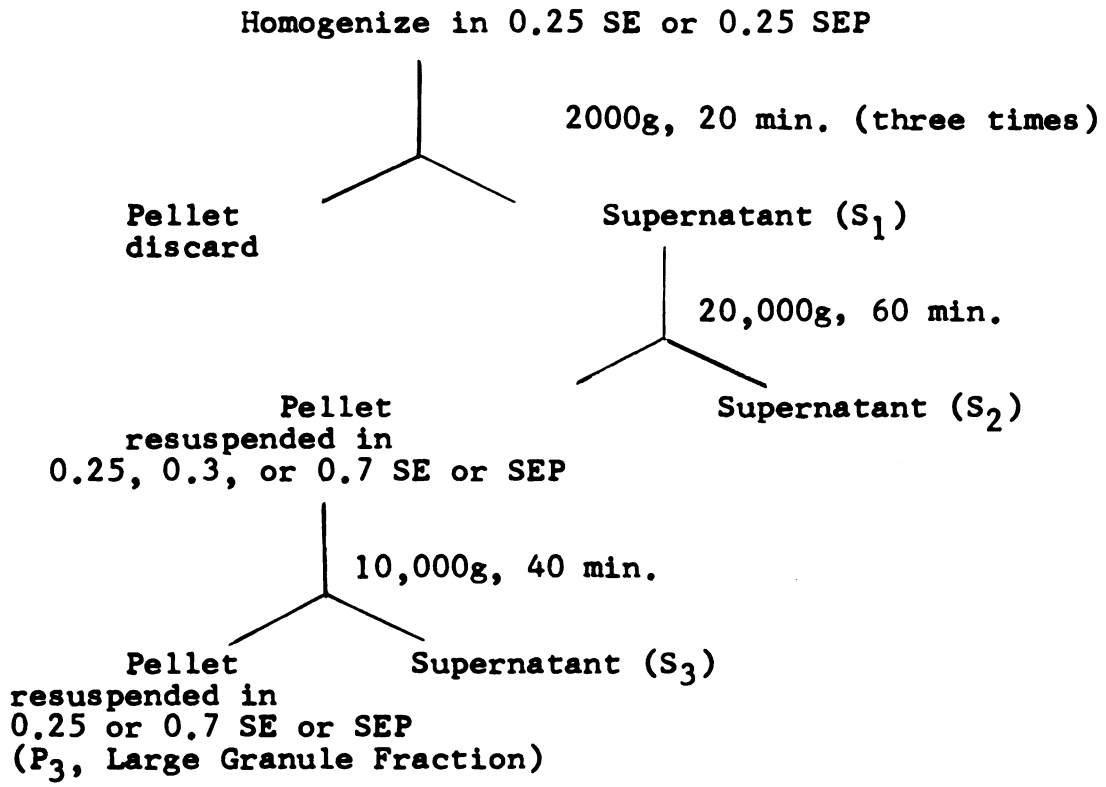
Either the SE or SEP media was used to prepare the sucrose density gradients.

C. Centrifugation

1. Differential Centrifugation - The differential centrifugations were performed with an International Refrigerated Centrifuge Model HR-1 equipped with the 856 angle head designed to hold 50 ml tubes. R_{max} was 10.7 cm and the volume of the suspension centrifuged was generally 35-40 ml. Variations in the fractionation scheme outlined below and in the composition of the medium used will be given in conjunction with the appropriate results.

Three centrifugations at 2000g for 20 minutes each were sufficient to remove all of the whole cells, nuclei, cellular debris, and most of the abundant pigment granules. The pigment granules which remained in the 2000g supernatant formed a compact mass adhering to the bottom of the tube after centrifuging at 20,000g. The main bulk of the 20,000g pellet could be carefully washed away from the adhering pigment mass with the resus-

pension medium.



The scheme outlined above involves centrifugations of longer durations than usual (e.g. see Allfrey, 1959, p. 254-255) in order to obtain a greater yield of larger granules.

Since the values for gravity and time referred to are exclusive of acceleration and deceleration they cannot be readily compared with the g min. values used by de Duve and his coworkers (1955).

2. Sucrose Density Gradient Centrifugation - Continuous sucrose density gradients were preformed by a gradient engine constructed with slight modifications according to the specifications given by Rosenbloom and Schumaker (1963).

By introducing into the engine 2.2 ml of 0.25M or 0.80M sucrose medium and 2.3 ml of 1.8M sucrose medium, a 4.5 ml continuous sucrose density gradient of variable steepness (0.25-1.8M sucrose or 0.8-1.8M sucrose) could be produced.

Six- to seven-tenths ml of the large granule fraction obtained by differential centrifugation was layered over the gradient. Three such gradients with overlayered samples were prepared in cellulose nitrate tubes and placed in an SW 39 swinging bucket rotor. Centrifugation was performed in a Spinco Model L preparative ultracentrifuge at 35,000 rpm for 2 hours. Deceleration occurred with the brake off in order to minimize disturbance of the gradients.

With the aid of an easily constructed tube-puncturing device (Fig. 1), 11 fractions of 30 drops each, unless otherwise noted, were collected from each gradient. Equivalent fractions from the three gradients were combined. With this apparatus the number and size of the drops obtained and their rate of flow (about 25 drops per min.) were very consistent from one gradient to another.

II. BIOCHEMICAL ASSAYS

A. Protein

Protein concentrations in appropriately diluted samples were determined by a modified version (Miller, 1959) of the Lowry technique (Lowry, et al., 1951). Reference was made to a bovine serum albumin standard.

B. Nucleic Acids

Following treatment of the fractions with cold trichloroacetic acid and lipid solvents, the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted and separated by the method of Schmidt and Thannhauser (1945). Modifications of the procedure recommended by Hutchison and Munro (1961, p. 794-795) were incorporated. Since the quantity of DNA in the sucrose gradient fractions is very small, bovine serum albumin was added to provide a bulky precipitate upon addition of perchloric acid to the alkaline digest.

The method described by Ceriotti (1952) involving the reaction of deoxypentose with indole was used to estimate the DNA content. The orcinol determination of ribose was used to estimate the RNA content (Ceriotti, 1955; see Webb and Levy, 1958).

C. Enzymes

Assays of enzymes were performed to obtain a quantitative estimate of the abundance of their respective host particles. If we may assume that studies on mammalian liver have general applicability, then a large granule or mitochondrial fraction from frog liver should contain predominantly mitochondria with lysosomes and peroxisomes (microbodies) as significant minor components. Thus, cytochrome oxidase was assayed as a marker for mitochondria, acid phosphatase and ribonuclease for lysosomes, and urate oxidase for peroxisomes.

Spectrophotometric measurements for determining the rates of cytochrome c oxidase and urate oxidase activity were performed with an Hitachi Perkin-Elmer Model 139 UV-VIS spectrophotometer

Figure 1. Tube puncturing device. Part A: The cellulose nitrate centrifuge tube (7) is fitted with a one-hole rubber stopper (2) into which is inserted a T-shaped glass tube (1). A solid rubber stopper (4) is bored ½ its length to receive one arm of the T-tube. The shank of a hypodermic needle (5) is passed through the solid portion of the stopper. The other end of the shank extending from the stopper is attached to a length of polyethylene tubing (6). This tubing is connected to the air syringe of the gradient machine. The other arm of the T-tube is provided with a piece of rubber tubing (3) which is closed with a clamp immediately after the centrifuge tube is punctured. The system is then essentially closed, and drops of the tube contents pass through the hole in the bottom of the tube only when pressure is supplied by a continuous and uniform depresion of the plunger of the air syringe (driven by a synchronous motor).

Part B: The puncturing device itself is constructed of ½ inch Plexiglas. The two upper shelves of the apparatus are provided with holes to hold the tube snugly in place. The bottom shelf (7) holds the puncturing needle, diagramed in cross-section. A short piece of the shank of a 17 gauge needle (4) is fitted into a hole in the shelf. It is held tightly in place by soldering (5, upper bead) it to a metal washer (6, head of thumb tack) and then rimming the upper end of the needle flush with the shelf surface. Into this sleeve is then inserted a somewhat longer piece of 20-22 gauge needle (2,3). The doubly tapered puncturing end of the needle (2) extends above the shelf about 2-3 mm. The puncturing needle (2,3) is held in place with a bead of solder (5, lower bead) which attaches it to the rigid sleeve (4). If the needle requires sharpening or replacement it can easily be removed by melting this solder bead. To receive the centrifuge tube and provide a good seal after it is punctured, the tip of the needle is circled with a ring of vaseline on a piece of Parafilm (1).

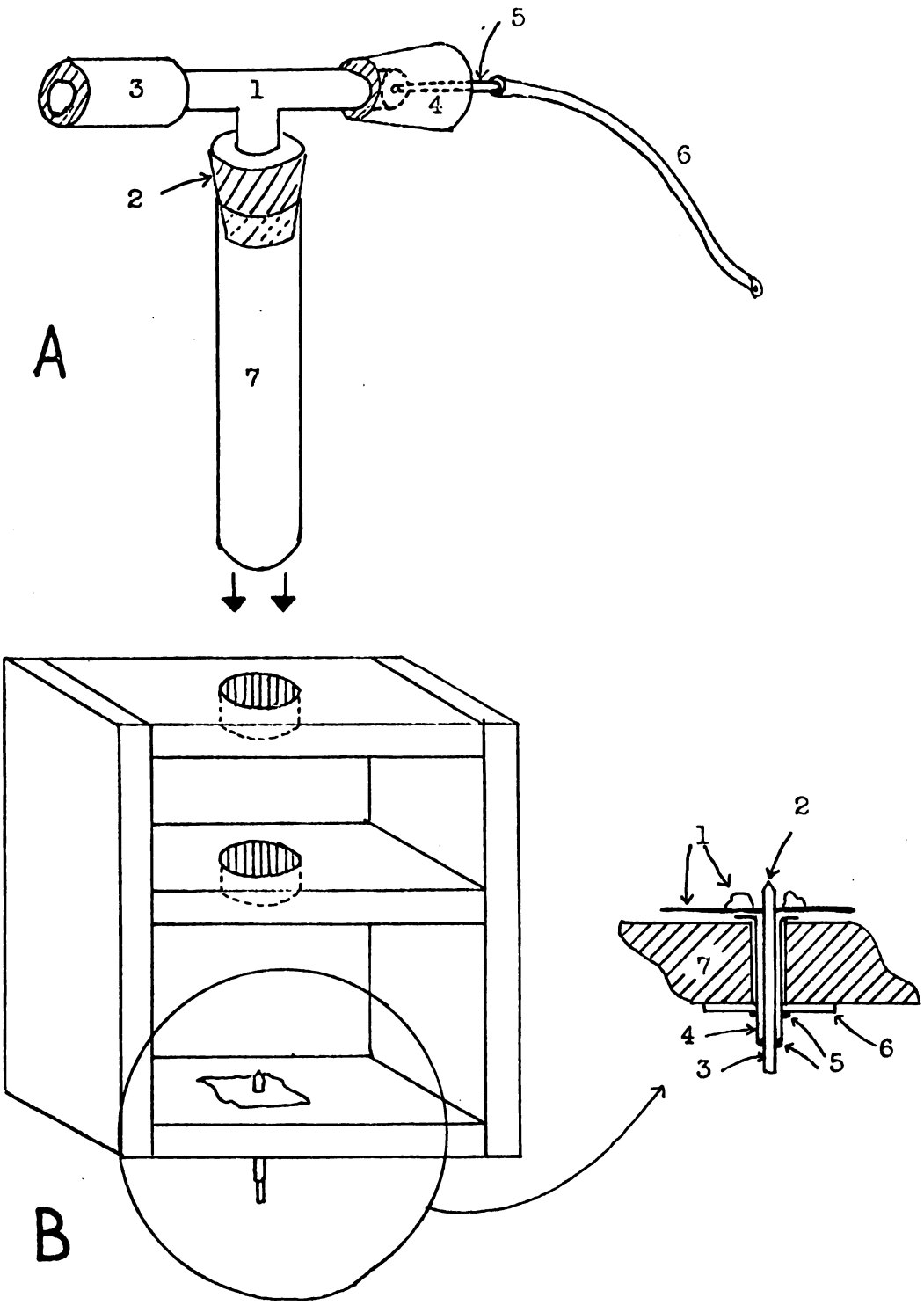


Figure 1

equipped with a photomultiplier detector unit (139-0250) and a model 159 recorder.

1. Cytochrome C Oxidase activity was determined spectrophotometrically according to the procedure outlined by L. Smith (1955). The cytochrome c (Sigma Type III, from horse heart) was reduced with hydrogen and palladium. The absorbance ratio A_{550}/A_{565} for the reduced cytochrome c was usually at least eight. The final concentration of reduced cytochrome c in the assay mixture was 15 μM. At this concentration the oxidation of reduced cytochrome c by oxidase is a first order reaction with respect to the cytochrome c concentration. The rate of oxidation of reduced cytochrome c was determined by measuring the decrease in absorbancy at 550mμ. The reaction was allowed to proceed for 3 minutes at 23°C.

A unit of cytochrome c oxidase activity is expressed as a first order velocity constant of 1×10^{-2} sec^{-1} ($k\ sec^{-1}$) for the oxidation under the specified conditions.

2. Urate Oxidase was assayed spectrophotometrically by the method outlined in the Worthington Manual No. 11 (E.C. 1.7.3.3, Worthington Biochemical Corp., Freehold, N.J.). Certain steps in the procedure were modified.

The uric acid substrate solution was prepared by mixing 4 volumes of borate buffer (0.125M sodium borate, pH 9.2) and 1 volume of uric acid stock (0.050 mg/ml, Stock No. 680-20 from Sigma Chemical Co., St. Louis, Mo.).

The reference cuvette was prepared by mixing 0.1 ml of the fraction to be tested, 0.2 ml of 0.1N KCN, 0.7 ml HOH, and 2.0

ml of uric acid substrate, in that order. The absorbancy at 293 mu was adjusted to 0.500.

To the test cuvette were added 0.2 ml uric acid substrate, 0.9 ml HOH, and 0.1 ml of the fraction. The decrease in absorbancy was recorded at 23°C for 6-7 minutes. The disappearance of 1.0 µg of uric acid/ml causes a drop in absorbancy at 293 mµ of 0.072 (1 cm light path). Using the molar absorbancy index or uric acid (1.22×10^4 cm^{-1}) the activity can readily be expressed in µM of uric acid oxidized per minute under the specified conditions. In this study a unit of activity is defined as that amount of enzyme which oxidizes 1 mµM of uric acid per minute.

3. Acid Phosphatase determinations were based on the direct measurement of p-nitrophenol which is liberated upon hydrolysis of the substrate p-nitrophenyl phosphate.

The assay mixture, with a final volume of 1.2 ml, contained 0.2 ml of the fraction to be tested, 2 mg of p-nitrophenyl phosphate (Sigma 104 Phosphatase Substrate), and citrate buffer at a concentration of 0.0375M, pH 4.3. Following the recommendation of Wattiaux and de Duve (1956), the final reaction mixture was made 0.1% (v/v) with respect to Triton X-100 in order to obtain a more accurate estimate of the total acid phosphatase activity. Calbiochem Acid Phosphatase Calsuls (capsules containing buffer and p-nitrophenyl phosphate) were used for some of the assays.

After 15 or 30 minutes of incubation in a 37°C water bath, the reaction was stopped by adding 5.0 ml of 0.1N NaOH. The

yellow color of the liberated p-nitrophenol develops immediately. The absorbance at 410 mμ was determined with a Bausch and Lomb Spectronic 20 and the micromoles of p-nitrophenol were determined from a standard curve prepared from a p-nitrophenol standard solution (10 mM/ liter, Sigma Stock 104-1). One unit of enzyme activity is expressed as 1.0 μM of p-nitrophenol liberated per hour at 37°C.

Preliminary experiments showed that the absorbance due to the 0.2 ml samples of fractions from sucrose density gradients could be ignored. Corrections were made only for the absorbancy of samples of whole large granule fractions.

4. Ribonuclease was assayed by the method of Kalnitsky and coworkers (1959) as described in the Worthington Manual No. 11 (E.C. 2.7.7.16, Worthington Biochemical Corp., Freehold, N.J.). The substrate used was a 1% commercial grade yeast RNA (Sigma) solution in 0.1M acetate buffer, pH 5.0. The assay depends on a spectrophotometric measurement at 260 mμ of the acid soluble oligonucleotides liberated in 4 minutes at 37°C. The activity is thus expressed in terms of the absorbancy increase at 260 mμ.

As a control on the procedure, standard RNase was processed in the same manner as the test fractions (RNase-A from bovine pancreas, Sigma Type III-A).

III. BIOLOGICAL ASSAY

Samples of the cell fractions obtained as described in a preceding section were injected into unfertilized eggs of the leopard frog (Rana pipiens) in order to determine whether the fractions contained material capable of inciting the eggs to cleave.

Eggs of Rana pipiens were obtained prior to the onset of breeding season by the method of Rugh (1934). Immediately before the microinjection of a fraction, 30 to 50 eggs were extruded onto a glass slide. Injections were performed as rapidly as possible and the slide with the injected eggs was placed in a finger bowl containing aerated tap water at room temperature (23-25°C). This operation required about 3 minutes to perform. As a control on the viability of the eggs, generally 100 to 200 eggs were artificially inseminated (Rugh, 1934). If fewer than 70% of the fertilized eggs cleaved, the results of that experiment were discarded. The sucrose medium alone, in which the fractions were suspended, was injected as a control. If any sperm, blood cells or other tissue debris were present which might initiate cleavage, their presence would be detected by this control.

Microinjections were performed with micropipettes fashioned from 1.0 mm O.D. capillary tubing with the aid of a Livingston pipette puller (Otto K. Hebel, Scientific Instruments, Rutledge, Pa.). A different micropipette was used for each fraction injected. A 30 ml B-D Plastipak Disposable Syringe equipped with a 21 gauge needle was attached to one end of a length of Intra-

medic polyethylene tubing (I.D. 0.030" x O.D. 0.048", Clay-Adams, Inc., N.Y.). The syringe and tubing were filled with distilled water to form a simple hydraulic system. The system was completed by attaching the micropipette to the other end of the tubing. Each micropipette was filled immediately prior to an injection by immersing the tip of the pipette into several drops of a fraction and withdrawing the plunger of the syringe.

Injections were performed under a binocular dissecting scope. If the tip of the pipette is fashioned properly, it will readily penetrate the jelly envelope and egg membrane. Penetration into the egg was made in the animal hemisphere apart from the area of the second meiotic metaphase spindle (Tchou Su and Chen, 1940). After the pipette was partially withdrawn from the egg, a brief pulse of firm pressure to the plunger of the syringe was generally sufficient to introduce about 0.04 µl of material into the egg.

The injection procedure itself activates the egg without stimulating it to cleave, unless cleavage-initiating material is introduced in the process. Following activation many eggs will exhibit a superficial wrinkling effect and some may undergo one or two irregular cleavages and then cease dividing. To avoid confusing true cell division with these aberrant forms, the embryos were observed at the blastula stage (Stages 8-9, Shumway, 1940). Following injection of fractions containing cleavage-initiating material, partial cleavage was frequently observed. In such embryos normal cleavage occurred in only part of the em-

bryo. The remaining surface was totally noncellular and there was an abrupt boundary between the fully cleaved and totally uncleaved regions (Fig. 2). The cells were typical in size and appearance of mid or late blastulae. Blastomeres in similar cases have been shown by Frazier (1951) and Ramirez and Huff (1967) to be almost entirely nucleated. Such embryos will be referred to as "partial blastulae". The types of embryos thus obtained ranged from partial blastulae with only a small cleaved patch of very few cells in the animal hemisphere to partial blastulae with a small uncleaved patch generally in the yolk region. Occasionally, normal appearing blastulae were also observed. In some experiments the blastulae were scored according to the approximate proportion of the surface area which cleaved, i.e. >0-1/4, >1/4-1/2, >1/2-3/4, and >3/4-total (Fig. 2).

IV. PRESENTATION OF DATA

The wet weight of the tissue processed was used as a crude reference standard to express the relative concentration of the material injected. For example, with reference to a particular subcellular fraction, the ratio 1:3 indicates that for each gram of tissue processed the fraction is suspended in 3 ml of sucrose medium. If 1 ml of this suspension is diluted with 3 ml of medium, the concentration is then denoted as 1:15.

Other details concerning the presentation of data will be given in the appropriate section on results.

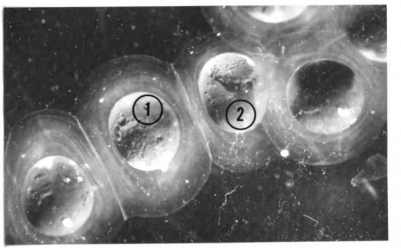
Figure 2. Parthenogenetic blastulae. Eggs were examined approximately 10-12 hours after being injected.

1 and 2: >0-1/4 blastulae. Blastomeres in both partial blastulae cover only a small portion of the total egg surface. Note the sharp demarcation between the blastomeres and uncleaved surface of the egg in embryo 2.

3: >0-1/4 blastula. Note the small patch of blastomeres.

4 and 5: >3/4-total blastulae. Most of the surface of the eggs is covered with blastomeres. A small uncleaved portion of the surface is visible in embryo 4.

6 and 7: No cleavage. Eggs exhibit superficial furrowing and aberrant cleavages. The photograph includes >1/2 blastulae.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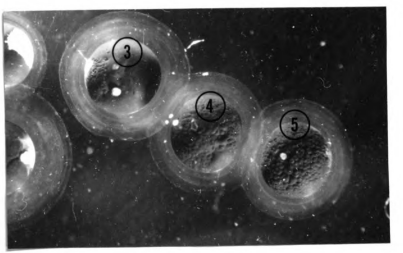
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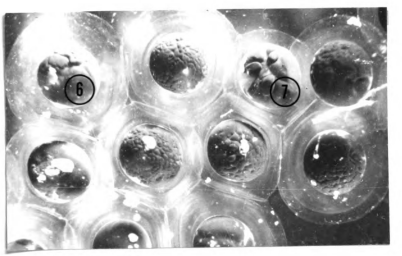
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RESULTS

The results obtained in this study are divided into two major sections. The first is concerned with the biochemical and biological assays of fractions obtained by differential centrifugation. The second considers the results of an analysis of fractions obtained by sucrose density gradient centrifugation.

I. FRACTIONATION BY DIFFERENTIAL CENTRIFUGATION

The cleavage-initiating (CI) capacity of the subcellular fractions of a variety of tissues has already been described (Shaver, 1953). The subsequent development of more refined isolation techniques using sucrose media made a more thorough study of this problem feasible.

Shaver (1953) showed that the large granule fraction (sedimented in 0.005M phosphate buffer, pH 7.3 at 3000-8000g, 20 min.) of cells was the most active in initiating cleavage. The results which follow support the conclusion that CI activity is associated only with cytoplasmic particles and predominantly or exclusively with the large granular elements. Most of the subsequent experiments are involved with an analysis of this large granular fraction.

Of the 674 eggs injected with suspension medium alone, none cleaved. Included in the media injected were the unbuffered sucrose-EDTA (SE) and phosphate buffered sucrose (SEP) media with the concentration of the sucrose at 0.04, 0.25, or 0.7 molar.

A. Selection of Liver as the Source of Cleavage-Initiating Material

Fractions from heart, brain, and liver tissue of the adult frog (Rana pipiens) were initially injected to test for the presence of CI material (Table I). Fractions from these three tissues were prepared in 0.25 SEP medium according to the differential centrifugation scheme outlined in the section on Methods. In all the tissues the greatest activity was found in

Table I. Cleavage-initiating activity of the large granule fraction and supernatant fluids prepared in 0.25 SEP from heart, brain, and liver of the frog.[a]

Tissue		Fractions injected[b]			
		S_1	S_2	S_3	P_3
Heart	No. eggs	26(1)[c]	---	49(1)	66(1)
	% cleaving	0		6.1	24.2
Brain	No. eggs	32(1)	52(1)	46(1)	338(4)
	% cleaving	18.8	1.9	6.5	30.2
Liver	No. eggs	---	---	---	285(3)
	% cleaving				30.2

[a] Fractionation by the differential centrifugation scheme in Methods.

[b] All fractions had a relative concentration of 1:10. The symbols represent the following: S_1, 2000g supernatant; S_2, 20,000g supernatant; S_3, 10,000g supernatant; P_3, 10,000g pellet (large granule fraction).

[c] Number in parentheses denotes the number of frogs from which eggs were obtained.

the final large granule fraction (P_3) sedimented at 10,000g.

Injection of P_3 from heart stimulated cleavage in 24% of the

eggs. The CI activity of P_3 from liver was identical to that from brain (30%). In later experiments injections of dilutions and subfractions of P_3 from liver frequently resulted in more than 40% of the eggs cleaving. Since liver is easier to homogenize than heart tissue and can be more readily obtained than brain tissue in large quantities, it was selected as the source of cleavage-initiating material for all subsequent experiments.

B. Localization of Cleavage-Initiating Material in the Large Particulate Cell Components

1. Cleavage-Initiating Activity - In a preliminary experiment brain tissue was fractionated by a more conventional scheme of differential centrifugation similar to the one proposed by Schneider (1948) and Schneider and Hogeboom (1950) for rat liver. The 0.25M sucrose medium used by these authors was modified by the addition of 0.0001M EDTA and 0.012M phosphate buffer, pH 7.3. The results in Table II show the drop in CI activity of the large granule suspension upon dilution. The injections in Tables III and IV were of large granules (P_3) obtained from liver by means of the scheme outlined in Methods. The percentages of cleavage obtained upon injecting dilutions of these suspensions indicate that a greater concentration of CI material is obtained by this method of fractionation. It was therefore the method used in all subsequent experiments, unless otherwise noted.

The data from Tables II, III, and IV demonstrate the lack of CI material in post large granule fractions. The 20,000g supernatants (S_2) of two preparations (Table III, 0.70 SEP;

Table IV, 0.25 SEP) and dilutions thereof were unable to initiate cleavage. The absorbancies of the various suspensions in Table

Table II. Cleavage-initiating activity and absorbancy at 550 mμ of large granule and small granule fractions prepared in a 0.25M sucrose medium from frog brain.[a]

	Fractions injected			
	8000g pellet			20,000g pellet
Rel. concn.	1:7	1:39	1:199	1:7
No. eggs	206(5)[b]	132(3)	109(3)	122(3)
% cleaving	34.0	16.7	3.7	0
A_{550} mμ	.975	.230	.045	.170

[a] See text for details of medium composition and fractionation procedure.

[b] See Table I.

II were measured at 550 mμ to give an estimate of the relative particle concentration. The data in this table show that the 1:7 and 1:39 suspensions of the 8000g pellet (large granules) were very active as CI agents. However, the pellet obtained by centrifuging the 8000g supernatant at 20,000g contained no CI activity in spite of the fact that the apparent particle concentration of the 20,000g pellet suspension (A_{550} = 0.170) approached that of the 1:39 suspension of the 8000g pellet (A_{550} = 0.230).

Centrifuging the 2000g supernatant, according to the usual scheme of fractionation (Methods), at 20,000g in either the 0.25

SEP or 0.7 SEP media yielded a pellet which was rich in CI activity. The activities of various dilutions of the supernatants (S_3) and pellets (P_3) obtained by a 10,000g centrifugation of the resuspended 20,000g pellets are shown in Tables III and IV. The data illustrate the effect that the density of the suspension

Table III. Cleavage-initiating activity of the large granule fraction and supernatant fluids prepared in 0.7 SEP medium from frog liver.[a]

Rel. concn.	Fraction injected[b]			
	S_1	S_2	S_3	P_3
1:3				0
1:15			32.4	8.9
1:29	4.1	0		
1:63				22.0
1:159			41.2	
1:255				24.4
1:299	32.5	0		
1:1023				17.2

[a] Fractionation by the differential centrifugation scheme in Methods. The data are expressed as the percentages of eggs which cleave after injection. Each percentage value is based on an average sample size of 40 eggs (31-49) from one frog.

[b] Fractionation symbols are explained in Table I and Methods.

medium has on the centrifugal distribution of the CI material. If the pellet (20,000g) is resuspended in 0.7 SEP and centrifuged at 10,000g, both the resultant supernatant (S_3) and the pellet (P_3) are very active in stimulating cleavage. On the other hand, if the suspension medium is 0.25 SEP, S_3 has little activity (max. of 20.5% at 1:11 concn.). Most of the activity sediments (max. of 50.0% cleavage at 1:447 concn.) in this medium.

Another phenomenon of interest is apparent from the data in Tables III and IV; namely, if the concentration of CI material injected is too great, its effectiveness in initiating cleavage is markedly reduced. This aspect of the problem will be dealt with separately in a subsequent section.

Table IV. Cleavage-initiating activity of the large granule fraction and supernatant fluids prepared in 0.25 SEP medium from frog liver.[a]

Rel. concn.	Fractions injected[b]			
	S_1	S_2	S_3	P_3
1:6				7.7
1:11			20.5	
1:24-1:27	8.6	0		22.9
1:47			0	
1:99-1:111	28.2	0		36.8
1:191			4.3	
1:399-1:447	39.0	0		50.0
1:767			0	
1:1599-1:1791	11.5			10.5
1:3071			0	

[a] Fractionation by the differential centrifugation scheme in Methods. The data are expressed as the percentages of eggs which cleave after injection. Each percentage value is based on an average sample size of 41 eggs (35-52) from one frog.

[b] Fraction symbols are explained in Table I and Methods.

2. Acid Phosphatase Assay - Assays for acid phosphatase in the supernatants (S_3) and pellets (P_3) of the 10,000g centrifugation (Tables III and IV) revealed that 48.8% of the activity sediments in 0.7 SEP medium whereas in 0.25 SEP medium 71.4% is sedimented (Table V). There is thus a marked difference in the partitioning of the enzyme in the two media. If one wishes to

compare the distribution of this marker enzyme with that of the cleavage-initiating activity, however, it is important to consider the activity per milliliter of the fractions rather than the total recovery, since the former represents each fraction as injected at its maximum concentration into the egg.

Table V. Effect of suspension medium on the distribution of acid phosphatase activity following centrifugation.

Medium	S_3[a]		P_3	
	Acid Pase units/ml[b]	Total activity[c]	Acid Pase units/ml	Total activity
0.25 SEP	0.72	57.6	3.55	142.0
0.7 SEP	0.37	29.6	1.88	28.2

[a] S_3 and P_3 represent the supernatants and pellets of the 10,000g centrifugations of Tables III and IV.

[b] Unit of activity defined in Methods. Assay performed with Calbiochem Calsuls, without Triton X-100.

[c] Units/ml times total ml of fraction.

The activity per milliliter in the pellet resuspended in 0.7 SEP (P_3) is 5.1 times the activity per milliliter in the supernatant (S_3), whereas in the 0.25 SEP medium the activity per milliliter of the resuspended pellet exceeds that of the supernatant by a factor of 4.9. The activities of the pellet and supernatant of the 0.25 SEP both exceeded the activities in the respective fractions in 0.7 SEP by a factor of two. No obvious correlation between the acid phosphatase concentration and cleavage-initiating activity of the fractions is apparent.

3. Assays for Cytochrome Oxidase Activity were performed on fractions S_1, S_2, S_3, and P_3 of Tables III. Table VI lists the total and specific activities of cytochrome oxidase as well as the activity per milliliter of each of the fractions. It is

Tables VI. Activity of cytochrome oxidase in subcellular fractions prepared in 0.7 SEP medium from frog liver by differential centrifugation.[a]

Fraction	Units/ml[b]	Tot. act.[c]	Sp. act.[d]
S_1	4.1	615.0	0.70
S_2	1.2	180.0	0.24
S_3	1.3	97.5	4.48
P_3	20.9	313.5	13.03

[a] Fractions analyzed are samples of the ones tested for cleavage-initiating activity in Table III.

[b] Units of cytochrome oxidase activity defined in Methods and Materials.

[c] Product of the units/ml of a fraction times the theoretical volume of the fraction.

[d] Units of activity per mg of protein in the fraction.

obvious from these data that the large granule pellet is enriched with mitochondria. It is significant, however, that following centrifugation for 1 hour at 20,000g the supernatant (S_2) still contained considerable cytochrome oxidase activity and thus presumably mitochondria. The level of activity per milliliter was comparable to that present in the S_3 fraction. As observed in Table III, however, S_2 contained no cleavage-initiating activity while considerable activity was present in S_3.

C. Cleavage-Initiating Material in Microsomal Fraction

Another fractionation scheme was employed and the results obtained are shown in Table VII. The procedure used is a modification of one described by Allfrey (1959) and based on the methods recommended by Hogeboom (1955) and Schneider and Hogeboom (1950). The scheme was intended to yield a small granule or microsomal fraction. The 0.25 SE medium was used throughout the entire procedure.

The sediments from three initial centrifugations at 700g, for 10 minutes each, were discarded. Centrifugations at 5000g for 10 minutes and 10,000g for 15 minutes yielded pellet fractions P_1 and P_2, respectively. The supernatant of the latter was further fractionated in the Spinco Model L ultracentrifuge equipped with the No. 40 rotor. A centrifugation at $56,593g_{max}$ for 60 minutes yielded a supernatant (S_3) and pellet which was resuspended in sucrose medium and centrifuged at $144,880g_{max}$. The resulting supernatant and pellet (resuspended) were designated S_4 and P_4, respectively.

The absorbancies at 260 mμ of the various fractions and the percentages of cleavage obtained by injecting these fractions into eggs are shown in Table VII.

The bulk of the cleavage-initiating activity again sedimented with the large granular material at 5000 and 10,000g. While dilutions of the various fractions were not injected to ensure that a maximum percentage of cleavage was obtained, there was some degree of certainty that a near optimum concentration was injected since the blastulae that resulted were of a gener-

ally high quality. The correlation between blastula quality and the concentration of cleavage-initiating material will be further analyzed in a subsequent section.

Table VII. Cleavage-initiating activity and absorbancy at 260 mμ of a microsomal and other subcellular fractions prepared in 0.25 SE medium from frog liver.[a]

	Fractions injected[b]				
	P_1	P_2	S_3	P_4	S_4
% cleaving[c]	44.1	50.4	0	12.7	0
A_{260}/mg protein	2.52	3.11	1.22	2.51	---

[a] See text for details of fractionation procedure.

[b] Symbols are explained in the text. The protein concentration of each fraction adjusted to 0.90 mg/ml by appropriate dilution prior to injection.

[c] Each percentage value is based on an average total of 134 eggs (126-141) from two frogs.

D. Cleavage-Initiating Activity of Nuclear Fractions

Several nuclear fractions were prepared in an effort to discount the possibility that fragments of the nuclear membranes or other nuclear constituents were the active factor in the large granule fraction by virtue of their adsorption to the components of that fraction.

A crude sample of nuclei was prepared from the pellet of a 2000g centrifugation of a liver homogenate in 0.25 SEP. The pellet, resuspended in 0.25 SEP, was centrifuged at 1000g for 10 minutes. The resulting pellet was again suspended in sucrose media and filtered through several thicknesses of cheesecloth.

The centrifugation at 1000g for 10 minutes and filtration was repeated twice more. The final suspension of nuclei in 0.25 SEP contained 0.11 mg protein per ml of suspension. As expected for a preparation of liver nuclei in sucrose medium, the suspension was heavily contaminated with whole cells and cytoplasmic fragments (Allfrey, 1959, p. 250). Of 50 eggs injected with this suspension, only one cleaved. In the same experiment, injection of a large granule fraction rich in cleavage-initiating material incited 26 of 77 eggs to cleave.

A very clean preparation of nuclei from frog liver was obtained in citric acid solutions by the method of Mirsky and Pollister (1946). Portions of the final nuclear pellet were resuspended in a variety of media including 2% citric acid at pH 2-3; 0.1M phosphate buffer, pH 7.0-7.2; and 0.25M sucrose, pH 3-4, plus combinations and dilutions of the preceding. In addition two of the suspensions were briefly treated with ultrsound. Material from a large granule fraction was injected into 52 eggs as a control. Of these, 24 cleaved. Of a total of 310 eggs injected with suspended nuclei or nuclear fragments, only 2 cleaved. As noted earlier by Shaver (unpublished observations) eggs injected with suspensions of nuclei displayed a higher frequency of superficial furrowing and abortive cleavages than those injected with only sucrose media or phosphate buffer.

E. Summary

Frog liver was selected as the source of cleavage-initiating material for experimental purposes. Fractionation of this material by differential centrifugation revealed that all detectable

cleavage-initiating activity sediments with granules in both the 0.25 and 0.7 SEP media when centrifuged at 20,000g for 1 hour. Centrifugation at 10,000g for 40 minutes also sediments most of the activity in the 0.25 SEP medium. In the 0.7 SEP medium, however, considerable activity remains in the supernatant. There was no obvious correlation between the centrifugal partitioning of the cleavage-initiating activity and the acid phosphatase activity (lysosomes) in these experiments. It may be recalled here that the reaction mixtures for the acid phosphatase assays did not contain Triton X-100.

The cleavage-initiating activity of a microsomal fraction sedimented at 56,593g (1 hour) was approximately one-fourth that of large granule fractions sedimented at 5000g (10 min.) and 10,000g (15 min.).

The injection of nuclear fractions prepared in sucrose or citric acid media did not stimulate cleavage.

II. TREATMENT OF THE LARGE GRANULE FRACTION

A. Freeze-Thaw Treatment

1. Preparation - In one experiment frozen-thawed material was prepared by freezing a sample of a 0.25 SEP large granule fraction (P_3, obtained by the standard procedure) in a dry ice-acetone mixture for 5 minutes. The frozen material was thawed at room temperature for 45 minutes to 20°C. The freeze-thawing was repeated once and a sample of this suspension (FT) was injected. The remaining suspension was centrifuged at 20,000g

for 45 minutes. The supernatant (FTS) and pellet, resuspended in 0.25 SEP, (FTP) were also injected.

2. Cleavage-Initiating Activity - A total of 756 eggs from four frogs were injected with non-treated P_3 material. The number of eggs cleaving was generally very low, except for one frog in which the maximum percentage of cleavage obtained was 34.6 (the relative concentration of cleavage-initiating material was 1:11). The maximum percentages of cleavage obtained in other batches of eggs injected with P_3 were 8.2, 4.3, and 0. Nevertheless, of the 652 eggs injected with the frozen-thawed material (FT, FTS, and FTP) over the concentration range of 1:2 to 1:767, none cleaved.

In a subsequent experiment a portion of the large granule fraction (P_3) from Table III was diluted with respect to the concentration of sucrose by adding an EDTA-Phosphate buffer solution to yield 0.04 SEP. This suspension in hypotonic medium was then treated by freeze-thawing and centrifuged in the same manner as the material in the preceding experiment.

As shown in Table III, eggs cleaved when injected with the P_3 suspension at the relative concentrations of 1:15, 1:63, 1:255, and 1:1023. The frozen-thawed fractions in hypotonic media were injected at the relative concentrations of 1:18, 1:75, and 1:303 into a total of 337 eggs. In spite of the fact that these concentrations are within the range of concentrations of the injected P_3, none of the eggs injected with this treated material cleaved.

3. Solubilization of Particle-Associated Constituents - Analyses for protein and acid phosphatase were performed on the treated material from both experiments described above to determine to what extent the particle bound molecules were released into solution by the treatment. In addition, cytochrome oxidase determinations were performed on the material of the second experiment. The pertinent results are tabulated in Table VIII.

Table VIII. Release of protein and enzymes from large granules by freeze-thaw treatment.

	I 0.25 SEP medium		II 0.04 SEP medium	
	FTS[a]	FTP	FTS	FTP
Protein				
Total[b]	5.33	20.0	3.00	6.00
% release[c]	21.0		33.3	
Acid Phosphatase				
Total act.[d]	6.24	29.1	2.10	9.60
% release	17.7		17.9	
Cyto. c Oxidase				
Total act.[d]	---	---	0	14.1
% release			0	

[a] FTS and FTP refer to the 20,000g supernatant and pellet, respectively.

[b] Expressed in milligrams of protein.

[c] FTS/(FTS + FTP) times 100.

[d] Enzyme activity expressed in units defined in Methods. Acid phosphatase assayed with Calbiochem Calsuls, without Triton X-100.

It is apparent from the data in Table VIII that the release of acid phosphatase from particles (presumably lysosomes) is not readily effected by freeze-thaw treatment. The concentrations of sucrose in the media apparently had no effect on the amount of acid phosphatase released. This is indicated by the fact that 17.7% of the enzyme was solubilized in the 0.25 SEP medium and 17.9% in the 0.04 SEP medium upon freeze-thawing, in spite of the fact that 1.5 times as much protein was released in the hypotonic medium as in the 0.25 SEP medium. None of the cytochrome oxidase was solubilized by the freeze-thaw treatments.

B. Ultrasound Treatment

1. Preparation - A portion of the large granule fraction (P_3) in 0.25 SEP from the experiment of Table IV was subjected to sonic disruption for 3 minutes in a Raytheon Sonic Oscillator, with a 250 watt output. The insonated suspension (son) was centrifuged at 20,000g for 60 minutes to yield a supernatnat (sonS) and pellet, which was resuspended in 0.25 SEP (sonP). The temperature of the suspension during insonation increased from 1° to 7°C.

2. Cleavage-Initiating Activity - The results obtained by injecting various dilutions of this material into eggs are shown in Table IX. The table shows that the CI activity of the untreated P_3 fraction was maximum when its relative concentration was 1:447. After being insonated, however, the P_3 material was most effective at a much higher concentration (1:27). Further dilution decreased its ability to initiate cleavage.

Table IX. Cleavage-initiating activity of insonated large granule fractions prepared in 0.25 SEP from frog liver.[a]

Rel. concn.	Fractions injected[b]			
	P_3	son	sonS	sonP
1:6	7.7	17.1	4.7	3.1
1:27	22.9	28.2	0	35.9
1:111	36.8	23.9	0	25.6
1:447	50.0	13.2	0	2.7
1:1791	10.5	8.2	---	---

[a] Large granule fraction obtained by the differential centrifugation scheme outlined in Methods. The data are expressed as percentages of eggs cleaving. Each percentage value for the injection of the insonated material is based on an average sample size of 40 eggs (32-49) from the same frog used in Table IV.

[b] P_3, son, sonS, sonP represent, respectively, the large granule fraction, insonated large granule fraction, and supernatant and pellet of the insonated material centrifuged at 20,000g for 60 minutes.

Subjecting the large granule fraction to sonic disruption for 3 minutes apparently either destroyed some of the cleavage-initiating material or released an inhibitor of the activity. The latter view is unlikely since the washed insonated particulate material (sonP) had a level of activity comparable to that of the uncentrifuged insonated material (son). The supernatant (sonS) contained very little activity (4.7% at 1:6). It is possible that the structural integrity of the particles, which is disrupted by insonation, is important to the CI capacity of the particles.

If we assume that the concentrations of CI agents in the 1:27 sonP suspension and the 1:447 P_3 suspension are about the

same, then it is reasonable to estimate that more than 90% of the CI material was destroyed by treatment for 3 minutes with ultrasound.

3. Solubilization of Particle-Associated Constituents - Determination were made of the extent to which some of the constituents associated with sedimentable particles were rendered soluble by sonic disruption.

A total 1.00 mg protein and 3.60 units of acid phosphatase activity were recovered in the supernatant and pellet of the insonated material described in Table IX. Of these, 55% and 52%, respectively, were released into the supernatant.

A more detailed analysis of the release of particle bound protein, acid phosphatase, and cytochrome c oxidase with time was performed on a large granule suspension prepared in 0.7 SEP medium. Treatment of this preparation with ultrasound was as described for the experiment in Table IX except that samples were taken at times 0, 30, 60, 120, and 240 seconds of insonation. The percentages of protein and enzymes released into the 20,000g supernatant are plotted versus time of insonation in Figure 3.

The total protein and enzyme activities/ml (combined pellet and supernatant values, sonP plus sonS), determined at insonation times of 0, 30, 60, 120, and 240 seconds were as follows: protein- 0.82, 0.85, 0.88, 0.88, and 0.88 mg; acid phosphatase- 2.67, 2.12, 2.09, 1.94, and 1.91 units; cytochrome c oxidase- 9.9, 7.7, 7.0, and 8.2 units. Determinations of these constituents were performed as described in Methods except that acid phosphatase assays were performed with Calbiochem Calsuls with-

Figure 3. Release of protein and enzymes from large granules by treatment with ultrasound. The data are recorded as percentages of protein, acid phosphatase, and cytochrome c oxidase released into the supernatant upon insonation of a large granule fraction prepared in 0.7 SEP from frog liver. The percentage release was calculated from the ratio of the activity per ml in the supernatant to the activity per ml in the pellet plus the supernatant, sonS/(sonS + sonP) times 100.

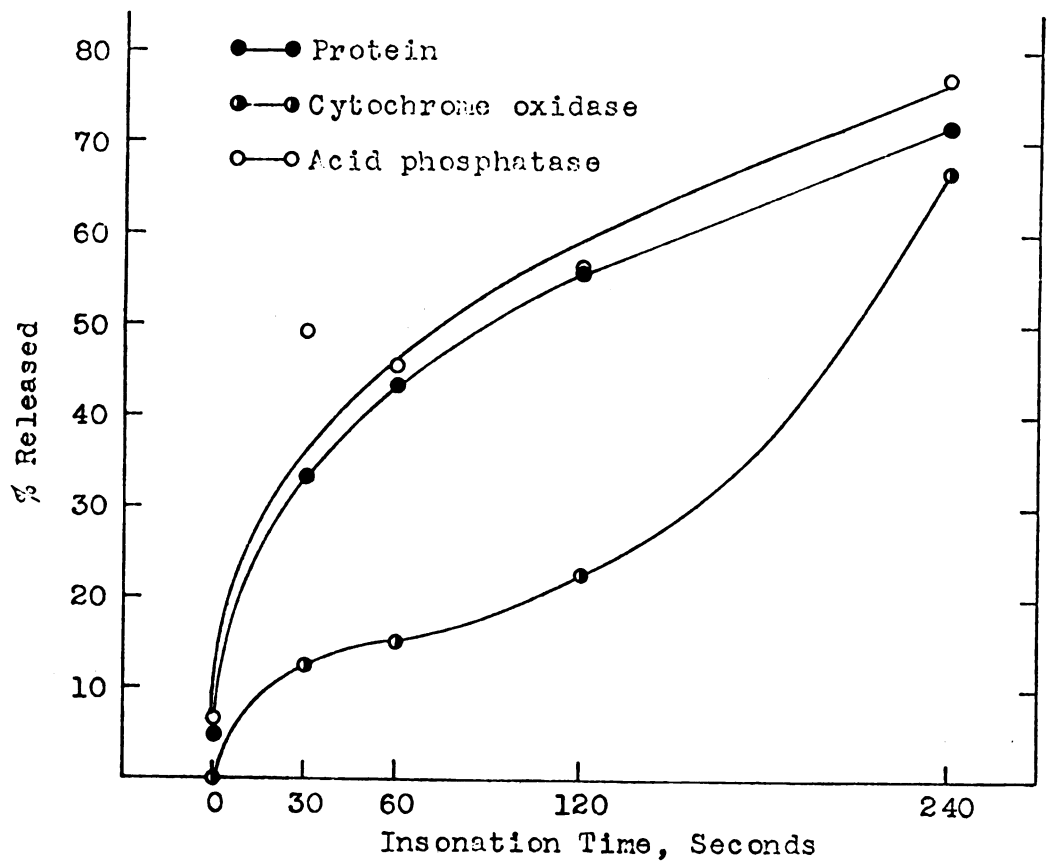
Protein
Cytochrome oxidase
Acid phosphatase
% Released
80
70
60
50
40
30
20
10
0
30
60
120
240
Insonation Time, Seconds

Figure 3

out Triton X-100.

The release of acid phosphatase closely parallels the release of protein whereas that of cytochrome oxidase is more resistant to sonic disruption. For example, after 2 minutes of insonation 56% of the protein and 57% of the acid phosphatase were released into the supernatant. Only 23% of the cytochrome oxidase, however, was released from the mitochondria. After 4 minutes of insonation the 3 constituents were solubilized to approximately the same extent with 72, 77, and 67% of the protein, acid phosphatase, and cytochrome oxidase, respectively, being released.

Since Triton X-100 was not used in the acid phosphatase assay mixture, estimates of the activity of this enzyme in the various pellets probably were somewhat low and the deviations from the real values more variable. This may account for the more erratic distribution of points for the acid phosphatase.

C. The Effect of Aging

1. Cleavage-Initiating Activity - The effect of storage at 2-3°C on the CI activity of the large granule fraction was investigated. A fraction was prepared at 1:34 relative concentration in 0.7 SE medium according to the procedure outlined in the section on Methods. This preparation was injected into the eggs of a different frog each day for three days. After one day of storage, the large granule fraction incited cleavage in 47.7% of the 44 injected eggs, after two days, 32.7% of 49 eggs, and after three days, 6.4% of 47 eggs. The deleterious effect of storage is apparent by the third day. These results must be

qualified, however, by the fact that different frogs were used as the source of eggs on different days. It is known that the cleavage response of batches of eggs from different frogs is variable.

In another experiment fractions were prepared similarly in 0.7 SE (rel. concn. 2:1) on four different days to yield large granule fractions that were 1, 10, 24, and 30 days old. The results of injecting these fractions at three concentrations each into the eggs of one frog are shown in Table X.

It is apparent from the data that there is a considerable decrease with age in the ability of the active fraction to initiate cleavage. The comparability of the results obtained by injecting the variously aged fractions is strengthened by the fact that all injections were made into eggs from one frog. Furthermore, while the time involved in injections spanned 2.5 hours, the 30 day old fraction was injected first and the 1 day fraction last. The possibility that a decrease in the viability

Table X. Cleavage-initiating activity of large granule fractions prepared in 0.7 SE from frog liver and aged for 1, 10, 24, and 30 days at 2-3°C.[a]

Rel. concn.	Age in days			
	1	10	24	30
2:1	14.0	9.1	1.6	0
1:5	35.3	12.8	0	0
1:23	59.2	0	2.2	0

[a] The data are expressed as percentages of eggs cleaving. Each value is based on an average sample size of 53 eggs (45-64) from one frog.

of the eggs during the injection period accounted for the above results is therefore unlikely.

2. Protein Hydrolysis during Storage - An analysis was performed to determine if any marked breakdown of protein constituents occurred during the storage period. Digestion of active materials by proteases from lysosomes, for example, might account for the decrease in activity with time.

Samples of the different age fractions were made 5% with respect to trichloroacetic acid (TCA). The cold sample-TCA mixtures were centrifuged to yield TCA-soluble and TCA-insoluble fractions. The fractions were colorimetrically analyzed for the content of materials sensitive to the Folin-Ciocalteu Phenol reagent by the method of Lowry, et al. (1951). If extensive hydrolysis of protein occurs during storage, it should be reflected in the ratio of the color intensity in the TCA-soluble fraction to that in the TCA-insoluble plus soluble fractions. The percentage of release of materials (e.g. tyrosine and tryptophan) sensitive to the reagent is calulated from this ratio. In the large granule fractions aged for 1, 10, 24, and 30 days the percentages of TCA-soluble material were 17.5, 23.5, 16.8, and 26.0 respectively. No definite relation of these percentages with the age and cleavage-initiating capacity of the samples is obvious.

This does not, however, preclude the possibility that such a correlation does exist. Small differences in the preparation of each large granule fraction could have overshadowed the differences from aging. Thus the same preparation should be the

source of samples at all ages. Furthermore, although protein hydrolysis may be a significant factor in reducing the cleavage-initiating activity its level may be too low to be readily detectable. Such an analysis would be most useful if performed on a more highly purified preparation of the active component or components.

D. The Effect of Heating on the Cleavage-Initiating Capacity of Large Granule Fractions

Shaver (1951) has shown that heating large granule fractions from frog testes to 55°C and 60°C for 1 minute will destroy their cleavage-initiating activity. Heating the fractions at 52°C for 10, 20, or 30 minutes, however, did not reduce but in fact increased their activity. Incubation for 60 minutes at this temperature destroyed most of the activity in the fraction.

Table XI. Cleavage-initiating activity of untreated and spermine treated large granule fractions prepared in 0.7 SE from frog liver and heated to 52°C.[a]

Rel. concn.	Time in minutes at 52°C					
	0		10	20	40	
	-S[b]	+S	-S	-S	-S	+S
1:9	17.0	3.8	0	0	0	0
1:37	31.6	17.5	0	0	0	1.0

[a] Percentage values from the injection of material at 1:37 concentration, heated for 0 and 40 minutes and untreated or treated with spermine, are based on an average sample size of 99 eggs (95-103) from two frogs. Other values are based on an average of 59 eggs (53-64) from one of the frogs.

[b] -S and +S signify untreated or spermine treated fractions, respectively.

An experiment was performed to determine the thermolability of the large granule fraction prepared in 0.7 SE from liver. The fraction at a relative concentration of about 1:1 was incubated in 4 ml quantities for 0, 10, 20, and 40 minutes in a 52°C water bath. As seen in Table XI, heating for 10 minutes was sufficient to completely destroy the effectiveness of the fraction at the concentrations injected. This apparent contradiction with Shaver's results will be considered in the discussion.

E. The Effect of Spermine on Cleavage Initiation

Shaver (1951) showed that when active cell fractions are incubated with 0.001% RNase for 10 minutes at 52°C their CI activity is completely lost. It is important to note here that this enzyme exerts a powerful antimitotic activity in a variety of systems, including amphibian eggs (Thomas, et al., 1946; Ledoux, et al., 1954; Ledoux, et al., 1955). Although the treated fractions in Shaver's experiments were washed prior to being injected into eggs, the possibility was entertained that RNase was still retained on the particles. Addition of RNase inhibitors to the treated and washed fraction prior to injecting it was therefore considered.

Briggs (personal communication) has found that bentonite and macaloid, known RNase inhibitors, are very toxic to amphibian eggs. Eggs injected with material treated with these inhibitors cleaved abortively. The effectiveness of spermine in inhibiting RNase activity has been demonstrated by Keister (1958). Before performing experiments which utilized the RNase inhibitory capacity of this amine it was necessary to determine its effect

on the cleavage response of the recipient egg. For this purpose a sample of the large granule fraction of Table XI was made 3×10^{-4}M with respect to spermine (Nutritional Biochemical Corp., Cleveland, Ohio), adjusted to pH 7.0-7.2, and incubated at 52°C for 0 and 40 minutes. The reduction in the percentage of eggs which cleave (Table XI) makes it apparent that spermine has a detrimental effect on either the cleaving ability of the egg or the cleavage-initiating capacity of the large granule fraction. Of the 61 control eggs injected with 3×10^{-4}M spermine in 0.7 SE adjusted to pH 7.0-7.2, none cleaved. A further analysis of this problem was deferred.

F. Summary

The cleavage-initiating activity of a large granule fraction in 0.25 SEP or 0.04 SEP is completely destroyed or inhibited by freeze-thaw treatment. The extent to which cell particulates are disrupted by this treatment (as indicated by the solubilization of protein, acid phosphatase, and cytochrome oxidase) does not correlate well with the destruction of cleavage-initiating activity. In 0.25 SEP 21% and 18% of the protein and acid phosphatase, respectively, are solubilized; in 0.04 SEP 33%, 18%, and 0% of the protein, acid phosphatase, and cytochrome oxidase, respectively, are released into solution.

Insonation for three minutes destroys more than 90% of the cleavage-initiating material, and releases into solution about 65-70% of the protein and acid phosphatase. Only about 40% of the cytochrome oxidase is solubilized, however.

Storage of the large granule fraction at 2-3°C for three or more days results in a marked decrease in its cleavage-initiating activity. No correlation of this reduction in activity with protein hydrolysis is apparent.

No cleavage-initiating activity is detectable in a 1:1 large granule fraction heated for 10 minutes at 52°C and injected at concentrations of 1:9 and 1:37.

Spermine added to a large granule fraction reduces the percentage of eggs which cleave when injected with this mixture.

III. RELATION OF PERCENTAGE OF EGGS WHICH CLEAVE AND THE QUALITY OF BLASTULAE TO THE CONCENTRATION OF LARGE GRANULES IN THE INJECTED FRACTIONS

Results presented thus far have indicated a dependency of the cleavage-initiating effectiveness of a large granule fraction on its concentration. Brief mention was also made of a relation between the quality of blastulae resulting from the injection of an active fraction and the concentration of granules in that fraction. A more detailed analysis of these observations follows.

A. Calculations and Recording of Data

Blastulae at 12-16 hours were scored as 1/4, 1/2, 3/4, or 1 according to whether blastomeres occupied 0< to 1/4, 1/4< to 1/2, 1/2< to 3/4, or 3/4< to all of the egg surface, respectively. The data presented in the graphs of Figure 4 were based on results pooled from all experiments in which the blastulae were scored in this manner. These include results from some of the

differential centrifugation experiments already reported. Most of the data included in this analysis were however based on the results obtained by injecting fractions from sucrose density gradient centrifugations. Results of this method of fractionation will be presented in detail in subsequent sections.

The analysis was based on the results of injecting CI material with relative concentrations ranging from 2:1 to 1:4007. The concentration of a particulate fraction obtained by differential centrifuation was determined as previously described in Part IV of Methods. It was necessary, however, to calculate the concentrations of particulate fractions obtained from sucrose density gradient centrifugations by an indirect method.

This approximation was dependent on the following known factors: 1) The relative concentration of the large granule fraction applied to the gradient. This was expressed as a fraction and denoted C_a; 2) The volume of the fraction applied to the gradient, V_a; 3) The volume of each of the fractions recovered from the gradient. Since the same number of drops was collected for each fraction, the volume of all fractions is assumed to be equal, and is denoted as $\underline{v}$; and 4) The percentage of the total recovered protein found in each gradient fraction.

From this information we can establish the equation $C_aV_a = v\sum c_i$, where c_i signifies the relative concentration (expressed as a numerical fraction) of CI material in each gradient fraction, and $\sum c_i = c_1 + c_2 + \ldots + c_{11}$ or c_{12}. For each gradient, then, C_aV_a/v equals a constant, k.

The assumption was made that the distribution of protein in

the gradient reflects the distribution of granular material. Thus, c_i can be calculated as the product of (% protein in fraction i/100)(k). A simple calculation follows: If the relative concentration of a large granule fraction is 1:1 ($C_a = \frac{1}{2}$) and 0.6 ml (V_a) of this material is layered over a gradient, then $C_aV_a = 0.30$. If 12 fractions of 0.4 ml each (v) are collected then $k = C_aV_a/v = 0.75 = \sum c_i$. If 20% of the total recoverable protein is found in fraction 5, then $c_5 = (20.0/100)(0.75) = 0.15 \simeq 1/7$. The relative concentration is then expressed as 1:6. Dilutions of all fractions were also injected. If the above fraction was diluted to 1/4 and 1/24 its original concentration, the respective relative concentrations would be 1:27 and 1:167.

B. Relation of Concentration to Blastular Quality

To illustrate the effect that the concentration of the injected large granule fraction has on the quality of the resulting blastulae, the concentrations were partitioned into the following three groups: a) 2:1-1:15, from 28 experiments; b) 1:16-1:32, from 21 experiments; and c) 1:33-1:4007, from 71 experiments. An average of 50 eggs were injected in each of the 120 experiments.

Of the eggs which cleaved, the percentage falling into each category of blastulae (1/4, 1/2, 3/4, or 1) was calculated for each experiment within each concentration group. The mean percentage of cleaved eggs in each category of blastulae and the standard error about this mean were calculated for each concen-

Figure 4. Quality of the blastulae as a function of the concentration of material injected. Each point on the graph represents the mean percentage of blastulae in a particular relative concentration group (A, 2:1-1:15; B, 1:16-1:32; or C, 1:33-1:4007) which displays either the 1/4, 1/2, 3/4, or 1 level of development. The vertical bars delimit the standard errors of the means. The means were calculated from n experiments based on about 50 eggs each.

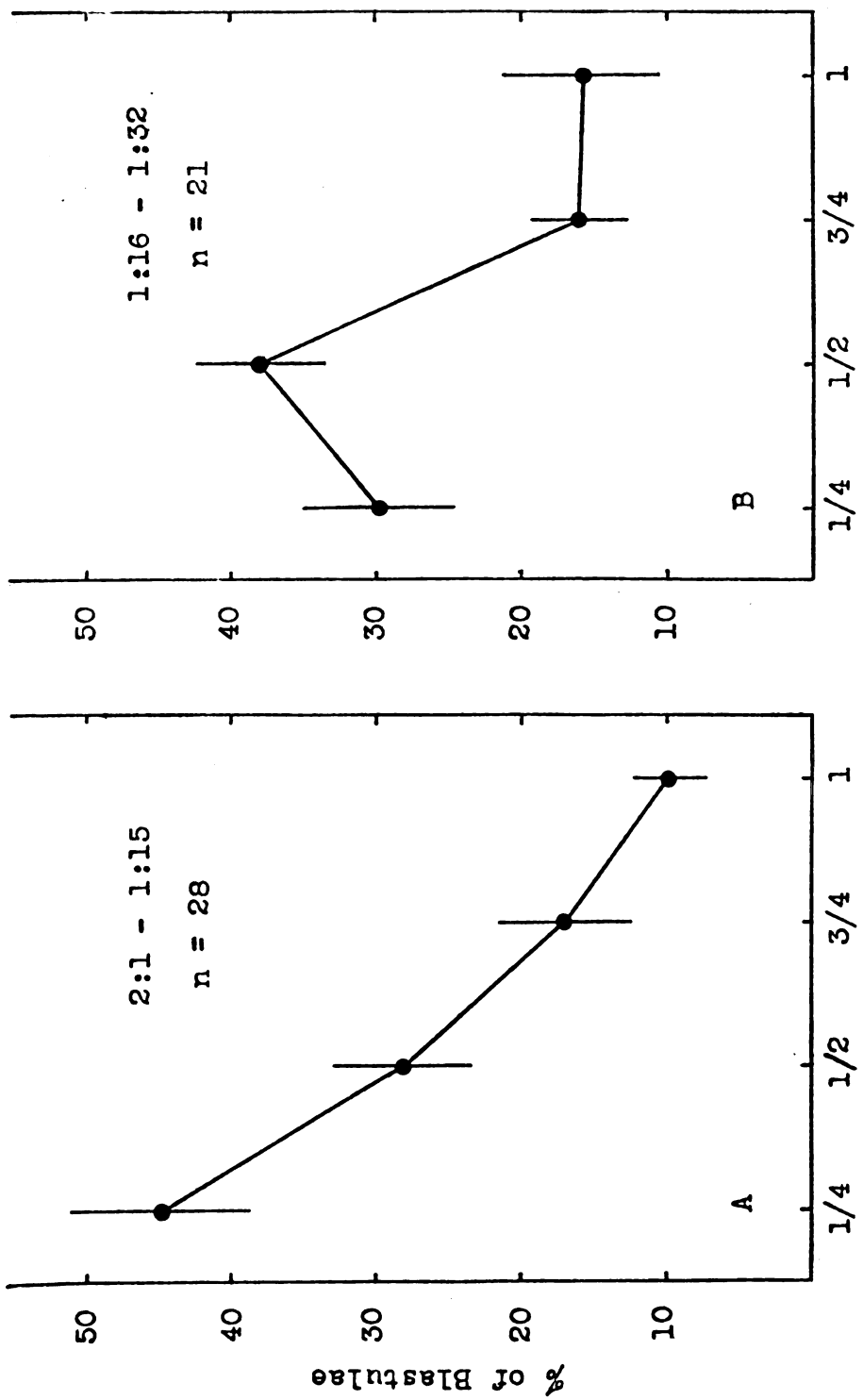
% of Blastulae
50
40
30
20
10
2:1 - 1:15
n = 28
A
1/4
1/2
3/4
1
1:16 - 1:32
n = 21
B
1/4
1/2
3/4
1
Quality of Blastulae

Figure 4

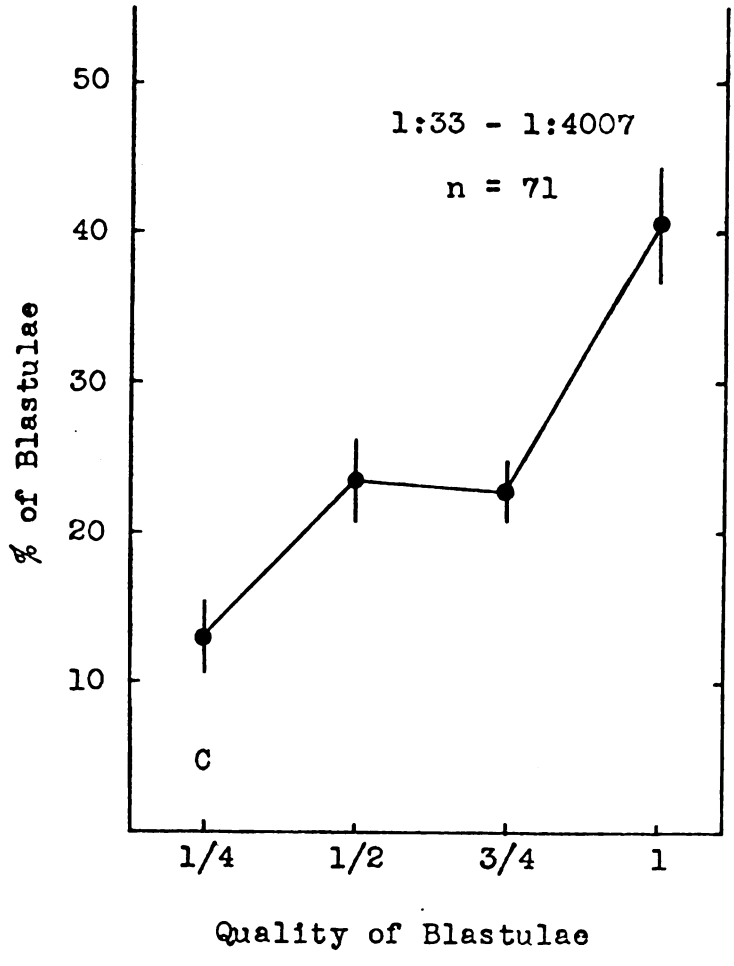

Figure 4 (cont.)

tration group. The results are shown in Figures 4A-C. It is obvious from Figure 4A that the injection of CI material in the concentration range of 2:1-1:15 results in most of the eggs cleaving over less than one-half of their surface. When more dilute material is injected, more of the eggs surface is involved in cleavage (Fig. 4B). Finally if dilutions are extended to yield concentrations of 1:33 and less, blastomeres cover more than one-half of most of the eggs which have undergone cleavage (Fig. 4C).

C. Relation of Concentration to Percentage of Injected Eggs which Cleave

In conjunction with the analysis of the blastular quality, the effect of the concentration of injected CI material on the percentage of the eggs which cleave was determined. Results of experiments in which the blastulae were not scored as required for the quality analysis could also be utilized in this analysis. The mean percentage of eggs cleaving when injected with each range of concentrations and the standard error about each mean were calculated. The concentration groups and the number of experiments on which each mean was based are as follows: 2:1-1:15, n = 35; 1:16-1:32, n = 25; 1:33-1:66, n = 15; 1:67-1:134, n = 24; 1:135-1:270, n = 18; 1:271-1:1084, n = 36; 1:1085- , n = 17. The average number of eggs injected in each experiment was 50.

In Figure 5 are shown the results of this analysis. The vertical bars represent the standard error about the mean for each concentration group. The effect that the concentration of the injected material has on the percentage of eggs which cleave

Figure 5. Percentages of eggs which cleave as a function of the concentration of the material injected. Each point represents the mean per cent cleavage in n experiments based on about 50 eggs each. The vertical bars delimit the standard errors of the means.

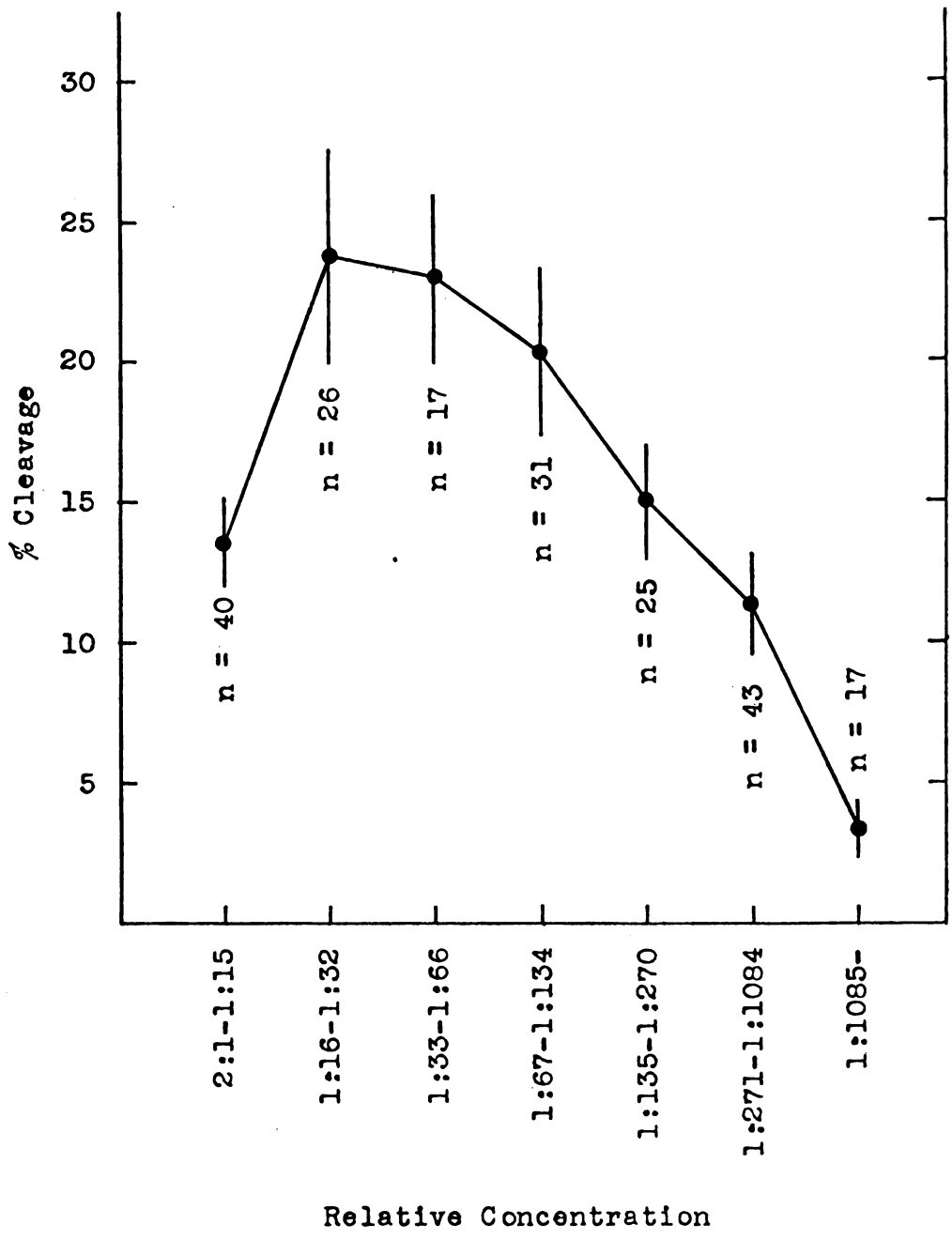
% Cleavage
30
25
20
15
10
5
n = 40
n = 26
n = 17
n = 31
n = 25
n = 43
n = 17
2:1-1:15
1:16-1:32
1:33-1:66
1:67-1:134
1:135-1:270
1:271-1:1084
1:1085-
Relative Concentration

Figure 5

is most obvious when data from each series of injections is considered separately as, for example, in Tables III and IV. Even with the errors introduced when analyzing pooled data, the existence of a concentration range of CI material of optimum effectiveness (roughly 1:16 to 1:134) is apparent from Figure 5.

D. The Effect of Dilution on the Protein Concentration and the pH of the Large Granule Fraction

The reason(s) for the effect of dilution on the blastular quality and on the percentage of cleavage initiation is unknown. Analyses were performed to determine if the pH and the relative concentrations of the soluble and sedimentable protein of the large granule fraction were affected by the dilution.

A large granule fraction (P_3) was prepared in 0.25 SEP by differential centrifugation in the usual manner. The relative concentration of the final suspension was 1:3 (designated as 1). Dilutions of P_3 were made with 0.25 SEP to yield additional concentrations 1/4, 1/16, 1/64 of the original.

Determinations of pH were made with a Metrohm Model E300 pH meter (Brinkmann Instruments, Westbury, N.Y.) on the above P_3 samples. Determinations were also made on the S_2 and final S_3 supernatant fractions and on the 0.25 and 1.8 SEP media. The latter was used in density gradient centrifugation experiments. All suspensions and solutions were at room temperature. No differences were noted in the pHs of S_2, S_3, and P_3 (1-1/64). All had a pH of 7.22. The pH of the 0.25 SEP medium was 7.20 and of the 1.8 SEP medium, 7.16.

The protein content of the P_3 samples was analyzed to determine whether the dilution caused a detectable release of protein bound on or within particles in suspension. Protein determinations were performed on the whole fractions (P_3) and on the supernatants (P_3S) and pellets (P_3P) resulting from an 18,000g centrifugation for 30 minutes of the whole fractions at each of the four concentrations (1, 1/4, 1/16, and 1/64). Prior to analysis the pellet was resuspended in a volume of 0.25 SEP equal

Table XII. The effect of dilution on the relative amounts of protein in the soluble and sedimentable phases of a large granule fraction.[a]

Fraction	Dilution			
	(1)	(1/4)	(1/16)	(1/64)
P_3	100.0(1336)[b]	27.3(365)	6.44 (86)	1.35 (18)
P_3P	100.0(1174)	24.5(288)	5.37 (63)	1.02 (12)
P_3S	100.0 (386)	14.5 (56)	3.89 (15)	0 (0)
Exp. % of (1)[c]	100.0	25.0	6.25	1.56

[a] Figures in the table, except those in parentheses, are percentages of the total protein of the undiluted (1) P_3, P_3S, and P_3P fractions that are found in their respective diluted fractions.

[b] Figures in parentheses are the protein concentrations of the fractions in μg/ml.

[c] Percentages which should theoretically be obtained when the fractions are diluted as indicated.

to that of the supernatant.

Table XII shows the results obtained. Protein concentrations are given in parentheses. The concentrations of protein

in fractions P_3, P_3S, and P_3P at the dilutions (1/4), (1/16), and (1/64) are expressed as percentages of the protein in the respective original fractions (1). The data in Table XII indicate that protein is not released from the particles upon dilution and that the percentage of protein observed in the supernatant at each dilution is in fact generally less than the expected percentage.

E. Summary

There is a range of concentration of large granular material (1:33-1:134) which is optimum for obtaining a high percentage of blastulae of good quality. Injecting material of progressively greater concentration results in an increasing number of blastulae which occupy less than one-half of the original egg surface. Injecting material of greater or lesser concentration also results in fewer of the eggs cleaving to blastulae.

The pH and the relative amounts of soluble and sedimentable protein of the large granule fractions were unaffected by the dilutions.

IV. FRACTIONATION BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

The differential centrifugation experiments have clearly demonstrated that the large granule fraction of frog liver contains most of the cleavage-initiating activity. Three types of particles are generally considered to be present in a comparable "mitochondrial" fraction from mammalian liver. Mitochondria

account for the bulk of the protein in this fraction. In addition lysosomes and microbodies are present as two important minor components (de Duve, 1964). Recently, de Duve (1965b) has proposed the name "peroxisome" for the microbodies of rat liver.

Considerable use has been made in recent years of density gradients for separating mammalian liver components (Thomson, 1959; de Duve, 1965a). Holter and coworkers (1953) were the first to use sedimentation equilibrium in a density gradient for cell fractionation work. In their work with Xenopus liver cells, they extended the range of the density gradient beyond the specific gravity of the heaviest cell component and prolonged centrifugation until all particles attained a level in the medium which corresponded to their density. Experimental evidence supporting the theoretical basis for this technique, termed "isopycnic gradient centrifugation" by Anderson (1955) is discussed by Beaufay and Berthet (1963).

Sucrose has been the most widely used material for making density gradients. Its high viscosity in dense solutions, however, is a disadvantage in that it retards sedimentation (see de Duve and Berthet, 1954, p. 227-229). It also makes the injection of fractions recovered from the bottom of the gradient very difficult.

In the account which follows, experiments will be described in which attempts were made to subfractionate the large granule fraction on sucrose density gradients. Assuming enzymic homogeneity as postulated by de Duve and coworkers (1955, and de Duve,

1964), cytochrome oxidase and acid phosphatase were routinely assayed as enzyme markers for mitochondria and lysosomes, respectively. In some experiments, urate oxidase was also assayed to indicate the position of the peroxisomes. Thus, curves of cell component frequency as a function of their position in the gradient tube (fraction number) could be constructed.

Roodyn (1965) suggests the calculation of the relative concentrations of various enzymes as one means of expressing cell fractionation data. The relative concentration of a particular enzyme in a fraction would be, according to the definition of Roodyn, the activity per mg protein in the fraction divided by the activity per mg protein in the homogenate. This value would be better described by relative specific activity (R.S.A., a term used by Baudhuin, et al. (1965) to describe a comparable value: % total recovered activity/% total recovered protein) since the value does not in fact reflect the concentration of any particular enzyme in a fraction. The only exception would be a situation in which the protein concentration in each fraction is the same. This is unlikely to occur in density gradient fractionation work. Data expressed in terms of the specific activities, total activities, and relative specific activities of the enzymes in each gradient fraction are given, when possible, in the Appendex. No reference is made to the homogenate since it was not considered in any aspect of this study.

In the present study we are concerned with determining in which fractions a particular cell component is the most abundant and the least contaminated with other species of cell components.

The results are therefore expressed in the text figures in terms of the percentage in each fraction of the total recovered activity of a particular enzyme in all the fractions. Curves constructed from such data portray the frequency distributions of the particles (marker enzymes) throughout the gradient.

The percentages of eggs which cleave when different batches are injected with the various fractions are graphed versus the fraction number to give a frequency distribution curve of the particles in the gradient which are active in initiating cleavage. This curve may then be compared to the corresponding curves which describe the distribution of cytochrome oxidase (mitochondria), acid phosphatase (lysosomes), and other marker enzymes.

The nature of the gradients was varied. Large granule material was layered over gradients preformed from 0.25 SEP and 1.8 SEP media, 0.8 and 1.8 SEP media, and from 0.8 and 1.8 SE media. The former is referred to as a steep gradient while the latter two are shallow gradients.

In all cases the fraction numbers given in the figures progress from the bottom (most dense) to the top (least dense) of the gradient tube.

Of the 775 eggs injected with the suspension media alone (0.25, 0.60, 0.70, and 1.8 SE or SEP), only 3 cleaved. One egg cleaved in each of three batches of eggs (total of 148 injected) injected with 0.25 SE. Blood, probably due to internal hemorraging of the female frog, was evident on two of these three batches of eggs.

A. Steep Gradients with SEP Media

The results of the first experiment performed to determine the distribution of CI material in a gradient are shown in Figure 6. These gradients were prepared by layering 1.0 ml of a 1:2 large granule suspension over preformed steep gradients of sucrose media containing 0.001M EDTA and 0.1M sodium phosphate buffer. The concentration of sucrose ranged from 0.25M to 1.8M yielding a range of density at 20°C from about 1.03 to 1.23. Centrifugation was performed at 25,000 rpm for 2 hours with the Spinco Model L Ultracentrifuge equipped with the SW 39L rotor. Ten fractions of 24 drops each were collected from each tube.

Samples of nine fractions were injected (omitting fraction 10) into an average of 63 eggs each (Fig. 6). No diluted fractions were injected. The depression of CI activity in fraction 3 coincided with the peak of maximum concentration of granular materials. Therefore in the subsequent analyses diluted fractions were also injected since previous differential centrifugation experiments had shown that the percentage of eggs which cleave is reduced if the concentration of the active material injected is too high.

An analysis for RNA showed the maximum concentration to be in fraction 3. Some of this RNA is probably mitochondrial, but adsorption of RNA to cell components may contribute to much of that found in fractions 3-5.

The data presented in Figure 7A, B, and C represent a summarization of results from three experiments. While the media used in forming the gradients were the same as for the preceding,

Figure 6. Frequency distribution in a steep SEP gradient of RNA and cleavage-initiating activity associated with large granules from frog liver.

The large granule suspension in 0.25 SEP (P_3, 1:2) was prepared according to the differential centrifugation procedure in Methods. 1.0 ml of P_3 was layered over a 0.25-1.8 SEP gradient and centrifuged at 25,000 rpm for 2 hours.

The distribution of RNA is expressed as the percentage in each fraction of the total (453 ug) recovered from the 10 fractions.

The other curve represents the percentage of eggs which cleave following injection with fractions 1-9. Each percentage value is based on an average of 63 (46-106) injected eggs.

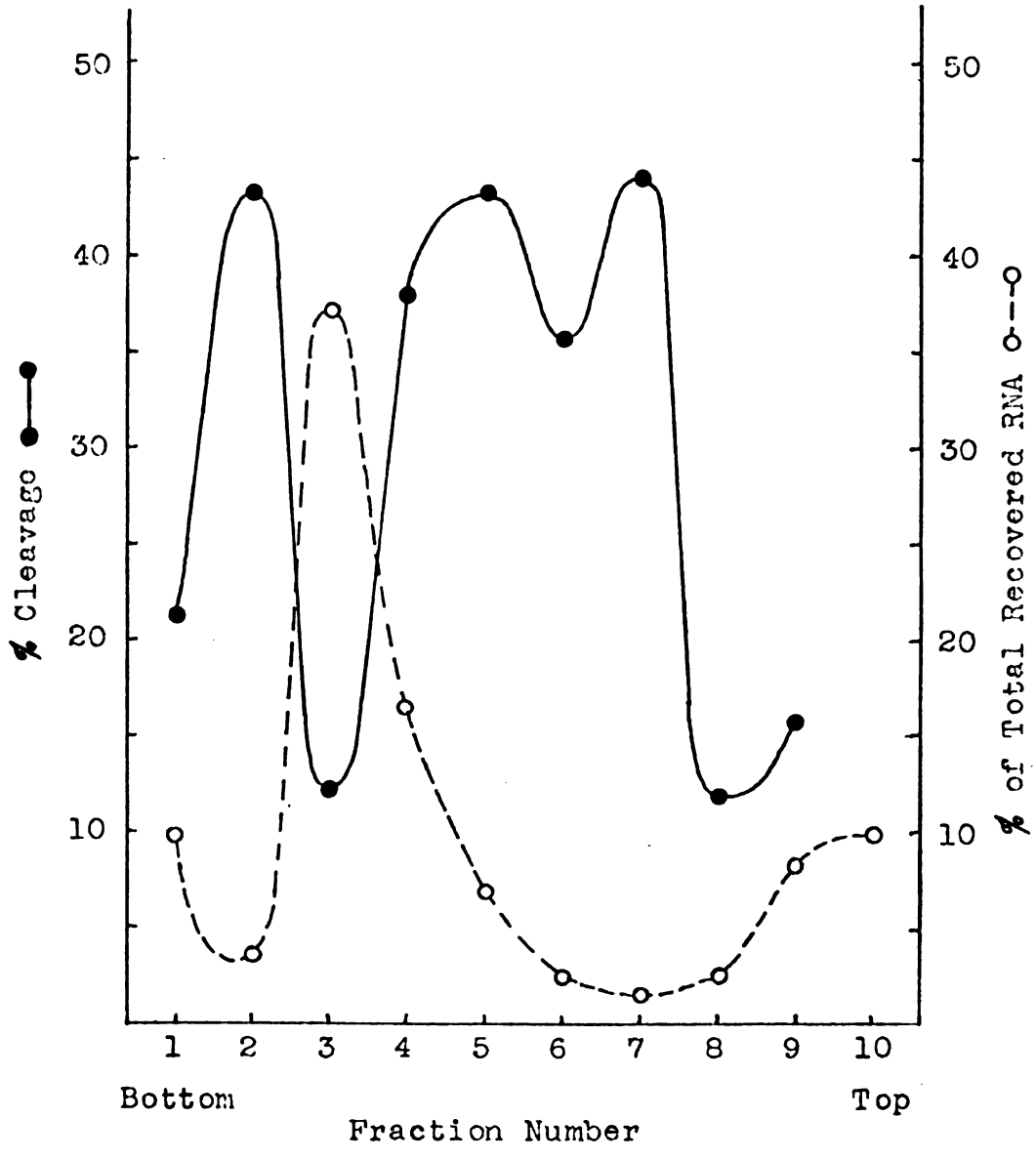
50
40
30
20
10
% Cleavage
% of Total Recovered RNA
1 2 3 4 5 6 7 8 9 10
Bottom
Top
Fraction Number

Figure 6

the procedure for the experiments in Figure 7 differed in the following respects: 1) a new gradient machine, referred to in Methods, was employed; 2) a 1:3 large granule fraction was layered over the gradients; 3) centrifugation was at 35,000 rpm for 2 hours; and 4) 12 fractions of 20 drops each were collected.

Figures 7A and 7B show the distribution of protein, cytochrome oxidase, acid phosphatase, and nucleic acids in the gradients. The protein distribution curve is based on data from three experiments, acid phosphatase on two of the three, and cytochrome oxidase and nucleic acids on one of the experiments. The protein and enzyme concentrations in each fraction are expressed in the graph as the average percentage, where applicable, of the total amount recovered in all the gradient fractions.

Figure 7C, which summarizes data from the three experiments, depicts the percentage of eggs cleaving when injected with the 12 gradient fractions. Fractions 1 through 12 were injected as recovered and after being diluted to 1/4 the original concentration. In two of the three experiments the fractions at 1/24 the original concentration were injected. Each percentage point on the graph is based on the injection of the 1, 1/4, and 1/24 concentrations into an average of 118, 106, and 91 eggs, respectively.

It is apparent from curve 1 (1:3) of Figure 7C that there is a general dispersion of CI material throughout the gradient. A depression of CI activity in the region of greatest particle concentration was again noted (Fractions 4 and 5, Figure 7 and Fraction 3, Figure 6). Injections of diluted fractions (1/4

Figure 7. Frequency distribution in a steep SEP gradient of protein, acid phosphatase, cytochrome oxidase, RNA, DNA, and cleavage-initiating activity associated with large granules from frog liver.

The large granule suspensions in 0.25 SEP (P_3, 1:3) were prepared by the differential centrifugation procedure in Methods. 1.0 ml of P_3 was layered over a 0.25-1.8 SEP gradient and centrifuged at 35,000 rpm for 2 hours.

A. Concentration of RNA and DNA in each fraction expressed as micrograms per ml of fraction.

B. Data are expressed as the percentages in each fraction of the total protein or enzyme recovered from all the fractions. Average total recoveries: protein, 3.14 mg; acid phosphatase, 7.9 units; and cytochrome oxidase, 2.2 units.

C. Data are expressed as percentages of eggs which cleave following injection with fractions 1-12. Each percentage value is based on an average of 118, 106, and 91 injected eggs for curves 1, 1/4, and 1/24, respectively.

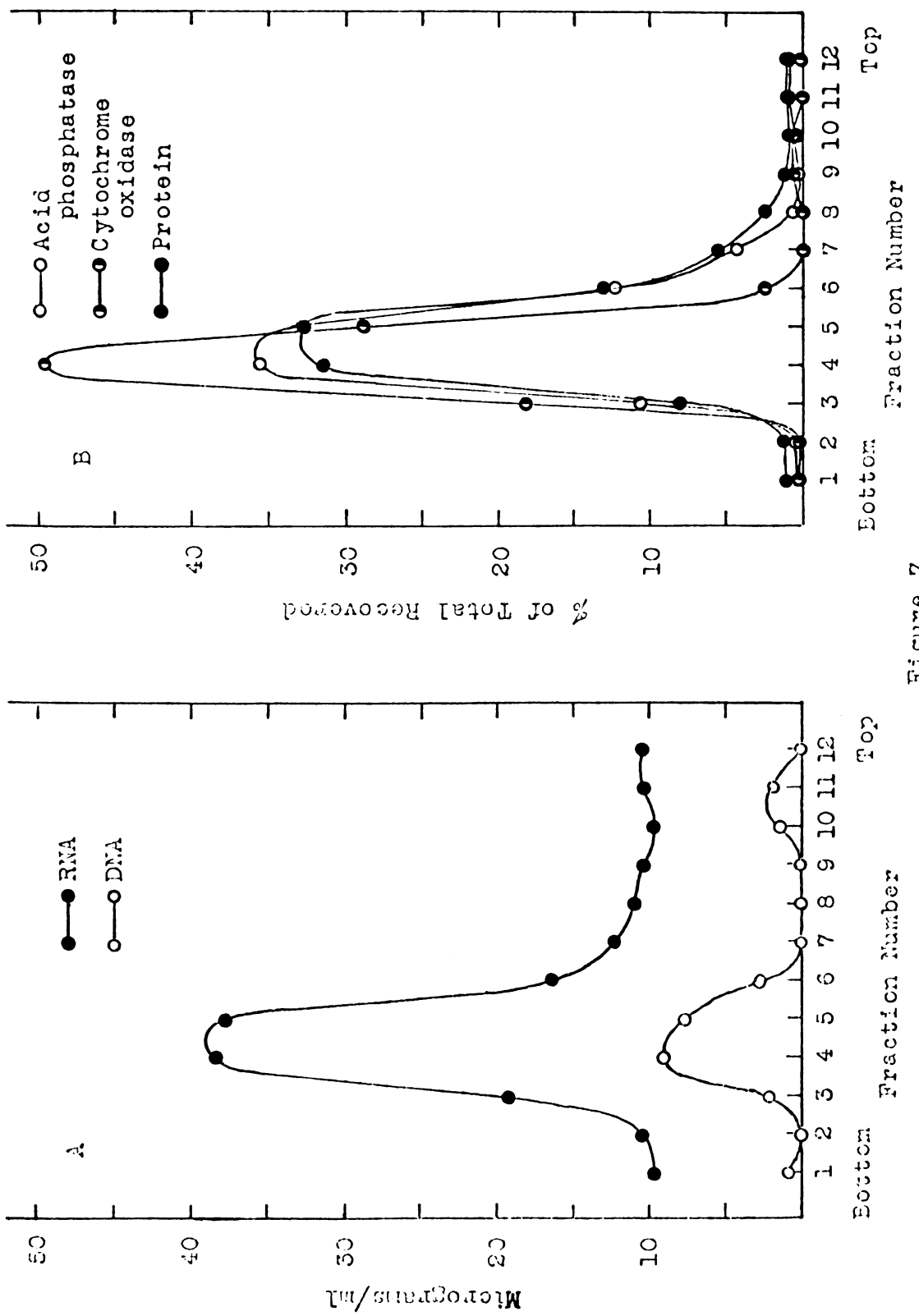
A
RNA
DNA
Micrograms/ml
50
40
30
20
10
Bottom
1 2 3 4 5 6 7 8 9 10 11 12
Top
Fraction Number
B
Acid phosphatase
Cytochrome oxidase
Protein
% of Total Recovered
50
40
30
20
10
Bottom
1 2 3 4 5 6 7 8 9 10 11 12
Top
Fraction Number

Figure 7

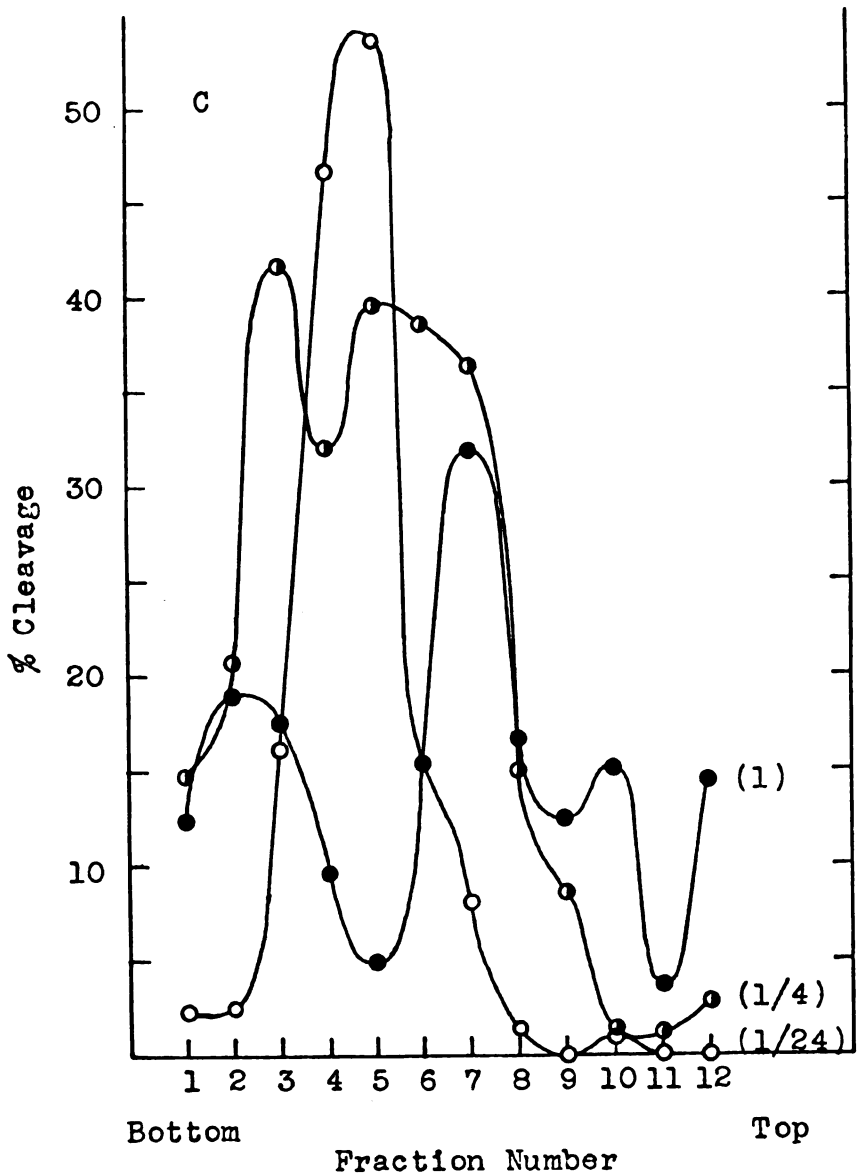

Figure 7 (cont.)

and 1/24, Figure 7) revealed that the greatest concentration of cleavage-initiating material did in fact reside in the same fractions in which the population of granules was the most dense.

By comparing all the graphs in Figure 7, it is apparent that the distribution of CI material is similar to that of protein, cytochrome oxidase, acid phosphatase, RNA, and DNA.

B. Shallow Gradients with SE Media

Little separation of mitochondria from lysosomes was obtained (Fig. 7B) with the steep gradients. Shallower gradients were therefore used in an effort to enhance the separation of these two populations of particles.

Large granule pellets were suspended in 0.7M sucrose medium and layered over gradients with sucrose concentrations ranging from 0.8M to 1.8M. The media were 0.001M with respect to EDTA·Na_2. Buffering with phosphate was omitted. In the experiments to be subsequently described the media were simply adjusted to pH 7.2 with NaOH. Although pH values lower than 6 have been found to cause extensive agglutination of particulate material (Claude, 1946; Hers, et al., 1951), liver preparations, when kept properly cooled, rarely reach critical pH values (de Duve and Berthet, 1954). There is also evidence suggesting that electrolytes in the isolation medium may cause cytoplasmic particles to agglutinate (Hers, et al., 1951; Dalton, et al., 1949; Hogeboom, et al., 1948). A disadvantage in using the sucrose-EDTA (SE) media is that the adsorption of soluble proteins on the surface of granules is a much more important phenomenon in

salt-free sucrose solutions than in media containing electrolytes (Beinert, 1951; Berthet, et al., 1951).

1. Biochemical Analyses on Gradient Subfractions of Large Granule Material from Livers of Frogs Injected with Triton WR-1339 - The first shallow gradient subfractionations were performed on large granule fractions of liver obtained from frogs injected with the non-ionic detergent Triton WR-1339 (WR-1339, an oxyethylated tert. octylphenol polymethylene polymer obtained from the Ruger Chemical Company, Inc., Irvington, N.Y.). These experiments were prompted by a report from Wattiaux and coworkers (1963) on the influence of the injection of this detergent on the properties of lysosomes of rat liver. The studies by the Louvain group revealed that intravenous injections of WR-1339 four days before the animals were killed resulted in the uptake of the detergent by the liver lysosomes, markedly shifting the distribution of this population of particles to a region of lower density. The distribution of cytochrome oxidase and urate oxidase were, however, unaltered.

Experiments were performed to determine whether this detergent, when injected into the dorsal lymph space of a frog, would accumulate within the lysosomes and thereby alter their density sufficiently to promote their clean separation from mitochondria. Quantities of WR-1339 were dissolved in a solution of 0.65% NaCl (isotonic amphibian saline) such that an injection of 0.1 ml per 10 grams of body weight represented a dosage of 0.8 mg of the detergent per gram of body weight in the first series of experiments (Fig. 8) and 1.0 mg/gram body weight in the second

series (Fig. 9). Control frogs were injected with 0.1 ml saline per 10 grams of body weight. The frogs were maintained at 17°C until killed.

To obtain the data in Figure 8, livers were excised from three treated frogs after 2 days and from three treated and three control frogs after 6 days. The large granule fractions were prepared as indicated in the legend to the figure. The final suspension in 0.7 SE was layered over a 0.8-1.8 SE gradient and centrifuged for 2 hours at 35,000 rpm.

A comparison of the gradient fractions from saline injected frogs with those from WR-1339 frogs was possible only with respect to the acid phosphatase distribution (Fig. 8C). The insufficient recovery of some gradient fractions in the saline experiment precluded their being analyzed for protein and cytochrome oxidase.

A comparison of the 2-day and 6-day samples from WR-1339 treated frogs shows that after 6 days a substantial proportion of the acid phosphatase associated with particles is distributed in a less dense portion of the gradient. This suggests that the detergent has decreased the density of the lysosomes. However, the acid phosphatase distribution in a sample from the 6-day saline injected frogs was very similar to that in the 6-day WR-1339 sample. As noted in the legend to the graphs, the differences in the differential centrifugation times of the 2-day and 6-day preparations may in part account for the discrepant results.

Figure 8. Frequency distribution in a shallow SE gradient of protein, acid phosphatase, and cytochrome oxidase associated with large granules from the liver of WR-1339 injected frogs.

A. The large granule suspension was prepared by differential centrifugation as follows: Liver tissue from frogs injected 2 days previously with WR-1339 was homogenized in 0.25 SE and centrifuged 3 times at 4200 rpm, 20 minutes. Final supernatant was centrifuged at 12,000 rpm, 30 minutes, to yield a pellet which was suspended in 0.7 SE and centrifuged at 9200 rpm, 40 minutes. Final pellet was suspended in 0.7 SE (P_3, 1:1). 0.5-0.6 ml of P_3 was layered over a 0.8-1.8 SE gradient and centrifuged at 35,000 rpm for 2 hours.

B and C. The large granule suspensions were prepared by differential centrifugation as follows: Liver tissue from frogs injected 6 days previously with saline or WR-1339 was homogenized in 0.25 SE and centrifuged 3 times at 4200 rpm, 20 minutes. The final supernatant was centrifuged at 12,000 rpm, 45 minutes, to yield a pellet which was suspended in 0.7 SE and centrifuged at 9200 rpm, 45 minutes. Final pellet was suspended in 0.7 SE (P_3, 1:1.7). 0.6-0.7 ml of P_3 was layered over a 0.8-1.8 SE gradient and centrifuged at 35,000 rpm for 2 hours.

The data are expressed as in Figure 7B. Total recoveries were as follows: A. protein, 3.08 mg; acid phosphatase, 24.3 units; and cytochrome oxidase, 17.7 units. B. protein, 3.68 mg; acid phosphatase, 37.4 units; and cytochrome oxidase, 9.7 units. C. acid phosphatase, 35.3 units.

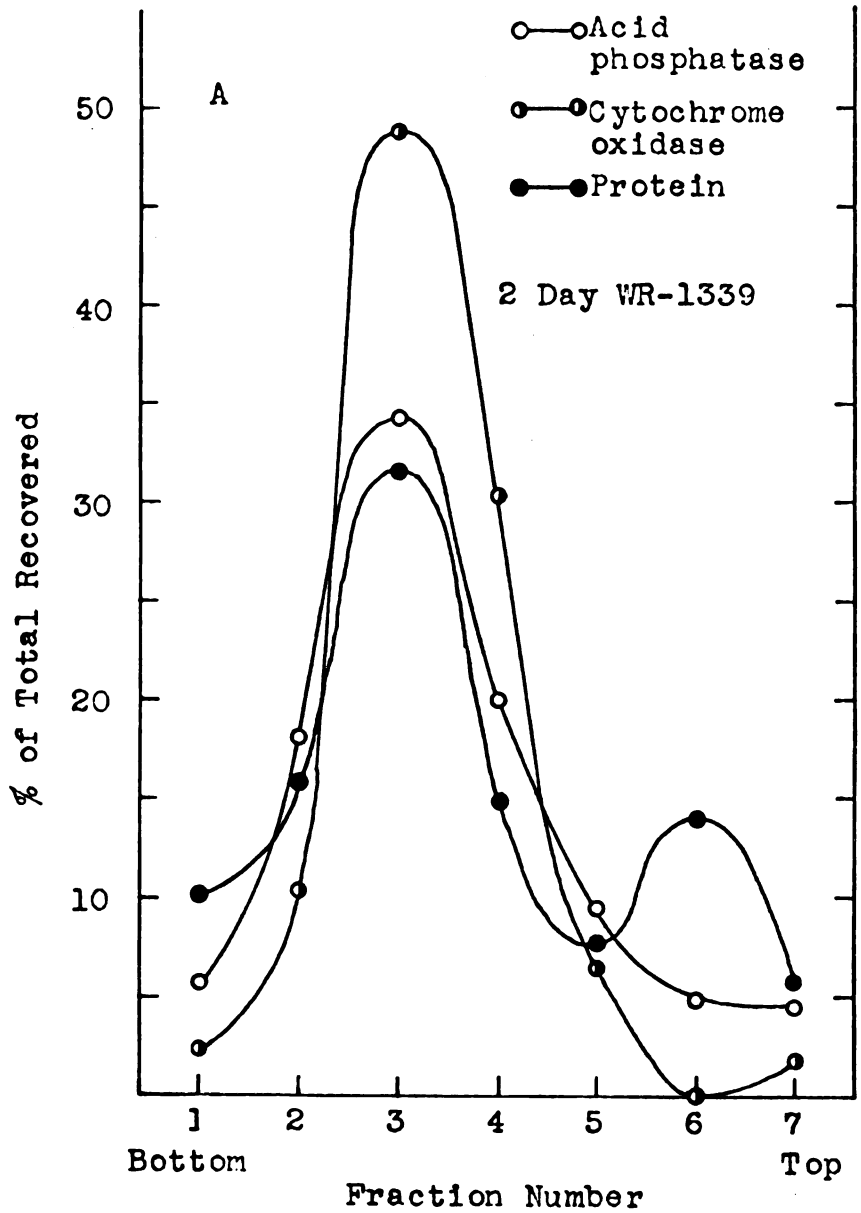
A
Acid phosphatase
Cytochrome oxidase
Protein
2 Day WR-1339
% of Total Recovered
50
40
30
20
10
1
2
3
4
5
6
7
Bottom
Top
Fraction Number

Figure 8

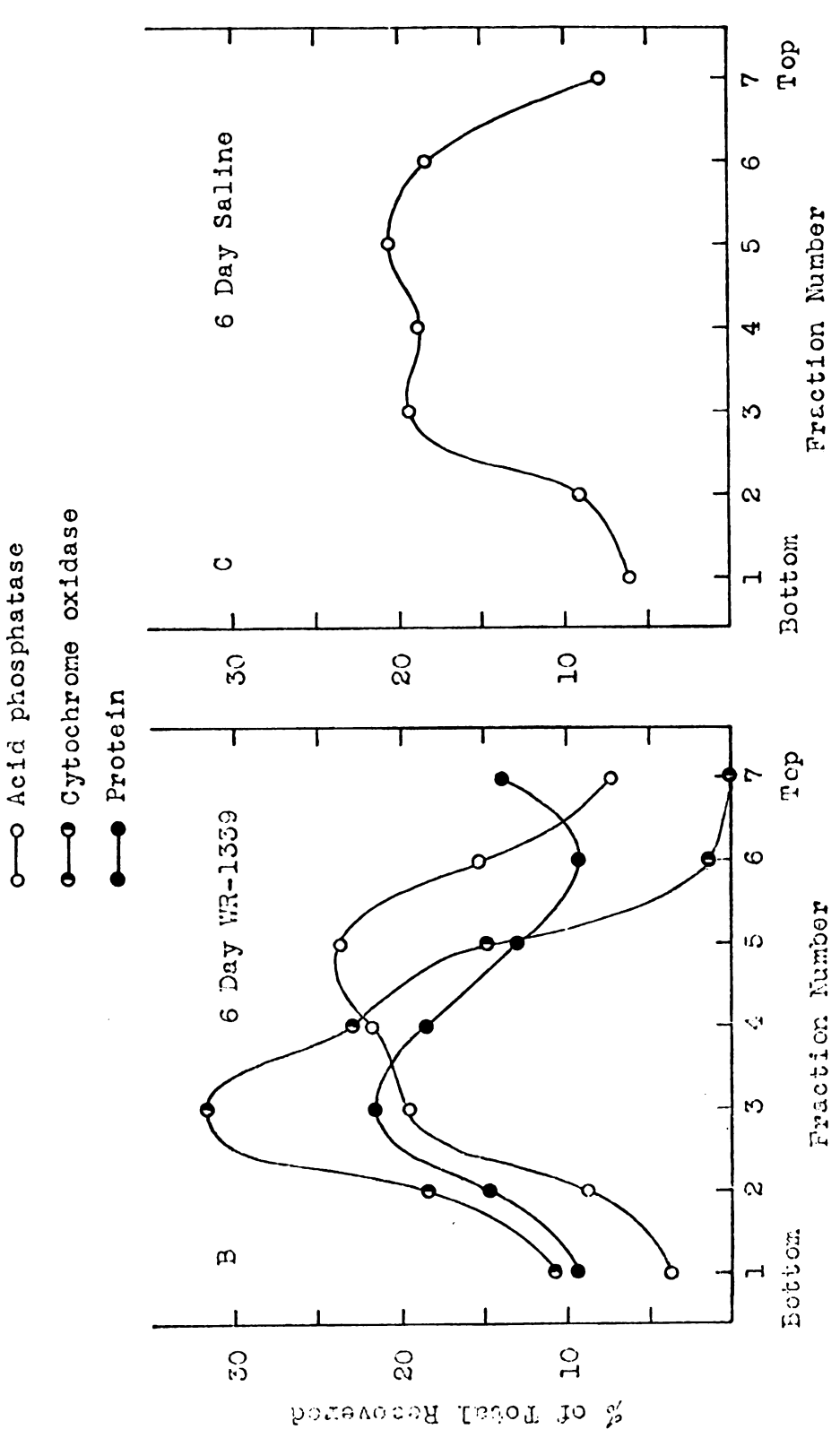

Figure 8 (cont.)

In the next series of analyses, therefore, a uniform centrifugation procedure was used throughout. The experiment yielded complete data from 6 saline injected control frogs as well as 6 frogs injected with WR-1339. Three frogs from each group were killed 3 and 6 days after injection. Large granule fractions were prepared by differential centrifugation as indicated in the legend to Figure 9. The suspensions of large granules in 0.7 SE were layered over 0.8-1.8 SE gradients. Following centrifugation for 2 hours at 35,000 rpm, 9 fractions of 30 drops each were collected from each gradient.

Figure 9 compares the frequency distribution in sucrose density gradients of protein, mitochondria, and lysosomes from liver of 3- and 6-day saline and WR-1339 injected frogs. The acid phosphatase distribution curves (Fig. 9) at both 3 and 6 days after detergent injection show a slight shift of maximum lysosome concentration toward the top of the gradient. Both the 3- and 6-day analyses showed a definite decrease in lysosome concentration of the WR-1339 material in the region occupied principally by mitochondria, namely fractions 3 and 4. In general, however, the differences in acid phosphatase distribution between material from detergent and saline injected frogs are not great enough to warrant the use of this technique. Repeated daily injections for 3-6 days may have accentuated the slight separation observed with a single dose. On the other hand, intravenous injections may be necessary to obtain results comparable to those of Wattiaux and coworkers (1963).

Figure 9. Frequency distribution in a shallow SE gradient of protein, acid phosphatase, and cytochrome oxidase associated with large granules from the liver of WR-1339 injected frogs.

All large granule suspensions (A-D) were prepared by differential centrifugation as follows: Liver tissue from frogs injected 3 and 6 days previously with WR-1339 and saline was homogenized in 0.25 SE and centrifuged 3 times at 2500 rpm and 5000 rpm, 10 minutes each. Final supernatant was centrifuged at 12,000 rpm, 45 minutes, to yield a pellet which was suspended in 0.3 SE and centrifuged at 9000 rpm, 10 minutes. Final pellet was suspended in 0.7 SE (P_3, 1:1). 0.6-0.7 ml of P_3 was layered over a 0.8-1.8 SE gradient and centrifuged at 35,000 rpm for 2 hours.

The data are expressed as in Figure 7B. Total recoveries were as follows: A. protein, 2.45 mg; acid phosphatase, 34.2 units; and cytochrome oxidase, 7.5 units. B. protein, 3.27 mg; acid phosphatase, 45.2 units; and cytochrome oxidase, 10.3 units. C. protein, 3.76 mg; acid phosphatase, 36.6 units; and cytochrome oxidase, 16.9 units. D. protein, 3.31 mg; acid phosphatase, 35.7 units; and cytochrome oxidase, 7.2 units.

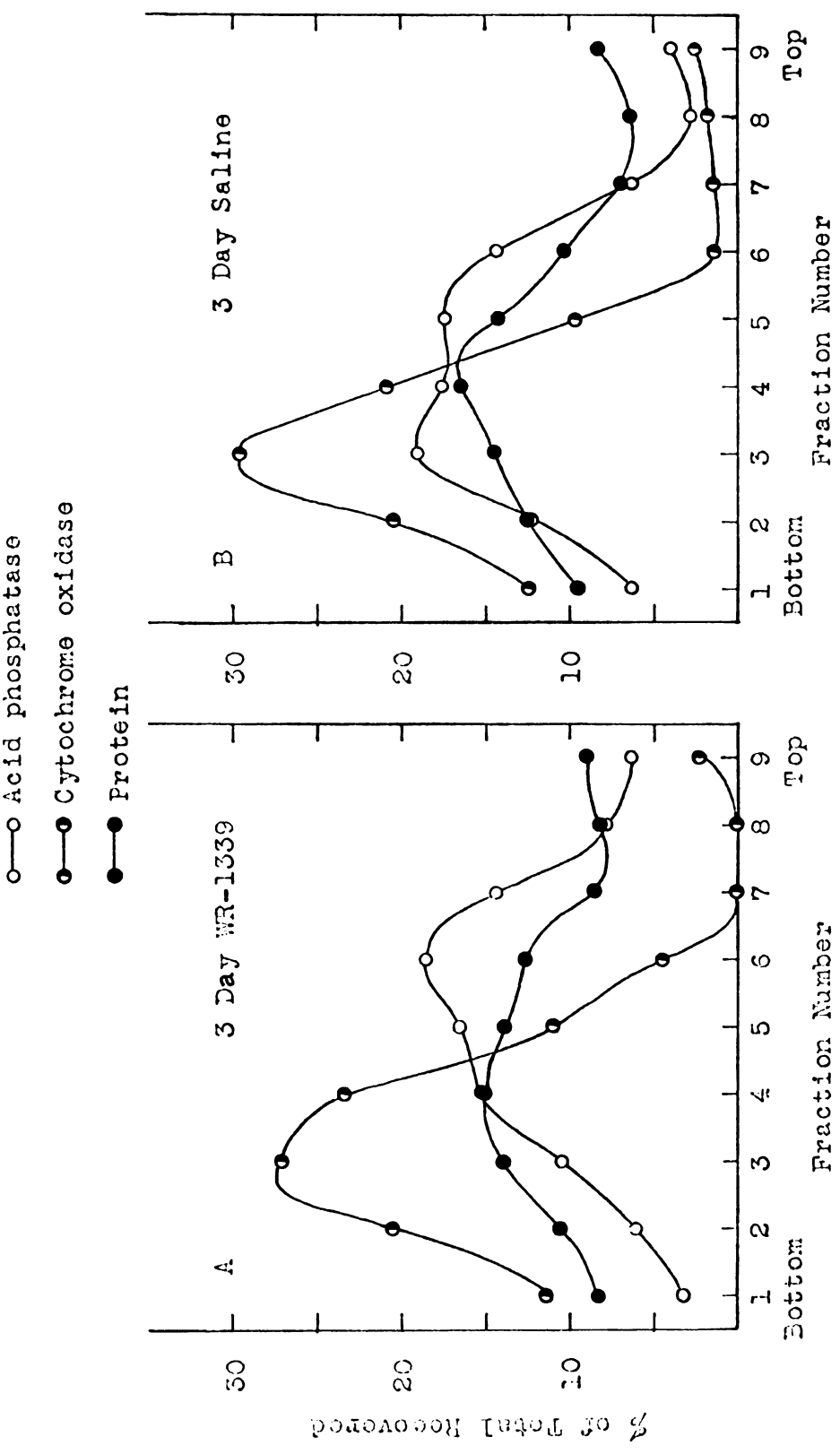
Acid phosphatase
Cytochrome oxidase
Protein
% of Total Recovered
50
20
10
A
3 Day WR-1339
30
20
10
B
3 Day Saline
Bottom
1 2 3 4 5 6 7 8 9
Top
Fraction Number

Figure 9

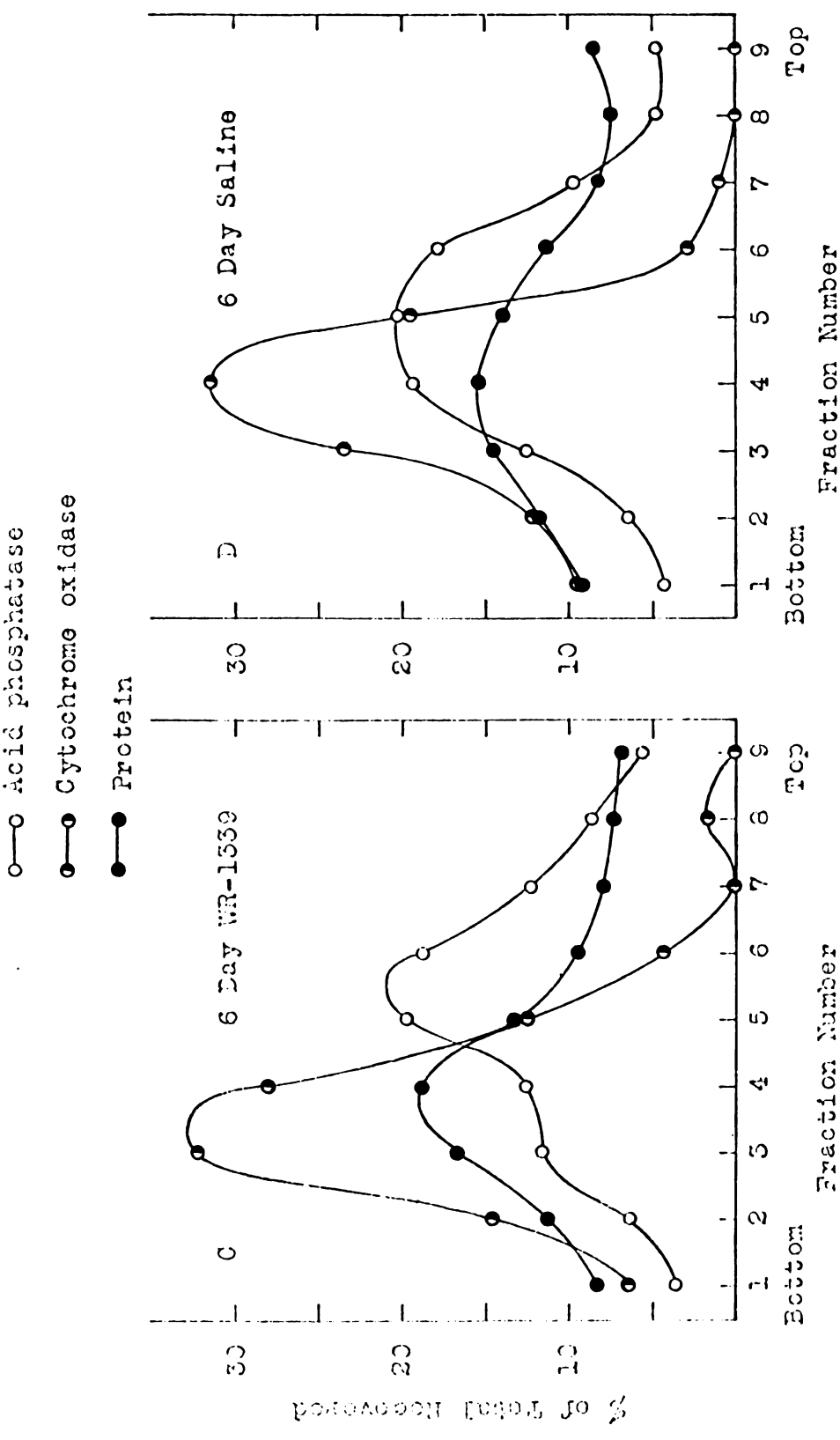

Figure 9 (cont.)

2. Biochemical Analyses and Bioassays for Cleavage-Initiating Material from Gradient Subfractions of Large Granule Material from Livers of Untreated Frogs - Two cell fractionations were performed on livers from untreated frogs. The 2:1 large granule fractions were obtained as indicated in Figure 10. Six- to eight-tenths ml of the suspensions in 0.7 SE were further fractionated on 0.8-1.8 SE density gradients as usual (Fig. 10 and 11). Eleven fractions of 30 drops each were recovered from each gradient. The drop-collecting apparatus described in Methods was used for this and all subsequent fractionations resulting in a more uniform drop size and no loss of material through leakage. The results of biochemical analyses are shown in the frequency distribution curves of Figure 10A and 11A-B. Each fraction in Figure 10 was analyzed for cytochrome oxidase, acid phosphatase, and protein. The material in Figure 11 was analyzed for urate oxidase, ribonuclease, acid phosphatase, and protein. Each fraction in Figure 10B was injected as recovered (1) and at two dilutions (1/5 and 1/25) of the original. In Figure 11C fractions were injected as recovered (1) and at a concentration 1/25 the original. All dilutions were made with 0.25 SE medium.

No correlation of cleavage-initiating activity with any particular type of cell component is apparent. The greatest concentration of cleavage-initiating material seems to be localized in the heavy end of the gradient. Some activity is generally found in fraction 11 from the top of the gradient. Although this activity at first appears to be non-sedimentable, it generally contained granular material. As the gradient is collected

Figure 10. Frequency distribution in a shallow SE gradient of protein, acid phosphatase, cytochrome oxidase, and cleavage-initiating activity associated with large granules from liver of untreated frogs.

The large granule suspension was prepared by differential centrifugation as follows: Liver tissue from untreated frogs was homogenized in 0.25 SE and centrifuged 3 times at 4200 rpm, 20 minutes. Final supernatant was centrifuged at 12,000 rpm, 30 minutes, to yield a pellet which was suspended in 0.7 SE and centrifuged at 9200 rpm, 40 minutes. Final pellet was suspended in 0.7 SE (P_3, 2:1). 0.6-0.7 ml of P_3 was layered over a 0.8-1.8 SE gradient and centrifuged at 35,000 rpm for 2 hours.

A. The data are expressed as in Figure 7B. Total recoveries: protein, 5.10 mg; acid phosphatase, 51.3 units; and cytochrome oxidase, 12.5 units.

B. The data are expressed as percentages of eggs which cleave following injection with fractions 1-11. Each percentage value is based on an average of 39 (24-57) injected eggs from one frog, except for the 1/25 curve, fractions 1-8, where each point is the average value from two batches of eggs from different frogs.

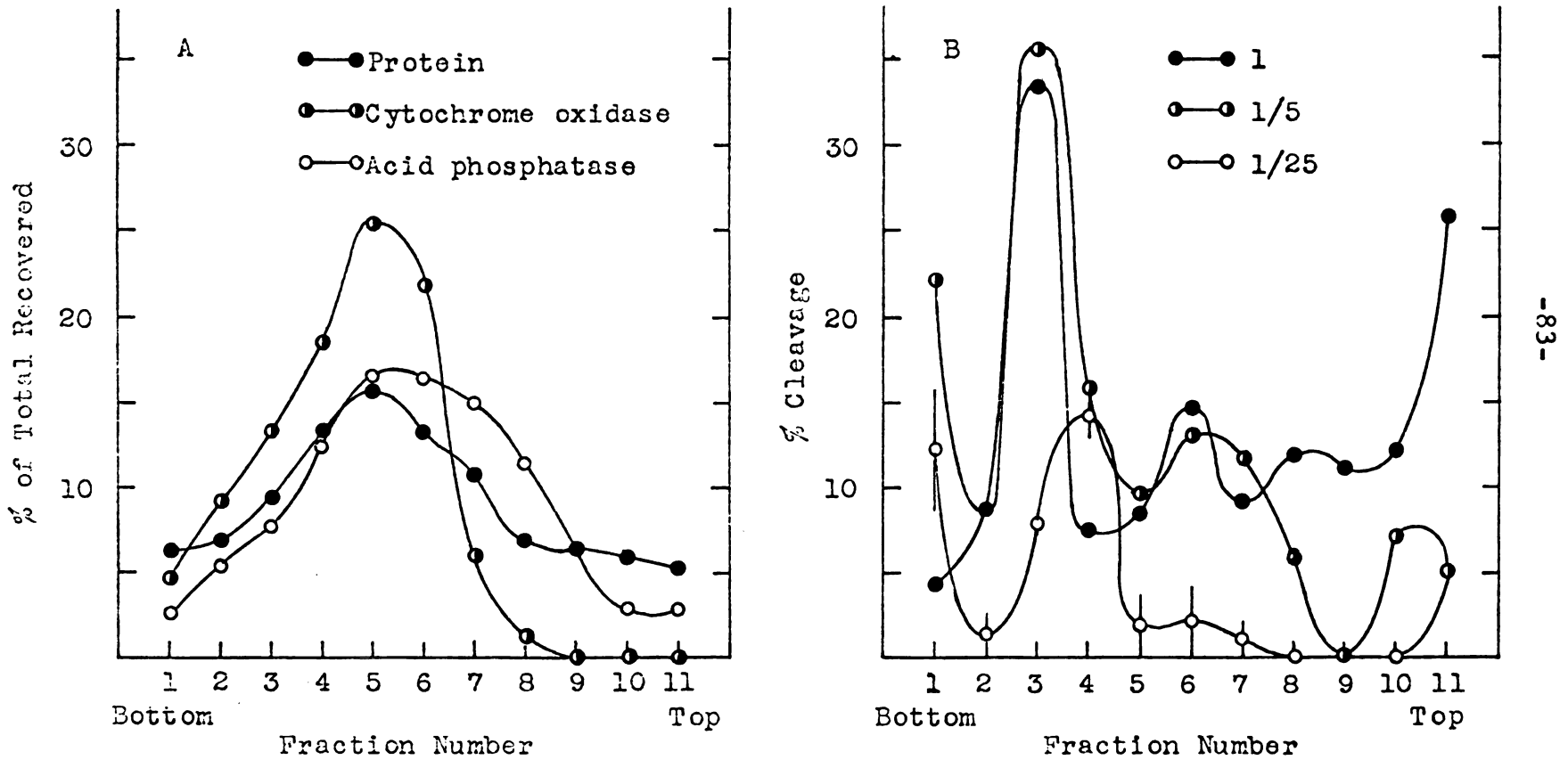
A
Protein
Cytochrome oxidase
Acid phosphatase
% of Total Recovered
30
20
10
1 2 3 4 5 6 7 8 9 10 11
Bottom
Top
Fraction Number
B
1
1/5
1/25
% Cleavage
30
20
10
1 2 3 4 5 6 7 8 9 10 11
Bottom
Top
Fraction Number

Figure 10

Figure 11. Frequency distribution in a shallow SE gradient of protein, acid phosphatase, urate oxidase, ribonuclease, and cleavage-initiating activity associated with large granules from liver of untreated frogs.

The large granule suspension in 0.7 SE (P_3, 2:1) was prepared as for Figure 10. 0.8 ml of P_3 was layered over a 0.8-1.8 SE gradient and centrifuged at 35,000 rpm for 2 hours.

A and B. The data are expressed as in Figure 7B. Total recoveries: protein, 4.34 mg; acid phosphatase, 45.8 units; urate oxidase, 49 units; and ribonuclease, 62 units.

C. The data are expressed as percentages of eggs which cleave following injection with fractions 1-11. Each percentage value is based on an average of 54 (32-75) injected eggs from one frog, except for the 1/25 curve, fractions 1-5, where each point is the average value from two batches of injected eggs from different frogs.

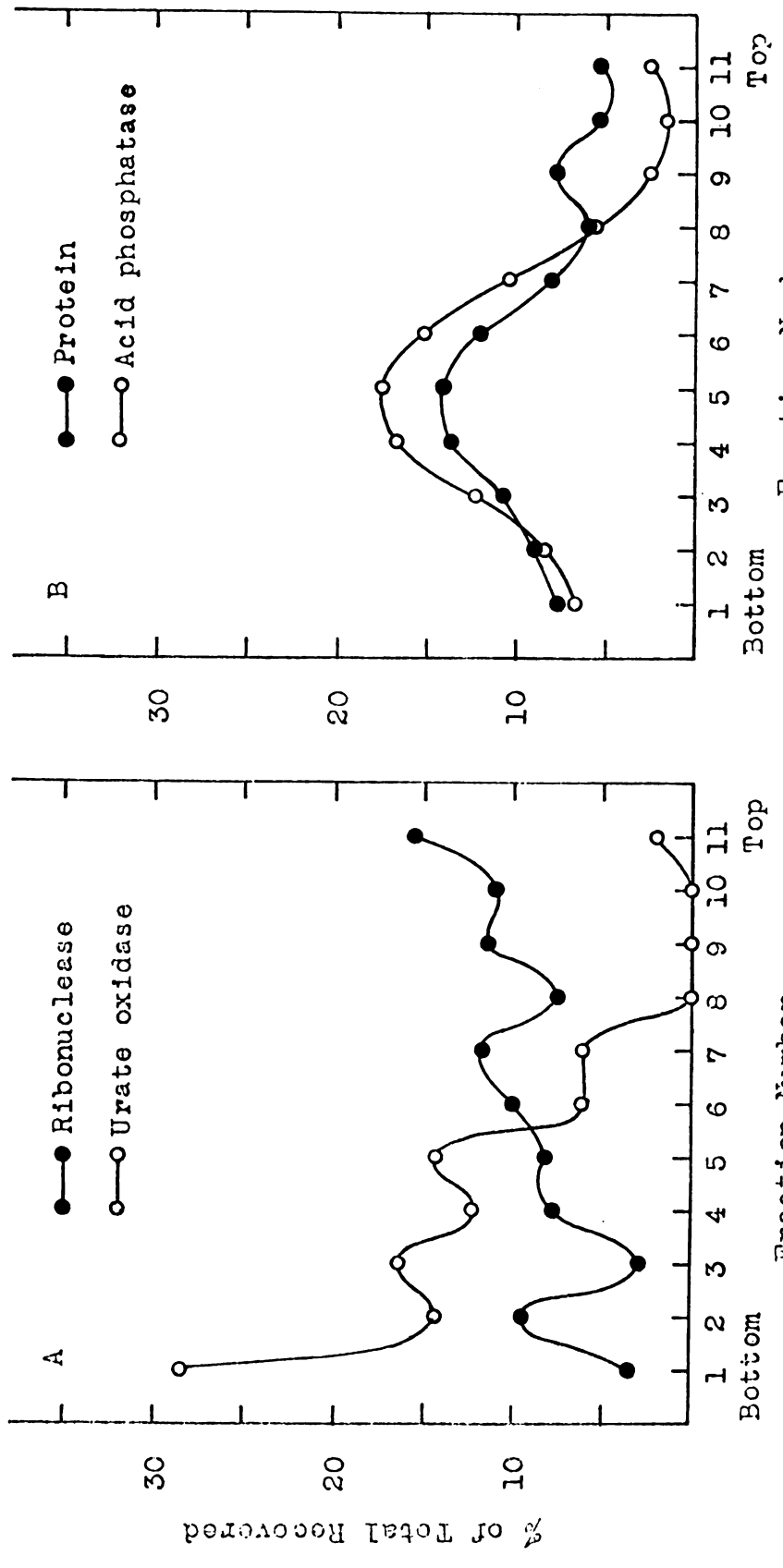
% of Total Recovered
A
Ribonuclease
Urate oxidase
Bottom
Top
Fraction Number
B
Protein
Acid phosphatase
Bottom
Top
Fraction Number

Figure 11

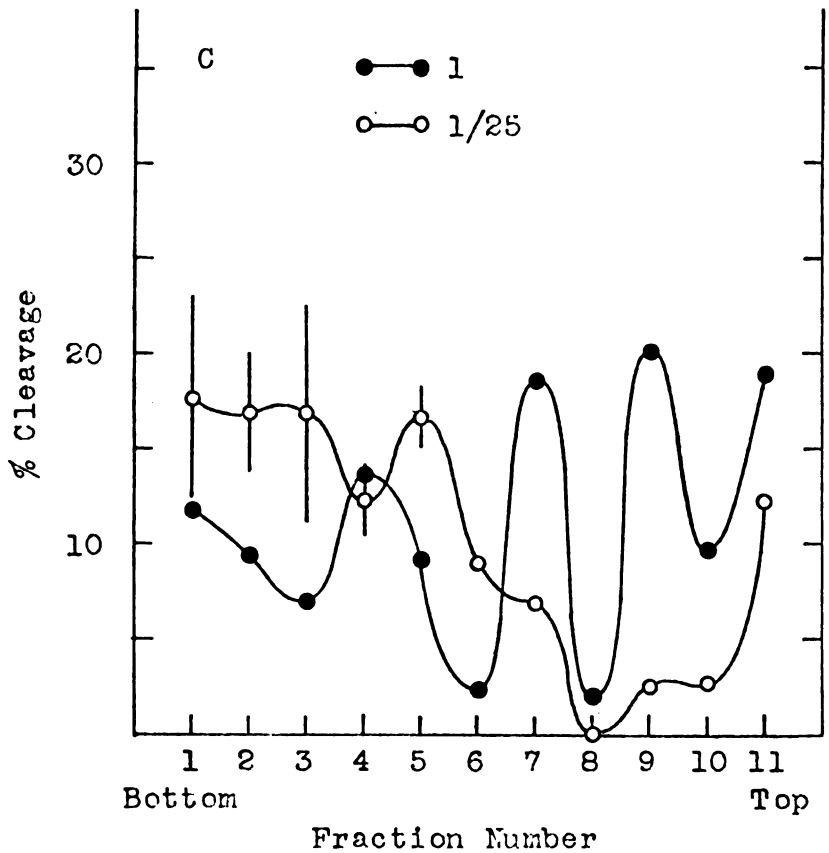

Figure 11 (cont.)

from the bottom of the tube, the meniscus collects particles adhering to the tube wall.

When fractions collected from the steep gradients were diluted and injected (Fig. 7) the distribution of cleavage-initiating material was clearly shown to be the same as that of the mitochondria and lysosomes. With the shallow gradients, however, such an association is not so evident (Fig. 10 and 11). If the material from the experiments in Figures 10 and 11 had been injected at a lower concentration it is possible that the area of the gradient with the greatest concentration of cleavage-initiating material would have been better defined. The large granule fraction layered over the shallow gradients had a relative concentration of 2:1 whereas the material layered over the steep gradient of Figure 7 had a relative concentration of 1:3. An average of 4.7 mg of protein was recovered from the shallow gradients (Fig. 10 and 11) and an average of 3.14 mg from the steep gradients (Fig. 7). On the other hand, the protein is more broadly distributed in the shallow gradient resulting in a lower maximum concentration per fraction in the shallow gradients than in the steep gradients. The most concentrated fraction in the latter in fact contained 2.6 times as much protein as the most concentrated fraction in the former (1.3 mg versus 0.5 mg). A dilution of 1/24 for the material in Figure 7 therefore resulted in a concentration of particles certainly low enough to demonstrate the region of maximum concentration of cleavage-initiating material, if such an area indeed existed in a clearly delimited fashion.

3. Gradient Subfractionation of Insonated Large Granule Fractions - From the experiment described in Table IX we know that insonation will destroy the cleavage-initiating activity of a suspension of cell particulates, and that the activity is not recoverable in the supernatant by centrifugation. By brief treatment with ultrasound it was hoped that lysosomes would be disrupted to a greater extent than mitochondria. An increase in the cleavage-initiating activity of the fractions from the less dense regions of the gradient might indicate that subparticulate elements of mitochondria are capable of inciting eggs to cleave.

Large granule fractions were prepared as in the preceding experiments in 0.7 SE at a 2:1 relative concentration. A 6 ml sample was placed in a Rosett cooling vessel (Branson Model 25) immersed in an ice bath and subjected to ultrasonic disruption with a Bronson S-75 sonfier set at a power level of 6 for 45 seconds. The 0.8-1.8 SE gradients were preformed and 0.7 ml of the insonated material was layered over each gradient. Following centrifugation for 2 hours at 35,000 rpm eleven 30-drop fractions were collected from each tube.

Fractionations were performed on two insonated large granule preparations. Figures 12A and 12B show the frequency distributions of protein, acid phosphatase, cytochrome oxidase, and urate oxidase in the gradients. The latter enzyme was assayed in only one of the experiments. Since the distributions of protein, cytochrome oxidase, and acid phosphatase in the two experiments were nearly identrical the results were combined and the curves for these constituents represent an average distribution

Figure 12. Frequency distribution in a shallow SE gradient of protein, acid phosphatase, cytochrome oxidase, urate oxidase, and cleavage-initiating activity associated with insonated large granule material from frog liver.

The large granule suspension in 0.7 SE (P_3, 2:1) was prepared as for Figure 10. 0.7 ml of P_3 was layered over a 0.8-1.8 SE gradient and centrifuged at 35,000 rpm for 2 hours.

A and B. The data are expressed as in Figure 7B. Average total recoveries for two experiments: protein, 4.87 mg; acid phosphatase, 63.3 units; and cytochrome oxidase, 43.9 units. Total urate oxidase recovered in one experiment was 84 units.

C. The data are expressed as the percentages of eggs which cleave following injection with fractions 1-11. Each percentage value is based on an average of 46 (27-64) injected eggs from a different frog for each curve.

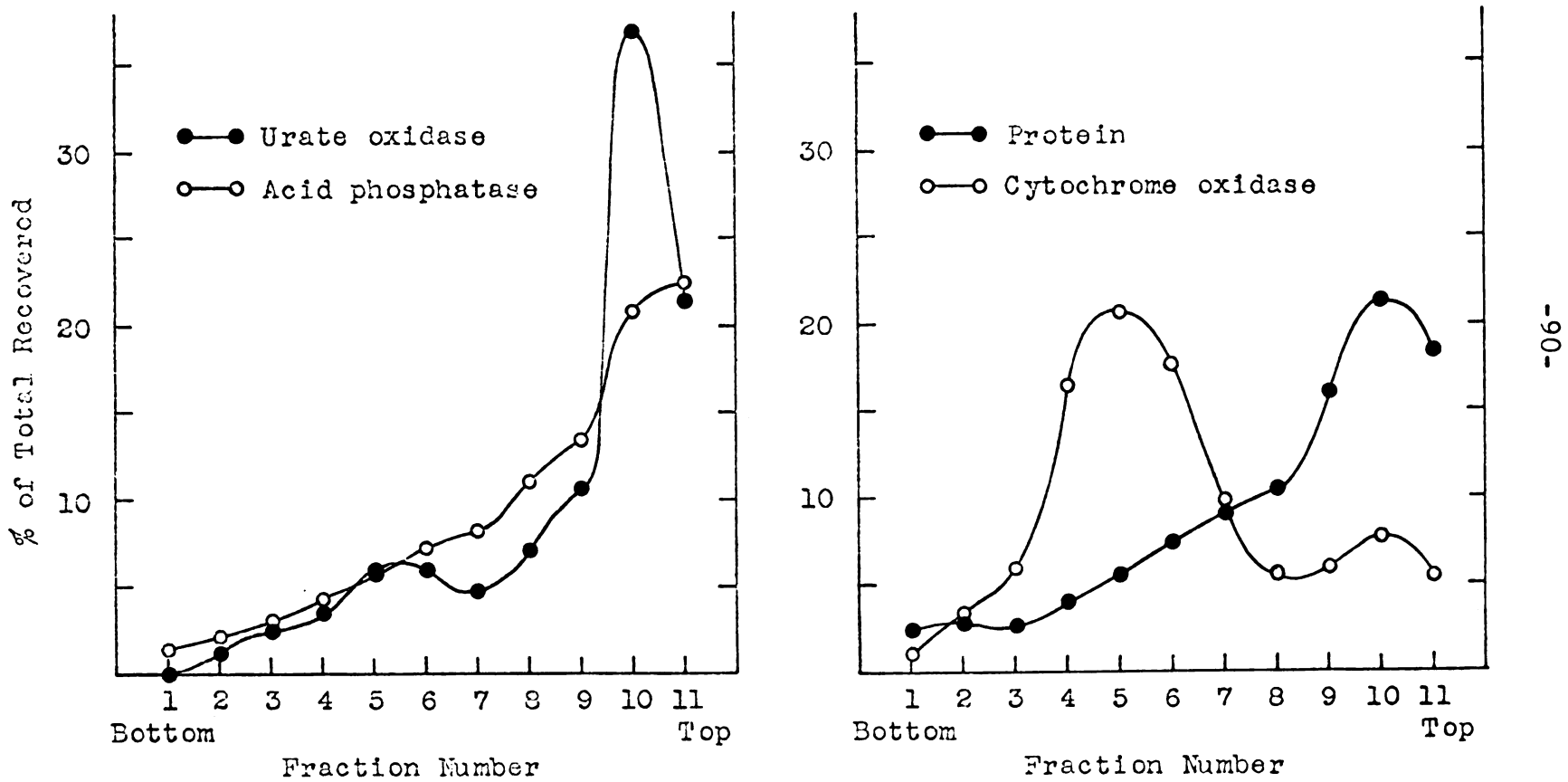
% of Total Recovered
30
20
10
Urate oxidase
Acid phosphatase
1 2 3 4 5 6 7 8 9 10 11
Bottom
Top
Fraction Number
30
20
10
Protein
Cytochrome oxidase
1 2 3 4 5 6 7 8 9 10 11
Bottom
Top
Fraction Number

Figure 12

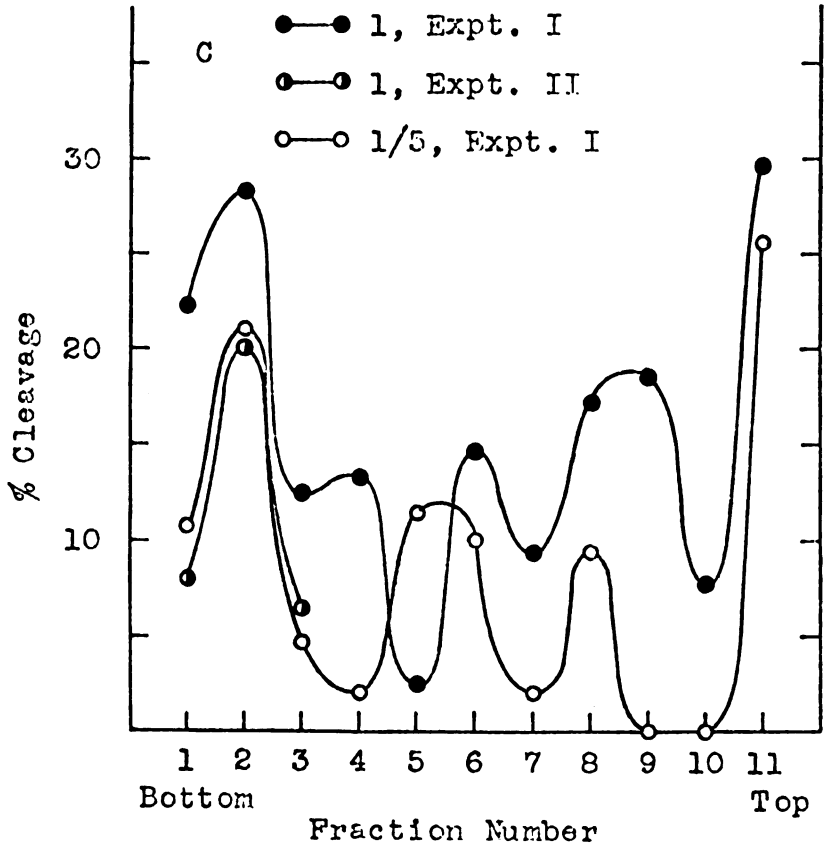

Figure 12 (cont.)

of the two experiments.

Using acid phosphatase and urate oxidase as indicators, the curves (A) show that the integrity of the lysosome and peroxisome structure was destroyed. Much of these enzymes as well as the total protein (B) was converted to a form less readily sedimented. The presence of these enzymes in the denser regions of the gradient may be due in part to their adsorption to membrane or particle fragments. The distribution of mitochondria was also altered (B), but to a lesser extent. There was a decrease of cytochrome oxidase in the denser portions (fractions 1, 2, and 3) of the gradient and a marked increase in the upper portions (fraction 8-11). No cytochrome oxidase activity was detectable in fractions 9-11 from non-insonated preparations. With the insonated preparations, however, 6.0, 7.8, and 5.4% of the activity was recovered in fractions 9, 10, and 11, respectively. The peak of cytochrome oxidase concentration was in fraction 5 for both the insonated and untreated preparations.

In view of the fact that de Duve (1964) attributes most of the protein in the "mitochondrial" fraction from rat liver to mitochondria, it seems somewhat surprising that the bulk of the mitochondrial enzyme cytochrome oxidase should appear in the usual fractions while the protein peak shifts to a region of lower density. This indicates that either there are components other than mitochondria present in substantial amounts or that insonation may have released substantial quantities of protein from the matrix of mitochondria without appreciably altering the density of the remaining structural shell. Without further bio-

chemical analyses and especially electron microscopic observations it is difficult to pinpoint the source of the bulk of the protein present in the upper fractions of the gradient.

Figure 12C shows the reactivity of eggs when injected with the gradient fractions. The fractions were injected as recovered (1) and after being diluted by 1/5. Good cleavage responses were obtained only with the injection of material from one (I) of the two experiments. The poor results of the other experiment (II) may be due to two factors. One, the low viability of the eggs, as determined from the percentage of eggs which cleaved when fertilized, may have reduced their responsiveness. Eggs from three frogs were used in this experiment. Less than 62% of the fertilized eggs from two of these three batches of eggs cleaved. Two, the material was injected two days after it was prepared. The decrease in cleavage-initiating activity of a fraction with time has been previously shown. This seems the most likely explanation for the following reasons: 1) The eggs from two of the three frogs were injected only with diluted cleavage-initiating material (1/5). Only fractions 1-3 were injected as recovered (1) into the eggs from the other frog and a good response was obtained in spite of the fact that only 61.4% of the fertilized control eggs cleaved. These results (II) are included in Figure 12C along with the results of the other experiment (I). They support the picture one gets of the distribution of cleavage-initiating material on the gradient. 2) The viability of one of the two batches of eggs injected only with diluted fractions was very good. Of the eggs fertilized, 90.8%

cleaved. Yet the injection of fractions 1-11 at a 1/5 concentration resulted in an average of only 2.0% cleavage. This may be compared to an average of 8.9 percent when a 1/5 concentration of material from the other experiment (I) was injected into eggs, although only 78.3% of the fertilized control eggs cleaved. The material in this latter experiment was less than one day old. Eggs from another frog were injected with undiluted gradient fractions (1 in experiment I). Of the fertilized control eggs from this batch, 92.9% cleaved.

As in the experiments of Figures 10 and 11, a definite correlation between any of the cell components studied and the presence of cleavage-initiating activity is not apparent. While most of the activity seems to be at the two extremes of the gradient, there is a considerable amount of active material spread throughout the gradient in spite of the previous ultrasound treatment. It may be of interest to note that the CI activity of all fractions, except fraction 5, decreased when the fractions were diluted to 1/5. Fraction 5, which showed an increase in CI activity, also contained the most cytochrome oxidase activity. Further dilution would have indicated with more certainty the amount of cleavage-initiating material in this fraction.

C. Comparison of Gradient Subfractionation in SE and SEP Media

When comparing the broad erratic distribution of the cleavage-initiating material in the shallow gradients with the more limited distribution in the steep gradients, it seems unlikely that the steepness of the gradient alone could account for this striking difference. The only other major difference in the two

procedures was the presence of phosphate buffer in the steep gradients. Further experiments were therefore performed using SEP media to construct the shallow gradients.

A large granule fraction was prepared as usual (see legend to Fig. 10) except that the media used were 0.25 and 0.7 SEP and the final suspension had a relative concentration of 1:1 rather than 2:1 as in the preceding experiments. Following centrifugation over 0.8-1.8 SEP gradients in a swinging bucket rotor (SW 39, Spinco) at 35,000 rpm for 2 hours, 11 fractions were collected and analyzed for protein and acid phosphatase activity. Samples were also injected into eggs to determine the presence of cleavage-initiating activity.

Figure 13A illustrates the distribution of protein and acid phosphatase in this shallow SEP gradient. The sedimentation profiles of these two constituents are strikingly different from those in the shallow SE gradients. The distributions of protein and acid phosphatase in the latter are much broader. In comparison to the amount of acid phosphatase and protein which is recovered in the denser portion of the SE gradient (fractions 1-4 in Fig. 10A and 11B), very little is recovered from similar fractions in the SEP gradient (fractions 1-4 in Fig. 13A). Table XIII compares the SE and SEP gradients of Figures 10, 11, and 13 with regard to the percentage of total protein and acid phosphatase recovered in three regions of the gradient: A, fractions 1-4; B, fractions 5-7; and C, fractions 8-11. It is obvious from these data that there is a greater concentration of protein (and hence presumably also mitochondria) and acid phosphatase

Table XIII. Comparison of the distribution of protein, acid phosphatase, and cleavage-initiating activity in three regions of shallow SE and SEP gradients.[a]

Medium	Protein			Acid Phosphatase			CI Activity[c]		
	A[b]	B	C	A	B	C	A	B	C
SE (I)	35.9	39.7	24.5	28.3	48.0	23.7	9.4	1.8	0.9
SE (II)	41.4	34.2	24.4	44.3	43.1	12.6	15.8	11.7	3.8
SEP	17.2	57.7	25.0	5.8	63.6	30.5	9.9	22.5	3.4

[a] Data are from experiments depicted in Figures 10 (SE,I), 11 (SE, II), and 13 (SEP) and are expressed as percentages in each of the three regions of the total protein and enzyme recovered from all fractions.

[b] Regions A, B, and C correspond to fractions 1-4, 5-7, and 8-11, respectively.

[c] Figures represent percentages of the total eggs in each group which cleaved when injected with the 1/25 concentration of the material from the SE and SEP gradients.

(lysosomes) in the mid-gradient region and also more acid phosphatase in the upper third of the SEP gradient than in the SE gradient.

The results obtained by injecting samples of the fractions from the shallow SEP gradient are shown in Figure 13B. Prior to injection the fractions were diluted to 1/12 their original concentration with 0.25 SEP. This was supposed to give a concentration of material comparable to the 1/25 concentration of the SE gradient fractions in Figures 10 and 11 on the basis of the following facts: 1) The relative concentration of the large granule fraction was 1:1 rather than 2:1 as in Figures 10 and 11; 2) 2.3 mg of protein were recovered from the SEP gradients in

Figure 13. Frequency distribution in a shallow SEP gradient of protein, acid phosphatase, and cleavage-initiating activity associated with large granules from frog liver.

The large granule suspension in 0.7 SEP (P_3, 1:1) was prepared as for Figure 10. 0.7 ml of P_3 was layered over a 0.8-1.8 SEP gradient and centrifuged at 35,000 rpm for 2 hours.

A. The data are expressed as in Figure 7B. Total recoveries of protein and acid phosphatase were 2.32 mg and 15.4 units, respectively.

B. The data are expressed as the percentages of eggs which cleave following injection with fractions 1-11. Each percentage value is based on an average of 124 (106-137) injected eggs from one frog.

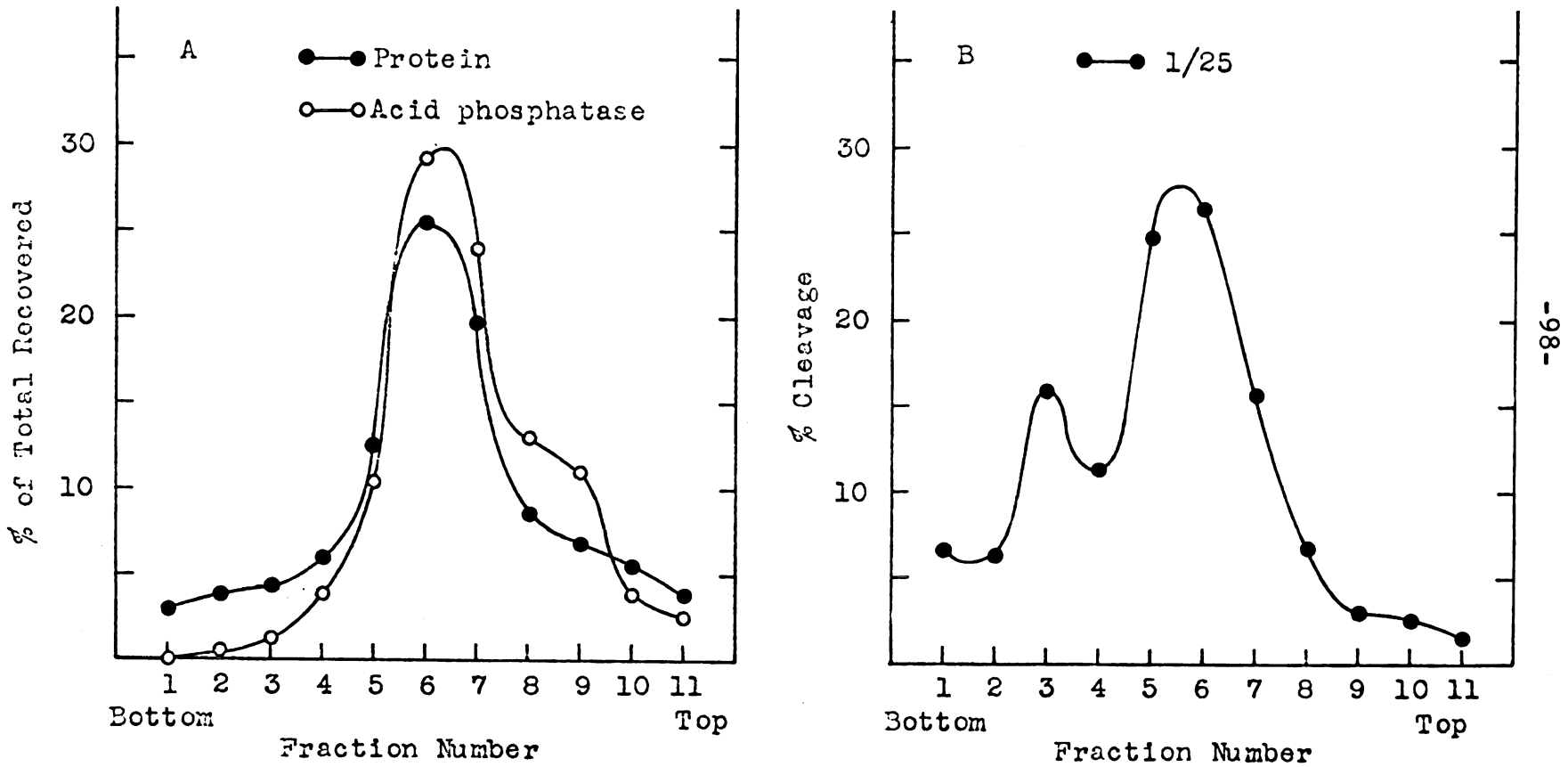
A
Protein
Acid phosphatase
% of Total Recovered
30
20
10
1 2 3 4 5 6 7 8 9 10 11
Bottom
Top
Fraction Number
B
1/25
% Cleavage
30
20
10
1 2 3 4 5 6 7 8 9 10 11
Bottom
Top
Fraction Number

Figure 13

Figure 13, while 5.1 and 4.3 mg total protein were recovered from the gradients in the two SE experiments of Figures 10 and 11, respectively.

Figure 13 shows that the more limited distribution of CI activity in the SEP gradient corresponds quite closely to the distributions of protein and acid phosphatase. If one assumes for the moment that lysosomes are the active components, it would be difficult to explain the substantial cleavage-initiating activity in fractions 1-3, on the basis of the acid phosphatase distribution alone. In considering this problem it is important to recall that the lysosomal population is very heterogeneous and that only acid phosphatase was assayed as a marker. This point will be given further consideration in the discussion. Since cytochrome oxidase was not assayed in this experiment no comparison of its distribution with that of the cleavage-initiating material is possible.

For the next and final gradient analysis, large granule fractions were prepared simultaneously in SE and SEP media. The final 1:1 suspensions in 0.7 SE and SEP media were layered over 0.8-1.8 SE and SEP gradients, respectively. Following centrifugation in the SW 39 rotor at 35,000 rpm for 2 hours, 11 fractions were collected and analyzed for protein, acid phosphatase, and cytochrome oxidase (Fig. 14). In addition, the absorbancies of the fractions at 260, 280, and 520 mμ were determined. Since A_{260} and A_{280} for all fractions were nearly identical, only A_{260} and A_{520} are graphically depicted (Fig. 15). The A_{520}'s were determined to provide a measureable indication of particle dis-

Figure 14. A comparison of the frequency distribution in shallow SE and SEP gradients of protein, acid phosphatase, and cytochrome oxidase associated with large granules from frog liver.

The large granule suspensions in 0.7 SE or SEP (P_3, 1:1) were prepared as for Figure 10. 0.7 ml of P_3 was layered over 0.8-1.8 SE or SEP gradient and centrifuged at 35,000 rpm for 2 hours.

The data are expressed as in Figure 7B. Total recoveries: SE medium - protein, 2.47 mg; acid phosphatase, 20.8 units; and cytochrome oxidase, 8.7 units. SEP medium - the total recoveries were as follows: protein, 4.16 mg; acid phosphatase, 22.1 units; and cytochrome oxidase, 25.2 units.

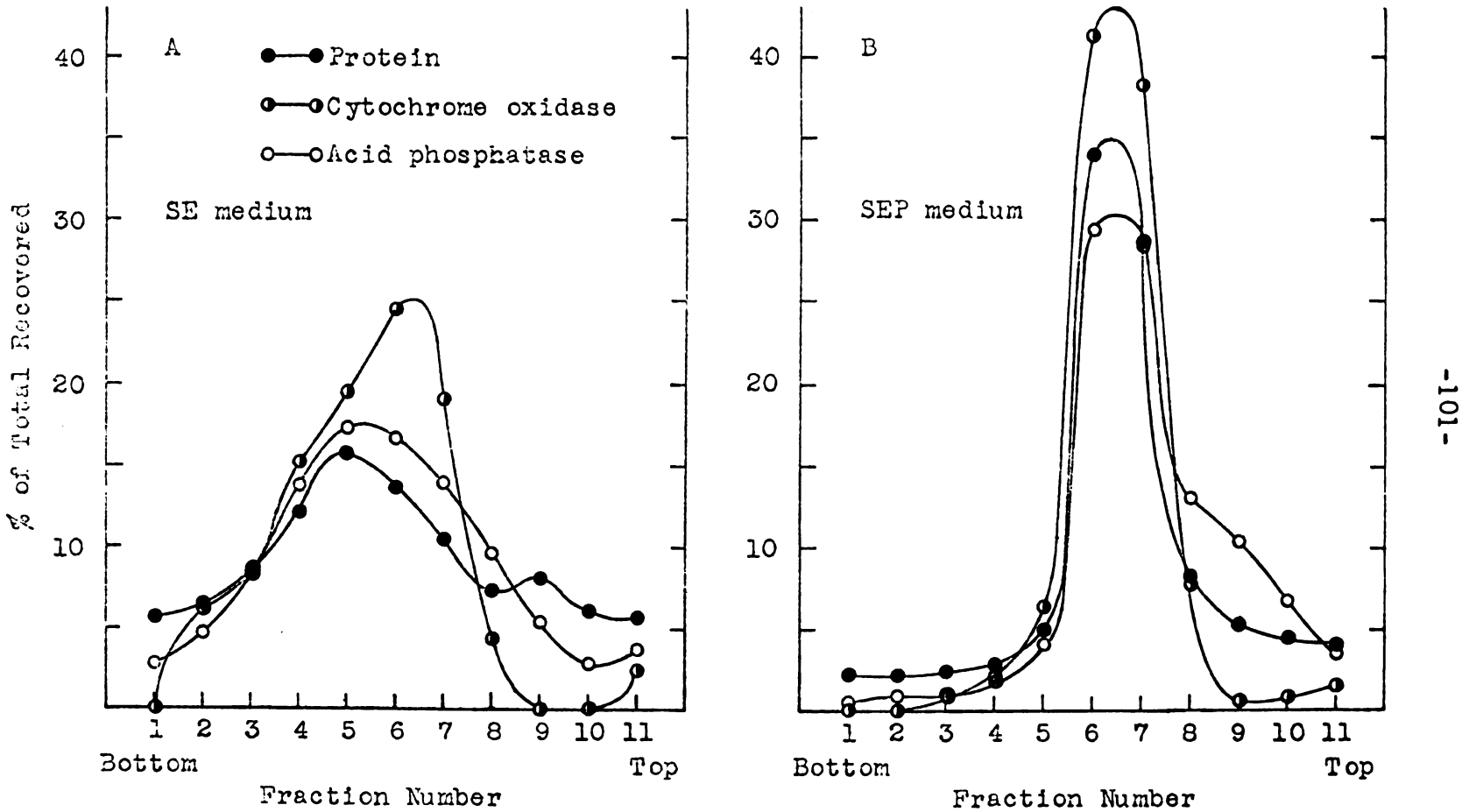
A
Protein
Cytochrome oxidase
Acid phosphatase
SE medium
% of Total Recovered
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11
Bottom
Top
Fraction Number
B
SEP medium
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11
Bottom
Top
Fraction Number

Figure 14

Figure 15. A comparison of the distribution in shallow SE and SEP gradients of 260 mμ and 520 mμ absorbing material associated with large granules from frog liver.

The fractions analyzed are from the same gradients described in Figure 14.

The relative absorbancy of a particular fraction is the ratio of its absorbancy to the average absorbancy. The latter value is obtained by dividing the sum of the absorbancies of all the fractions by the number of fractions collected from the gradient. The average absorbancies per fraction were as follows: SE medium - $A_{260\ m\mu} = 0.509$, $A_{520\ m\mu} = 0.048$; SEP medium - $A_{260\ m\mu} = 0.736$, $A_{520\ m\mu} = 0.101$.

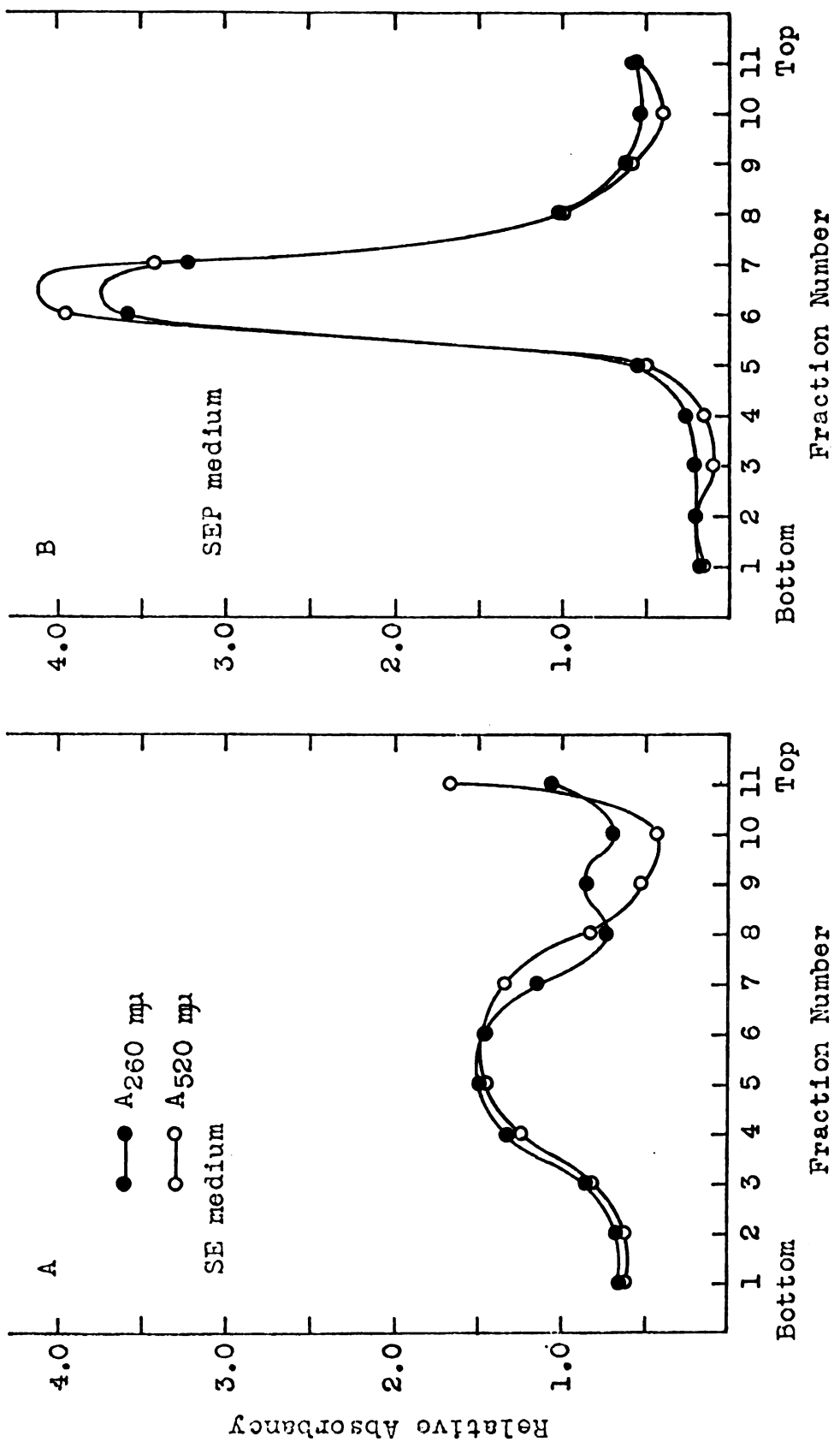
A
SE medium
A_{260} mμ
A_{520} mμ
B
SEP medium
Relative Absorbancy
4.0
3.0
2.0
1.0
Bottom
1 2 3 4 5 6 7 8 9 10 11
Top
Fraction Number

Figure 15

tribution in the gradient.

Figures 14 and 15 emphasize the marked differences between the SE and SEP gradients with respect to their distributions of protein, acid phosphatase, cytochrome oxidase, and the 260 and 520 mμ absorbing materials. As noted before, the distribution of particles in the SE gradient is much broader than in the SEP gradient. Table XIV compares the percentages of protein and enzymes recovered in the three regions (fractions 1-4, 5-7, and 8-11) of the two types of gradients. Compared to the SE gradient, there is again a marked diminution in fractions 1-4 from the SEP gradient of all the components analyzed.

Table XIV. Comparison of the distribution of protein, acid phosphatase, and cytochrome oxidase in three regions of shallow SE and SEP gradients.[a]

Medium	Protein			Acid Phosphatase			Cytochrome Oxidase		
	A[b]	B	C	A	B	C	A	B	C
SE	32.8	40.1	27.2	30.3	48.0	21.6	30.1	63.1	6.8
SEP	9.7	68.2	22.2	4.1	62.0	33.9	3.1	85.9	10.8

[a] Data are from the experiment depicted in Figure 14 and are expressed as percentages in each of the three regions of the total protein and enzyme recovered from all fractions.

[b] Regions A, B, and C correspond to fractions 1-4, 5-7, and 8-11, respectively.

The photographs in Figure 16 show the granule distribution in two types of gradients following centrifugation at 35,000 rpm for 2 hours. In the SEP gradient the granule distribution ap-

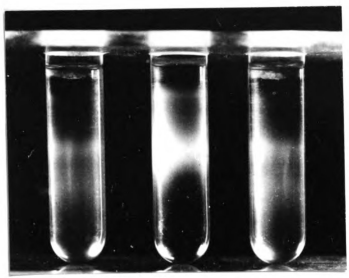

Figure 16. A photograph comparing the appearance of the particle distribution in shallow SE and shallow SEP gradients following centrifugation for 2 hours at 35,000 rpm.

pears to be concentrated in one dense band corresponding to fractions 6 and 7 with a general diffuse distribution from this band to a smaller band near the top of the gradient, corresponding to fractions 9 and 10. In the SE gradient, on the other hand, an additional band of granules appears. The two upper bands correspond with those in the SEP gradient and are collected in fractions 6-7 and 9-10 (the uppermost band is not apparent in the photograph). The dispersion of granules in the SE gradient is however much broader and more evident throughout the bottom half of the SE gradient than the SEP gradient. The additional broad diffuse band in the former appears in the lower half of the gradient, centered approximately in a position corresponding to fraction 4.

Thus the presence of 0.1M Na_2HPO_4/NaH_2PO_4 in the media has a significant and reproducible effect on the sedimentation behavior or equilibrium density of the particles. In contrast to the suggestion made earlier that the presence of salt in the medium may enhance agglutination of the particles (Hers, et al., 1951; Dalton, et al., 1949; and Hogeboom, et al., 1948), the preceding experiments suggest that agglutination may in fact be a more predominant phenomenon in the salt-free medium (SE) than in the phosphate buffered medium (SEP).

V. OTHER EXPERIMENTS

A. Attempts at Isolating Lysosomes by Differential Centrifugation

Sawant, et al. (1964b) were able to obtain relatively pure lysosomal fractions from rat-liver homogenates by a combination of differential and density-gradient centrifugation. Their preparations contained no detectable succinoxidase (mitochondria), trace amounts of urate oxidase (peroxisomes), and a content of glucose-6-phosphatase or cytochrome b_5 equivalent on a protein basis to 6-10% microsomal contamination. Other preparations were obtained free of microsomes.

This fractionation technique was applied unsuccessfully to liver tissue from frogs. Several modifications of the method were introduced in an effort to obtain a preparation of lysosomes free of other particles, particularly mitochondria. The homogenate and samples from all steps of the fractionation were assayed for protein, urate oxidase, acid phosphatase, cytochrome oxidase, and glucose-6-phosphatase. Specific activities were determined to monitor the purification procedure. The percentage yield at each step was calculated on the basis of the total activity of the homogenate. In one analysis the final pellet was somewhat enriched with lysosomes. The specific activity of acid phosphatase was increased 14-fold over that of the homogenate and the percentage yield was 1.1%. This compares unfavorably with the 65-67-fold increase and 11% yield obtained by Sawant and coworkers. Furthermore, in the present work, there was a 15-fold enrichment of urate oxidase and a lesser increase

(3-fold) in the specific activity of cytochrome oxidase. The only significant reduction occurring was that of microsomal contamination. The final yield was only 0.03%. These experiments were only exploratory in nature. To achieve the desired purpose of such a fractionation of frog liver, numerous trial and error fractionations would be required with enzyme assays and protein determinations performed at each step in the scheme.

To ensure complete reproducibility of a particular procedure the centrifugation conditions must be carefully controlled. These conditions include the temperature and volume of the sample (determines R_{min}) and the time and force of centrifugation, including acceleration and deceleration. The time and force of centrifugation are preferably expressed compositely as g-min according to de Duve and Berthet (1953).

In general, the centrifuge used in the above experiments (IEC Refrigerated High-Speed Centrifuge, Model HR-1, not equipped with a revolution meter) was ill-suited for precise cell fractionation by differential centrifugation.

B. Injections of Membranes, Thymidine and ATP, Separately and in Mixture

Membranous components are obviously present in particulate fractions which contain mitochondria and lysosomes. To test the possibility that membranes nonspecifically serve in some physical capacity to stimulate egg cleavage, a suspension of fat globule membranes obtained from washed cream of unpasteurized cows milk (Courtesy of J.R. Brunner, Dept. of Food Science, Mich. State Univ.) was injected into eggs at several concentrations.

Since Stevens (1966) has shown that thymidine plus adenosine-5'-triphosphate stimulates cytokinesis in ascites tumor cells, a mixture of these compounds alone and in combination with the fat globule membranes was injected into eggs.

The concentration of membranes injected was somewhat arbitrary. An attempt was made to use an initial concentration which was optically comparable to an effective concentration of the large granule fraction from liver. The choice of 1.00 mg/ml for the initial concentration of both ATP and thymidine was based on the results of a previous investigation (Pfohl, 1962) which showed that the injection of this concentration of ATP was effective in enhancing the cleavage of parthenogenetically stimulated eggs.

All suspensions and solutions were in 0.25 SE medium adjusted to pH 7.0-7.2. An average number of 47 eggs (38-60) was injected with each of the suspensions and solutions separately or mixed. Injections were also made of 1/10 and 1/100 dilutions of the above. With one exception, none of the eggs injected with the above materials cleaved. Of 48 eggs injected with a solution of 0.01 mg each of ATP and thymidine/ml, one egg cleaved.

DISCUSSION

If the appropriate biological material is introduced into the mature egg of a frog, the egg will undergo a sequence of normal cleavages. The aim of this study, as stated in the introduction, was to elucidate the nature of the factor (second factor of Bataillon, cleavage-initiating substance of Shaver, or formative stimulus of Loeb) which regulates the cleavage response of the egg.

Before pursuing this line of thought I will first briefly describe the preparations a cell must make before its division response can be elicited by a proper stimulus. This description is based largely on the comprehensive review of mitosis and cell division by Mazia (1961). Subsequent to this, with these prerequisites in mind, we will examine in more detail the state of preparedness of the mature unfertilized frog egg and the reactions it is capable of undergoing in response to stimuli other than those which normally incite cleavage.

I. PREPARATIONS OF THE CELL FOR DIVISION

The most obvious preparations that the cell makes for division are dissociable to the extent that they can be separately identified and studied. These include the doubling of chromosomal material, reproduction of division centers, and synthesis and assemblage of mitotic apparatus. They are, however, interdependent to the extent that successful cell division is a pro-

duct of their coordination. Conversely, their continuance over a period of time, where an extended sequence of cell divisions normally occurs, is dependent on normal cell division.

The idea that the provision of an energy supply is a prerequisite for mitosis (Swann, 1954) has been abandoned by Mazia (1963) since it has been shown, at least for sea urchin embryos (Epel, 1963), that all phases of mitosis require the continuous synthesis of ATP.

The doubling of chromosome substance is apparently necessary for cell division to occur. Mazia (1963) has stressed the structural association of DNA and DNA polymerase as critical for the initiation and termination of DNA synthesis. Control of cell division may then be associated with the regulation of the level of DNA polymerase in nuclei. Inhibition of DNA synthesis before doubling of chromosomes has occurred precludes cell division. Intense DNA synthesis has been shown to occur in frog eggs, however, without accompanying cleavage (Grant, 1965).

The division center may be best regarded in a functional sense; it describes an agency responsible for polarizing the mitotic apparatus. According to Wilson (1966), "Cell centers, in the physical sense...cannot be considered essential universal parts of the mitotic apparatus. Nonetheless, even in plants highly localized regions act as focal points for the operational spindle. Furthermore, observations of 'split' figures involving multipolar spindles suggest that these focal points are real entities rather than ill-defined zones." Centrioles, when observed, provide a structural basis for the activity of the cen-

ters. Splitting of the center is an event necessary for the establishment of the poles. This event is parallel to but independent of chromosome reproduction (Mazia, et al., 1960).

Another prerequisite to division is an adequate supply of the proteins which are necessary for forming the mitotic apparatus. The mitotic apparatus as defined by Mazia and Dan (1952) includes spindles, asters, centrioles, nuclei (before breakdown), and chromosomal structures (after breakdown of nuclear membrane).

II. THE FROG EGG--ITS STATE OF PREPAREDNESS

We will assume that at least the above preparations have significance for cell division in general. What, then, is the status of the frog egg with regard to these preparations? In some cases direct answers can be obtained, while in others only inferences can be made. In this connection we will be most interested in what responses can be elicited from the egg by simple activation (i.e. the "first factor" or puncturing of the egg). Having this information we may gain some insight into the nature and function of the inoculated or injected "cleavage-initiating material."

The egg is an exceptional cell in that it has differentiated a complex cytoplasmic organization during oogenesis. The mature egg is initially equipped to elaborate a multicellular system which is itself capable of morphogenesis. The state of the amphibian egg is summarized by Grant (1965). The activation of the

amphibian egg at fertilization initiates the patterns of DNA replication, spindle formation, and energy cycling associated with mitosis and cell division. Since division of the egg can occur in the absence of a nucleus as well as under anaerobiosis and in the presence of actinomycin D, nucleic acid analogs, and nitrogen mustard, Grant (1965) suggests that functional protein-forming systems and an energy source sufficient to carry the egg through cleavage are preformed in the egg.

A. Store of Cytoplasmic DNA and DNA Precursors

There are many reports dealing with the quantity of DNA in egg cytoplasm (see review by Grant, 1965). Most recently, for example, Dawid (1965) has shown that the eggs of Rana pipiens and Xenopus laevis contain 300-500 times more DNA than the somatic cells of these animals. He further concludes (1966) that two-thirds of the DNA of eggs is of mitochondrial origin. However, many other workers have reported much greater quantities of DNA per egg than that estimated by Dawid (1965). Haggis (1964), for example, estimates the DNA content per egg (R. pipiens) to be 0.2 μg while Dawid (1965) obtains a value of 4.5 mμg. The latter author also rejects the generally accepted hypothesis that cytoplasmic DNA functions as a storage material for the assembly of chromosomes during early development. Whatever the case may be, a number of workers (Grant, 1958; Bieber, et al., 1959; Hoff-Jørgensen and Zeuthen, 1952) have, by direct measurement, demonstrated large stores of deoxypentose derivatives in Rana pipiens eggs. A large store of non-specific precursors for the synthesis of chromatin material has also been

shown indirectly to exist in the eggs of Rana pipiens (Briggs and King, 1955) and Xenopus laevis (Graham, et al., 1966). This synthesis, according to Grant (1965), "...is dependent on the state of the egg cytoplasm rather than any factor introduced by sperm, because artificial activation and development is possible in most eggs. The egg seems primed to initiate rounds of DNA replication but is somehow inhibited and can be readily released from this inhibition by chemicals and physical changes occurring in the cytoplasm.... It is possible that enzymes...essential for DNA replication are 'masked' or inhibited and are released upon activation."

That the egg need not be incited to cleave by fertilization or injection of active material in order to initiate DNA replication can be inferred from the early cytological studies of Bataillon (1929) which showed that mere activation of the egg by pricking resulted in an increase in the size and number of chromosomes in the egg. In a brief report, Huff and Ramirez (1966) also observed nuclear replication occurring as early as ten hours after activation. By 16 hours 8 to 14 nuclei were distributed throughout the uncleaved egg (Ramirez and Huff, 1967).

More definitive proof comes from a recent study by Graham (1966) with unfertilized eggs of Xenopus laevis. The nuclei of eggs which he had injected with H^3-thymidine were all labeled within 60 minutes. Puncture of the egg in the course of injection activates the egg. As in the eggs of Rana pipiens, cleavage does not ensue. This result demonstrates that the factor which initiates mitosis and cell division is not the same as that which

induces DNA synthesis. Finally, cleavage of amphibian eggs can occur in the absence of chromatin material (Briggs, et al., 1951; Fankhauser, 1934).

B. Synthesis of Proteins

For cleavage to occur the egg must either contain or be able to synthesize as required a large store of essential proteins or protein subunits. These would include spindle and aster proteins, cell membranes, and probably histones (in regard to the last, see Moore, 1963).

Evidence is rapidly accumulating in support of the idea that the unfertilized egg contains all the synthetic machinery necessary for the production of the special proteins required by rapidly dividing cells.

For example, Actinomycin D, known to inhibit DNA dependent RNA synthesis, exerts little effect on cleavage (Brachet, et al., 1964). Other workers have, however, been able to stop cleavage by injecting this agent at lower concentrations than those used by Brachet and his coworkers (L.D. Smith and R. Ecker, personal communication). Nonetheless, the RNA molecules and ribosomes necessary for protein synthesis are present in adequate amounts. Brown and Caston (1962) have shown that eggs and early embryos of Rana pipiens contain relatively small but apparently sufficient amounts of cytoplasmic ribosomes for the protein synthesis required during embryogenesis up to Shumway stage 18.

That protein synthesis is necessary for continued cell divisions is demonstrated by the fact that amphibian eggs injected with puromycin soon cease cleaving (Brachet, et al., 1964). In-

teresting in this regard is the finding of Ficq (1964) which demonstrated that Actinomycin D could inhibit cleavage of Xenopus laevis eggs if the oöcytes were exposed to the agent during oögenesis 6 weeks prior to fertilization. Apparently the RNA types necessary for protein synthesis during the cleavage period are produced during oögenesis. In this connection, Spirin (1966) reviews the evidence supporting his hypothesis of "masked" messenger RNA in unfertilized eggs and its utilization during cleavage and early development.

According to Brown and Littna (1964) the mature oöcyte does not synthesize RNA. During ovulation, however, when meiosis is known to occur, the mature oöcyte synthesizes a small amount of heterogeneous RNA. No detectable synthesis of ribosomal or sRNA occurs during ovulation. Fertilization does not result in any obvious additional synthesis of RNA until late cleavage (heterogeneous RNA and 4s RNA). Ribosomal RNA synthesis begins at gastrulation along with the first appearance of definitive nucleoli.

Another preliminary to cleavage involves the maturation of the germinal vesicle. Contents of the mature vesicle must be mixed with the cytoplasm of the egg before cleavage can successfully be induced by sperm or artificial means (Detlaff, et al., 1964). Following germinal vesicle maturation and breakdown (induced by intracoelomic injection of pituitary hormone) protein synthesis begins to increase (Smith, et al., 1966) until it reaches a maximum level at least 10 times higher than that observed in full grown ovarian oöcytes from uninjected frogs. The

incorporation of tritiated leucine remains at a maximum level for a period of days, regardless of whether or not the egg is fertilized or activated. The same level and rate of incorporation occurs in eggs that are fertilized, simply activated or even enucleated (Smith and Ecker, 1965; Ecker and Smith, 1966). These observations suggest to the authors (Smith and Ecker, 1965) "...that fertilization and subsequent cleavage divisions are not prerequisites for continued protein synthesis. Moreover, the results obtained from enucleated eggs indicate that protein synthesis can take place in the absence of continuous nuclear control, and thus would not be due to the continued production or release of new 'messenger RNA' by the nucleus."

These authors have also studied the synthesis of proteins in defolliculated ovarian eggs matured in vitro by treatment with progesterone (personal communication). The pattern of protein synthesis is identical to that in eggs maturing in vivo. The frog egg is remarkable in still another way. F. Greenslade (personal communication), using the in vitro system of Smith and Ecker, has enucleated the oöcytes (removed the germinal vesicle) at the time of progesterone treatment. His preliminary results indicate that 24-48 hours after progesterone treatment RNA synthesis is occurring in the enucleated eggs at the same level as in the nucleated controls.

C. Energy Production

If the results of experiments on sea urchin eggs have general validity, then the conventional idea that the provision of an energy supply is a prerequisite for mitosis, as proposed by

Swann (1954), should be abandoned (Mazia, 1963). In a sense the sea urchin egg does have a reserve of energy that will carry it through some stages of mitosis after its supply of energy has been cut off. Epel (1963) has shown that mitosis can be blocked at any stage if the inhibition is applied at an appropriate time before that stage. The results obtained by this author (1963; see also Mazia, 1963) indicate that mitoses in the sea urchin egg depend on the continuous functioning of the oxidative phosphorylation system, and hence the continuous synthesis and utilization of ATP. The following discussion will indicate that this may not be strictly true for the frog egg in which the glycolytic cycle may play a significant role.

The frog egg contains an abundant store of energy-rich materials. Glycogen accounts for about 8% of the dry weight of the mature egg, lipids about 25% and the proteins of the yolk platelets even more (Barth and Barth, 1954). The egg contains a total of about 10-12 micrograms of phosphate which is stored in the form of phosphoprotein, nucleic acids, phospholipids, and acid-soluble phosphate (including such compounds as ATP)(Grant, 1953).

Biochemical studies show that the usual glycolytic, tricarboxylic and energy transfer systems are present in the amphibian egg (see reviews by Barth and Barth, 1954, and Boell, 1955). The respiratory mechanisms are functional in the unfertilized egg; a relatively high respiratory activity was observed in the eggs of Bufo immediately after their removal from oviducts (A.H. Legname, 1962, cited in Barbieri and Salomón, 1963). Experi-

ments by others (Spiegelman and Steinbach, 1945; Gregg and Ray, 1957) have established through analyses of homogenates of frog embryos that enough respiratory machinery is available at the onset of development to support oxidation rates greater than any exhibited by intact prehatching embryos.

Phase contrast and electron microscopical studies of amphibian oöcytes reveal that the morphological counterparts (mitochondria) of at least the TCA and energy transfer systems are present in large numbers (Balinsky and Devis, 1963; Wischnitzer, 1964; Ward, 1962a, 1962b; Kemp and Istock, 1967; and L.D. Smith and M. Williams, personal communication).

The energy requirements for the clevage process in amphibian embryos are apparently rather low, since the respiratory scheme of energy production can be interfered with by anaerobiosis and cyanide without any observable effects on cleavage up to the blastula stage (Parnas and Krasinka, 1921; Salomón and Barbieri, 1964; Barnes, 1944; and Spiegelman and Moog, 1945). Although sodium fluoride interfered with the metabolism of pyruvate in the eggs of Bufo arenarum, it did not effect cleavage or glycolysis as demonstrated by the continued production of lactic acid (Salomón and Barbieri, 1964).

Under anaerobic conditions development of the frog egg proceeds from early cleavage to the early gastrula stage. Since the ATP content during this period decreases only about 3% while the inorganic phosphate increases about 11%, Barth and Barth (1954) conclude that the low energy demands during cleavage come from the breakdown of other esters in addition to ATP. Harrison (1965)

suggests that phosphorylation of creatine may provide a store of phosphate bond energy.

The production of some high energy intermediates may occur through the breakdown of glycogen and passage of glucose through the glycolytic pathway. A buildup of lactic acid has been observed in the absence of oxygen (Barbieri and Salomón, 1963). Cohen (1954) has shown that frog eggs, even by first cleavage, contain the enzymes and coenzymes necessary to convert glycogen to lactic acid.

Many authors have emphasized the role of ATP in cleavage (see Pfohl, 1962, for a review of the pertinent literature). Brachet (1954), for example, has shown that dinitrophenol, which uncouples phosphorylation from oxidation, will eventually inhibit cleavage of amphibian eggs. Gelation, which occurs in a cyclic fashion in cleaving eggs of Rana pipiens, is an endothermic process (Marsland and Landau, 1954). Presumably ATP could serve as an energy source for this process.

Details are lacking on the production and utilization of energy in eggs which are merely activated. That energy producing mechanisms are active can be inferred from the fact that processes known to require energy do occur in activated but uncleaving eggs of the frog. The synthesis of protein in activated and enucleated eggs (Smith and Ecker, 1965) and of DNA in activated eggs (Graham, 1966) are pertinent examples. Other evidence, perhaps less convincing, comes from the observation by Huff and Ramirez (1966) that activated but uncleaved eggs contain many cyasters. From the work of Marsland and Landau (1954), previously

mentioned, we must assume that energy is required for the formation of these cytasters.

To what extent these events can proceed with the available stores of ATP or other high-energy intermediates is unknown. From the preceding discussion, however, it is readily apparent that the sperm or cleavage-initiating material can add nothing of significance to the already abundant store of energy-rich materials in the egg. It seems unlikely, furthermore, that some constituent of the sperm or the cleavage-initiating material is necessarily involved in activating the energy-producing machinery of the egg.

D. Division Centers

The division center associated with the nucleus of the frog egg does not normally become involved in the cleavage process. During fertilization the spermatozoon carries into the egg an active division center which replaces or renders ineffectual the one initially present in the egg (Boveri, 1895). The amphiaster (achromatic apparatus) for the first cleavage of a fertilized egg arises by the division of the center (centriole) around which the sperm aster forms. For detailed discussions of the origin and function of the division centers and their associated astral figures in fertilized and artificially activated eggs, the reader is referred to reviews by Wilson (1925), Tyler (1941), Chambers and Chambers (1961), and Briggs and King (1959).

The inability of the frog egg to cleave normally in the complete absence of a division center from the sperm or egg nucleus suggests that the egg cytoplasm cannot independently form

effective centers (Briggs, et al., 1951). A recent cytological study of the eggs of Rana pipiens (Ramirez and Huff, 1967) reveals the presence of a large number of cytasters in activated and enucleated eggs. This suggests that division centers may arise de novo in the egg cytoplasm. However, the actual origin of these cytasters is still unknown. To date, no electron microscopical studies have been published which demonstrate that division centers are in fact associated with the cytasters in frog eggs. On the other hand, the cytasters in artificially activated sea urchin eggs are apparently biochemically identical to the mitotic apparatus of fertilized eggs (Dirksen, 1964). Electron microscopical studies have further revealed the presence of a centriole in the center of each cytaster (Dirksen, 1961).

Successful artificial parthenogenesis of frog eggs implies that the center associated with the egg nucleus may become functional (Herlant, 1913), or alternatively, a functional center is introduced with the cleavage-initiating material (Bataillon, 1929). According to Bataillon the inoculated material catalyzes the formation of an aster contemporaneous with the appearance of the spermaster (about 40 minutes after inoculation or fertilization, 17-18°C) in a fertilized egg. The female pronucleus, situated at the periphery of the egg, moves down the rays of the aster of inoculation. Before it arrives at the center of this aster, the latter divides to form a dicentric system upon which the chromosomes of the female pronucleus are released. This behavior is identical with the sequence of events in a fertilized egg (Bataillon, 1929). Of course in the absence of a sperm, fu-

sion of the pronuclei does not occur.

On the other hand, an aster does not begin to form for about 2 3/4 to 3 hours (18°C) after an egg has been simply activated (puncture, electric shock). By this time the migration of the female pronucleus has carried it toward the center of the egg and the pronuclear membrane has broken down. While mitosis can and does occur, it is always monocentric. After 3½ hours some accessory cytasters may also appear which occasionally give rise to "pseudoamphiasters" resulting in an irregular segmentation of the egg, if the former are near the egg surface.

E. Summary

The frog egg seems able to synthesize DNA, RNA, and protein when subjected to appropriate stimuli.

Maturation of the egg with concommitant protein and RNA synthesis is initiated by hormone treatment. Completion of the maturation process, replication of chromatin material, and formation of cytasters occur when the egg is simply activated by puncture or electric shock.

These stimuli, however, do not lead to the formation of an amphiasteral system which functions harmoniously in mitosis. Normal cleavage never occurs unless the appropriate material is inoculated or injected into the egg.

In the sections which follow I will discuss, together with the work of others, the results I have obtained from investigations on the nature of this cleavage-initiating material.

III. THE NATURE AND ROLE OF THE CLEAVAGE-INITIATING MATERIAL: RESULTS OF THE PRESENT INVESTIGATION

A. Source and Particulate Nature of the Cleavage-Initiating Material

The experimental results tabulated in a preceding section demonstrate the presence of cleavage-initiating (CI) material in the heart, brain, and liver tissues of Rana pipiens. Active material is obtainable from a wide variety of tissues (see Bogucki, 1923, 1926; Einsele, 1930; Parat, 1933; Rostand, 1938; Shaver, 1953; and Huff and Preston, 1965).

Tissue fractionation studies using sucrose media in conjunction with differential and gradient centrifugation techniques revealed that the CI activity sediments with the large granules of a cell. These results confirm similar observations made earlier by Shaver (1953).

Assays for marker enzymes showed that the large granule fractions contain at least mitochondria, lysosomes, and peroxisomes. Although Shaver (1953) did not assay specifically for marker enzymes, his procedure for obtaining large granule fractions is comparable to that used by other workers to obtain a "mitochondrial" fraction (Allfrey, 1959, p. 254).

Suspensions of nuclei at various concentrations were ineffective as CI material. There was also no detectable activity in the supernatants of tissue homogenates (in 0.25 or 0.7 SEP media) centrifuged at 20,000g. This would suggest that small granules or microsomes were inactive. To specifically test this idea, a microsomal pellet was prepared from the supernatant of a 10,000g, 10 minute centrifugation of a liver homogenate freed of

whole cells and nuclei. The final suspension of small granules contained about one-fourth the CI activity of a large granule suspension prepared from the same homogenate (Table VII). Most likely, however, this activity was due to contamination with large granules. The volume of the material centrifuged and the conditions of centrifugation were such that complete sedimentation of the large granules was unlikely. Table VI, for example, shows that even after the S_1 supernatant (0.7 SEP medium) was centrifuged at 20,000g for 1 hour, almost 1/3 of the cytochrome oxidase activity remained in the supernatant. It is possible that such contamination could account for the small amount of CI activity in the small granule fraction prepared by Shaver (1953).

The absence of detectable CI activity in S_2 (Tables III and IV) could be explained by assuming that the large granules were dispersed in too great a volume of medium, and were thus too "diluted".

This brings us to some interesting observations on the correlations of the concentration of the injected material with the percentages of eggs which cleave and the quality of the blastulae obtained.

B. The Effect of the Concentration of the Cleavage-Initiating Material on the Frequency and the Quality of Cleavage

The fact that the concentration of the material inoculated or injected into eggs is very important in determining whether or not the egg will cleave normally was already ascertained by some of the earliest workers in the field. Bataillon (1929, p.

735), for example, states: "Ce n'est pas l'inoculation la plus riche qui donne les développements les meilleurs, au moins quand elle encombre l'énergide principale."

When inoculating whole cells or whole homogenates the danger usually lies in introducing too much material rather than too little. However, sufficient amounts of active material probably do not get into the egg when cell-free extracts or biochemical preparations are inoculated (Einsele, 1930; Voss, 1923). With his injection technique Einsele (1930) could be more certain that adequate amounts of material were introduced. The results obtained by Einsele (1930) and Parat (1933) following the injection of cell-free testis and blood extracts are very similar to those obtained in the present study. These authors reported that if the material injected was too concentrated either very few or no eggs cleaved. As the material was diluted an optimum concentration of active material was attained, resulting in the cleavage of a high percentage of the injected eggs. Shaver (1953) has suggested that the lack of normal cleavage or variability in the percentages of cleavage obtained after injecting extracts of various tissues is due to variations in the concentration of the granules injected. The present investigation has extended these results and includes an analysis of the quality of the blastulae obtained following the injection of R. pipiens eggs with subcellular fractions of liver tissue from the frog.

The data clearly show (Figures 4 and 5) that there is a range of concentration of large granules which is optimum for obtaining a high percentage of good quality blastulae. About

17% to 28% of the eggs can be expected to cleave when injected with material in the 1:16 to 1:134 relative concentration range. The percentages in Figure 5 are averages, with their standard errors, obtained from pooled data. In individual experiments over 40% cleavage was frequently observed. When material of greater or lesser concentration than the 1:16 to 1:134 range was injected fewer of the eggs cleaved to blastulae.

Most of the blastulae which developed from eggs injected with CI material were partial to varying degrees. The extent of this "partialness" was shown to be dependent on the concentration of the injected active material (Figure 4). If material of 1:33 or less relative concentration was injected, 60% of the blastulae which were obtained consisted of blastomeres which covered over one-half of the egg surface. Injection of 2:1 to 1:15 material resulted in fewer than 27% of the blastulae with blastomeres covering more than one-half of the egg surface. About 45% of the blastulae in this case had blastomeres only over one-fourth or less of the surface.

The blastomeres composing the partial blastulae obtained through artificial means by other workers were of regular size and contained nuclei (Frazier, 1951; Ramirez and Huff, 1967).

The pH of the large granule suspensions was unaltered by the dilution procedure. Furthermore, the proportion of nonsedimentable protein to total protein did not increase upon dilution. It is unlikely therefore that proteins released during dilution (beyond the 1:134 concentration) could act as inhibitors of the CI activity.

Shaver (1953) observed marked differences in the CI activity of extracts from various tissues. Liver tissue apparently contained the least CI material. He suggests, however, that under the conditions of his experiments the differences in CI activity may not reflect actual variations in the content of active material in the cells. That they do not, is suggested by the high level of activity obtained from liver tissue in the present study. It is possible that the extracts of liver injected by Shaver contained too great a concentration of CI material.

C. Speculations Concerning the Role of the CI Material

> "...il est de tradition trop constante chez les chercheurs d'attribuer à la dernière version des faits le maximum d'exactitude et de vertu explicative." Bataillon, 1929.

The number of cytasters produced by parthenogenetically stimulated sea urchin eggs is dependent on the strength of the parthenogenetic stimulus. A persisting monaster is the result of an insufficient stimulus; a monaster followed by an amphiaster, of an optimum stimulus; and accessory cytasters of an overstimulus (Fry, 1925). Both Fry (1925) and Chambers (1921) have demonstrated that one aster associated with the nucleus may lead to normal development of the egg. The presence of accessory cytasters leads to abnormalities in the further development of the activated egg.

Much the same situation exists in an artificially stimulated frog egg (Bataillon, 1929). An insufficient stimulus, such as merely puncturing the egg, results only in the delayed formation of a monaster which subsequently undergoes cyclic sol-gel trans-

formations. Inoculation or injection of the optimum amount of active material results in the formation of an aster comparable in morphology, physiological functions, and time of appearance to that of the sperm aster in a fertilized egg. Introducing too much CI material into the egg is detrimental since an abundance of small accessory cytasters are formed which interfere with the normal growth and functioning of a principal one. Under optimum conditions the latter would normally become associated with the female pronucleus.

Although numerous cytasters may appear after simple activation of the frog egg, their appearance is delayed until a time when the egg is no longer capable of cleaving.

Huff and Ramirex (1966) suggest "that the main difference between parthogenesis in sea urchins and frog is the time required for activated eggs of these two animals to produce cytasters capable of forming cleavage spindles." Extracts of activated eggs known to contain cytasters are capable of inducing cytaster formation and cleavage in other unfertilized frog eggs (Huff and Preston, 1965; Huff and Ramirex, 1966).

On the basis of the above and preceding discussions it is apparent that the egg is a self-contained unit capable of cleavage and development upon being adequately stimulated. With the onset of maturation and subsequent activation one finds the egg capable of chromatin replication, protein and RNA synthesis, and even aster formation. Deficiencies may exist in all of these processes, but the only deficiency obvious at the present time is in the formation of a normal aster. To reiterate, although

a monaster may form in a pricked egg, its appearance is delayed when compared to the time of formation of the monaster (sperm-aster) in a fertilized egg. The former has never been observed to give rise to an amphiaster. Pseudo-amphiasters may appear as a result of the union of a monaster with one or more of the aces-sory cytasters which appear after the monaster has formed; how-ever, they do not give rise to normal cleavages.

It is my opinion that the role of the CI material injected into the egg of a frog resides in its ability to catalyze the formation of a monaster around a center which is capable of di-viding and giving rise to a functional amphiaster. Either the material injected serves as a center for the monaster formation, as suggested by Bataillon (1929), or it activates something al-readly present in the egg to serve in this capacity. The nature of the active material will be discussed further in a subsequent section.

Opposition to this proposed role might be inferred from the work of others which suggests that asters are not essential for cleavage. For example, Dietz (1966) after studying the spermato-cyte divisions in a crane-fly concludes that asters are of no importance for chromosome movement and cell division. They merely represent the mechanism by which centrioles, essential in the formation of cilia, sperm tails, etc., are distributed to daughter cells. The centrioles and their associated asters may, however, play a role in morphogenesis by determining the orien-tation of the spindle axis within the cell.

On the other hand, the results of studies on amphibian and sea urchin material impel one to assign a much more important

role to asters in the cleavage of eggs of these species. Chambers and Chambers (1961) review the results of a number of studies on this problem in marine invertebrate material. They conclude (p. 250) that "while the furrowing process occurs independently of the late mitotic figure, there is no question that the ultimate location and timing of cleavage depends on influences emanating from the early mitotic figure.... As is well known, the cleavage plane is located at right angles to the long axis of the developing spindle. Evidently the mitotic figure exerts the determinative influence responsible for this relationship comparatively early, so that by the time the later stages of the mitotic figure are attained, the differentiation in the cortical region responsible for the furrowing process has already been well established."

The above conclusions may very well be true for amphibian material. The importance of asters for regulating the rhythm of division and assuring normal cleavage to the blastula stage was noted by Fankhauser (1934).

Dan and Kuno-Kojima (1963) and Waddington (1952) have demonstrated an independence of furrow formation in the cortex of a cleaving amphibian egg from the underlying endoplasm. Once the furrowing process has begun, it proceeds autonomously by some self propagating mechanism. The authors envision some factor within the cell as the force initiating the furrowing. In some very preliminary experiments Waddington (1952) was able to modify the position of the cleavage furrow by shifting the position of the anaphase spindle. These results suggest that the mitotic

apparatus may have an influence on the early stage of furrow development. Zotin (1964) believes that the formation of an internal wall (diastema) at the anaphase stage and its growth toward the egg surface is the first step in the cleavage process. The furrow is initiated and determined when the diastema approaches the surface of the animal hemisphere. Zotin (1964) suggests that the diastema is an adaptation developed in large eggs to bring furrow -inducing substances to the site of the local action at the surface of the egg.

The importance of asters may lie in directing the formation of an effective diastema.

D. Attempts at Characterizing the Active Particle

The results discussed thus far confirm the previous observation by Shaver (1953) that the CI activity extractable from animal tissues is associated with the large granules in cells. The role proposed for CI material upon being introduced into the egg is essentially the one set forth years ago by Bataillon (1929).

Until now reference has been made in general terms to the localization of the CI activity in the large granule fraction. Reference was made earlier to the fact that this fraction contains at least the following particles: mitochondria, lysosomes, and peroxisomes.

The following discussion will describe attempts I have made to characterize the active component.

1. Necessity of Particulate Structural Integrity - Treatment by freeze-thawing and insonation are techniques commonly

used to disrupt the structure of cellular particles.

When large granule fractions were treated two times by freezing in a dry ice-acetone mixture and thawing at room temperature, the CI activity was completely destroyed. Insonation of a large granule fraction for 3 minutes destroyed about 90% of its CI activity. The release of an inhibitor of the CI agent or of the cleavage process itself by these treatments is one possible explanation for their observed effect. This is unlikely, however, in view of the fact that neither the supernatants nor resuspended pellets of centrifuged frozen-thawed material were active, and the resuspended pellet of centrifuged insonated material contained about the same level of activity as the uncentrifuged insonated fraction.

Determinations of protein and marker enzymes were made on the treated material in hopes that loss of CI activity could be correlated with the breakdown of either lysosomes or mitochondria. Following the freeze-thaw treatment (Table III) in two experiments, 21% and 33% of the protein were released into solution. In both experiments only 18% of the acid phosphatase was solubilized. All cytochrome oxidase activity remained in association with sedimentable particles.

Protein and acid phosphatase were released to about the same extent when the large granule fractions were insonated. After 3 minutes of insonation in one experiment 52% and 55% of the acid phosphatase and protein, respectively, were released. In another experiment (Fig. 3), after 3 minutes of treatment, about 64% and 68% of the protein and acid phosphatase, respec-

tively, were released. Only about 37% of the cytochrome oxidase, however, was recovered in the supernatant.

The low extent to which protein, acid phosphatase, and cytochrome oxidase are released by freeze-thaw treatment does not lend much support to the idea that either mitochondria or lysosomes are the active components. The results of treatment with ultrasound could be interpreted to support an active role for lysosomes. On the basis of these experiments, one cannot exclude mitochondria, however. Cytochrome oxidase unfortunately was not a wise choice in this instance as a marker enzyme for mitochondria, since it is firmly bound in the membrane structure of the mitochondrion. Mitochondrial disruption would therefore not be accurately reflected by the relative amount of this enzyme in the soluble phase.

The fact that most of the acid phosphatase was sedimentable after two treatments by freeze-thawing may perhaps be attributed to the adsorption of released enzyme to intact particles or particle fragments. Direct evidence to support or refute this possibility is lacking. The assay conditions (37°C, ½ hour) precluded the possibility of distinguishing between the activity of adsorbed enzyme and the activity of enzyme within intact lysosomes (bound or "latent" activity)(Appelmans and de Duve, 1955). Nonetheless, it is unlikely that adsorption of free enzyme to sedimentable particles obscured the extent to which the bound enzyme was released. Consider the following facts: 1) The presence of 0.1M phosphate in the medium (SEP) would have minimized the adsorption of soluble proteins to particles (Beinert,

1951; Berthet, et al., 1951). 2) Insonation of a large granule fraction, suspended in SEP medium, for two minutes resulted in the solubilization of more than half of the total acid phosphatase (Figure 3). One would expect adsorption to be equally important following the freeze-thaw or insonation treatment. Either it apparently is not as important following insonation or, more likely, the lysosomes are in fact not extensively disrupted by two freeze-thaw treatments.

The large granule fractions were also subjected to storage at 2-3°C for various periods of time and to high temperature (52°C). These treatments could feasibly cause disruption of lysosomes and mitochondria. The release of acid phosphatase from rat liver lysosomes is effected by aging and elevated temperatures (Berthet and de Duve, 1951; Berthet, et al., 1951). Very likely the lysosomes in the large granule fraction from frog liver also gradually break down during storage and when subjected to 52°C. As a result of the breakdown of lysosomes, previously "latent" hydrolytic enzymes become active. As a consequence mitochondria as well as other subcellular particles may be attacked by acid proteases (Sawant, et al., 1964a). Determinations of TCA-soluble material sensitive to the Folin-Ciocalteu Phenol reagent were performed on large granule fractions aged for 1-30 days. No definite increase in hydrolytic products with age was apparent.

Both aging and heating had a detrimental effect on the CI capacity of the fractions. Heating at 52°C for 10 minutes completely destroyed the CI activity. A gradual but definite decrease in the CI activity of the fractions occurred with an in-

crease in the time of storage.

The absence of a correlation between the amount of protein hydrolyzed and the age and CI activity of the fractions does not preclude the possibility that undetectable amounts of protein important to CI activity were hydrolyzed or denatured.

Previous workers have reported that heating active material to 55°C or more completely destroys its activity (Bogucki, 1921; Parat, 1933; Shaver, 1951). Shaver (1951) noted, however, that heating large granules to 52°C for as much as 30 minutes increased rather than decreased their CI activity. His results conflict with those obtained in the present study. This conflict may be resolved if we assume that the material Shaver was working with was much more concentrated than that employed in the present study. The concentration of the material injected by Shaver is estimated to be about 2:1. Concentrations this high in the present study demonstrated a low level of cleavage-initiating ability. Destruction of active material by heating might reduce its concentration to a more effective level.

The preceding results demonstrate that some integrity of the particle structure must be retained if the particle is to remain active in initiating cleavage. This interpretation could be extended to mean that a particular biochemical must remain structurally integrated with an intact particle in order to express its cleavage-initiating capacity. The results of the heating experiments suggested to several authors (Bogucki, 1921; Parat, 1933) that enzymes are the active constituents. The fact that the injection of a variety of enzyme preparations has proven in-

effective (Bogucki, 1922; Shaver, 1953) does not preclude the possibility that an enzyme or group of enzymes is the effective CI factor.

2. Analyses Using Sucrose Density Gradient Centrifugation - In order to determine if a particular type of cell component in the large granule fraction was responsible for the CI activity, the large granule material was further fractionated by sucrose density gradient centrifugation. Both the SE and SEP media were used in forming the gradients. The steep gradients ranged in concentration of sucrose from 0.25 to 1.8M, the shallow gradients, 0.8 to 1.8M.

a. Steep gradients with SEP media - The first fractionations of large granule material from frog liver were performed with steep SEP gradients (Fig. 6 and 7).

The CI material was initially found in all the gradient fractions, but when the fractions were appropriately diluted, the greatest concentration of active material was found in the same fractions which contained the greatest concentrations of mitochondria, lysosomes, RNA, DNA, and protein.

b. Shallow gradients with SE media - In view of the similar densities of mitochondria and lysosomes, shallower gradients were used in an effort to enhance their separation. The shallow gradients were formed with SE media since several workers (Hers, et al., 1951; Dalton, et al., 1949; Hogeboom, et al., 1948) had reported that the presence of electrolytes in the isolation medium may cause cytoplasmic particles to agglutinate.

The shallow SE gradients yielded some degree of separation between mitochondria, lysosomes, and peroxisomes (Fig. 9-11). In general the particles were much more broadly distributed in these gradients than in the steep SEP gradients. The lysosomal distribution was broader than the mitochondrial and tended more toward the less dense region of the gradient. The peroxisomes were largely distributed in the heavy end of the gradient, the maximum concentration being in the bottom fraction (No. 1). Assays for ribonuclease revealed that the distribution of this lysosomal enzyme was erratic. This distribution may in part be explained by adsorption of the enzyme to cytoplasmic particles, especially microsomes (Schneider and Hogeboom, 1952). The fact that the media (SE) contained no electrolytes would contribute to this phenomenon (Beinert, 1951; Berthet, et al., 1951).

Injection of samples from each of the fractions into frog eggs revealed an even broader distribution of CI material in the shallow SE gradients than in the steep SEP gradients. Furthermore, injection of diluted fractions did not demonstrate any correlation of CI activity with any particular type of cell component. Injection of the most dilute fractions indicated that most of the CI material was localized in the lower half of the gradient. The significant activity frequently observed in the uppermost fraction (No. 11) may in part be attributed to the collection of particles adhering to the tube wall by the meniscus as the tube is emptied.

3. Subfractionation of Insonated Material on Shallow SE Gradients - Since lysosomes are more labile particles than mitochon-

dria one might expect that brief treatment with ultrasound would preferentially disrupt the lysosomes. The experiments performed (Fig. 12) show a significant release of enzymes from lysosomes and peroxisomes upon treatment with ultrasound. Most of the protein was likewise converted to a non-sedimentable form. The mitochondrial distribution was not as markedly affected (compare Fig. 10A and 12B). The bulk of the cytochrome oxidase was present in fractions 4-6 in both the treated and untreated material. Whereas no activity was recovered in fractions 9-11 of the non-insonated preparations, almost 20% of the total recovered activity was found in these fractions of insonated material. Some of this gain in activity was reflected by the considerable loss of activity in fractions 1-3.

In their gradient fractionations of rat liver "mitochondrial" preparations, de Duve (1964) and his coworkers consider the protein distribution to be essentially the same as that of the mitochondria. The marked difference obtained in the present study between the distribution of protein and cytochrome oxidase from insonated material is therefore surprising. Although the reason for this difference is unknown, two possible explanations come to mind. One, lysosomes or peroxisomes may be present in substantially greater amounts than at first assumed. Two, the bulk of the protein present in the top of the gradient may have come from soluble proteins present in the matrix of intact mitochondra. Presumably 56% of the protein of rat liver mitochondria is in the soluble form (Hogeboom and Schneider, 1950). If the latter alternative is correct one would expect the majority of

the cytochrome oxidase bearing particles to be mere mitochondrial shells. Insonation would presumably disrupt the osmotic space of the mitochondria while releasing soluble proteins from the matrix with the result that the membranes of such particles would be freely permeable to water and sucrose. These particles would have a greater density than the intact mitochondria (Beaufay and Berthet, 1963). Using the equations formulated by Beaufay and Berthet (1963) to calculate the theoretical densities of the intact mitochondria and the insonated mitochondria it was estimated that the equilibrium densities of the two types of particles should be two gradient fractions removed from each other. A review of Figures 10A and 12B shows that such is not the case.

The distribution of CI activity in this shallow SE gradient again defies explanation (Fig. 12C). The distribution of activity shows no correlation whatsoever with the distribution of any of the enzymes. For example, the quantity of protein and enzymes was very low in fraction 2; yet considerable CI activity was present. One might suspect that the high level of CI activity in fraction 11 may be due to soluble factors released from the disrupted particles. The levels of protein and enzyme, however, are nearly as high or higher in fraction 10. Yet this fraction is nearly devoid of CI activity.

The sedimentation profiles of CI activity observed in shallow SE gradients leave one with the impression that the active factor is randomly adsorbed to particles or particle fragments throughout the gradient or, alternatively, that active particles are agglutinated in various sized clumps.

4. A Comparison of Shallow SE and SEP Gradients - When comparing the more sharply defined distribution of CI material in the steep SEP gradients with the erratic distribution in the shallow SE gradients, it seems inconceivable that the steepness of the gradient alone could account for this difference. Subsequent experiments (Figures 13-16, Tables XIII and XIV) clearly showed that the broad distribution of the protein and enzymes and the erratic distribution of CI material in the shallow SE gradient were due solely to the absence of the phosphate buffer. The tables and figures demonstrate that the particles sediment to a denser level in the SE gradient. This strongly suggests that particles in the SE medium tend to aggregate more than in the SEP medium. This observation contrasts with the findings of others (Hers, et al., 1951; Dalton, et al., 1949; Hogeboom, et al., 1948) which suggest that the presence of electrolytes in the isolation medium enhance clumping of cytoplasmic particles.

When the fractions from one of the shallow SEP gradients were injected (Fig. 13B), a correlation of CI activity with the distributions of cell particulates was again observed as had been previously in the steep SEP gradients.

IV. CONCLUSIONS

Experiments in which eggs were injected with subcellular fractions obtained by differential centrifugation of frog liver homogenates clearly show that the cleavage-initiating (CI) activ-

ity is associated with the large granules of cells. The results of injecting large granule fractions treated by freeze-thawing and insonation and the sedimentation behavior of the CI activity of the granules in steep and shallow SEP gradients also support this conclusion. Preparations of nuclei and microsomes from liver tissue were unable to initiate cleavage.

The mass of data accumulated by analyzing and injecting liver tissue fractions seems, however, to offer little positive evidence for the association of CI activity with any particular cell component in the large granule fraction. Most of the data do, in fact, suggest that the activity is not associated with any of the three components this study has been largely concerned with, namely mitochondria, lysosomes, and peroxisomes.

That mitochondria are not likely to be the active particles is demonstrated by the data in Tables III and VI. The results show that although the cytochrome oxidase activity, and thus presumably the concentration of mitochondria, was similar in the supernatant fractions S_2 and S_3, the S_2 contained no CI activity whereas considerable activity was present in S_3. That the presence of an inhibitor in S_2 may have accounted for its lack of CI activity is unlikely in view of the fact that the supernatant, S_1, from which S_2 was obtained, was very active in initiating cleavage. Furthermore, analyses involving shallow SE gradient centrifugations revealed a striking lack of correlation between the distribution of CI activity and mitochondria. The results obtained using the SE media render fortuitous the positive correlation between the distribution of CI activity and mitochondria

in SEP gradients.

Assays for urate oxidase were performed in only two gradient centrifugation experiments. One of these experiments involved the fractionation of insonated material. From the results of this experiment one must conclude that peroxisomes are not the active particles.

The fact that freeze-thawing completely destroyed the CI activity of a fraction but did not effectively disrupt the lysosomes, as indicated by the release of acid phosphatase, does not favor the view that they constitute the active factor. Since the lysosomes are a very heterogeneous group of particles the results of gradient centrifugation analyses must be interpreted with caution. The data in this thesis do not indicate the lysosomal heterogeneity as fully as is desirable. Since only acid phosphatase was assayed as a marker, only the less dense portion of the lysosome population is indicated in the sedimentation profiles. In order to define the densest limits of these particles, assays should also have been performed for acid deoxyribonuclease (Beaufay, et al., 1959). Furthermore, the conditions of the assays for lysosomal marker enzymes were such that it was impossible to discriminate between the activity of enzymes randomly adsorbed to subcellular particles and the activity of enzymes within intact lysosomes. For the above reasons the distribution of acid phosphatase as portrayed in the graphs may not be a true reflection of the distribution of lysosomes. Even with these reservations in mind, however, the results obtained by fractionating insonated material on a gradient do not support

the idea that the active component is a lysosome.

The results of gradient fractionations in SE media lead one to the conclusion that the active material may be adsorbed in a non-specific manner to the various intact or fragmented cell components. Membrane fragments might behave in this fashion. However, the view that membrane fragments comprise the active material is not supported by the available evidence. For example, microsomal fractions (containing intracellular membranes) and suspensions of nuclear fragments displayed little or no CI activity. Injections of lipoprotein membranes from milk fat globules likewise did not stimulate cleavage.

On the basis of the available evidence it is doubtful that a sound hypothesis concerning the nature of the cleavage-initiating material can be proposed. The results of studies by Shaver (1951, 1953; Shaver, et al., 1952) suggest that hypotheses concerned with the nature and role of the CI material should consider the following factors: its possible thromboplastic and enzymic nature; the inhibition of its activity by sodium azide and p-chloromercuribenzoate; and its sensitivity to ultraviolet radiation at 2537 A.

From reports in the literature, its seems apparent that whatever the nature of the CI material may be, its role must be intimately associated with the establishment of a functional dicentric astral system in the egg. Whether the material introduced into the egg serves as a division center or catalyzes the formation of such a center in the egg cannot be determined at this time.

SUMMARY

Homogenates of liver tissue from Rana pipiens were fractionated in sucrose media by means of differential centrifugation or density gradient centrifugation. Assays for cytochrome oxidase and acid phosphatase were performed on each fraction to indicate the relative concentrations of mitochondria and lysosomes, respectively. In some instances urate oxidase was assayed to indicate the presence of peroxisomes. A sample of each of the fractions was injected into the eggs of Rana pipiens to test for cleavage-initiating (CI) activity.

The CI activity was invariably associated with the large granules of the liver cell. Microsomal and nuclear fractions contained little or no CI activity.

Treatment of the large granule fraction by freeze-thawing or by heating to 52°C completely destroyed its activity. Subjecting the large granule fraction to insonation for 3 minutes or to storage at 2-3°C for 3 or more days resulted in a marked decrease in its CI activity.

The distribution of cytochrome oxidase and acid phosphatase in the various fractions obtained by differential centrifugation failed to correlate consistently with that of the CI activity.

In most experiments injections were made of a series of dilutions of the fractions. The results obtained indicate that there exists a range of concentration of large granules which is optimum for obtaining a high percentage of blastulae of good quality. The pH and the relative amounts of soluble and sedi-

mentable proteins of the large granule fractions were unaffected by the dilutions.

Further fractionation of the large granule suspensions was achieved by subjecting them to sucrose density gradient centrifugation. If the gradients used were formed from sucrose-EDTA media buffered with 0.1M phosphate, no separation of lysosomes from mitochondria was obtained. The distribution of CI activity in such a gradient was essentially the same as that of the granules. If, however, the gradients were formed from salt-free sucrose-EDTA media, much broader and slightly separated distributions of mitochondria and lysosomes were obtained. The peroxisomes were most heavily concentrated in the bottom of the gradient. The distribution of RNase, presumably a lysosomal enzyme, was quite irregular and did not correspond to the distribution of any other constituents. The CI activity was distributed throughout the whole gradient, with the greatest concentration of activity in the bottom half of the gradient.

Insonation of the material prior to gradient centrifugation did not markedly affect the cytochrome oxidase distribution. The lysosomes and peroxisomes appeared to be extensively disrupted.

In all the gradients formed from salt-free sucrose-EDTA media, the distribution of CI material did not consistently correspond to the distribution of any of the cell components studied. The tentative conclusion is that none of the cell components which were studied, namely the mitochondria, lysosomes, or peroxisomes, can be individually considered as the active factor. The nature of the cleavage-initiating factor thus remains unknown.

The metabolic state of the frog egg and the processes it is able to undergo without entering cleavage are discussed. It is proposed that the CI material functions either as a division center or catalyzes the formation of such a center in the egg.

APPENDIX

Supplementary Compilation of the Biochemical Data Graphed in Figures 8-14

Each fraction consists of material collected from corresponding positions in three identical gradients. The total mg protein (Tot. mg) and total units of enzyme activity (Tot. Act.) recovered per fraction are given. Specific activity (S.A.) and relative specific activity (R.S.A.) are also listed. The R.S.A. of a fraction is the % total recovered activity/% total recovered protein in that fraction. The data in the text figures are expressed in terms of the per cent total recovered activity. The total protein and enzyme activity recovered from all fractions are also compared below with the total protein and activity in the P_3 material layered over the gradients. The units of enzyme activity are defined in the section on Methods.

Figure 8A

Fraction Number	Protein	Acid Phosphatase			Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0.31	1.4	4.7	0.57	0.41	1.33	0.23
2	0.49	4.4	9.1	1.14	1.82	3.73	0.65
3	0.97	8.3	8.5	1.09	8.64	8.89	1.55
4	0.46	5.6	12.2	1.54	5.37	11.72	2.03
5	0.24	2.3	9.8	1.22	1.15	4.88	0.83
6	0.43	1.2	2.8	0.35	0	0	-
7	0.18	1.1	6.4	0.78	0.32	1.80	0.31
P_3			6.8			3.47	

Protein: Of 2.67 mg layered, 3.08 mg or 115.4% recovered.
Acid phosphatase; Of 18.1 units layered, 24.3 units or 134.3% recovered.
Cytochrome oxidase; Of 9.25 units layered, 17.71 units or

191.5% recovered.

Figure 8B

Fraction Number	Protein	Acid Phosphatase			Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0.37	1.4	4.1	0.40	1.05	3.12	1.17
2	0.54	3.3	6.1	0.60	1.77	3.27	1.24
3	0.79	7.3	9.2	0.91	3.08	3.88	1.48
4	0.68	8.1	11.8	1.17	2.23	3.26	1.24
5	0.48	8.9	18.8	1.83	1.44	3.03	1.15
6	0.34	5.7	16.5	1.65	0.12	0.36	0.13
7	0.50	2.7	5.3	0.52	0	0	-
P_3			4.6			1.31	

Protein; Of 5.15 mg layered, 3.68 mg or 71.3% recovered.
Acid phosphatase; Of 23.8 units layered, 37.4 units or 157.1% recovered.
Cytochrome oxidase; Of 6.75 units layered, 9.69 units or 143.6% recovered.

Figure 8C

Fraction Number	Protein	Acid Phosphatase		Cytochrome Oxidase	
	Tot. mg.	Tot. Act.	S.A.	Tot. Act.	S.A.
1	0.11	0.7	6.2	0.24	2.24
2	0.20	1.0	5.0	0.24	1.23
3	-	-	-	-	-
4	0.27	2.1	7.6	0.37	1.35
5	-	-	-	-	-
6	0.14	2.0	14.2	-	-
7	0.19	0.9	4.6	-	-
P_3			4.2		0.95

Figure 9A

Fraction Number	Protein	Acid Phosphatase			Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0.20	1.1	5.4	0.39	0.85	4.20	1.39
2	0.26	2.1	7.9	0.57	1.54	5.83	1.94
3	0.34	3.9	11.2	0.82	2.02	5.87	1.95
4	0.37	5.2	14.1	1.01	1.72	4.66	1.54
5	0.34	5.7	16.6	1.20	0.82	2.39	0.79
6	0.31	6.4	20.9	1.47	0.34	1.16	0.36
7	0.20	4.9	23.9	1.66	0	0	-
8	0.20	2.7	13.5	0.96	0	0	-
9	0.22	2.2	10.2	0.71	0.16	0.75	0.23
P_3			11.1			3.29	

Protein; Of 3.15 mg layered, 2.45 mg or 77.8% recovered.
Acid phosphatase; Of 34.9 units layered, 34.2 units or 98.0% recovered.
Cytochrome oxidase; Of 10.34 units layered, 7.46 units or 72.1% recovered.

Figure 9B

Fraction Number	Protein	Acid Phosphatase			Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0.31	2.8	8.9	0.65	1.27	4.10	1.30
2	0.41	5.5	13.4	0.98	2.08	5.11	1.62
3	0.47	8.6	18.3	1.32	3.04	6.50	2.06
4	0.54	8.0	15.0	1.07	2.15	4.01	1.27
5	0.46	7.9	17.1	1.24	1.01	2.18	0.70
6	0.37	6.5	17.6	1.27	0.12	0.34	0.12
7	0.23	2.8	12.4	0.88	0.14	0.62	0.20
8	0.21	1.3	6.1	0.45	0.18	0.85	0.28
9	0.26	1.8	6.9	0.48	0.27	1.03	0.31
P_3			11.8			2.34	

Protein; Of 4.52 mg layered, 3.27 mg or 72.3% recovered.
Acid phosphatase; Of 53.4 units layered, 45.2 units or 84.6% recovered.
Cytochrome oxidase; Of 10.60 units layered, 10.27 units or 96.9% recovered.

Figure 9C

Fraction Number	Protein	Acid Phosphatase			Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0.31	1.4	4.4	0.46	1.08	3.47	0.79
2	0.42	2.3	5.5	0.56	2.50	5.99	1.32
3	0.63	4.3	6.8	0.70	5.44	8.58	1.92
4	0.71	4.6	6.4	0.67	4.72	6.64	1.48
5	0.50	7.3	14.5	1.50	2.12	4.24	0.95
6	0.36	6.9	19.0	1.97	0.72	1.99	0.45
7	0.30	4.5	14.9	1.54	0	0	-
8	0.27	3.2	12.2	1.21	0.29	1.10	0.24
9	0.26	2.1	8.0	0.83	0	0	-
P_3			8.2			1.32	

Protein; Of 4.77 mg layered, 3.76 mg or 78.8% recovered.
Acid phosphatase; Of 39.1 units layered, 36.6 units or 93.6% recovered.
Cytochrome oxidase; Of 6.29 units layered, 16.88 units or 268.4% recovered.

Figure 9D

Fraction Number	Protein	Acid Phosphatase			Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0.30	1.5	4.8	0.46	0.69	2.26	1.05
2	0.39	2.3	6.0	0.54	0.87	2.24	1.02
3	0.48	4.5	9.5	0.87	1.68	3.54	1.61
4	0.51	6.9	13.6	1.25	2.26	4.44	2.04
5	0.46	7.2	15.5	1.45	1.41	3.05	1.41
6	0.37	6.4	17.1	1.60	0.21	0.57	0.26
7	0.27	3.5	12.6	1.20	0.07	0.27	0.12
8	0.25	1.7	6.7	0.63	0	0	-
9	0.28	1.7	6.1	0.56	0	0	-
P_3			8.7			1.72	

Protein; Of 4.15 mg layered, 3.31 mg or 79.8% recovered.
Acid phosphatase; Of 36.1 units layered, 35.7 units or 98.9% recovered.
Cytochrome oxidase; Of 7.13 units layered, 7.19 units or 100.8% recovered.

Figure 10A

Fraction Number	Protein	Acid Phosphatase			Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0.32	1.4	4.5	0.44	0.58	1.85	0.76
2	0.35	2.7	7.8	0.77	1.14	3.24	1.33
3	0.48	4.0	8.3	0.82	1.65	3.42	1.40
4	0.68	6.4	9.5	0.94	2.31	3.41	1.40
5	0.80	8.5	10.6	1.06	3.16	3.94	1.62
6	0.67	8.4	12.5	1.24	2.71	4.05	1.65
7	0.55	7.7	14.1	1.39	0.75	1.36	0.56
8	0.35	5.9	16.8	1.67	0.14	0.41	0.17
9	0.33	3.3	9.9	0.98	0	0	-
10	0.30	1.5	5.1	0.49	0	0	-
11	0.26	1.5	5.6	0.56	0	0	-
P_3			9.6			2.23	

Protein: Of 5.74 mg layered, 5.10 mg or 88.9% recovered.
Acid phosphatase: Of 55.1 units layered, 51.3 units or 93.1% recovered.
Cytochrome oxidase: Of 12.78 units layered, 12.45 units or 97.4% recovered.

Figure 11A

Fraction Number	Ribonuclease			Urate Oxidase		
	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	2.3	6.7	0.47	14	43	3.67
2	5.9	15.3	1.07	7	19	1.59
3	1.8	3.9	0.28	8	18	1.51
4	4.9	8.1	0.57	6	10	0.88
5	5.1	8.3	0.58	7	12	1.01
6	6.3	12.0	0.84	3	6	0.51
7	7.3	20.7	1.46	3	8	0.75
8	4.8	18.2	1.28	0	0	-
9	7.1	20.8	1.47	-	-	-
10	6.8	29.2	2.08	0	0	-
11	9.7	41.8	2.94	1	6	0.38
P_3		1.0			20	

Ribonuclease: Of 6.7 units layered, 62.0 units or 925.4% recovered.
Urate Oxidase: Of 138 units layered, 49 units or 35.5% recovered.

Figure 11B

Fraction Number	Protein	Acid Phosphatase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.
1	0.34	3.1	9.2	0.87
2	0.39	3.9	10.0	0.94
3	0.47	5.6	11.8	1.13
4	0.60	7.7	12.9	1.22
5	0.61	8.0	13.1	1.24
6	0.52	6.9	13.3	1.26
7	0.35	4.8	13.7	1.30
8	0.26	2.6	9.8	0.95
9	0.34	1.2	3.5	0.33
10	0.23	0.8	3.2	0.32
11	0.23	1.2	5.4	0.49
P_3			7.7	

Protein: Of 6.88 mg layered, 4.34 mg or 63.1% recovered.
Acid phosphatase: Of 52.9 units layered, 45.8 units or 86.6% recovered.

Figure 12A (I)

Fraction Number	Urate Oxidase			Acid Phosphatase		
	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0	0	-	0.9	8.9	0.61
2	1.0	9.6	0.52	1.4	13.3	0.96
3	1.7	12.8	0.80	2.0	14.7	1.03
4	3.1	17.6	0.88	3.0	16.9	1.12
5	4.9	21.7	1.13	3.9	17.3	1.13
6	5.0	17.7	0.94	5.0	18.0	1.20
7	3.8	11.0	0.60	5.4	15.6	1.05
8	5.9	13.8	0.72	7.4	17.3	1.16
9	8.8	12.4	0.66	8.8	12.4	0.83
10	30.8	30.7	1.61	12.5	12.5	0.84
11	17.8	21.0	1.10	14.3	16.9	1.13
P_3		28.4			17.6	

Urate oxidase: Of 146 units layered, 84 units or 57.5% recovered.
Acid phosphatase: Of 90.0 units layered, 64.6 units or 71.8% recovered.

Figure 12A (II)

Fraction Number	Acid Phosphatase		
	Tot. Act.	S.A.	R.S.A.
1	0.9	7.0	0.62
2	1.6	9.5	0.66
3	1.4	10.9	1.29
4	2.4	11.4	1.00
5	3.5	11.8	1.02
6	4.1	9.1	0.78
7	4.9	9.1	0.78
8	6.6	11.2	0.97
9	8.3	9.7	0.85
10	13.9	13.2	1.14
11	14.1	15.0	1.30
P_3		10.8	

Acid phosphatase: Of 63.3 units layered, 61.9 units or 97.8% recovered.

Figure 12B (I)

Fraction Number	Protein	Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.
1	0.10	0.42	4.06	0.43
2	0.10	0.99	9.66	1.04
3	0.13	2.78	20.93	2.27
4	0.18	6.59	37.21	3.90
5	0.23	9.27	40.84	4.24
6	0.28	7.53	26.88	2.86
7	0.35	4.35	12.58	1.32
8	0.43	2.42	5.62	0.60
9	0.71	2.23	3.15	0.33
10	1.00	2.62	2.62	0.28
11	0.85	1.91	2.25	0.24
P_3			7.88	

Protein: Of 5.11 mg layered, 4.35 mg or 85.3% recovered.
Cytochrome oxidase: Of 40.28 units layered, 41.11 units or 102.1% recovered.

Figure 12B (II)

Fraction Number	Protein	Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.
1	0.13	0.50	3.84	0.46
2	0.17	1.99	11.98	1.34
3	0.13	2.40	18.21	2.12
4	0.21	7.91	37.15	4.33
5	0.30	8.90	29.66	3.41
6	0.45	7.97	17.82	2.04
7	0.54	4.29	7.98	0.91
8	0.58	2.47	4.23	0.48
9	0.85	3.07	3.60	0.42
10	1.06	4.30	4.07	0.47
11	0.94	2.90	3.09	0.35
P_3			7.39	

Protein: Of 5.84 mg layered 5.37 mg or 92.0% recovered.
Cytochrome oxidase: Of 43.20 units layered, 46.71 units or 108.1% recovered.

Figure 13A

Fraction Number	Protein	Acid Phosphatase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.
1	0.07	0	0	-
2	0.09	0.1	1.00	0.15
3	0.10	0.2	2.24	0.30
4	0.14	0.6	4.50	0.65
5	0.29	1.6	5.58	0.83
6	0.59	4.5	7.67	1.15
7	0.46	3.7	7.94	1.21
8	0.20	2.0	9.89	1.51
9	0.16	1.7	10.18	1.59
10	0.13	0.6	4.65	0.70
11	0.09	0.4	4.63	0.67
P_3			5.75	

Protein: Of 2.50 mg layered, 2.32 mg or 92.8% recovered.
Acid phosphatase: Of 14.4 units layered, 15.4 units or 106.9% recovered.

Figure 14A

Fraction Number	Protein	Acid Phosphatase			Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0.14	0.6	4.24	0.51	0	0	-
2	0.16	1.0	6.42	0.74	0.55	3.36	0.97
3	0.21	1.8	8.53	1.02	0.75	3.57	1.01
4	0.30	2.9	9.63	1.15	1.33	4.46	1.26
5	0.38	3.6	9.38	1.09	1.71	4.45	1.24
6	0.34	3.5	10.32	1.22	2.14	6.30	1.78
7	0.26	2.9	11.21	1.32	1.66	6.31	1.81
8	0.18	2.0	11.13	1.32	0.38	2.17	0.60
9	0.20	1.1	5.71	0.65	0	0	-
10	0.15	0.6	4.33	0.48	0	0	-
11	0.14	0.8	5.87	0.67	0.21	1.45	0.42
P_3			7.3			2.95	

Protein: Of 2.78 mg layered, 2.47 mg or 88.8% recovered.
Acid phosphatase: Of 20.3 units layered, 20.8 units or 102.5% recovered.
Cytochrome oxidase: Of 8.20 units layered, 8.73 units or 106.5% recovered.

Figure 14B

Fraction Number	Protein	Acid Phosphatase			Cytochrome Oxidase		
	Tot. mg.	Tot. Act.	S.A.	R.S.A.	Tot. Act.	S.A.	R.S.A.
1	0.09	0.1	1.4	0.23	0	0	-
2	0.09	0.2	1.6	0.41	0	0	-
3	0.10	0.2	2.5	0.38	0.25	2.58	0.42
4	0.12	0.4	3.7	0.62	0.54	4.49	0.72
5	0.21	0.9	4.4	0.82	1.62	7.60	1.28
6	1.43	6.5	4.6	0.85	10.42	7.27	1.20
7	1.20	6.3	6.2	0.99	9.64	8.01	1.33
8	0.34	2.9	8.6	1.60	1.98	5.88	0.96
9	0.22	2.3	10.6	1.96	0.18	0.81	0.11
10	0.19	1.5	7.9	1.48	0.21	1.13	0.17
11	0.17	0.8	4.7	0.88	0.38	2.24	0.36
P_3			5.5			5.58	

Protein: Of 4.12 mg layered, 4.16 mg or 101.0% recovered.
Acid phosphatase: Of 22.8 units layered, 22.1 units or 96.9% recovered.

Cytochrome oxidase: of 22.99 units layered, 25.22 units or 109.7% recovered.

LITERATURE CITED

Allfrey, V. 1959. The isolation of subcellular components, p. 193-290. In J. Brachet and A.E. Mirsky (eds.), The cell, vol. I. Academic Press, New York.

Anderson, N.G. 1955. Studies on isolated cell components VII. High resolution gradient differential centrifugation. Exptl. Cell Res. 9:446-459.

Appelmans, F., and C. de Duve. 1955. Tissue fractionation studies. 3. Further observations on the binding of acid phosphatase by rat-liver particles. Biochem. J. 59:426-433.

Balinsky, B.I., and R.J. Devis. 1963. Origin and differentiation of cytoplasmic structures in the oocytes of Xenopus laevis. Acta Embryol. Morphol. Exptl. 6:55-108.

Barbieri, F.D., and H. Salomón. 1963. Some experimental studies on glycogen and lactic acid in the developing eggs of Bufo arenarum. Acta Embryol. Morphol. Exptl. 6:304-310.

Barnes, M.R. 1944. The metabolism of the developing Rana pipiens as revealed by specific inhibitors. J. Exptl. Zool. 95: 399-417.

Barth, L.G., and L.J. Barth. 1945. The energetics of development. A study of metabolism in the frog egg. Columbia University Press, New York.

Baudhuin, P.H., H. Beaufay, and C. de Duve. 1965. Combined biochemical and morphological study of particulate fractions from rat liver: Analysis of preparations enriched in lysosomes or in particles containing urate oxidase, D-amino acid oxidase, and catalase. J. Cell Biol. 26:219-243.

Bataillon, E. 1911. Les deux facteurs de la parthenogénèse traumatique chez les amphibiens. Compt. Rend. Acad. Sci., Paris. 152:920-922.

--- 1912. La parthenogénèse des amphibiens et la "fécondation chimique" de Loeb (étude analytique). Ann. Sci. Nat. Zool. 16:249-307.

--- 1929. Analyse de la fécondation par la parthenogénèse expérimentale. Arch. Entwicklungsmech. Organ. 115:707-778.

Beaufay, H., D.S. Bendall, P. Baudhuin, R. Wattiaux, and C. de Duve. 1959. Tissue fractionation studies 13. Analysis of mitochondrial fractions from rat liver by density-gradient centrifuging. Biochem. J. 73:628-637.

Beaufay, H., and J. Berthet. 1963. Medium composition and equilibrium density of subcellular particles from rat liver, p. 66-85. In J.K. Grant (ed.) Biochem. Soc. Symp. No. 23. Cambridge University Press, London.

Beinert, H. 1951. The extent of artificial redistribution of cytochrome c in rat liver homogenates. J. Biol. Chem. 190:287-292.

Berthet, J., and C. de Duve. 1951. Tissue fractionation studies. 1. The existence of a mitochondria-linked, enzymatically inactive form of acid phosphatase in rat-liver tissue. Biochem. J. 50:174-181.

Berthet, J., L. Berthet, F. Appelmans, and C. de Duve. 1951. Tissue fractionation studies. 2. The nature of the linkage between acid phosphatase and mitochondria in rat-liver tissue. Biochem. J. 50:182-189.

Bieber, S., J.A. Spence, and G.H. Hitchings. 1959. Nucleic acids and their derivatives and the development of Rana pipiens. I. Oogenesis. Exptl. Cell Res. 16:202-214.

Boell, E.J. 1955. Energy exchange and enzyme development during embryogenesis. In B.H. Willier, P.A. Weiss, and V. Hamburger (eds.) Analysis of development. W.B. Saunders Co., Philadelphia.

Bogucki, M. 1921. Przyczynek do analizy dzieworództwa traumaticznego. Prace Inst. Nenckiego 1(6):1-12. (Read in English translation).

--- 1922. Dalsze badania nad dzieworództwem sztucznem. Prace Inst. Nenckiego 1(16):1-12. (Read in English translation).

--- 1923. Rola krwi w dzieworodztwie traumatycznem. Prace Inst. Nenckiego 2:1-10. (Read in English translation).

--- 1926. Z badań nad dzieworództwen doświadczalnem. Prace Inst. Nenckiego 3(50):1-25. (Read in English translation).

Boveri, T. 1895. Ueber des Verhalten der Centrosomen bei der Befruchtung des Seeigel-Eies nebst allgemeinen Bemerkungen über Centrosomen und Verwandtes. Verh. Phys. Med. Ges. Würzburg, 29:1-75.

Brachet, J. 1934. Étude du métabolisme de l'oeuf de Grenouille (Rana fusca) au cours du développement 1. La respiration et la glycolyse, de la segmentation a l'eclosion. Arch. Biol. 45:611-727.

--- 1954. Constitution anormale du noyau et métabolisme de l'embryon chez les Batraciens. Arch. Biol. 65:1-71.

Brachet, J., H. Denis, and F. de Vitry. 1964. The effects of actinomycin D and puromycin on morphogenesis in amphibian eggs and Acetabularia mediterranea. Develop. Biol. 9: 398-434.

Briggs, R., E.U. Green, and T.J. King. 1951. An investigation of the capacity for cleavage and differentiation in Rana pipiens eggs lacking "functional" chromosomes. J. Exptl. Zool. 116:455-499.

Briggs, R., and T.J. King. 1955. Specificity of nuclear function in embryonic development, p. 207-228. In E.G. Butler (ed.) Twelfth Symposium of the Society for the Study of Development and Growth. Princeton, New Jersey.

Briggs, R., and T.J. King. 1959. Nucleocytoplasmic interactions in eggs and embryos, p. 537-617. In J. Brachet and A.E. Mirsky (eds.) The cell, vol. I. Academic Press, New York.

Brown, D.D., and J.D. Caston. 1962. Biochemistry of amphibian development. I. Ribosome and protein synthesis in early development of Rana pipiens. Develop. Biol 5:412-434.

Brown, D.D., and E. Littna. 1964. RNA synthesis during development of Xenopus laevis, the South African clawed toad. J. Mol. Biol. 8:669-687.

Ceriotti, G. 1952. A microchemical determination of desoxyribonucleic acid. J. Biol. Chem. 198:297-303.

Chambers, R. 1921. The formation of the aster in artificial parthenogenesis. J. Gen. Physiol. 4:33-38.

Chambers, R., and E.L. Chambers. 1961. Explorations into the nature of the living cell. Harvard Univ. Press, Massachusetts.

Claude, A. 1946. Fractionation of mammalian liver cells by differential centrifugation. II. Experimental procedures and results. J. Exptl. Med. 84:61-89.

Cohen, A.I. 1954. Studies on glycolysis during early development of the Rana pipiens embryo. Physiol. Zool. 27:128-141.

Dalton, A.J., H. Kahler, M.G. Kelly, B.J. Lloyd, and M.J. Striebich. 1949. Some observations on the mitochondria of normal and neoplastic cells with the electron microscope. J. Natl. Cancer Inst. 9:439-449.

Dan, K., and M. Kuno-Kojima. 1963. A study on the mechanism of cleavage in the amphibian egg. J. Exptl. Biol. 40:7-14.

Dawid, I.B. 1965. Deoxyribonucleic acid in amphibian eggs. J. Mol Biol. 12:581-599.

--- 1966. Evidence for the mitochondrial origin of frog egg cytoplasmic DNA. Proc. Natl. Acad. Sci. U.S. 56:269-276.

de Duve, C. 1964. Principles of tissue fractionation. J. Theoret. Biol. 6:33-59.

--- 1965a. The separation and characterization of subcellular particles, p. 45-87. In The Harvey Lectures, series 59. Academic Press, New York.

--- 1965b. Functions of microbodies (peroxisomes). J. Cell Biol. 27(2):25A-26A.

de Duve, C., and J. Berthet. 1953. Reproducibility of differential centrifugation experiments in tissue fractionation. Nature, 172:1142.

--- 1954. The use of differential centrifugation in the study of tissue enzymes. Intern. Rev. Cytol. 3:225-275.

de Duve, C., B.C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J. 60:604-617.

Dettlaff, T.A., L.A. Nikitina, and O.G. Stroeva. 1964. The role of the germinal vesicle in oocyte maturation in anurans as revealed by the removal and transplantation of nuclei. J. Embryol. Exptl. Morphol. 12:851-873.

Dirksen E.R. 1961. The presence of centrioles in artificially activated sea urchin eggs. J. Biophys. Biochem. Cytol. 11:244-247.

Dirksen, E.R. 1964. The isolation and characterization of asters from artificially activated sea urchin eggs. Exptl. Cell Res. 36:256-269.

Ecker, R.E., and L.D. Smith. 1966. The kinetics of protein synthesis in early amphibian development. Biochim. Biophys. Acta, 129:186-192.

Einsele, W. 1930. Entwicklungserregung von Froscheiern durch Injektion zellfreier Organextrakte. Arch. Entwicklungsmech. Organ. 123:279-300.

Epel, D. 1963. The effects of carbon monoxide inhibition on ATP level and the rate of mitosis in the sea urchin egg. J. Cell Biol. 17:315-319.

Fankhauser, G. 1934. Cytological studies on egg fragments of the salamander Triton. IV. The cleavage of egg fragments without the egg nucleus. J. Exptl. Zool. 67:349-393.

Ficq, A. 1964. Effets de l'actinomycine D et de la puromycine sur le métabolisme de l'oocyte en croissance. Étude autoradiographique. Exptl. Cell Res. 34:581-594.

Frazier, R.L. 1951. A cytological and cytochemical study of parthenogenetic embryos of Rana pipiens. Master's Thesis. Univ. Missouri.

Fry, H.J. 1925. Asters in artificial parthenogenesis. I. Origin of the amphiaster in eggs of Echinarachnius parma. J. Exptl. Zool. 43:11-47.

Graham, C.F. 1966. The regulation of DNA synthesis and mitosis in multinucleate frog eggs, J. Cell Sci. 1:363-374.

Graham, C.F., K. Arms, and J.B. Gurdon. 1966. The induction of DNA synthesis by frog egg cytoplasm. Develop. Biol. 14: 349-381.

Grant, P. 1953. Phosphate metabolism during oogenesis in Rana temporaria. J. Exptl. Zool. 124:513-543.

--- 1958. The synthesis of deoxyribonucleic acid during early embryonic development of Rana pipiens. J. Cellular Comp. Physiol. 52:227-248.

--- 1965. Informational molecules and embryonic development, p. 483-593. In R. Weber (ed.) The biochemistry of animal development, vol. I. Academic Press, New York.

Gregg, J.R., and F.L. Ray. 1957. Respiration of homogenized embryos: Rana pipiens and Rana pipiens ♀ x Rana sylvatica ♂. Biol. Bull. 113:382-387.

Guyer, M.F. 1907. The development of unfertilized frog eggs injected with blood. Science, 25:910-911.

Haggis, A.J. 1964. Quantitative determination of deoxyribonucleic acid in embryos and unfertilized eggs of Rana pipiens. Develop. Biol. 10:358-377.

Harrison, M.N. 1965. The role of creatine and its phosphate in amphibian development. Biol. Bull. 128:230-240.

Herlant, M. 1913. Etude sur les bases cytologiques du mécanisme de la parthénogénèse expérimentale chez les Amphibiens. Arch. Biol. 28:505-608.

Hers, H.G., J. Berthet, L. Berthet, and C. de Duve. 1951. Le système hexose-phosphatasique. III. Localisation intracellulaire des derments par centrifugation fractionée. Bull. Soc. Chim. Biol. 33:21-41.

Hoff-Jørgensen, E., and E. Zeuthen. 1952. Evidence of cytoplasmic desoxyribosides in the frog's egg. Nature, 169: 245-246.

Hogeboom, G.H. 1955. Fractionation of cell components of animal tissues, p. 16-19. In S.P. Colowick and N.O. Kaplan (eds.) Methods in enzymology, I. Academic Press, New York.

Hogeboom, G.H., and W.C. Schneider. 1950. Sonic disintegration of isolated liver mitochondria. Nature, 166:302-303.

Hogeboom, G.H., W.C. Schneider, and G.E. Pallade. 1948. Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. J. Biol. Chem. 172:619-635.

Holter, H., M. Ottesen, and R. Weber. 1953. Separation of cytoplasmic particles by centrifugation in a density-gradient. Experientia, 9:346-348.

Huff, R.E., and J.T. Preston. 1965. The production of a cleavage-initiating factor by artificially activated eggs of Rana pipiens. Tex. J. Sci. 17:206-212.

Huff, R.E., and S.A. Ramirez. 1966. Cytological changes in artificially activated eggs of Rana pipiens. Am. Zool. 6: 611.

Kalnitsky, G., J.P. Hummel, and C. Dierks. 1959. Some factors which affect the enzymatic digestion of ribonucleic acid. J. Biol. Chem. 234:1512-1516.

Kemp, N.E., and N.L. Istock. 1967. Cortical changes in growing oocytes and in fertilized or pricked eggs of Rana pipiens. J. Cell Biol. 34:111-122.

Keister, D.L. 1958. Some aspects of the metabolic function of the naturally occurring amines. Ph.D. Thesis. Univ. Maryland, Medical School.

Ledoux, L., J. LeClerc, and J. Brachet. 1955. Action de la ribonucléase sur le développement embryonnaire des Batraciens. I. Expériences de micro-injection. Exptl. Cell Res. 9: 338-347.

Ledoux, L., J. LeClerc, and F. Vanderhaeghe. 1954. Influence of ribonuclease on the division of amphibian eggs. Nature, 174:793-794.

Lillie, F.R. 1919. Problems of fertilization. The Univ. of Chicago Press, Illinois.

Loeb, J. 1912. The mechanistic conception of life. D. Fleming (ed.), Reprinted by Harvard Univ. Press, Mass. 1964.

--- 1913. Artificial parthenogenesis and fertilization. The Univ. of Chicago Press, Illinois.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Marsland, D.A., and J.V. Landau. 1954. The mechanism of cell division; temperature - pressure experiments on the cortical gel system in various marine eggs. J. Exptl. Zool. 125:507.

Mazia, D. 1961. Mitosis and the physiology of cell division, p. 77-412. In J. Brachet and A.E. Mirsky (eds.) The cell, vol. III. Academic Press, New York.

--- 1963. Synthetic activities leading to mitosis. J. Cellular Comp. Physiol. Suppl. 1:123-140.

Mazia, D., and K. Dan. 1952. The isolation and biochemical characterization of the mitotic apparatus of dividing cells. Proc. Natl. Acad. Sci. U.S. 38:826-838.

Mazia, D., P.J. Harris, and E. Bibring. 1960. The multiplicity of the mitotic centers and the time-course of their duplication and separation. J. Biophys. Biochem. Cytol. 7:1-20.

Miller, G.L. 1959. Protein determination for large numbers of samples. Anal. Chem. 31:964.

Mirsky, A.E., and A.W. Pollister. 1946. Chromosin, a desoxyribose nucleoprotein complex of the cell nucleus. J. Gen. Physiol. 30:117-147.

Moore, B.C. 1963. Histones and differentiation. Proc. Natl. Acad. Sci. U. S. 50:1018-1026.

Needham, J. 1942. Biochemistry and morphogenesis. Cambridge Univ. Press, London.

Parat, M. 1933. L'acrosome du spermatozoïde dans la fécondation et la parthénogénèse expérimentale. Compt. Rend. Soc. Biol. 112:1134-1137.

Parnas, J.K., and Z. Krasinska. 1921. Über den stoffwechsel der Amphibienlarven. Biochem. Z. 116:108-137.

Pfohl, R.J. 1962. The effect of adenosine triphosphate on the stimulation of cleavage in the eggs of Rana pipiens. Master's Thesis. Michigan State Univ.

Pierce, C.H., R.J. Dubos, and W.B. Schaefer. 1953. Multiplication and survival of tubercle bacilli in the organs of mice. J. Exptl. Med. 97:189-206.

Ramirez, S.A., and R.E. Huff. 1967. A cytological study of parthenogenetically activated eggs of Rana pipiens. Tex. J. Sci. 19:41-56.

Roodyn, D.B. 1965. The classification and partial tabulation of enzyme studies on subcellular fractions isolated by differential centrifuging. Intern. Rev. Cytol. 18:99-190.

Rosenbloom, J., and V.N. Schumaker. 1963. Analytical ultracentrifugation of T4r bacteriophage DNA in preformed sucrose density gradients. Biochemistry, 2:1206-1211.

Rostand, J. 1924. Parthénogénèse traumatique par le sperme sec. Compt. Rend. Soc. Biol. 90:931.

--- 1928. Propriétés du catalyseur-noyau en parthénogénèse traumatique. Compt. Rend. Soc. Biol. 98:1207-1208.

--- 1938. La parthénogénèse des vertébrés. In J. Rostand Actualites scientifiques et industrielles, 651. Hermann and Co., Paris.

Rugh, R. 1934. Induced ovulation and artificial fertilization in the frog. Biol. Bull. 66:22-29.

Salomón, H., and F.D. Barbieri. 1964. Some effects of anaerobiosis and fluoride on the developing eggs of Bufo arenarum. Acta Embryol. Morphol. Exptl. 7:21-28.

Sawant, P.L., I.D. Desai, and A.L. Tappel. 1964a. Digestive capacity of purified lysosomes. Biochim. Biophys. Acta, 85:93-102.

Sawant, P.L., S. Shibko, U.S. Kumta, and A.L. Tappel. 1964b. Isolation of rat-liver lysosomes and their general properties. Biochim. Biophys. Acta, 85:82-92.

Schneider, W.C. 1948. Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat-liver fractions. J. Biol. Chem. 176:259-266.

Schneider, W.C., and G.H. Hogeboom. 1950. Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome c in rat-liver homogenates. J. Biol. Chem. 183:123-128.

Schneider, W.C., and G.H. Hogeboom. 1952. Intracellular distribution of enzymes. X. Desoxyribonuclease and ribonuclease. J. Biol. Chem. 198:155-163.

Schmidt, G., and S.J. Thannhauser. 1945. A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem. 161:83-89.

Shaver, J.R. 1951. An experimental study of artificial parthenogenesis in the frog egg. Ph.D. Thesis. Univ. Pennsylvania.

--- 1953. Studies on the initation of cleavage in the frog egg. J. Exptl. Zool. 122:169-192.

Shaver, J.R., S. Subtelny, and A. Wania. 1952. Studies on initiation of cleavage in the frog egg. Biol. Bull. 103:282.

Shumway, W. 1940. Stages in the normal development of Rana pipiens. I. External form. Anat. Rec. 78:139-147.

Smith, L. 1955. Spectrophotometric assay of cytochrome c oxidase, p. 427-434. In D. Glick (ed.) Methods of biochemical analysis II. Interscience Publishers, New York.

Smith, L.D., and R.E. Ecker. 1965. Protein synthesis in enucleated eggs of Rana pipiens. Science, 150:777-779.

Smith, L.D., R.E. Ecker, and S. Subtelny. 1966. The initiation of protein synthesis in eggs of Rana pipiens. Proc. Natl. Acad. Sci. U. S. 56:1724-1728.

Spiegelman, S., and F. Moog. 1945. A comparison of the effects of cyanide and azide on the development of frogs' eggs. Biol. Bull. 89:122-130.

Spiegelman, S., and H.B. Steinbach. 1945. Substrate-enzyme orientation during embryonic development. Biol. Bull. 88: 254-268.

Spirin, A.S. 1966. On "masked" forms of messenger RNA in early embryogenesis and in other differentiating systems, p. 1-38. In A. Monroy and A.A. Moscona (eds.) Current topics in developmental biology, vol. I. Academic Press, New York.

Stevens, D.F. 1966. Mechanisms influencing cytokinesis in hamster ascites multinucleated cells. The effect of some citrate cycle inhibitors and nucleotides. Exptl. Cell Res. 41:492-501.

Swann, M.M. 1954. The mechanism of cell division: experiments with ether on the sea urchin egg. Exptl. Cell Res. 7:505-517.

Tchou SE, and Chen Chao Hai. 1040. The technique of Prof. Bataillon and some three-year-old parthenogenetic frogs. Chinese J. Exptl. Biol. 1:303-333.

Thomas, J.A., J. Rostand, and J. Gregoire. 1946. Action inhibitrice de doses minimes de ribonucléase sur la segmentation de l'oeuf de la Grenouille rousse. Compt. Rend. Acad. Sci. 222:1139-1141.

Thomson, J.F. 1959. Isolation of cytoplasmic particulates from animal tissues by density-gradient centrifugation. Anal. Chem. 31:836-838.

Tyler, A. 1941. Artificial parthenogenesis. Biol. Rev. Cambridge Phil. Soc. 16:291-336.

Voss, H. 1923. Studien zur Kunstlichen Entwicklungserregung des Froscheies. I. Wirking und Wesen der Einimpfung von Zellelementen in das Froschei bei der traumatischen Parthenogenese. Arch. Entwicklungsmech. Organ. 97:616-640.

Waddington, C.H. 1952. Preliminary observations on the mechanisms of cleavage in the amphibian egg. J. Exptl. Biol. 29: 484-489.

Ward, R.T. 1962a. The origin of protein and fatty yolk in Rana pipiens. I. Phase microscopy. J. Cell Biol. 14:303-308.

--- 1962b. The origin of protein and fatty yolk in Rana pipiens. II. Electron microscopical and cytochemical observations of young and mature oocytes. J. Cell Biol. 14:309-341.

Wattiaux, R., and C. de Duve. 1956. Tissue fractionation studies. 7. Release of bound hydrolases by means of Triton X-100. Biochem. J. 63:606-608.

Wattiaux, R., M. Wibo, and P. Baudhuin. 1963. Influence of the injection of Triton WR-1339 on the properties of rat-liver lysosomes, p. 176-200. In CIBA Foundation Symposium:Lysosomes. Little, Brown, and Co., Boston.

Webb, J.M., and H.B. Levy. 1958. New developments in the chemical determination of nucleic acids, p. 1-30. In D. Glick (ed.) Methods of biochemical analysis, VI. Interscience Publishers, New York.

Wilson, E.B. 1925. The cell in development and heredity (third edition). The Macmillan Co., New York.

Wilson, G.B. 1966. Cell division and the mitotic cycle. Reinhold Publishing Corp., New York.

Wischnitzer, S. 1964. Ultrastructural changes in the cytoplasm of developing amphibian oocytes. J. Ultrastruct. Res. 10: 14-26.

Zotin, A.I. 1964. The mechanism of cleavage in amphibian and sturgeon eggs. J. Embryol. Exptl. Morphol. 12:247-262.