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The proteins from ovaries of the cockroach, Leucophaea maderae, were analyzed in different stages of development. Most of the protein in mature ovaries consisted of a large lipoprotein with a sedimentation coefficient of 27S and a molecular weight of 1, 450, 000 as determined by high-speed equilibrium centrifugation. A lipid content of .094 mg lipid/mg protein was found. This lipid appeared to be primarily phospholipid. In the early stages of development, most of the protein was found in a component which sediments at 14S and has a molecular weight of 581, 000 as determined by the high-speed equilibrium centrifugation method.

Female cockroaches were injected with C^{14}-leucine and the ovaries were removed after different time intervals. Yolk was prepared from these ovaries and the incorporation of radioactivity was determined. The 14S fraction was labeled first and the 27S became radioactive after prolonged exposure. It appeared that the 27S was formed by an active polymerization of the 14S components.

A method of separating the 14S component from the 27S component by sucrose density gradient centrifugation was devised and no interconversion was observed when the isolated fractions were stored in 0.4 M NaCl. Preliminary investigations suggest considerable potential for agarose as a medium for column chromatography.

An amino acid analysis of each component was performed. The amino acid compositions of the two components appear to be identical. The 27S component was disaggregated to a fraction which also sedimented at 14S by heating at 65° C for 10 minutes, treatment with alkaline solutions of pH 9, and by 4 M urea. Reaggregation of the 14S formed was observed after dissociation with urea but not after dissociation with base or heat.

Partial Characterization of the Yolk Protein Of *Leucophaea Maderae* (Fab.) With Emphasis on Changes During Oocyte Development

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PARTIAL CHARACTERIZATION OF THE YOLK PROTEIN OF *LEUCOPHAEA MADERAE* (FAB.) WITH EMPHASIS ON CHANGES DURING OOCYTE DEVELOPMENT

I. INTRODUCTION

The formation and deposition of yolk in the eggs of many species of insects is under the control of hormone secreted by neurosecretory cells and by the corpora allata. The subject has been extensively investigated for many years, and the results have been obtained with a variety of methods carried out on a correspondingly large number of insect species.

In general the approach has been to measure the effect of the presence or absence of a particular endocrine gland on some facet of the reproductive cycle. Most often measurement has been made in terms of the increase in size of developing oocytes which is largely the result of the deposition of yolk. Several additional physiological changes are manifested during the period of vitellogenesis in insects. Among these are the hypertrophy of the fat body, variation in concentration of protein in the hemolymph and variation in the number of microscopic invaginations of the egg membrane. Claims have been made that these physiological changes are the direct result of the secretions of one gland or the other and the claims are often contradictory. There is evidence now that the physiological, biochemical, and morphological events associated with ovarian maturation can be related by a scheme in which yolk material is

synthesized outside of the ovary, transported by the hemolymph, and incorporated into the egg by pinocytosis. Most of these events may be mutually influential and therefore not require the intervention of a hormone.

In Leucophaea maderae the egg increases in length from 1 to 5 or 6 mm with an estimated 200-fold increase in volume. The growth is the result of a 70-fold increase in protein, a 160-fold increase in lipid (29) and a smaller but unmeasured increase in carbohydrates and other materials. There are many species of proteins in the immature eggs, but most of the proteins of a mature egg consist of a single lipoprotein. This lipoprotein has been designated as the yolk protein.

The results of earlier work in this laboratory have suggested that the formation and deposition of yolk as well as other events related to ovarian maturation are controlled by secretions from the corpora allata. These glands produce neotenin or juvenile hormone which regulates the metamorphic development of immature stages. The question was asked whether or not secretions of corpora allata act by inducing the synthesis of proteins specific to the yolk.

The present work is concerned with the isolation and characterization of the yolk protein and was done in order that the action of hormone could be measured in terms of a specific protein rather than complex physiological phenomena. In the course of these studies

it was found that the protein was made up of structural subunits that may play an important part in its deposition as yolk.

II. HISTORICAL REVIEW

Yolk Production in Animals

The subject of vitellogenesis has received much attention during the past 50 years. Both biochemical and cytological methods have been employed to identify the type of materials present and the sequence of events during maturation. When the many reports in the literature are examined, three types of yolk production are indicated: a) intraoocyte synthesis of yolk, b) extraoocyte synthesis of yolk, and c) a combination of the two. It is not always clear when yolk material is transported into the oocyte or when yolk precursors are incorporated. Often the content of the oocyte is not characterized clearly enough to define what is actually yolk.

Ward, in his paper on Rana pipiens (79), reviews evidence of some early investigators which implicates four organelles in the intraoocyte origin of yolk of different organisms. These four organelles are the mitochondria, the Golgi bodies, nucleolar extrusions, and the ground substance of the cytoplasm. He also points out the variations that can occur between such closely related organisms as two different species of frogs. This variation within the same genus shows the need for caution when extrapolation of results between species is made. His detailed electron microscope study on Rana pipiens indicates that part of the yolk is synthesized by the

mitochondria in the oocyte. The electron microscope was also used by Kessel in studies that indicate that the Golgi apparatus is involved in yolk formation of jellyfish (38) and an echinoderm, Thyone (40). Similar investigation of several crustaceans by the same author (39) demonstrated that the protein yolk arises from the rough endoplasmic reticulum. The endoplasmic reticulum was also found to be the source of synthesis of the yolk protein in crayfish (4).

In a wide variety of organisms in the animal kingdom, evidence has been reported that indicates that all or most of the yolk materials are made outside of the ovary, transported to the ovary, and incorporated into the egg in some way. Specific examples of this process can be found in mammals (46), birds (60, 62), amphibians (26, 30, 51, 54, 75, 76, 77) and insects which will be treated in detail in this paper.

Two organisms that seem to combine the two types of yolk development described above are the spider crab (35) and the horseshoe crab (22). In these two organisms, both the endoplasmic reticulum and Golgi participate in intraoocyte yolk synthesis in early vitellogenesis. Later in vitellogenesis yolk appears to be synthesized at an extraoocytic site and yolk spheres are formed by pinocytosis (22, 35). This type of development was also found in guppies by Droller and Roth (21). Early in oogenesis the Golgi system of the egg proliferates. Vesicles containing electron opaque material

apparently pinch off from the Golgi and form one type of yolk droplet. Different types of yolk droplets are formed from material from the endoplasmic reticulum and the two types of droplets fuse. Later in development two different types of vesicles are formed by pinocytosis. These are postulated to be involved with the selective uptake of dissimilar extracellular proteid materials.

Yolk Formation in Insects

In general, the oocytes of insects are characterized by the absence of organelle systems conventionally associated with synthetic activity (35). However, the follicular cells of Schistocerca gregaria have been reported to play an active role in the intraoocytic synthesis of yolk (45) and in Rhodnius both the nurse cells and the follicular cells may play a part in the synthesis of some of the ovarial proteins (72).

Intraoocytic synthesis of yolk material also may be found in the fruit fly. Drosophila ovaries have 15 nurse cells per egg cell. These are arranged in a nest surrounded by an envelope of follicle cells. Early in development all 16 cells develop at the same rate. When vitellogenesis begins, the egg cell grows at a much faster rate than the nurse cells, dense material streams from the nurse cells into the egg cell and the nurse cells degenerate (42). After the nurse cells have degenerated, no substantial increase in egg cell

volume occurs (42). These data suggest that a substantial amount of the synthesis may relate to the role of the nurse cells (42).

Several observations of insect development reported in the literature can be explained by a scheme of extraovarian synthesis of yolk. Three of these were evident in the investigation of the mosquito by Roth and Porter (58). They list the following reasons to believe the yolk is made at a site outside of the ovary: a) yolk develops rapidly, synthesis and storage are completed in as little as 25 hours after a blood meal; b) none of the usual synthetic or packaging machinery appear in the oocyte or follicle cells; c) a series of pits or wells forms in the surface of the oocyte during its growth.

The earliest evidence that insect ovaries incorporate proteins from the hemolymph was reported by Wigglesworth (81). In a series of experiments with the blood-sucking insects Rhodnius and Pediculus he observed hemoglobin or a derivative of hemoglobin in the hemolymph and in the eggs. The amount of hemoglobin in the eggs was increased by injections of laked blood. A greater concentration of oxy hemoglobin was found in the eggs of Pediculus than in the hemolymph. This observation led to the hypothesis that the ovaries could concentrate this protein.

In a series of experiments analyzing the blood and eggs of saturniid moths, Telfer further demonstrated the uptake of blood

proteins by the eggs (67). He discovered a specific sex-linked blood protein in the female moths. The concentration of this female protein in the blood decreased during yolk formation in normal moths. However, in ovariectomized moths the protein concentration in the blood increased instead of decreased during the period when yolk should have been formed. The protein was found in the eggs at 20-30 times the concentration that occurred in the blood. To demonstrate that the protein was not synthesized in the eggs, cecropia blood was transfused to a polyphemus pupa. Immunochemical analysis of the polyphemus egg showed the incorporation of cecropia female protein.

To state that the yolk protein is synthesized outside of the oocyte almost automatically raises the next question: How does the yolk protein cross the layer of follicle cells, basement membrane and tunica propria and become incorporated in the oocyte? The uptake of yolk in Caliphora was traced by the use of tritiated histidine and two stages of incorporation of radioactivity in the oocytes were shown (5). Within 15 minutes after injection radioactivity was found in the cytoplasm. After 40 minutes a concentrated band of radioactivity was noted adjacent to the follicle cells at the periphery of the oocyte. The label had moved into the yolk spheres after 80 minutes. The early stage of incorporation was interpreted as the entry of free histidine and the second stage as the entry of yolk

protein that had been synthesized after the injection of the label.

A study of the mechanism of blood protein uptake by Telfer and Melius (70) showed that the surface of the oocyte assumed a brush border configuration at the time of yolk deposition. The follicle cells had spaces between them and fluorescein-labeled antibodies to blood protein stained the material in these spaces in the same manner as they stained blood. The yolk spheres also were stained by these antibodies. Smaller spheres were found near the periphery of the oocyte and larger spheres were seen toward the center. These authors concluded that pinocytosis is the mechanism of yolk protein uptake in cecropia. Later studies on cecropia with the electron microscope showed that the fine structure of the oocyte surface and the presence of vesicles and yolk spheres were consistent with pinocytosis as a mechanism of yolk protein uptake (65). After ferritin was injected into the hemocoel of the moth it was found in the surface pits and in the outermost vesicles. This was further confirmation of pinocytotic activity.

The mechanism of pinocytosis was further implicated by the presence of the series of pits or wells observed by Roth and Porter in the mosquito oocyte (58). These authors propose three reasons for associating the pits with pinocytosis: a) pit development is at a peak when yolk uptake is in progress, b) other mechanisms of uptake are not evident and simple diffusion of yolk through the

membrane is improbable, c) the vesicles deeper in the cortex show a relationship to the pits in that they have the same content and same order of bristles. Pinocytosis has also been indicated on the basis of ultrastructure in the milkweed bug (41) and in Periplaneta (1).

Anderson concludes that yolk formation in Periplaneta is independent of any outside organelle system (1). In contrast to this, Bombus combines yolk synthesized outside the ovary with that formed in the ovary (36). Early in development the lipid yolk is formed under the influence of the nurse cells. Later albuminous yolk is incorporated by pinocytosis. Still later glycogen yolk particles are formed from vesicles derived from the nurse cells.

Pinocytosis as a mechanism for protein uptake in oocytes helps to explain two previously cited observations. In the cecropia moth every protein detected in the blood was also found in the eggs (66) but one protein called the female protein had a concentration in the egg that was about 20 to 30 times the concentration in the blood (69). When foreign proteins were injected into the hemocoel of polyphemus pupae, these proteins were incorporated in the egg at a concentration about one-tenth of that in which they were found in the blood (69). A selective adsorption on the surface would explain the increased concentration of a specific protein and other proteins would be deposited as they were trapped when the pits developed into vesicles. This selectivity is further supported by observations

on the deposition of certain pigments in moth oocytes (67).

Morphology of Orthopteran Ovaries

In the cockroach Leucophaea, a specific vitellogenic protein was shown to be synthesized in the fat body and incorporated into the oocyte (12). For this reason, a description of the morphology of the ovaries will be given and the literature pertaining to this type of egg development will be considered with an emphasis centered on investigations dealing with insects. Results obtained from other organisms will also be included for amplification.

Insect ovaries are classified as panoistic or meroistic. Meroistic ovaries include trophic cells (nurse cells) which either join the oocyte directly (polytrophic) or lie at the anterior end of the ovariole and are joined to the oocyte by cytoplasmic connections (teleotrophic). The panoistic type ovaries are found in the Orthoptera among other orders and are characterized by the absence of trophic cells (7).

The panoistic ovary in Periplaneta americana has been described in detail by Bonhag (8) and a description of the ovaries of Schistocerca gregaria has been written by Lusis (45). These two papers indicate that the ovaries of the American cockroach and locust are quite similar so it is probably reasonable to assume that the ovaries of Leucophaea have the same basic structure. The earliest stages

of oogenesis are found in the anterior portion of the ovariole and the most mature oocytes are found toward the posterior portion. The oocyte that is at the distal end of the ovariole is often called the terminal oocyte but is more appropriately called the basal oocyte. In the vitellogenic stage of Leucophaea, this oocyte is many times larger than the penultimate oocyte and is the site of most of the yolk deposition. When the basal oocyte of Leucophaea reaches about 1 mm in length, rapid deposition of yolk occurs. This deposition continues until a length of about 5 to 6 mm is reached and at that stage, the basal oocytes from the ovarioles are enclosed by an egg case or ootheca (14). We have used the length of the basal oocyte as an index of the age or stage of growth in these studies.

Yolk Platelets and the Chemical Composition of Yolk

The term "yolk" has had wide usage in the literature and little definition. Studies on the frog reveal hexagonal plates in the early stages of ovary growth (80). These plates enlarge as the ovary matures and in large eggs, bodies are formed with hexagonal crystaline centers surrounded by globular material (79).

These yolk platelets are described as the principal component of the mature oocyte (75). In the amphibian, egg yolk platelets comprise better than 80% of the protein-nitrogen (31) and more than 90% of the protein-phosphorus (32). In the last stages of oocyte

maturation, the size of the oocyte is greatly enlarged in a relatively short period of time. It is during this stage that the yolk platelets are formed (32). Yolk is defined by Wallace (75) as the principle conjugated protein components of the yolk platelet. Various other components such as ferritin, glycogen, and free ribosomes will fall outside of this definition.

The chemical composition of yolk in several animals has been studied. The hen's egg yolk has been studied in detail and two major components called phosvitin and lipovitellin have been isolated (16). Frog's eggs have also been the object of biochemical investigation. The isolated platelets of Rana pipiens consist of two protein components. A phosphoprotein makes up about 10% of the protein and most of the rest of the protein is found in one other component (79). More than 90% of the phosphorus is found in the phosphoprotein. Reports of lipid content in Rana pipiens range from 1. 62% to 18. 9% depending on the method of extraction (79). In the yolk platelets of Rana fusca 70% of the phosphorus is found in the phosphoprotein and the rest is in a phospholipid (53). Small platelets in this animal contain more RNA-phosphorous than the larger platelets, but the large platelets contain a greater proportion of phospholipid per gram of nitrogen. Recently the yolk of the South African toad, Xenopus, has been analyzed (76, 77). The yolk protein of this animal also consists of two major components, phosvitin and lipovitellin.

From a comparative study of the proteins of the hen, Rana esculenta, trout, dogfish and a marine mollusk, Fujii (28) reported that the principle protein constituent of each vertebrate species was a high density lipophosphoprotein. These proteins were insoluble in dilute salt solution but were soluble in concentrated salt solutions or in alkaline solution. The principle protein from the mollusk was also a lipophosphoprotein but it was soluble in water. Additional investigations of the trout (2) and six crustaceans (78) have shown that in these animals the major yolk protein is a lipoprotein.

Some insects also contain platelets or yolk spheres. Yolk protein is found as yolk spheres in the cecropia moth (68) and yolk granules with a crystalline pattern are the major form of yolk protein in mosquito eggs (58). Yolk globules containing albuminous yolk have also been reported in Periplaneta (52). These are incorporated into the oocyte mainly in the later stages of egg maturation.

Although many histological investigations of ovaries have been reported, few chemical studies on the composition of yolk material in insects have been attempted. The proteins of cecropia (66) have been investigated immunologically. Several protein species were present in low concentration with one fraction forming the major portion of the protein. No direct report of a crystalline yolk platelet is available for Leucophaea, but it is known from electrophoretic analysis (19) and immunological analysis (25) that the young

oocyte contains many proteins. In the mature egg, however, almost all of the protein is found in the form of one lipoprotein which is soluble in 0.4 M NaCl and very slightly soluble in 0.2 M NaCl (19). This difference in solubility was used in the investigations reported in this paper to isolate the yolk protein from the supernatant of ovary homogenates in order to further characterize this protein.

III. MATERIALS AND METHODS

The cockroaches were reared in a chest of drawers with screened fronts and glass tops as described by Chambers (13). Each of two drawers contained from 75-100 adults. The young nymphs produced each week were removed by aspiration and 50-60 were placed in each of two 2.5 gallon ice cream tubs with screen centers in the lids. Both young and adult insects were nourished by dog food (Gaines Meal) and water. Breeding drawers and rearing tubs were checked weekly and food and water added as needed. The presence of mature males in the rearing tubs signaled the approaching final molt of the female nymphs, because the males attain maturity after approximately five months and seven instars, whereas the females pass through eight nymphal instars over a period of approximately six months. All the insects were then sexed and the large female nymphs accumulated in additional modified drawers. The mature females were removed weekly and placed 30 per 2.5 gallon tub with an equal number of males. In this way there was a constant supply of recently mated females of similar ages. All insects were held in a room with temperature controlled at 80^{o} F and relative humidity at 50%.

The ovaries were dissected from the cockroaches with the aid of a binocular microscope. Measurement of the oocytes was made

with a calibrated ocular scale. The length of the basal oocyte at ecdysis averages 1.0 mm. Eggs which are smaller than 1.1 mm are not considered to be in a stage of active yolk protein uptake. The average of the mature eggs is 5.6 mm (13).

Yolk protein was prepared by homogenizing ovaries in a Duall glass tissue grinder with 10 or 20 volumes of 0.4 M NaCl. The homogenate was centrifuged at 0° C in Corex tubes at 12,000 g for 30 minutes. When the homogenates of the larger ovaries were centrifuged, a lipid cap formed at the top of the centrifuge tube and a pellet of cell debris was deposited at the bottom of the tube. The aqueous supernatant was removed with a pipette or syringe. An equal volume of water was added to the supernatant and the yolk protein was precipitated inthe 0.2 M NaCl resulting from this dilution. After 15 minutes at 0° C the precipitate was collected by centrifugation for 30 minutes at 12,000 g. This precipitate was redissolved in 0.4 M NaCl and was designated yolk protein.

Yolk protein was measured quantitatively by the amount of absorbance at 280 mμ. The mg of protein can be calculated by the Warburg Christian method (43).

A second method of analysis made use the amino acid analysis data. The formula:

$$\text{gm protein} = (\text{moles amino acid}) \times (\text{molecular weight of amino acid} - 18)$$

was used. This formula allowed a comparison of the OD_{280} of the protein that was used in the amino acid analysis with the weight of amino acids obtained. It is necessary to subtract 18 from the molecular weight of each amino acid to account for the molecule of water lost when the peptide bond is formed.

Protein content was also measured by use of the Lowry method (44). Values were obtained by comparison with standard curves of crystaline bovine serum albumen.

Gel filtration was performed with agarose (Bio-Gel A, Bio·Rad Laboratories) in 2. 5 cm diameter columns of 50 and 100 cm lengths (Pharmacia Fine Chemicals). The columns were packed at the same temperature as they were used. A large funnel was sealed in the top of the column with a one-hole rubber stopper, the outlet tube was clamped shut and the column filled to about two-thirds capacity with buffer. A slurry containing a sufficient amount of gel to fill the column was added to the funnel and stirred with a mechanical stirring apparatus. After about 12 cm of gel had settled at the bottom of the column, the clamp was loosened to allow a flow rate of about ten ml of buffer per hour. . After all of the gel material was packed, the funnel was removed and the column was washed with buffer before use. The void volume of the column was estimated to be approximately 40% of the column volume.

Samples were carefully layered on the top of the gel surface and

the eluting buffer was either pumped through the column at a constant flow rate with a Mini-pump (Milton Roy Co.) or was allowed to flow by gravity. Fractions were collected with a fraction collector (Gilson Medical Electronics). The size of the fraction was regulated by a specified number of drops when a gravity flow system was used or a specified time interval when the pump was used. The absorbance at 260 mμ and 280 mμ was measured in either a Beckman DB or Gilford Model 2000 Spectrophotometer.

Sucrose gradients were formed by mixing two solutions of different densities in a chamber of the same general design as that of Britton and Roberts (11). Gradients were 5 to 20% or 10 to 30% sucrose in 0.4 to 0.8 M NaCl. The combining solutions in the mixing chamber were stirred with either a vibrating metal stirrer or a stream of air bubbled from a Dispo (Scientific Products Co.) pipette. Gradients were deposited in 1 inch by 3 inch cellulose nitrate centrifuge tubes. The sample was layered on top of the sucrose solution with a pipette, and the tubes were placed in an SW 25 or SW 25.1 rotor and spun for 16 hours at 25,000 rpm in a Model L, L2, or L65B Spinco ultracentrifuge.

Three methods of collection were used during the sequence of experiments. The first method consisted of puncturing the bottom of the tube with a 21 gauge needle which had been cut off, sharpened to a double bevel and inserted in a rubber stopper. Forty drops

(0. 7 ml) were then collected per tube and read on a Beckman DB Spectrophotometer.

In the second method, an Isco gradient fractionater was used with the Gilson Medical Electronics (GME) UV flow monitor connected to a Heath servo recorder. The samples were collected by a GME 2000 fraction collector. The percent transmittance was obtained instead of the optical density.

The third and best method made use of a Harvard Apparatus Company Peti-pump (50 ml capacity; 2 ml/min flow rate) connected by a tygon tube to a 16 gauge hypodermic needle. The centrifuge tube was held in an Isco centrifuge tube holder with a teflon top and a stainless steel bottom. The tube was clamped between the two parts of the holder with a wooden frame. The pump was filled with 88% glycerol, the tube punctured at the bottom, and glycerol pumped into the cellulose nitrate tube to displace the sucrose. The gradient was conducted from the teflon top by PE 100 polyethylene catheter tubing (Clay Adams) to a Gilford 2000 recording spectrophotometer equipped with a flow cell. The second and third systems allowed visualization of the bands of absorbance as the gradient was removed from the tube. The protein components were isolated in separate tubes or collected in equal fractions with the GME fraction collector. The tubes containing fractions of interest were pooled and dialyzed against 0. 4 M NaCl to remove the sugar and excess NaCl.

The method of amino acid analysis was that of Moore and Stein (50) and was performed by Dr. Robert Becker and Robert Howard of the Department of Biochemistry and Biophysics. Separated components were transferred to lyophilization tubes (Kontes Glass Co., 5 ml capacity) and after evaporation at reduced pressure, 1 ml of constant boiling HCl was added. The atmosphere in the vial was alternately flushed with nitrogen and evacuated ten times and the vial was sealed while evacuated. The protein was then hydrolyzed for 20 hours or 68 hours in boiling toluene (110^{o} C). The HCl was removed after hydrolysis by rotary evaporation at reduced pressure and after rinsing with 1 ml of water and re-evaporation under the same conditions, the sample was taken up in 2.5 ml of pH 2.2 citrate buffer. Aliquots of the hydrolysate were chromatographed on a Beckman-Spinco 120B Amino Acid Analyzer. Labeled yolk protein was prepared by injecting female cockroaches with developing ovaries with 50 µl (2.5 µc) of uniformly labelled leucine-C^{14} (250 mc/mmole, New England Nuclear). After a suitable interval the yolk protein was prepared as described. This yolk protein was fractionated by the sucrose density gradient method and 0.8 ml fractions were collected. A sample of unlabeled yolk protein was added to each fraction and the total fraction was precipitated in 10% trichloroacetic acid (TCA). The precipitate was

collected on membrane filters (B-6, Schleicher & Schuell Company). The membranes were added to scintillation vials together with 1 ml of 0.1 N NaOH and the samples were counted in Bray's solution (10) in a Packard liquid scintillation counter.

Lipids were extracted using a modified method of Bligh and Dyer (6). Yolk protein dissolved in 0.4 M NaCl was mixed in a mixing cylinder with chloroform and methanol in proportions of 4:5:10, respectively. This mixture was shaken vigorously for two minutes and then five parts of chloroform were added. After one minute of shaking, five parts of water were added and the mixture was shaken for an additional minute. This mixture was filtered and the filter paper and apparatus washed with about 25 ml of chloroform. The filtrate and wash were stored at 4° C overnight in a stoppered mixing cylinder and the volume of the chloroform layer was recorded. A rotary evaporator was used to evaporate an aliquot of the chloroform layer from a tared round bottom flask. The flask containing the lipid was stored in a desiccator over Granusic (J. T. Baker Co.) and weighed periodically until a constant weight was obtained. The total weight of lipid was calculated from the weight per aliquot and the total volume of the chloroform layer. This weight was compared to the mg of yolk protein and the results were expressed as a ratio of mg lipid/mg protein.

Thin-layer chromatography was carried out using prepared

silica gel plates (Eastman Chromagram Sheets 6061) according to a slightly modified method of Mangold (47). The plates were activated by heating at 105° C for at least one hour. A hexane-ether (85:15) solvent system was used to separate the classes of lipids and a more polar solvent system of chloroform, methanol and ammonium hydroxide (65:25:4) was used to separate the phospholipids. Iodine vapor was used as a non-specific lipid stain and Dragendorff reagent was used as a specific stain for phospholipids (20).

Sedimentation studies were performed at 20° C with a Spinco Model E ultracentrifuge with standard and wedge single sector cells and Schlieren optics. Photographs were taken on Kodak metallographic plates, and the boundary positions were measured with a Nikon comparator. A plot of the log of the migration of the protein as a function of time was used to obtain S_{20} by the following formula (59):

$$S = \frac{1}{\omega^2} \frac{d \ln \bar{x}}{dt}$$

where S = sedimentation coefficient
ω = angular velocity
t = time
x = the distance from the center of rotation.

These values were not corrected to standard conditions. The relative amounts represented by the peaks were determined by cutting out and weighing tracings of the peaks. The data were

corrected for radial dilution using the formula:

$$\frac{C_o}{C_a} = \frac{r_a^2}{r_o^2}$$

where C_o = concentration at the miniscus
C_a = concentration at the point a in the cell
r_a = distance of point a from the axis of rotation
r_o = distance of the miniscus from the axis of rotation

A Johnston-Ogston correction was not made.

Molecular weights were determined at 20°C by the high-speed equilibrium method of Yphantis using Rayleigh interference optics (86). Protein concentrations of 0.6-1.3 mg/ml were used and the Spinco Model E analytical ultracentrifuge was run at 8,000-10,000 rpm for 10-18 hours. Initial overspeeding and underspeeding were employed to hasten the attainment of equilibrium. The fringes were measured on a Nikon comparator. The formula for calculating the molecular weight is given in the Results section.

Partial specific volumes ($\bar{v}$) were determined by pycnometry in a 25° C water bath. Pycnometers were made in the Oregon State Department of Chemistry glass blowing shop by drawing out the top of a 25-ml erlenmeyer flask and attaching a neck of 1 mm capillary tubing. A line was etched on the neck and the vessel was filled to this line with distilled water. The weight and density of the water was used to calculate the volume of the pycnometer. A 25-ml syringe with a six-inch needle was used to fill the vessels with

protein or buffer solutions and the weights of the solutions were obtained. From these weights and the known volumes, the densities of the solutions were calculated. Aliquots of the buffer and protein solutions were frozen in liquid nitrogen, dried in a vacuum, and weighed to determine the salt and protein concentrations. The apparent specific volume, ϕ, could be calculated by the formula:

$$\phi = \frac{1}{\rho s}\left(1 - \frac{\Delta \rho}{C_1}\right)$$

where ρs is the buffer density, $\Delta \rho$ = (density of solution - density of the buffer), and c_1 = concentration of the protein in the solution (mg/ml). For most proetins $\phi = \bar{v}$ at concentrations of 20-30 mg/ml or lower (17).

In order to compare the density of the two components of the mixture used in pycnometric measurements, a banding experiment was performed as described by Ifft and Vinograd (37). About 0.2 mg/ml of each component of the yolk protein was dissolved in cesium chloride solution with a density of 1.32 g/ml, the density of the protein as found by pycnometry. These solutions were centrifuged in synthetic boundary cells with standard and wedge windows. The

centrifuge was run at 20°C at 48,000 rpm and Schlieren optics were used to visualize the position of the protein band in the cell.

IV. RESULTS

Qualitative and Quantitative Changes in Yolk Protein During Vitellogenesis

The analysis by analytical ultracentrifuge of yolk protein from small ovaries is compared to that of more nearly mature ovaries in Figure 1. Two major components were found in yolk protein from the smaller ovaries, but in the larger ovaries, most of the yolk was composed of the faster sedimenting peak. The sedimentation values for the two peaks were found to be 27S and 14S. In addition, a larger compound with sedimentation values ranging between 39S and 44S was seen consistently at pH 7 and lower. This fraction, which will be designated 40S, constituted a very small proportion of the yolk protein fraction and had an OD_{280}/OD_{260} ratio of one or smaller. The 40S peak had already sedimented to the bottom of the cell and is not seen in Figure 1.

Yolk from ovaries at several stages of development was analyzed using Bio-Gel A-15 m agarose and the results are presented in Figure 2. In spite of the poor resolution the change in proportion between the two components can be seen by the change of the direction of the skew of the initial peak. The OD_{280}/OD_{260} ratio of this peak was 1.5 - 1.6 in the small ovaries and increased to 1.8 - 1.9 in the more nearly mature ovaries. Another peak occurred after

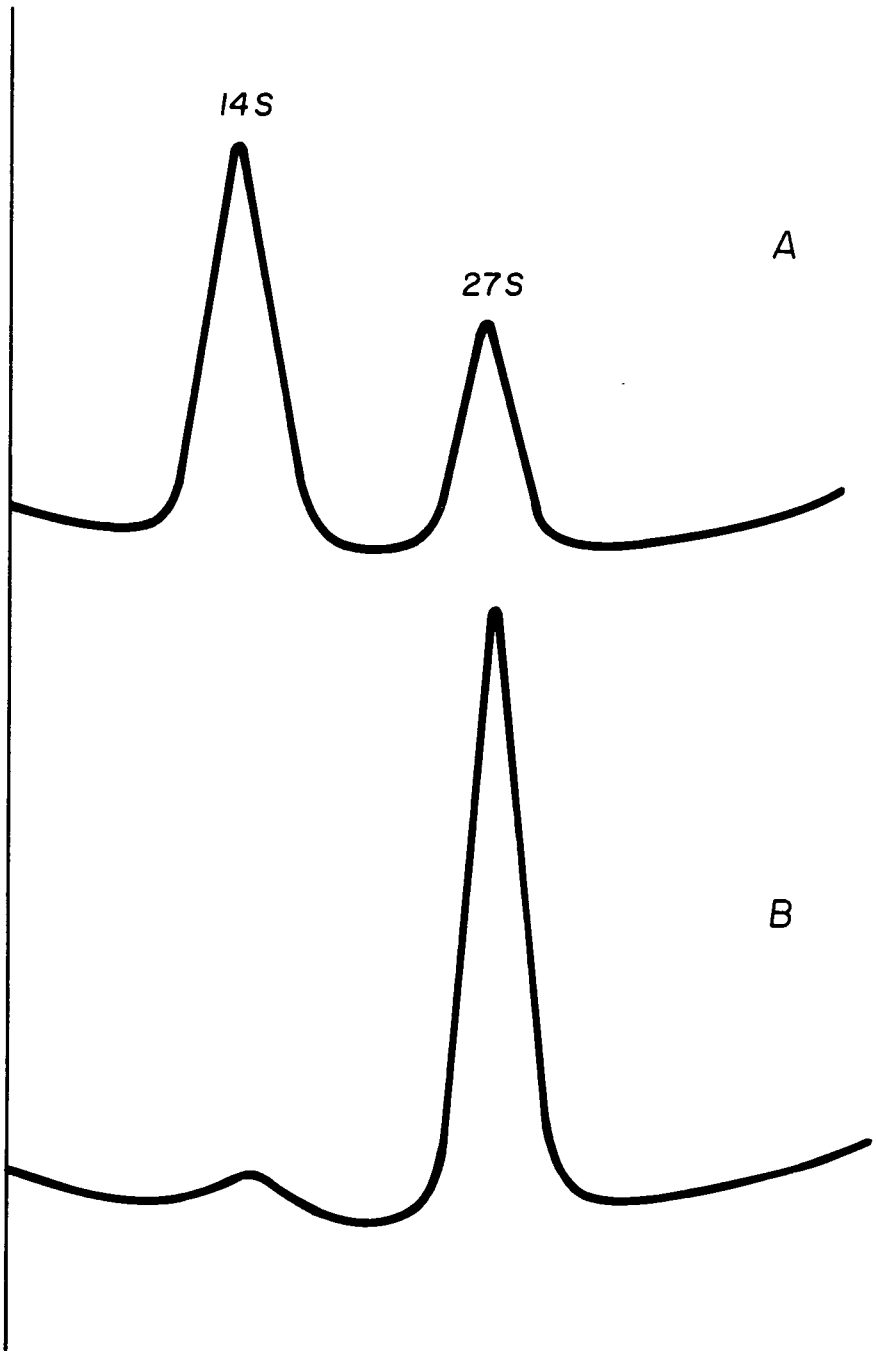


Figure 1. Schlieren patterns of the yolk protein from ovaries in two different stages of development. This picture was taken 59 minutes after the rotor had reached 31, 410 rpm at 20°C. The direction of sedimentation was to the right.

A. Basal oocyte 2.33 mm, 4.8 mg/ml.

B. Basal oocyte 5.08 mm, 5.7 mg/ml.

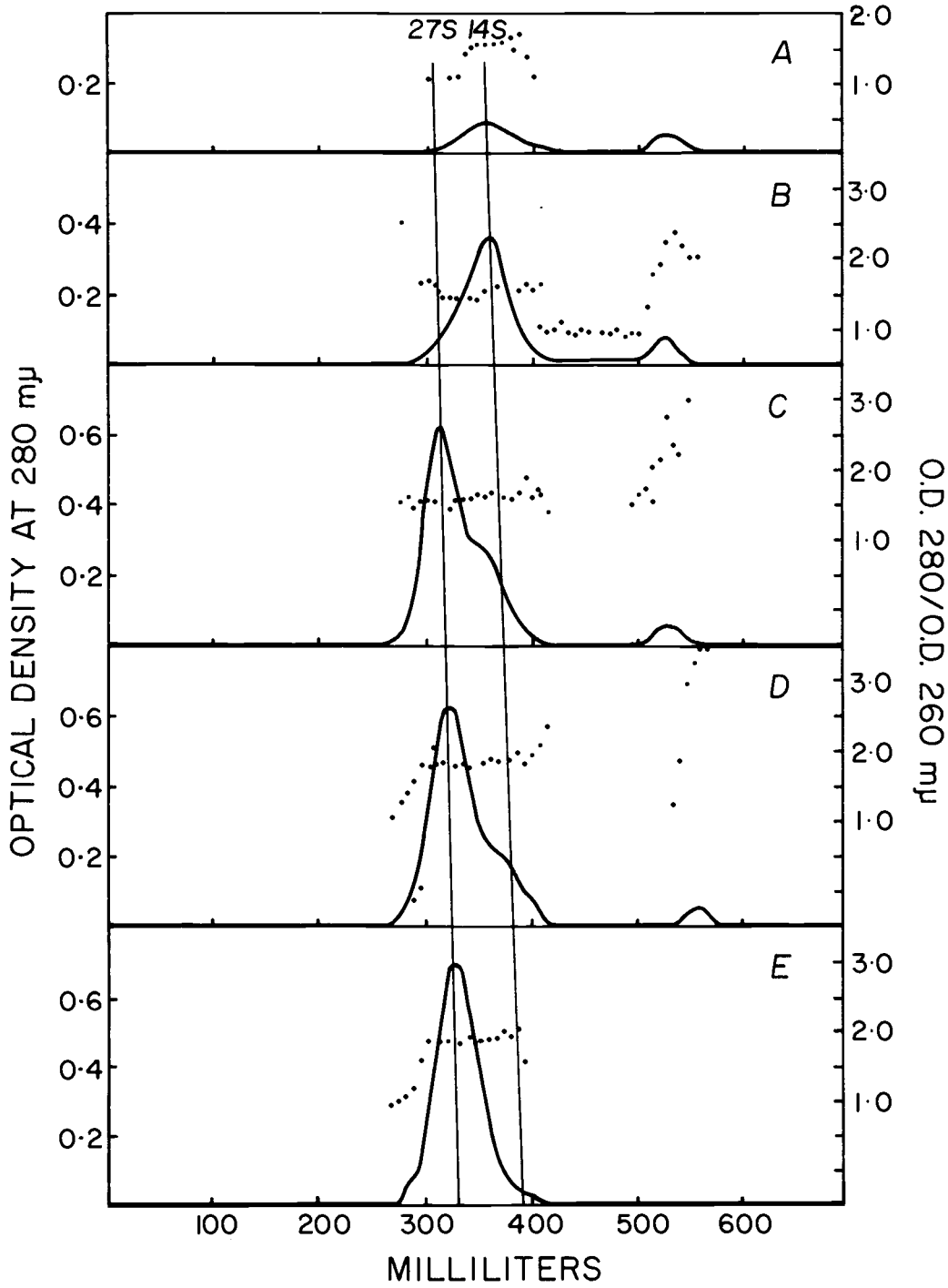


Figure 2. A comparison of the yolk protein profiles from a range of ovary sizes on a long column of Bio-Gel A-15m eluted at 26 ml/hr with 0.4 M NaCl at 2°C.

Ovary sizes and estimated column load:

A. 1.83 mm, 12 mg
B. 2.67 mm, 32 mg
C. 3.25 mm, 40 mg
D. 3.75 mm, 40 mg
E. 4.45 mm, 40 mg

further elution which often had an OD_{280}/OD_{260} ratio of 2.5 or greater. More information on the later peak (the third peak) is given in the section pertaining to the separation properties of different gels.

A similar type of experiment on ovaries at different stages of development was performed using sucrose density gradient separation. The resolution was much better and a quantitative description of the 14S and 27S peaks is given in Figures 3 and 4. In yolk from one group of ovaries with eggs smaller than 1.0 mm, neither the 14S nor 27S protein was found. When the ovaries first began to mature, the 14S fraction formed the major portion of the protein. At an egg length of about 2.5 mm, the two fractions were present in equal amounts. By the time the eggs reached 3 mm, the 27S accounted for the major part of the protein and when a length of 4 mm was reached, about 90% of the protein was 27S. Included in Figure 3 are the results of an analysis (by the analytical ultracentrifuge) of two pairs of ovaries with terminal oocytes of 5.1 mm and 2.3 mm. The relative proportions of the 14S and 27S were found by comparing the areas under the Schlieren peaks. The results obtained in this manner fit well into the pattern of results found by sucrose gradient analysis.

In an earlier study the Kjeldahl method was employed to analyze nitrogen and determine the relationship between the length of the

basal oocyte and the protein content per ovary pair (19). The results of that study were used with the results expressed in Figure 3 to find the amount of protein in each component in a series of ovaries of different sizes (Figure 4). The amount of both of the components increased until the eggs reached a length of about 2.5 mm. At that length the content of 14S reached a level of 2 to 3 mg per pair of ovaries and showed little change from this amount during the rest of the period of egg maturation. The amount of 27S protein continued to increase and accounted for the large overall increase in protein content of the eggs.

Female cockroaches with developing ovaries were injected with C^{14}-leucine. The ovaries were removed at intervals after injection and yolk protein was prepared and fractionated by density gradient centrifugation. Rapid incorporation of leucine into protein began about four hours after injection. Most of the radioactivity incorporated during the first eight hours was found in the 14S fraction. The results from animals prepared 8, 24, and 72 hours after injection are given in Figure 5. During the first 24 hours, the specific activity of the 14S component substantially increased with a slower increase in the 27S component. In the interval between 24 and 72 hours, the specific activity of the 27S component continued to increase, whereas that of the 14S decreased. In separate experiments it was found that the initial incorporation of the C^{14}-leucine took

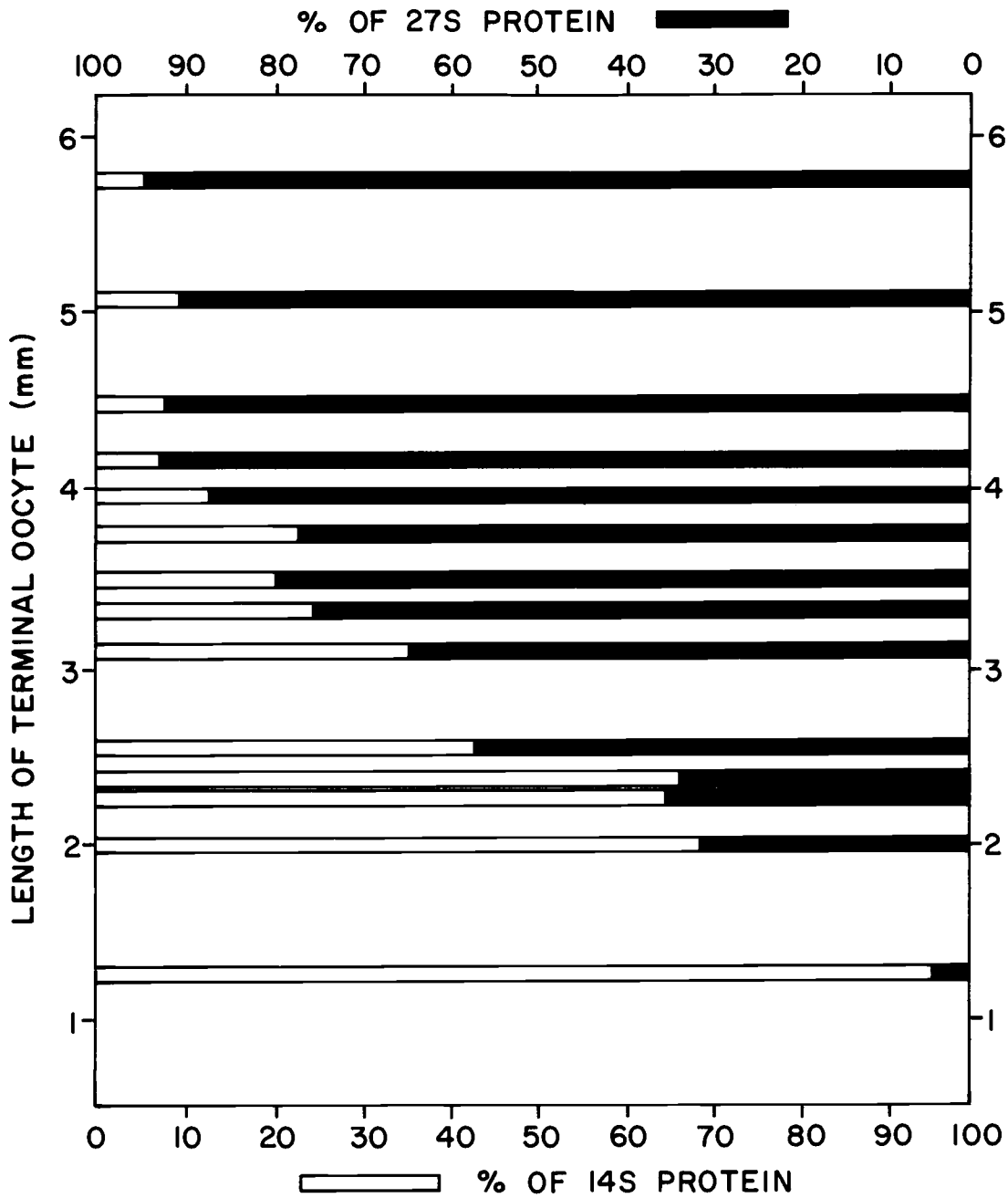


Figure 3. Change in relative proportions of 14S and 27S proteins with respect to ovary size. Analyses of ovaries with 5.1 and 2.4 mm oocytes were made by comparison of the areas under Schlieren peaks in an analytical ultracentrifuge run. All other analyses were based on sucrose gradient measurements.

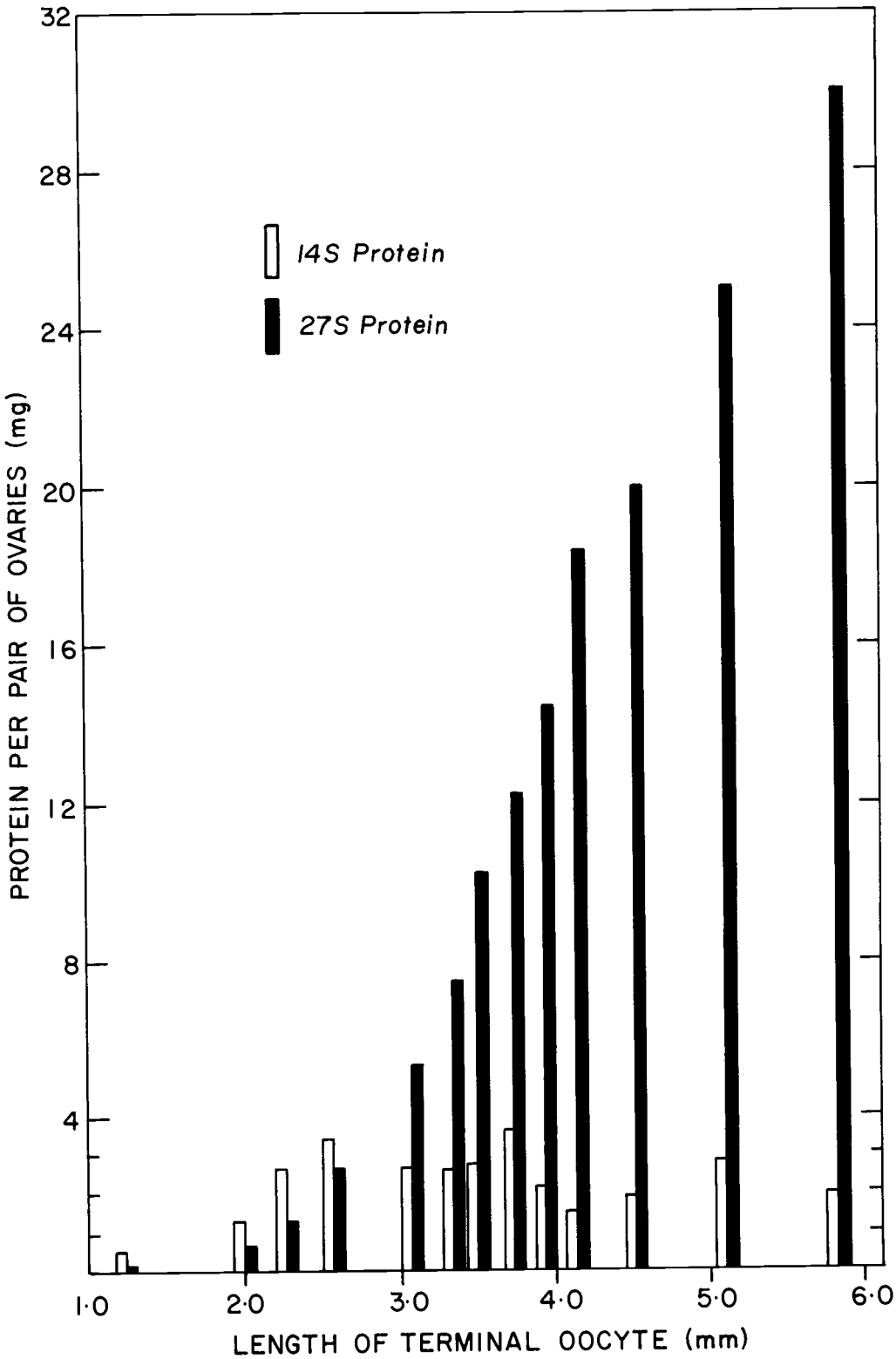
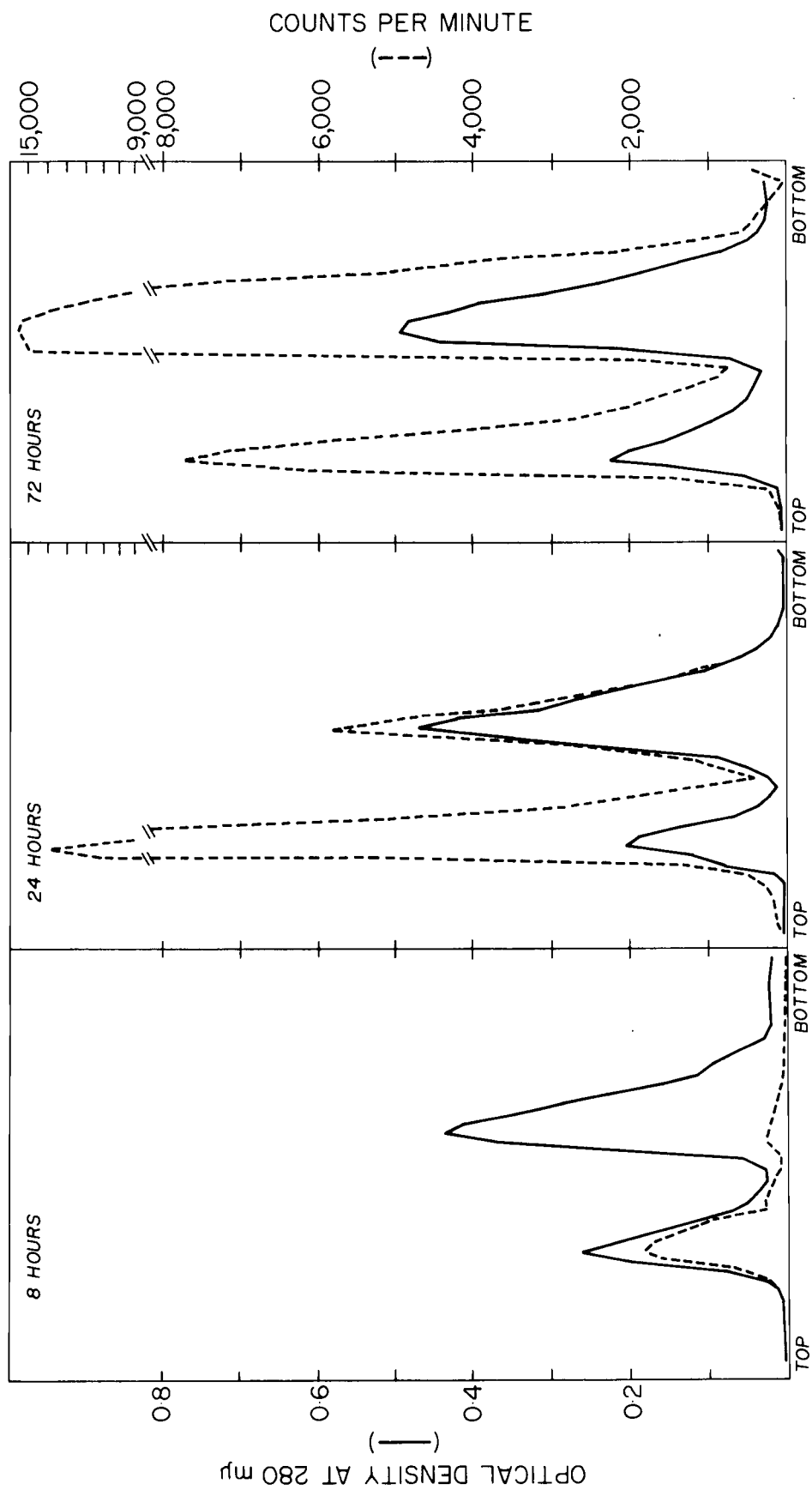


Figure 4. Amount of 14S and 27S protein found in ovaries in different stages of development. Calculations were based on the relative proportion of each peak shown in Figure 3 and on the total protein per ovary pair (19).


COUNTS PER MINUTE
(---)
15,000
9,000
8,000
6,000
4,000
2,000
72 HOURS
24 HOURS
8 HOURS
BOTTOM
TOP
BOTTOM TOP
BOTTOM TOP
TOP
OPTICAL DENSITY AT 280 mμ
(——)
0·8
0·6
0·4
0·2

place in the 14S peak regardless of the relative proportions of the two components.

Physical and Chemical Characteristics of Yolk Protein

The above biological data point out that an understanding of the relationship between the 14S and the 27S components is essential to an understanding of vitellogenesis in this insect. For this reason, further analytical studies were undertaken and these are presented below.

The method most often used in this paper to estimate the quantity of protein was that of optical density at a wavelength of 280 mμ (OD_{280}). The method of Warburg and Christian (43) utilizes a ratio of the OD_{280}/OD_{260}. This ratio which was usually about 1.65 - 1.80 for the protein yolk solution indicates that there is less than 0.25% nucleic acid and that the mg protein/OD_{280} ratio is 0.92 - 0.90. Since many compounds absorb at 280 mμ and could cause erroneous values, two other methods of protein analysis were used and compared to the OD_{280} (Table I). These two methods were that of Lowry and a calculation of the total weight of amino acids from the amino acid analysis. For details of this calculation see the Materials and Methods section. Unfortunately, a significant amount of loss occurred during the amino acid analysis of one of the 27S samples so the values of only one of the two 27S fractions will

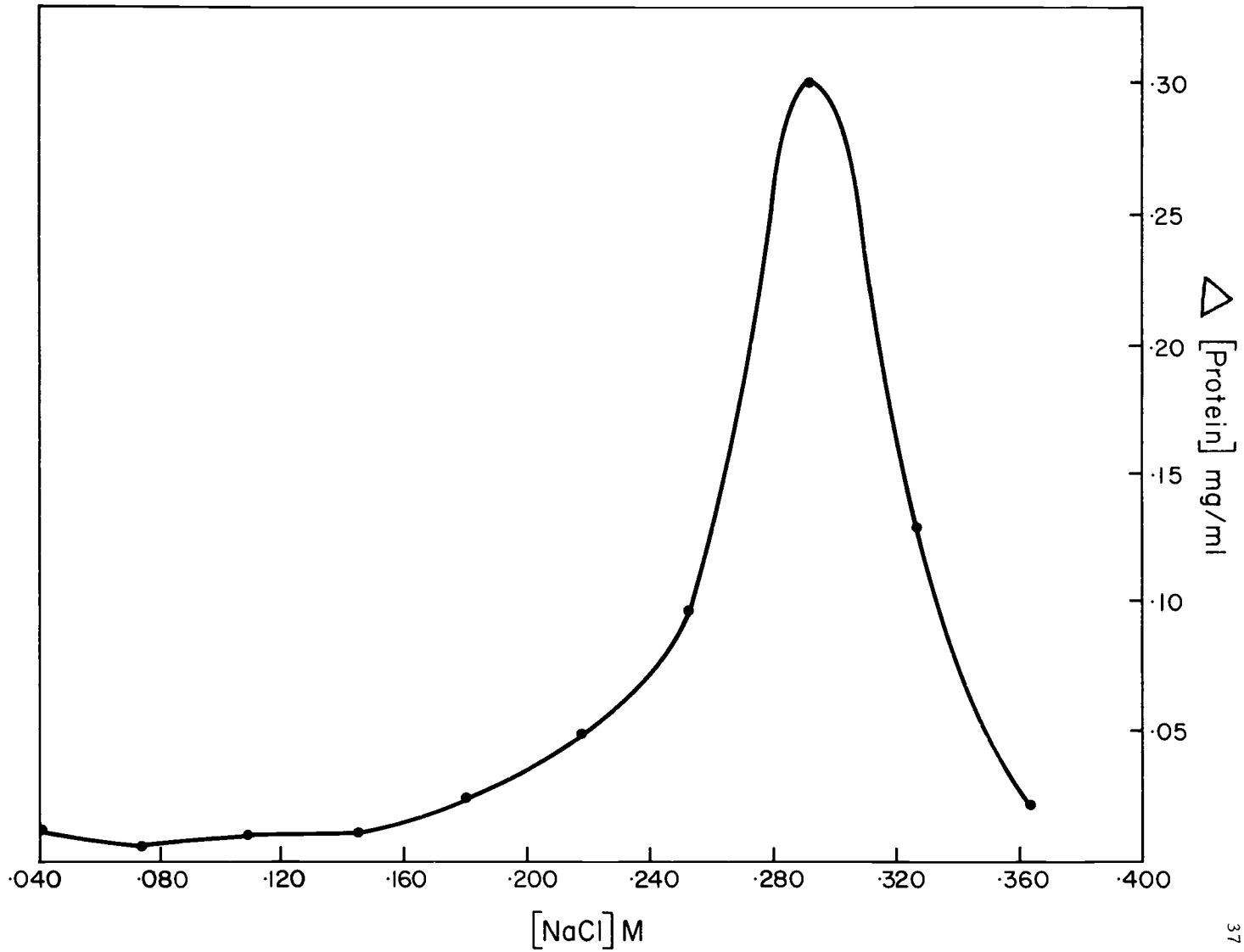
be useful. In the case of the Lowry method, the mg protein/OD_{280} ratio was 0. 83 compared to a value of 0. 86 for the amino acid analysis method. An average mg protein/OD_{280} ratio of 0. 86 will be used for conversion of OD_{280} to mg protein.

Table I. Comparison of Three Different Methods of Protein Analysis

Protein Source	OD_{280}	Lowry method mg protein	Amino acid analysis mg protein	mg protein per OD_{280}
27S Peak	3. 0		2. 51	0. 84
14S Peak	3. 0		2. 64	0. 88
27S Peak	2. 0		1. 13	0. 57*
14S Peak	2. 0		1. 72	0. 86
5. 4 mm oocyte	. 587	. 467		0. 80
5. 6 mm oocyte	. 545	. 470		0. 86
5. 3 mm oocyte	. 539	. 457		0. 85
5. 8 mm oocyte	. 585	. 473		0. 81

*Sustained loss during analysis

In order to determine the effect of ionic strength on the solubility of yolk protein, 6. 24 mg of yolk protein were suspended at 0° C in centrifuge tubes containing 5. 0 ml of solution ranging from 0. 364 M to 0. 04 M NaCl. These tubes were stored on ice overnight and were centrifuged the following day. The OD_{280} of the supernatants were measured and used to estimate the protein concentrations. The difference in protein concentration between successive tubes as the concentration of NaCl was lowered indicated the amount of


·30
·25
·20
·15
·10
·05
Δ [Protein] mg/ml
·040
·080
·120
·160
·200
·240
·280
·320
·360
·400
[NaCl] M

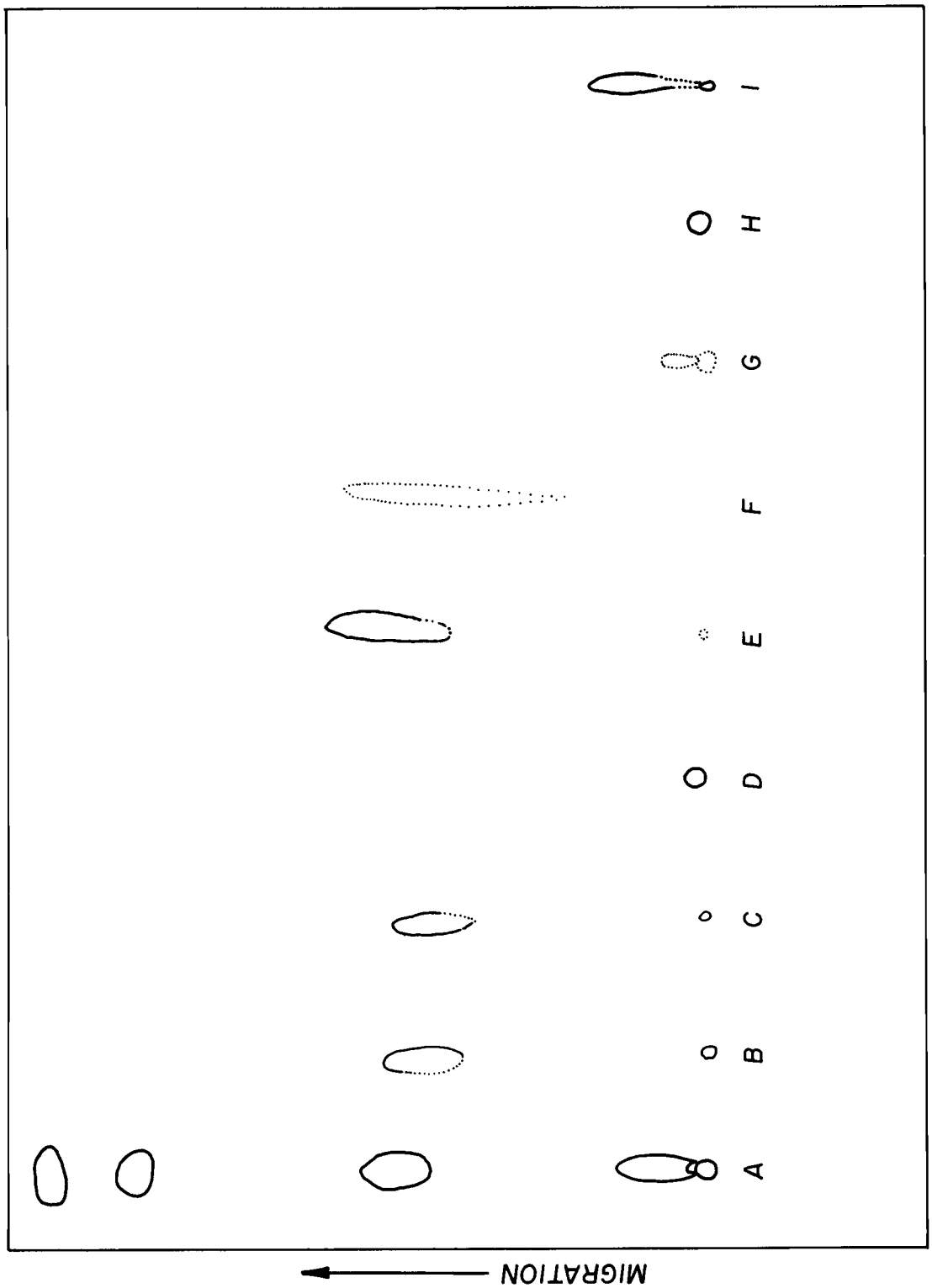
protein which was precipitated due to the lower ionic strength. This is pictured as a derivative curve in Figure .6. The results show that most of the protein is precipitated as the NaCl concentration drops from 0. 325 M to 0. 250 M. The maximum solubility of the protein was not determined at concentrations of NaCl greater than 0. 328 M but protein solubilities of 20 mg/ml or higher were routinely obtained in 0. 4 M NaCl.

The results of a quantitative analysis of the lipid content of nearly mature ovaries is given in Table II. The average ratio of mg lipid/mg protein was 0. 0943 in yolk protein from ovaries of the size analyzed.

Table II. Weight of Lipid in Yolk From Nearly Mature Ovaries

Pairs of Ovaries	Average length mm	Range mm	Protein analyzed mg	Lipid mg	mg lipid per mg protein
12	4. 5	3. 5-5. 3	196. 1	18. 8	0. 0955
9	4. 7	4. 2-5. 2	306. 0	28. 7	0. 0938
6	4. 7	4. 2-5. 2	219. 5	20. 5	0. 0935

Thin layer chromatograms of the lipids from extracts of large (5. 00 mm) and small (2. 17 mm) ovary homogenates were developed in a solvent system of hexane and ether (85:15) (Figure 7). No difference in composition of the samples from large and small eggs was observed. Both contained at least two components. On the


I
H
G
F
E
D
C
B
A
MIGRATION

basis of staining with Dragendorff reagent, the component which remained at the origin in Figure 7 appeared to be a phospholipid. Phospholipids would be expected to remain at the origin with the solvent system used. The second spot was thought to be a triglyceride because it migrated the same distance as the triglyceride standards which were included. A chloroform extract of yolk protein is also shown in Figure 7. The triglyceride was not present in the yolk protein and only the phospholipid was detected. A chloroform extract of the lipid cap which forms at the top of the tube when ovary homogenates are centrifuged, appeared to contain mainly triglyceride.

An attempt to fractionate the phospholipid with a solvent system of chloroform, methanol, and ammonium hydroxide (65:25:4) separated the lipid of the yolk into five spots and the lipid of the egg into two spots (Figure 8). With this system neutral lipids were expected to move at the front. No further attempt was made to identify these components.

Separation of the 14S and 27S Components of Yolk Protein

Further studies of the relationships of yolk protein structure to yolk deposition required a reliable means for separation of the various components in quantities sufficient for further analysis. An attractive method is that of molecular sieving which if successful provides not only a means of separation but also for obtaining several

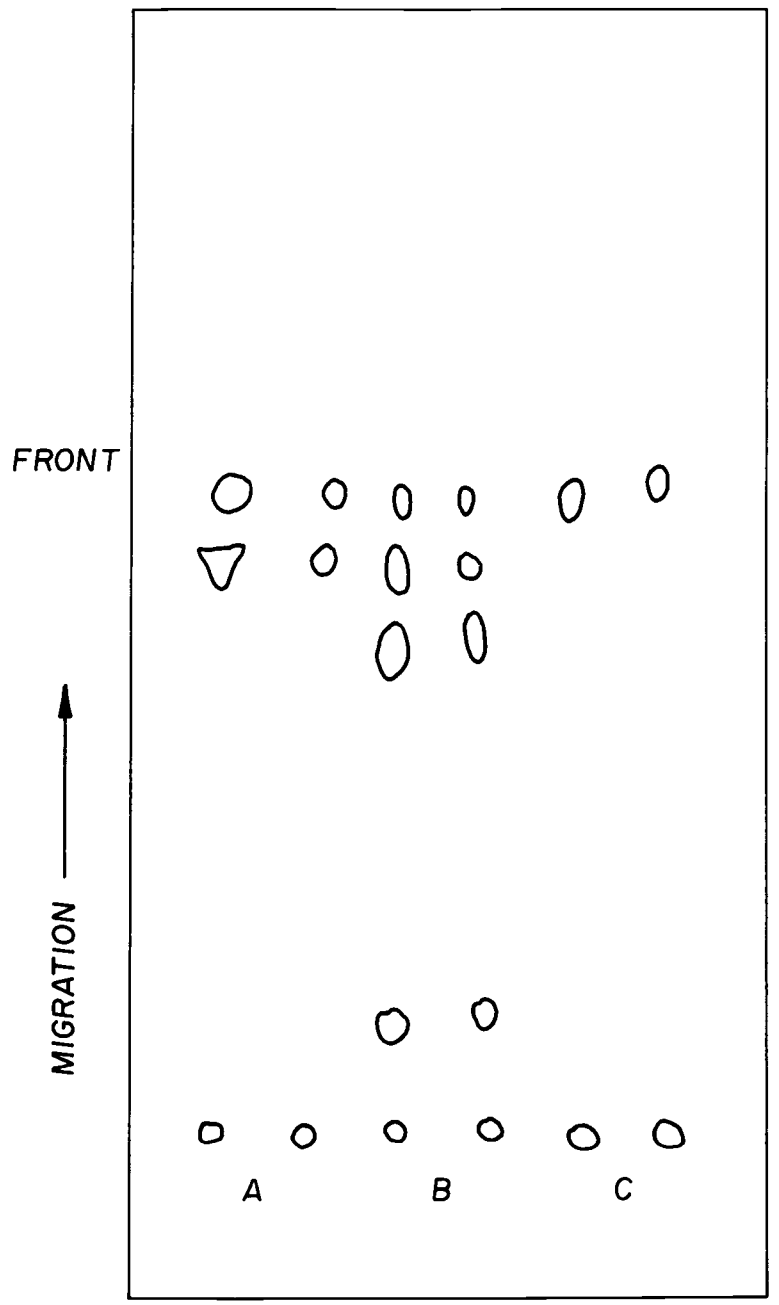


Figure 8. Separation of lipids on a thin-layer chromatogram developed in chloroform, methanol and ammonium hydroxide (65:25:12) and stained with iodine vapor.

A. Cholesterol oleate
cholesterol
triolein
oleic acid
hydrogenated lecithin

B. Chloroform extract of yolk from 5.00 mm oocytes

C. Chloroform extract of 5.00 mm oocytes

physical constants, Stokes ratio for example, useful in characterizing the protein. Several of the gels commercially available at the time were tested. During the course of these experiments, the density gradient method was devised and since that method seemed to be suitable for the immediate requirements of the research, the work with the gels was discontinued. There are several limitations associated with the density gradient method and a column separation would substantially facilitate the characterization of the yolk protein and the associated subunits. The information below is presented as a basis for further studies with the method.

Analysis using Sephadex G-200 showed that all of the material absorbing at 280 mμ was eluted with the void volume. Agarose gels were then tested since they are suited for separation of very large macromolecules. Initially the A-5m gel and the A-15m gel were chosen so that both the 14S and the 27S proteins would fall within the separation range of the gel. This condition would allow the detection of any aggregate larger than the 27S which might be present. With Bio-Gel A-1.5m and perhaps with Bio-Gel A-0.5m better separation of the 14S from the 27S would be expected. However, the 27S would be eluted at the void volume along with any other larger molecules which might occur and these larger molecules would not be detected. The last two gels A-1.5m and A-0.5m were not tested.

The preliminary column determinations were made using

phosphate buffer. The yolk protein dissolved in its usual solvent of 0.4 M NaCl was measured to be pH 5.8, therefore a sodium phosphate buffer at this pH was used. The ionic strength of 0.2 of the buffer was increased by adding NaCl to a concentration of 0.2 M.

A comparison of the results obtained with the two sizes of agarose is given in Figure 9. Bio-Gel A-5m and A-15m were used in 45 by 2.5 cm columns at room temperature to obtain Figure 9A and B. For graph C, Bio-Gel A-5m was used in a 95 by 2.5 cm column and the filtration was carried out in a cold room at 2^{o} C. The 45 cm and 95 cm columns will be referred to as short and long columns respectively.

A comparison of Figure 9A (A-5m) and 9B (A-15m) indicates that under the same conditions the peaks were sharper when A-15m was used but the difference in void volumes was somewhat greater when A-5m was used. With both gels in a short column, the 14S and 27S fractions could easily be distinguished, but were eluted together. The comparison between A-5m in a short column (Figure 9A) and in a long column (Figure 9C) demonstrated the increased separation obtained by the use of the longer column.

With A-5m the ratio of elution volume of the third peak to that of the 27S peak is about 3. The literature supplied by Bio-Rad (price list T - July 1, 1968, p. 44) indicates molecular weight values only to a ratio of 2.4 for elution volume/void volume. The

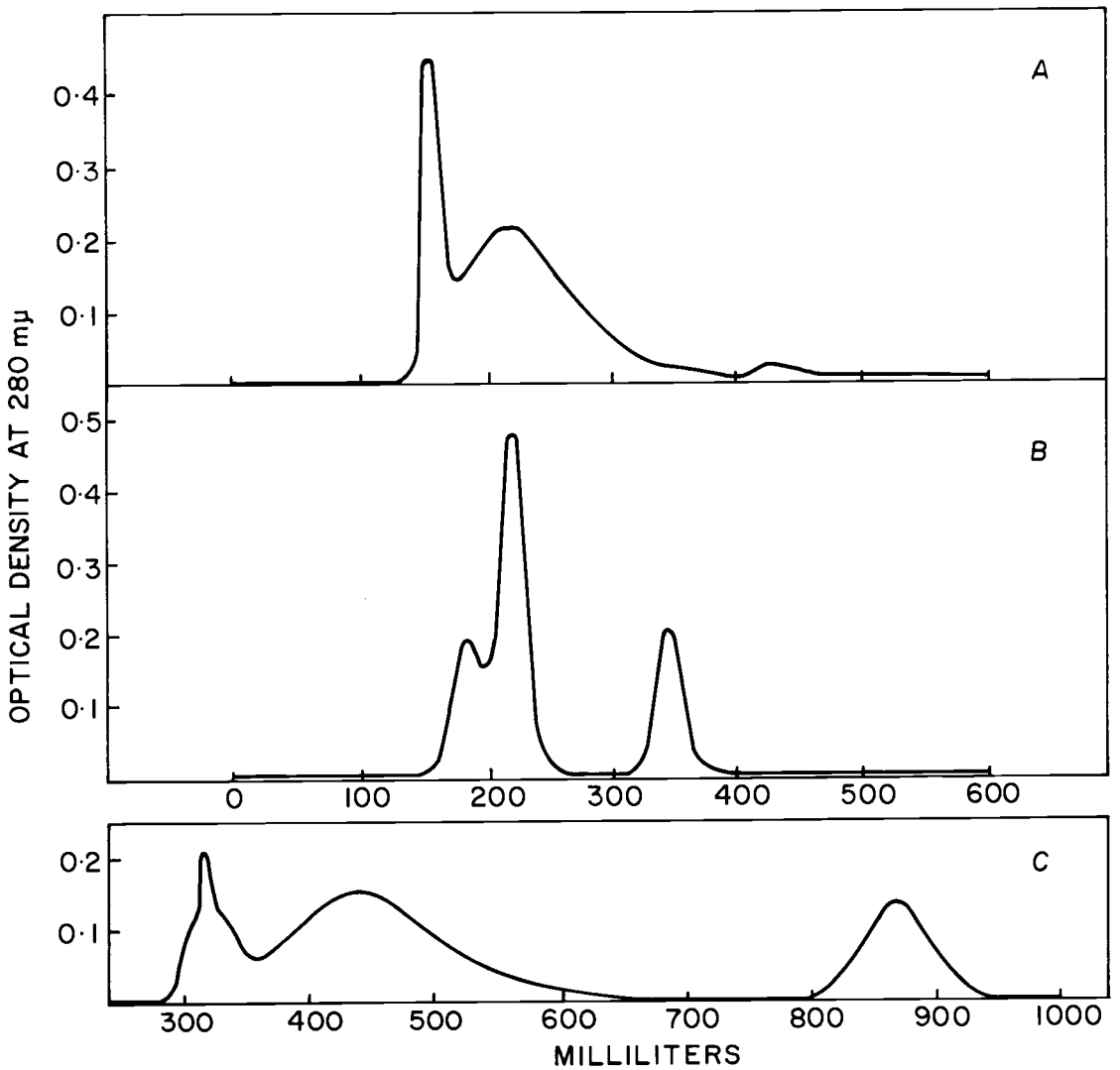


Figure 9. Comparison of the separation of yolk protein from small ovaries on Bio-Gel A-5m and A-15m in long and short columns eluted with pH 5. 8 phosphate buffer.

A. Bio-Gel A-5m, 24°C short column, 38 mg yolk from ovaries with 2. 33 mm average basal oocyte length.

B. Bio-Gel A-15m, 24°C, short column, 20 mg yolk from ovaries with 1. 75 mm average basal oocyte length.

C. Bio-Gel A-5m, 2°C, long column, 32 mg yolk from ovaries with 1. 75 mm average basal oocyte length.

ratio of the third peak elution volume to that of 27S obtained with the A-15m gel was about 2. 2; however, the 27S peak would not be expected to be eluted with the void volume when this gel is used so the results are not contradictory. In both cases the third peak was eluted in a position indicating that the material comprising the peak was retarded on the gel by some factor other than molecular size. A possible explanation for the late elution of this peak could be partial adsorption of the material on the agarose gel.

Flow rates between 13 ml/hr and 75 ml/hr were tested on a short column of A-15m. Somewhat better resolution was obtained with the slowest rate. However, at this rate, the pump would occasionally cease to function so rates of 25 or 50 ml/hour were used in most experiments.

Bio-Gel A-50m was tested but the 14S and 27S fractions came off in one peak. A very small amount of a large molecular weight fraction was separated from the 14S and 27S fractions in a preparation which had been heated to 65^{o} C for five minutes. This fraction accounted for less than 5% of the total protein and may be the 40S or some denaturation product. Additional experiments involving heating of the protein are detailed later.

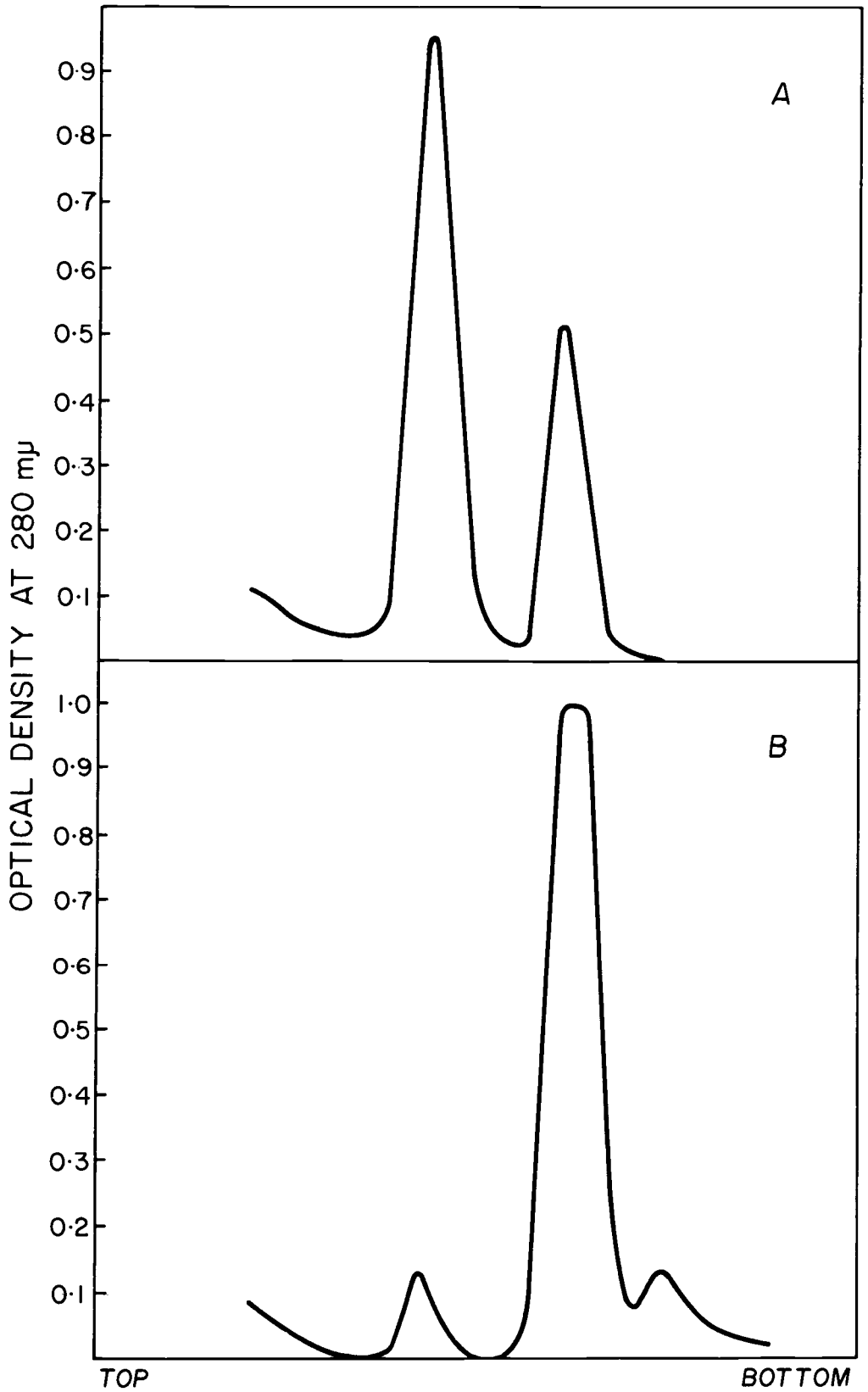
Bio-Gel P-300 was also used in an attempt to separate the components of the yolk, but the resolution obtained from a short column was inferior to that obtained with both Bio-Gel A-5m and

A-15m.

Excellent separation of 14S from 27S was obtained using gradients of 5% - 30% sucrose in 0.4 - 0.8 M NaCl. An example of the separation by this method can be found in Figure 10. This technique was used as our preparative method. Occasionally a small peak of material heavier than 27S was resolved in these gradients. This peak is thought to correspond to the 40S peak. If such a peak was not resolved, the faster sedimenting edge of the 27S peak usually showed a lower OD_{280}/OD_{260} ratio than the remaining portion of the peak. When the 27S fraction was isolated for further analysis, this portion of the peak was not included. Separated components obtained in this manner were dialyzed against 0.4 M NaCl in the cold room to remove excess salt and sucrose and the protein was precipitated by the addition of one to three volumes of water. Some of the protein was lost during the dialysis step, presumably by adsorption to the dialysis membrane. The Model E analytical ultracentrifuge was also useful for analyzing the preparations as has been demonstrated in Figure 1.

Amino Acid Analysis of the Yolk Protein

Yolk for amino acid analysis was prepared from 40 pairs of small ovaries (0.92-2.67 mm; ave 2.24 mm). The 14S and 27S peaks were isolated by density gradient centrifugation and dialyzed


OPTICAL DENSITY AT 280 mμ
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
A
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
B
TOP
BOTTOM

against 0.4 M NaCl to remove excess NaCl and sucrose. Two sets of samples were used. Samples containing 3.0 OD_{280} units were hydrolyzed for 68 hours and samples containing 2.0 OD_{280} units were hydrolyzed for 20 hours. The hydrolysates were analyzed and the data are reported in μmoles amino acid per sample (Table III, columns 1-4). These values were converted to mole fractions and are also given in Table III (columns 5-8).

In the case of the 14S 68-hour analysis, proline was not resolved. Therefore, the amount of proline was estimated by taking an average of the ratios of corresponding amino acids from the 14S and 27S analyses and correcting the proline value found for 27S by that average ratio.

A convenient way to express the data for comparison of the two proteins is to choose one of the amino acids and to calculate the ratio of each amino acid to the chosen one. Arginine was chosen due to its stability and the ratios are given in Table III (columns 9, 10, 12 and 13). If the two proteins have identical amino acid compositions, the corresponding ratios (individual amino acid to arginine) should be identical, The ratios from the 14S protein were divided by the ratios from the 27S protein, and the quotients are in fact very close to 1.00 (Table III, columns 11 and 14).

Table III. Results of Amino Acid Analysis

Amino Acid	27S (68)	14S (68)	27S (20)	14S (20)	27S (68)	14S (68)	27S (20)	14S (20)	27S (68)	14S (68)	14S/ 27S (68)	27S (20)	14S (20)	14S/ 27S (20)	27S(68) / 27S(20)	14S(68) / 14S(20)
	--Micromoles per sample--				--mole fraction x 10^2--				Res. /res. Arg.			Res. /res. Arg.				
Lysine	1.15	1.37	0.58	0.89	5.26	5.98	5.92	5.98	0.82	0.91	1.12	0.92	0.89	0.97	0.88	1.02
Histidine	0.72	0.72	0.28	0.47	3.30	3.14	2.86	3.11	0.51	0.48	0.94	0.44	0.46	1.04	1.16	1.04
Arginine	1.41	1.51	0.63	1.00	6.46	6.59	6.43	6.69	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Aspartic Acid	3.39	3.50	1.50	2.21	15.52	15.28	15.31	14.79	2.40	2.32	0.97	2.38	2.21	0.93	1.01	1.05
Threonine	1.08	1.22	0.51	0.77	4.94	5.32	5.20	5.15	0.76	0.81	1.06	0.81	0.77	0.95	0.94	1.05
Serine	1.45	1.65	0.79	1.22	6.64	7.20	8.06	8.17	1.03	1.09	1.06	1.25	1.22	0.98	0.83	0.89
Glutamic Acid	2.56	2.63	1.09	1.68	11.72	11.48	11.12	11.25	1.82	1.74	0.96	1.73	1.68	0.97	1.05	1.04
Proline	1.06	1.12*	0.49	0.75	4.85	4.89*	5.00	5.02	0.75	0.74*	0.99*	0.78	0.75	0.96	0.96	0.99*
Glycine	0.76	0.78	0.33	0.47	3.48	3.40	3.37	3.15	0.54	0.52	0.96	0.52	0.47	0.90	1.04	1.11
Alanine	1.24	1.25	0.52	0.80	5.68	5.46	5.31	5.36	0.88	0.83	0.94	0.82	0.80	0.98	1.07	1.04
Half Cystine	trace	trace	trace	trace	trace	trace	trace	trace								
Valine	1.65	1.52	0.67	1.02	7.55	6.63	6.84	6.83	1.17	1.01	0.86	1.06	1.02	0.96	1.10	0.99
Methionine	0.43	0.47	0.20	0.31	1.98	2.07	2.04	2.08	0.31	0.31	1.00	0.32	0.31	0.97	0.97	1.00
Isoleucine	1.14	1.19	0.47	0.71	5.22	5.19	4.80	4.75	0.81	0.79	0.98	0.75	0.71	0.95	1.08	1.11
Leucine	2.04	2.12	0.87	1.34	9.34	9.25	8.88	8.97	1.45	1.40	0.96	1.38	1.34	0.97	1.05	1.04
Tyrosine	0.82	0.90	0.47	0.70	3.75	3.93	4.80	4.69	0.58	0.60	1.03	0.75	0.70	0.93	0.77	0.86
Phenylalanine	0.94	0.96	0.40	0.60	4.30	4.19	4.08	4.02	0.67	0.64	0.96	0.63	0.60	0.95	1.06	1.07

*Estimated
(68) = 68-hour hydrolysis
(20) = 20-hour hydrolysis

Molecular Weight Determination of the Yolk Protein

The calculation of a minimum molecular weight from an amino acid analysis is based on the assumption that one mole of the least prevalent amino acid is present in one mole of the protein. The amino acid which occurs at lowest measurable concentration in both the 27S and 14S peaks is methionine. (A trace of cysteine was detected but not enough is present to be quantitated.) The following formula was used:

$$\text{Minimum molecular weight} = \frac{\mu\text{moles amino acid}}{\mu\text{moles methionine}} (\text{molecular weight of amino acid} - 18)$$

The amino acid values were taken from Table III. Eighteen represents the weight of water lost in peptide bond formation. The results of the calculations and molecular weights are given in Table IV. The average of the four values found is 5, 640.

Molecular weights of the 27S peak and the 14S peak were determined by the high-speed equilibrium method of Yphantis (86). The molecular weights were determined using the formula

$$\ln (c(r) - c(a)) = \frac{M (1 - \bar{v}\rho) \omega^2}{RT} \quad \frac{r^2 - a^2}{2}$$

where $c(r)$ = concentration at r
$c(a)$ = concentration at the miniscus
M = molecular weight
$\bar{v}$ = partial specific volume
ω = speed of rotation

Table IV. Grams of Each Amino Acid Contributing to the Minimum Molecular Weight as Calculated by Amino Acid Analysis

	Grams of respective amino acid per mole methionine			
Amino Acid	27S (68)	14S (68)	27S (20)	14S (20)
Lysine	341.2	370.5	371.7	369.7
Histidine	228.6	208.3	192.0	205.7
Arginine	509.8	497.6	492.0	503.9
Aspartic Acid	903.3	849.9	863.3	820.6
Threonine	252.8	260.3	257.9	251.2
Serine	292.3	303.2	344.0	342.7
Glutamic Acid	765.2	716.5	703.8	699.8
Proline	238.3	229.5	238.0	235.0
Glycine	100.4	93.9	94.2	86.5
Alanine	204.1	187.5	184.8	183.5
Half Cystine	trace	trace	trace	trace
Valine	378.7	317.9	332.1	326.2
Methionine	131.2	131.2	131.2	131.2
Isoleucine	298.6	284.1	266.0	259.2
Leucine	534.4	506.2	492.3	489.2
Tyrosine	309.7	309.8	383.5	368.5
Phenylalanine	320.3	298.1	294.4	284.9
Total	5,809.0	5,564.6	5,641.2	5,557.8

R = 8.314
T = temperature in ^{o}K
r = distance from center of rotation
a = distance of miniscus from center of rotation
ρ = density of solvent

When interference optics are used, the displacement of fringes is proportional to the change in concentration. Therefore, when the log of the number of fringes displaced at a point r is plotted against $(r^2 - a^2)$, the slope of the line will equal $\frac{M(1 - \bar{v}\rho)\omega^2}{2RT(2.303)}$.

Five values for each component are given in Table V. The average molecular weights found for the 27S was 1,450,000 and for the 14S was 581,000, a ratio of 2.50.

The partial specific volume ($\bar{v}$) used in calculating the values found in Table V was 0.756 cc/gm. This value was found by pycnometry of yolk which contained a mixture of 27S and 14S protein. A mixture was used because of the difficulty of separating enough of the individual proteins for individual $\bar{v}$ determinations. The individual components were banded in cesium chloride solution (ρ = 1.32) on the analytical ultracentrifuge to determine if there was any difference in density between them. Figure 11 gives the results of this experiment and demonstrates that the two components were very close to the same density.

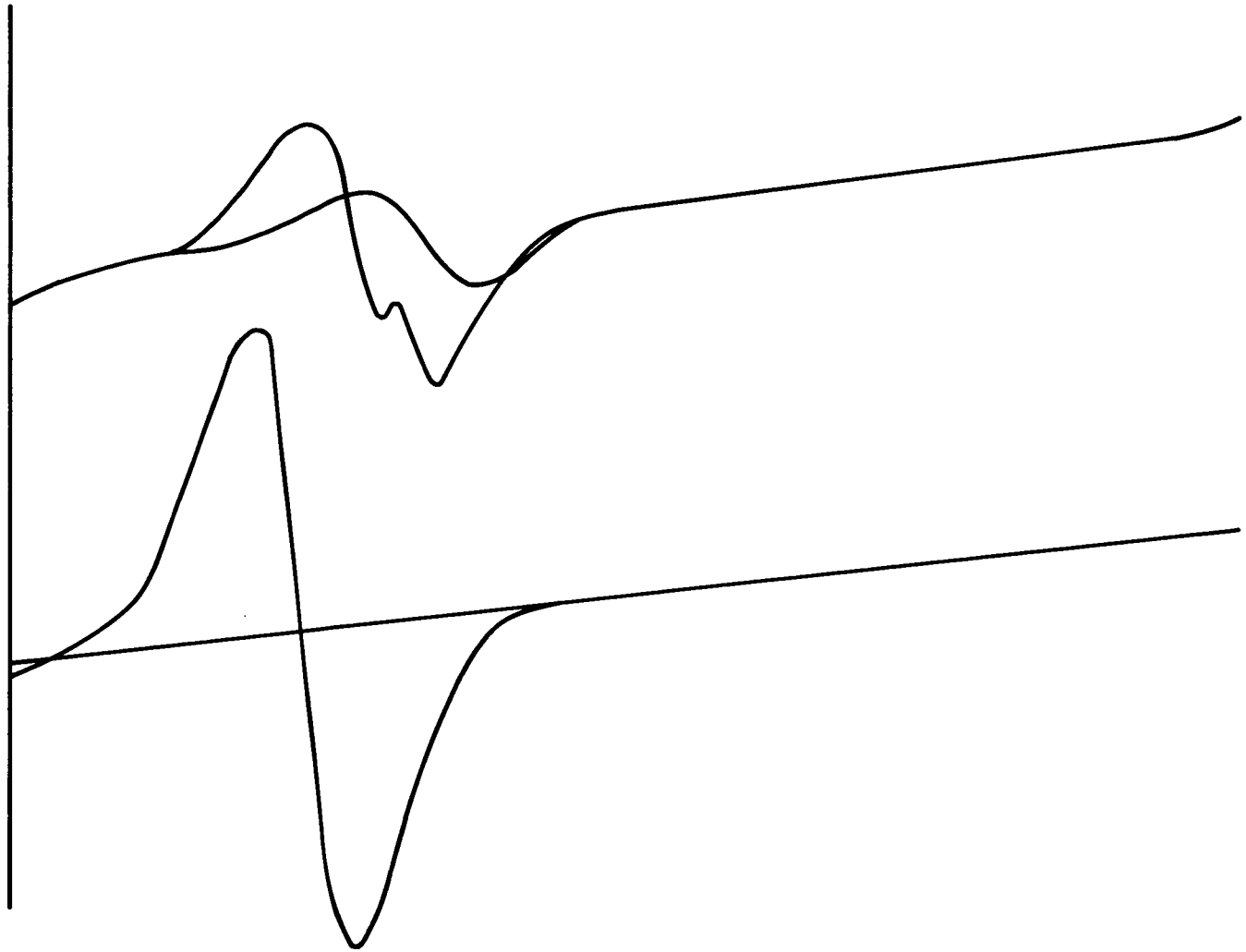


Figure 11. Schlieren pattern of 14S component (top, 0.32 mg/ml) and 27S component (bottom, 0.29 mg/ml) of yolk protein banded in cesium chloride solution (ρ = 1.32) at 48,000 rpm and 20° C.

Table V. Molecular Weights Found by the High-Speed Equilibrium Method.

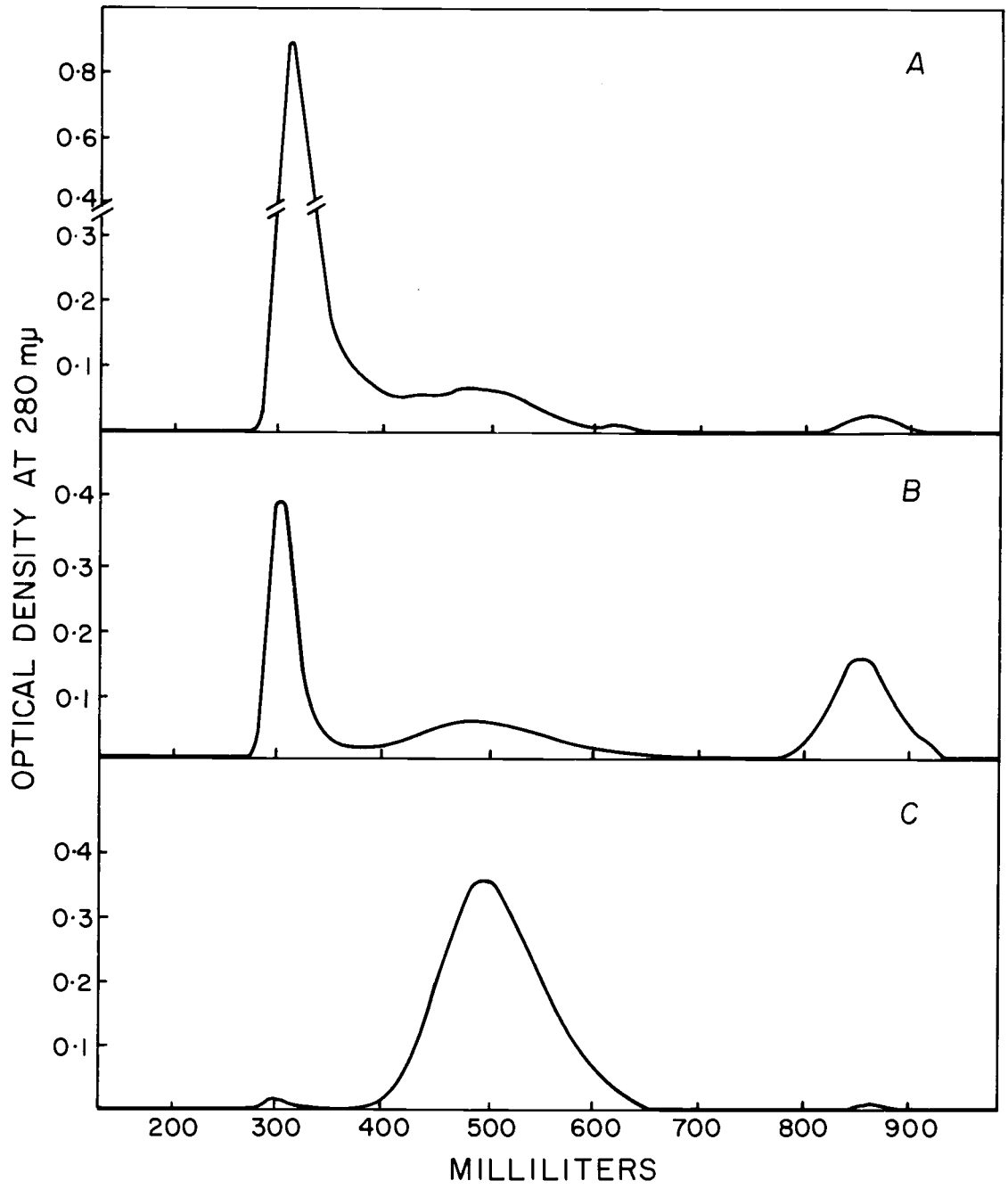
14S	27S
586, 000	1, 420, 000
578, 000	1, 400, 000
612, 000	1, 540, 000
588, 000	1, 450, 000
542, 000	1, 460, 000
581, 000 ave.	1, 450, 000 ave.

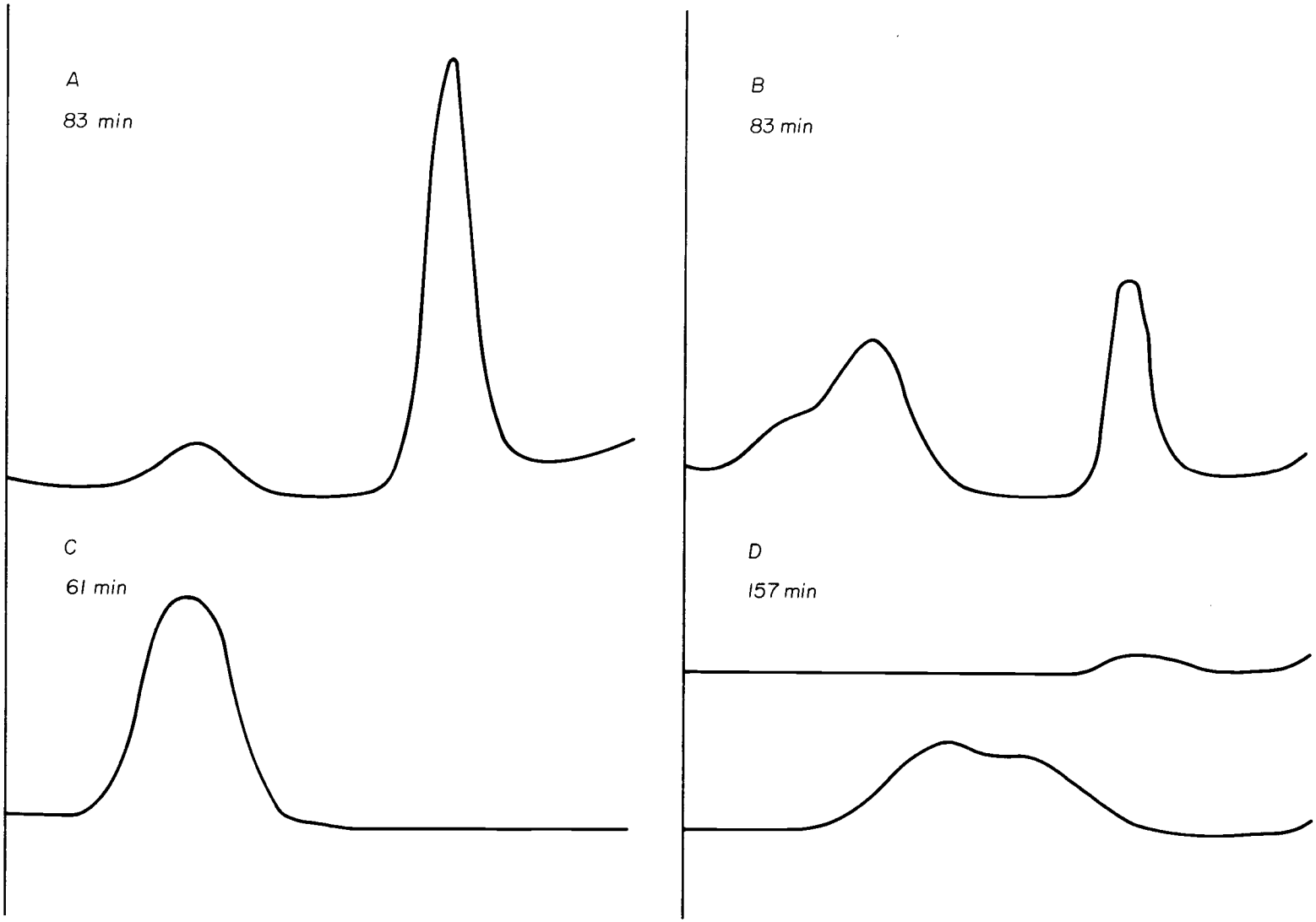
$$\frac{\text{27S ave.}}{\text{14S ave.}} = 2.50$$

Disaggregation of the 27S Component

The relative proportions of the 27S and 14S components can be changed *in vitro* by changing the pH of the yolk fraction. The results of column chromatography on agarose at different pH values are given in Figure 12. Slow breakdown occurred when the sample was held at pH 7. 8 at a concentration of 19 mg protein/ml for a week (Figure 12A, B). When enough NaOH was added to the yolk to bring it to pH 9. 0, almost all of the 27S was broken down and eluted near the position of 14S (Figure 12C).

In Figure 13A, B, are shown the results of an experiment in


A
B
C
0·8
0·6
0·4
0·3
0·2
0·1
0·4
0·3
0·2
0·1
0·4
0·3
0·2
0·1
OPTICAL DENSITY AT 280 mμ
200
300
400
500
600
700
800
900
MILLILITERS


A
83 min
B
83 min
C
61 min
D
157 min

which yolk protein fraction was divided into two parts and each part dissolved in phosphate buffer, (I = 0.4) so that one part was pH 5.6 and the other pH 8.1. These fractions were held at 4°C at a concentration of 5.7 mg protein/ml for at least 24 hours and then centrifuged on the analytical ultracentrifuge as described in Figure 13. At the higher pH the amount of the 27S component was reduced, the 14S increased and a new lighter fraction appeared. Similar experiments were performed at pH 6.4, 7.4, 7.8, and 9.0 (Table VI). At pH 7.4 and below there was no change in the relative proportions of the two peaks. The centrifugal pattern at pH 7.8 was similar to that shown in Figure 13B including the small shoulder which appeared on the 14S component. Adjusting the yolk protein fraction to pH 9 resulted in the further dissagragation of the yolk proteins. As shown in Figure 13 C and D, the 27S and 14S components no longer existed and in their place was a fraction of lighter weight which on prolonged centrifugation proved to be heterogeneous. Part of this material may correspond to that which appeared as a shoulder on the 14S component (Figure 13 B) but this cannot be proved on the basis of the data available. At pH 7.8 and 8.1, an incubation period was necessary to disaggregate the protein. Centrifugation performed within two hours of pH adjustment revealed little change in the relative peak proportions. The changes observed at pH 9.0 took place within a two-hour period and the centrifugal patterns after 61 minutes and

157 minutes are shown in Figure 13C, D.

Table VI. Changes in the Relative Proportions of the Yolk Protein Fractions as a Function of pH, as Determined by Analysis of Peak Area of Schlieren Pattern on Model E. Corrected for radial dilution.

Mean Length of Basal Oocyte (mm)	pH	27S	14S and Smaller	Concentration (mg/ml)
5.08	5.8	89	11	4.8
4.33	5.6	87	13	5.7
4.25	5.7	80	20	6.6
4.25	6.4	79	21	6.6
4.25	7.4	82	18	6.6
4.25	7.8	65	35	6.6
4.33	8.1	59	41	5.9
3.70	9.0	0	100	5.1

The amount of aggregation in many subunit systems is a function of the concentration of the components. To test this possibility in the yolk protein system, yolk preparations were analyzed by sucrose density gradient centrifugation at concentrations ranging from 1.5 mg/ml to 18 mg/ml. The proportions of the components were unchanged as a result of these different concentrations. Next a study of the effect of storage at different concentrations was implemented. A yolk preparation was stored on ice at concentrations of 8, 15, and 30 mg/ml. Each concentration was analyzed by density gradient centrifugation on the day it was prepared, after two weeks, and after five weeks. No significant change in the proportions could

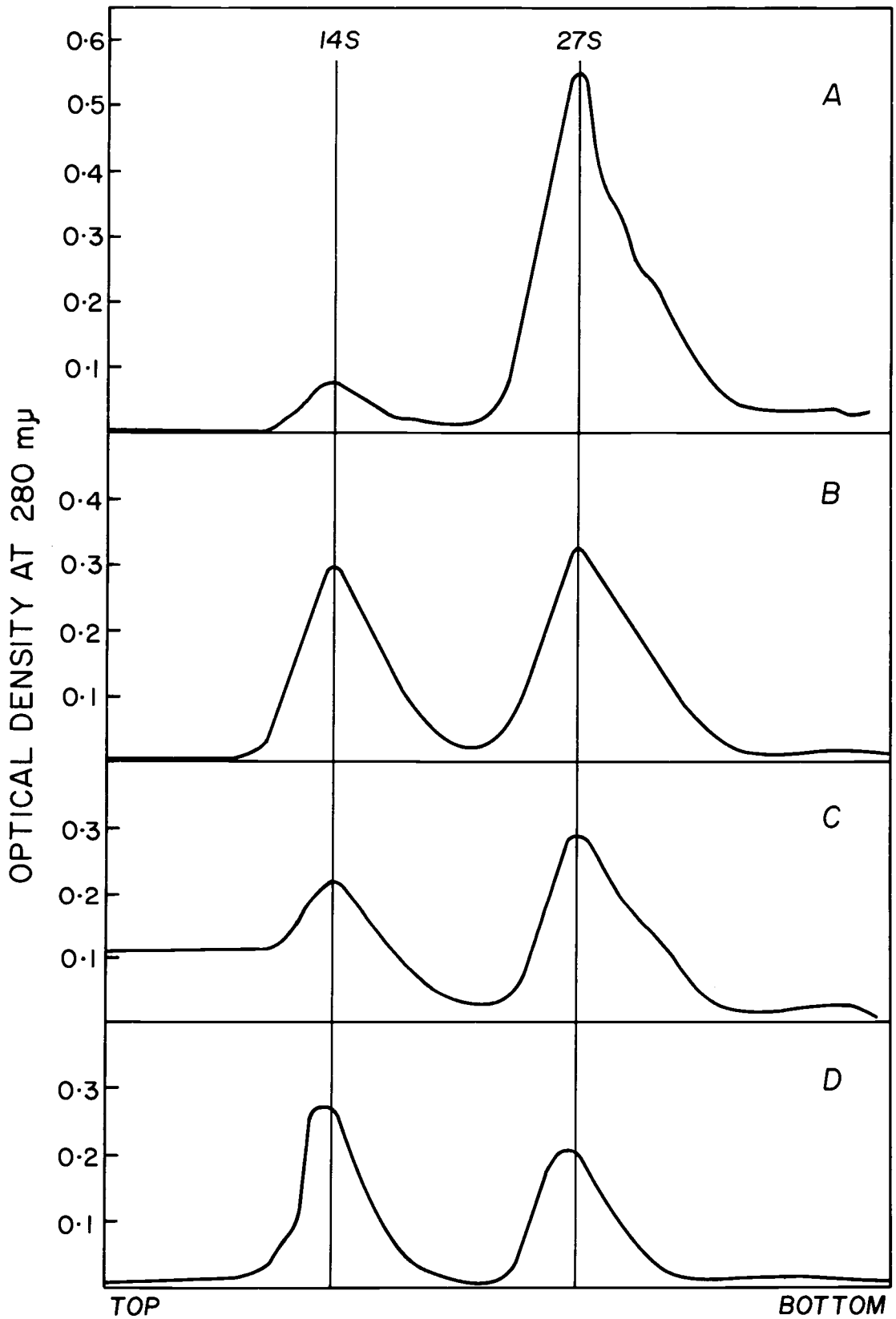
be seen.

Some microbial growth was seen in the most dilute preparation after five weeks. In order to prolong the storage time 0.02% sodium azide was added to a freshly-made preparation but after four hours a precipitate was observed. Sucrose gradient analysis of a sample containing azide showed that the proportion of 27S had decreased. Therefore sodium azide was considered unsuitable for use as a microbial inhibitor in this system.

The 27S and 14S components were separated by density gradient centrifugation. The fractions were dialyzed against 0.4 M NaCl to remove the sucrose and excess NaCl. Water was added and the precipitated protein was collected and analyzed by density gradient centrifugation. Both peaks are stable when stored on ice and no interconversion was detected.

When yolk from nearly mature ovaries was heated in a 65° C water bath, the 27S fraction was converted to a smaller molecule. Comparison of an untreated yolk with preparations treated for five and ten minutes is made in Figure 14. The yolk preparations in both C and D of Figure 14 were heated for ten minutes at 65° C. Some of the material in Figure 14 C appears to have broken down to units smaller than 14S.

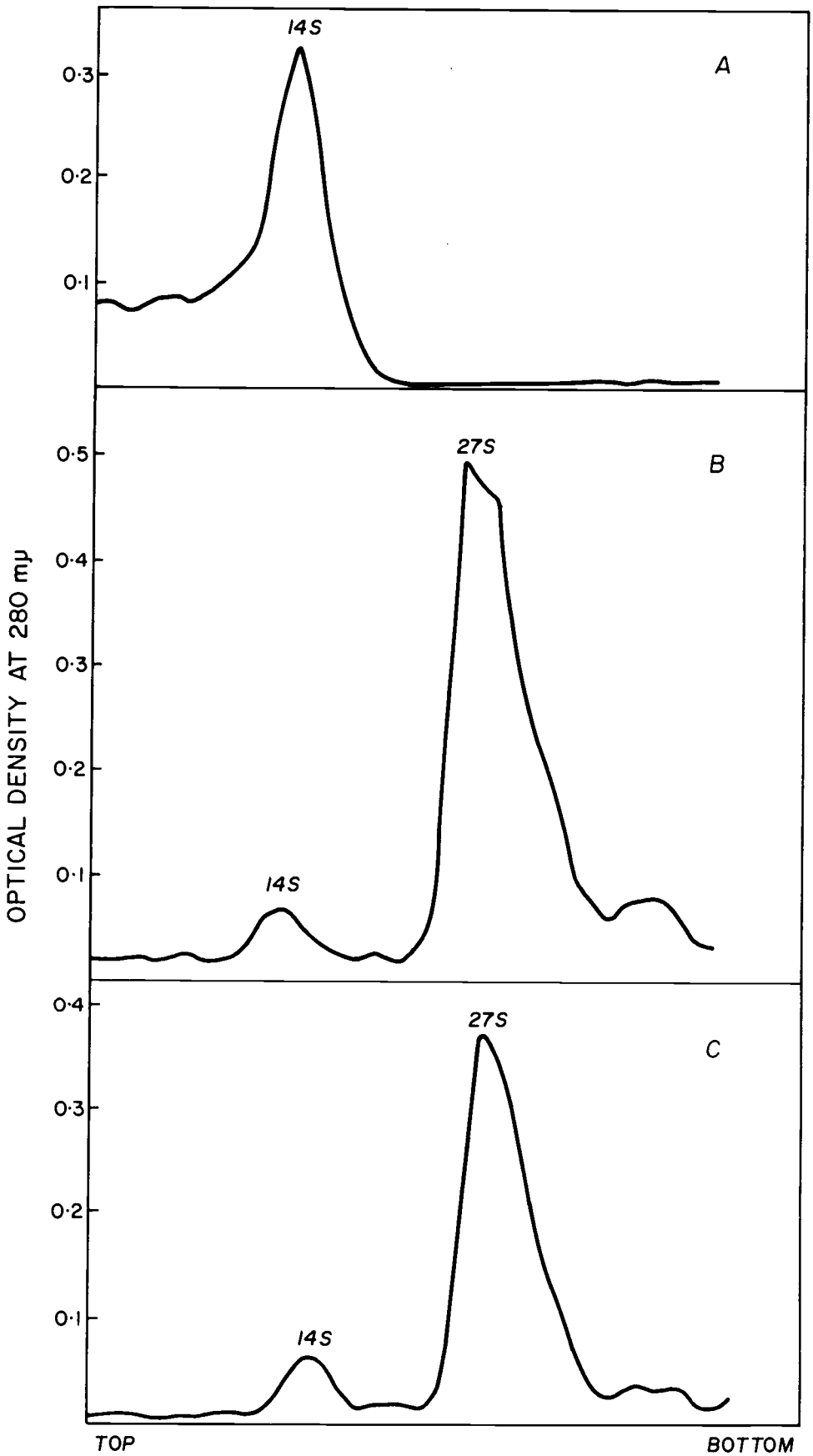
Urea is often used to dissociate protein into subunits and its action on yolk protein was examined. Yolk protein was prepared


14S
27S
A
B
C
D
0·6
0·5
0·4
0·3
0·2
0·1
OPTICAL DENSITY AT 280 mμ
TOP
BOTTOM

from ovaries containing large eggs so that most of the protein was present as the 27S fraction. Solutions containing 7.3 mg/ml were made up in solutions of 1, 2, and 4 M urea and analyzed by sucrose density gradient centrifugation. Little or no effect was observed on the protein in the presence of 1 and 2 M urea. However, in 4 M urea the 27S peak disappeared and the 14S peak increased in size along with the appearance of some material that sedimented slower than 14S. (Figure 15A). When the dissociated protein solution in 4 M urea was dialyzed against 0.4 M NaCl, a precipitate was observed in less than an hour. A more satisfactory method of removing urea proved to be dialysis against solutions of successively lower concentrations of urea. Disaggregated protein solutions were dialyzed against 3, 2, 1, 0.5 and 0.0 M urea made up in 0.4 M NaCl for intervals of 2, 2, 1, 1, and 1 hours respectively. Aliquots of the dialyzed solution were analyzed by sucrose density gradient centrifugation. It was found that removal of urea resulted in the restoration of the proportion of 14S and 27S that existed prior to urea treatment.

All previous results had indicated that there was no spontaneous aggregation of the 14S component. It was of interest, therefore, to find out whether or not the 14S would aggregate when combined with the subunit which is obtained after treatment of 27S with urea.

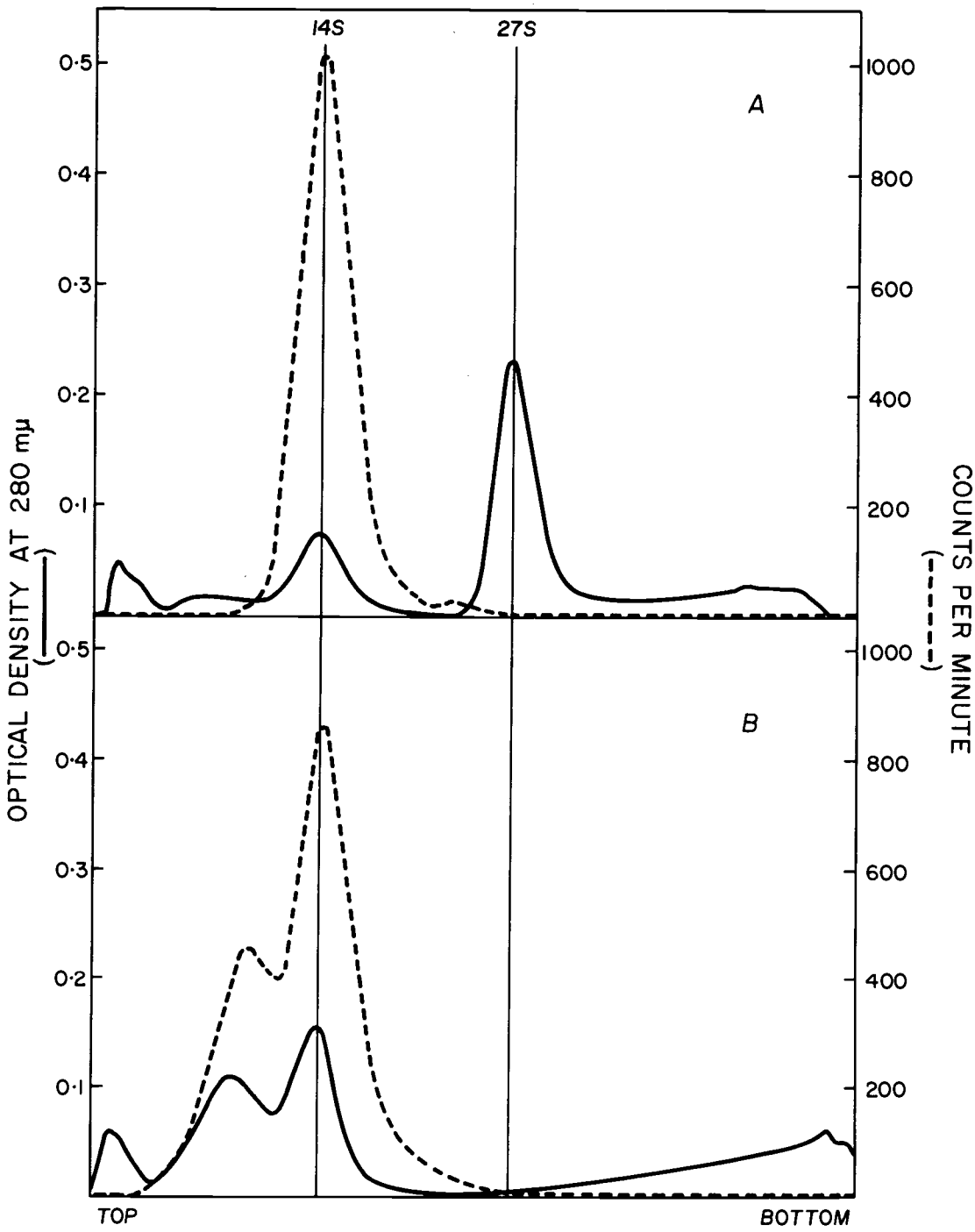
A group of cockroaches was injected with C^{14}-leucine and after


14S
A
0·3
0·2
0·1
27S
B
0·5
0·4
0·3
0·2
0·1
14S
OPTICAL DENSITY AT 280 mμ
27S
C
0·4
0·3
0·2
0·1
14S
TOP
BOTTOM

24 hours the ovaries were removed and yolk was prepared in the usual manner. The 14S and 27S fractions were separated by density gradient centrifugation and the labeled 14S peak obtained was combined with a non-radioactive 27S peak prepared in the same way. This mixture which had a protein concentration of 0.83 mg/ml was dialyzed for an hour against 0.4 M NaCl to remove sucrose; then dialyzed overnight against 4 M urea. After urea was removed by stepwise dialysis as explained above, a sample was analyzed by density gradient centrifugation (Figure 16).

Figure 16B shows that the 27S had disappeared and the 14S fraction had increased. An additional peak of slower sedimentation than the 14S was found and none of the 27S had reassociated upon the removal of the urea. The experiment was repeated with a similar result. Since the successful reassociation experiment had been carried out on a whole yolk from large ovaries, the possibility was raised that some factor present in the yolk preparation was absent in the combination of the 14S and the 27S fractions. To test this possibility a fraction containing only 27S protein (1.62 mg/ml) was treated with urea and the urea was removed. Partial reaggregation resulted from this treatment (Figure 17) as well as some 14S and some of the slower component.

In order to remove the variable introduced by mixing 27S and 14S fractions from two different ovary preparations, it was decided


14S
27S
A
B
0·5
0·4
0·3
0·2
0·1
1000
800
600
400
200
OPTICAL DENSITY AT 280 mμ
(——)
COUNTS PER MINUTE
(- - - -)
TOP
BOTTOM

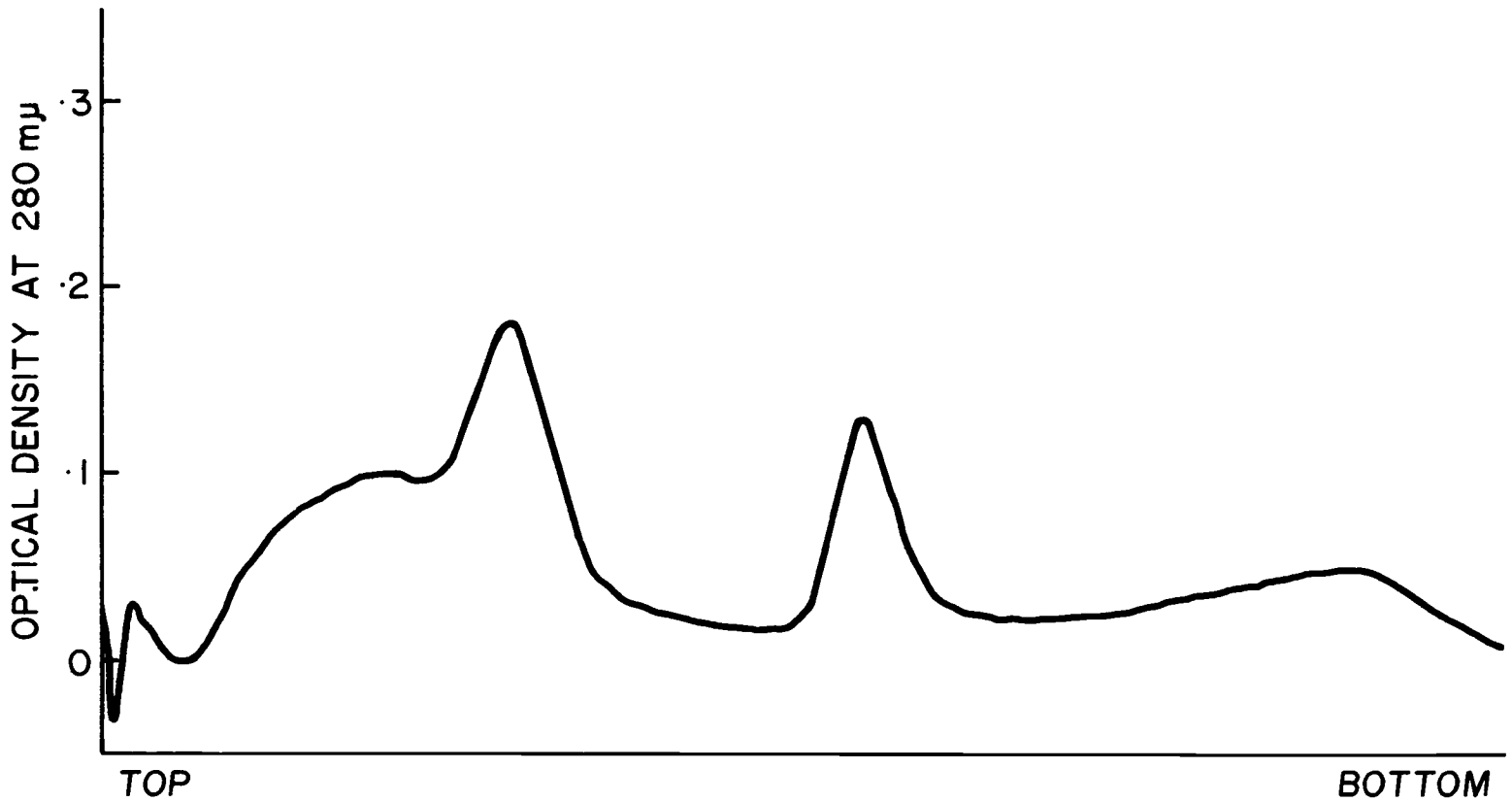
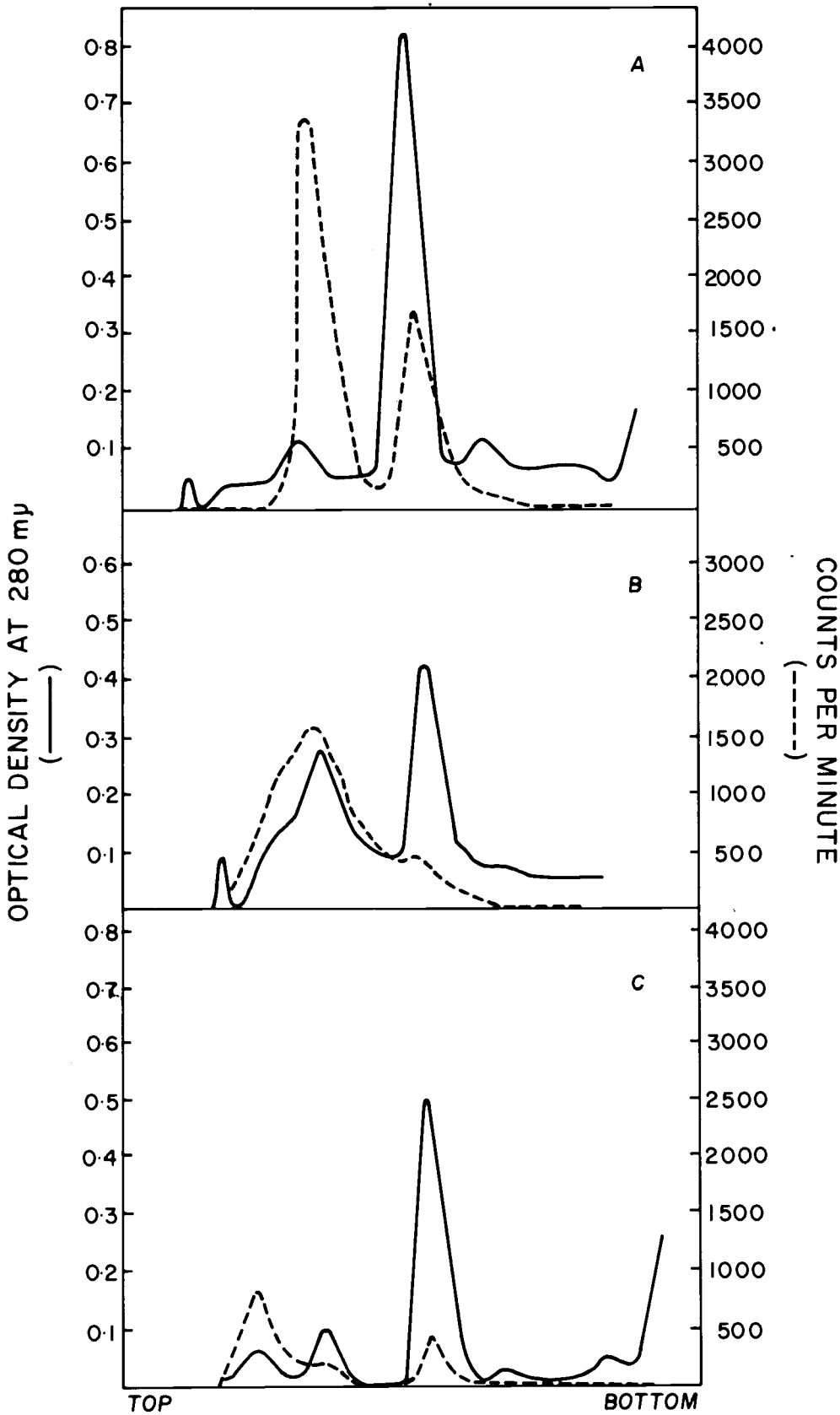


Figure 17. Pattern of a sucrose density gradient centrifugation of 27S component which had been dialyzed overnight against 4 M urea then dialyzed against 3, 2, 1, 0.5, and 0 M urea in 0.4 M NaCl.

to use a preparation from insects which had been injected for 16 hours with C^{14}-leucine. The yolk prepared from these insects had a high specific activity in the 14S fraction and a low specific activity in the 27S fraction (Figure 18A). If after dissociation by urea, reassociation occurred only between 14S particles which had been formed by dissociation of the 27S protein, the specific activity of the reassociated 27S would remain low. On the other hand, if reassociation occurred at random between the 14S particles formed from the low specific activity 27S and the high specific activity 14S of the yolk, the resulting reassociated 27S would have a higher specific activity. This experiment was carried out twice and the results are shown in Figure 18 B, C.

No explanation can be given for the differences between B and C. Much variation was observed in samples thought to be treated with identical procedures. Evidently some unrecognized factor is involved. However, the specific activity of the 27S fraction did not increase and it can be concluded that reassociation to 27S particles only occurs between protein subunits which had previously been 27S particles.

The slower sedimenting component was estimated by the ratio of its mobility to the mobility of the 14S peak (49) to have a sedimentation coefficient of between 5 and 6 (Table VII). This protein can be formed from the 27S peak (Figure 17) or from the 14S peak


A
B
C
OPTICAL DENSITY AT 280 mμ
(——)
COUNTS PER MINUTE
(- - - -)
TOP
BOTTOM

(cpm curve of Figure 16).

Table VII. Estimation of S-Value of Small Peak in Urea by Use of the 14S Peak and the Respective Mobilities in Sucrose Density Gradients

Mobility *	S-value calculated	Mobility *	S-value reference	Mobility *	S-value calculated
8.0±0.5	5.7±0.3	19.7	14.0	37.4	26.6
7.7±0.7	5.4±0.5	20.0	14.0	38.0	26.6
7.5	5.4	19.4	14.0	37.2	26.8

*In arbitrary units from the miniscus

V. DISCUSSION

The absorbance at 280 mμ is a very convenient method of analysis. Its value lies in its rapidity and in the fact that the protein is not damaged by the analysis. Alone, it provides only a means for relative comparison of protein concentration. For this reason, two alternate methods of analysis were compared with the results of the absorption at 280 mμ. The methods of Lowry and amino acid analysis gave ratios of 0.83 and 0.87 OD_{280}/mg protein. It must be remembered that these proteins are lipoproteins and the quantities obtained by these methods will only relate to the weights of the amino acid chains and not to that of the lipid or other moiety.

The results of the brief survey of the lipids reported here agree and amplify the summary of unpublished data reviewed by Gilbert (29). He found in Leucophaea that 90-92% of the egg lipid consisted of "neutral lipid" and 8-10% was phospholipid. Of the neutral lipid, about 90% was triglyceride. The results reported here indicate that the phospholipid is found associated primarily with the protein of the yolk and that 0.094 mg lipid is found per mg of yolk protein in large ovaries.

The sucrose density gradient centrifugation method of separation of the peaks proved to be very useful. Complete separation was achieved between the 14S and the 27S components. Contamination

of the 27S peak due to lack of separation between the 40S and the 27S can be prevented by not including the faster sedimenting portion of the 27S peak in a purified 27S fraction but some of the 27S fraction is lost in this manner. Other disadvantages of this method of separation are the 16 hours required for the actual centrifugation and the time required in making the gradients and fractionating the centrifuge tubes after centrifugation. After the peaks are separated they must be dialyzed to remove the sucrose and excess salt. Some protein is lost in the dialysis step. In order to layer the sample to be analyzed on the miniscus of the tube, the sample must be lighter than the least dense portion of the gradient. This is a disadvantage when urea or other heavy material is present in the sample to be analyzed.

If a satisfactory separation could be achieved by column chromatography, several of the above disadvantages would be minimized. While the actual time of separation would not be decreased greatly, the number of man hours could be shortened considerably if an automatic scanner and fraction collector were used. The dialysis step would be eliminated and the protein would not be subjected to a high sucrose concentration. The preliminary experiments reported here indicate that agarose might be suitable as a chromatography medium for this protein.

The analysis of the series of ovaries in different stages of development revealed important quantitative and qualitative changes

which occur during maturation of the oocytes. In ovaries smaller than 1.0 mm neither 14S nor 27S protein was found. This biochemical observation that the level of yolk material in eggs of this size was below the limit of detection can be correlated with earlier morphological work showing that eggs smaller than 1.0 mm are not in the process of vitellogenesis (14). The high proportion of 14S in the small ovary shortly after oocyte maturation had begun compared to the high proportion of 27S in the mature ovary suggested that the 27S may be formed from 14S. Additional evidence of this is discussed later.

The OD_{280}/OD_{260} ratio of the 14S and 27S peaks in mature ovaries (1.7 - 1.8) is consistent with that reported for pure protein (43). The low OD_{280}/OD_{260} ratio of the 40S component suggested that it may contain both nucleic acid and protein. Anderson has reported the presence of many ribonucleoprotein particles in the cytoplasm of Periplaneta oocytes (1). The procedure used here to extract the yolk protein will also extract nucleic acid so that nucleic acid may occur in our yolk preparations. The OD_{280}/OD_{260} ratio of 1.6 reported in the yolk from small ovaries (Figure 2) indicates a content of about 0.25% nucleic acid (43). The increase in the ratio (and thus the relative decrease in nucleic acid) as the oocytes grow can be explained by simple dilution of the nucleic acids as the oocytes add protein yolk material. Since such a small proportion of

the yolk was found in the 40S peak, no further analysis was carried out on this component.

In contrast to the 40S component, the peak which was eluted from the agarose columns later than the 14S peak exhibited a very high OD_{280}/OD_{260} ratio. Such a ratio is not characteristic of the absorbance of phenylalanine, tyrosine, and tryptophane found in side chains of proteins and cannot be explained at present. As much as three void volumes are required to elute this peak from agarose (Figure 9). Material eluting this late from molecular sieving columns is thought to interact with the column packing gel and the elution position cannot be relied upon for molecular size determinations.

The observation that the amount of 14S material in the ovary remains fairly constant after the basal oocyte reaches lengths greater than 2 mm while the 27S material increases manyfold in this same period of development can be explained in one or two ways. One explanation is that the 14S material is deposited during the early part of maturation and the 27S material is deposited later. A second explanation is that the 14S molecules aggregate in some manner to form the 27S. One method of testing these hypotheses was to inject female cockroaches with C^{14}-leucine and to remove the ovaries at varying time intervals after the injection. In this manner the fate of the amino acid could be followed as it was incorporated into the proteins. Regardless of the stage of development in which the

C14-leucine was injected the radioactivity always entered the 14S peak soon after injection and was found in the 27S component after prolonged exposure. This observation coupled with the observation that the activity of the 27S peak increases in the period from 8 to 72 hours after injection indicates that the 27S is made from the 14S.

After finding that the 27S protein is made from the 14S protein, we explored some of the relationships between the two proteins. One major question concerns the composition of the 27S molecule. Is it merely a polymer of 14S molecules or is it one or more 14S molecules plus an additional moiety?

One way to determine if the 27S contains an additional protein moiety of different composition is to compare the amino acid composition of the two proteins. The results of the amino acid analysis are given in Table III. The ratios of the amount of each amino acid to the amount of arginine were calculated and these ratios were used to compare the 14S protein with the 27S protein. These ratios are given in Table III, columns 9, 10, 12 and 13. The ratios obtained for the 14S component were divided by the corresponding ratios from the 27S component and, if the two proteins contain identical amino acid compositions, these quotients will equal 1.00. The values obtained (Table III, columns 11, 14) are very close to 1.00. The accuracy of the amino acid analysis is $\pm 3\%$. The two components can be said to have identical amino acid compositions.

The two different times of hydrolysis in the amino acid analysis may give some indication of the location of certain amino acids. If more of a specific amino acid is detected upon prolonged hydrolysis, this suggests that the amino acid is in the interior of the molecule and is not released immediately. Two such instances may occur in the 27S molecule as reflected in the values of 1.16 and 1.10 for histidine and valine (Table III, column 15). In the 14S molecule only isoleucine has a ratio which may be significantly greater than 1.00 (Table III, column 16) and the isoleucine value is also slightly higher than 1.00 in the 27S peak. From these data it might be weakly speculated that the polymerization involves valine and histidine.

Lower values of tyrosine and serine in the 68-hour hydrolysis suggest that these two amino acids are decomposed during prolonged hydrolysis. A low value for lysine in the 27S protein cannot be explained.

With the above evidence that the 27S protein contained the same amino acid profile as the 14S particles, an attempt was made to learn more about the sizes of the two proteins.

Analysis of the molecular weights of the two components showed that the 27S was 2.5 times the molecular weight of the 14S. The $\bar{v}$ used in calculations was determined by pycnometry of a mixture of the two proteins. A small difference in the $\bar{v}$ would influence the molecular weight greatly so the two components were banded in a

cesium chloride gradient in the Model E analytical ultracentrifuge. This experiment demonstrated that the difference in $\bar{v}$ values is not large enough to change the ratio of the molecular weights so that a dimer or trimer structure may be assigned on the basis of the molecular weights. Since 14S will not aggregate spontaneously as shown by storage as a separate component, the possibility exists that there is some smaller unit added to the two 14S molecules to link them together. However, amino acid analysis data indicate that there is no difference in amino acid composition between the 14S and 27S.

The yolk proteins of birds (61), amphibians (77, 79, 80) and six crustaceans (78) have been studied biochemically. These proteins have been shown to be made up of subunits. The precursors to the yolk protein are synthesized elsewhere in the body of the organism and transported to the ovary where they are incorporated and assembled to the final yolk form. In a recent study of the toad, Xenopus, by Wallace and Jared, a single serum lipophosphoprotein was shown to be the precursor for both lipovitellin and phosvitin. This precursor is incorporated by the ovary and in 8-20 hours it is converted to the two components of the yolk platelet (77). In this study Leucophaea yolk protein has been shown to be made up of 14S subunits, but so far the rapid clotting of the hemolymph has rendered experiments on the transport of the material impractical. A protein

which sediments in the 14S position and on the basis of immunology is identical to the 14S yolk protein is synthesized in the fat body of Leucophaea (12) and it is assumed that these subunits are transported to the ovary via the hemolymph with little or no change.

The yolk platelets of Rana pipiens consists of two phosvitin molecules associated with the surface of a lipovitellin dimer (75). Phosvitin is a phosphoprotein containing about 50% serine (74) and lipovitellin is a large lipophosphoprotein (16). Phosvitin can be dissociated from lipovitellin by increasing the ionic strength of the medium (73). The dimers of lipovitellin can be reversibly dissociated by alkaline conditions (16).

The yolk of Leucophaea also requires a high ionic strength for solubility. The resulting 27S protein from mature ovaries can be broken down to 14S material by alkaline pH, heating at 65° C and 4 M urea. However, neither the heating nor alkaline dissociation have proved to be reversible. The 14S fraction which is a precursor to the 27S of mature yolk is stable by itself and does not spontaneously aggregate. The 14S yolk protein subunits which have formed due to treatment with urea do reaggregate to form the 27S material. This would indicate that there is some active process causing the formation of 27S from 14S.

The experiments involving reaggregation after urea treatment were inconsistent in their outcome. Different results were observed

between samples which were thought to be treated in the same manner. Samples in which reaggregation did occur were at a concentration of 5 mg/ml or greater. The results reported here suggest the possibility that the protein concentration may be an important factor in reaggregation. Aggregation was obtained at 6-7 mg/ml but no aggregation was obtained at 0.8 mg/ml and only partial aggregation at 1.6 mg/ml. However, in another experiment with yolk at 6-7 mg/ml reaggregation did not occur.

The active process which occurs to link the molecules may explain why the 27S is 2.5 times the molecular weight of the 14S. It must be kept in mind that the "glue" is either non-protein or is protein of the same amino acid composition as the 14S molecules.

Three suggested possibilities for the "glue" are carbohydrate, lipid or phosphate. The discrimination of pinocytotic uptake would seem to be achieved by the selective adsorption of protein on the membrane (57). The lining of the pits which form into vesicles in Periplaneta stain with the periodic acid Schiff reagent indicating the presence of carbohydrate (1). It is possible that a carbohydrate could act in some way to link the two molecules. Carbohydrate has been reported in the yolk of many organisms (38, 45, 52, 58) and has recently been found by Brookes (unpublished) in Leucophaea. Marshall (48) speculates that the material which lines the pinocytotic pits of amoeba could be mucoid or lipoid material. The lipid

moiety may also serve as a link in Leucophaea.

Phosphate has been implicated in the linking of protein subunits in the frog egg. The water insoluble protein found in the frog egg yolk platelet can be split by pH extremes to form water soluble 6S and 11S fractions. The 6S fraction is a phosphoprotein which selectively binds calcium. If the two water soluble forms are mixed in distilled water, a precipitate forms which resembles the original protein. If, however, calcium is bound to the 6S or if the phosphate is removed, the 6S - 11S complex does not form and the protein remains soluble (27). In view of the role suggested for phosphate in linking the units of the yolk protein in the frog egg, it would be of interest to follow the route of P^{32} in the fat body, blood, and ovaries.

The ratio of the molecular weights obtained coupled with the fact that the 27S dissociates to 14S suggests that the 14S may be a dimer and that two 14S molecules are linked by a monomer to form the 27S molecule. The 5-6S fraction observed after denaturation with urea (Figure 16) would also support such a mechanism if this fraction is homogeneous. The single peak eluted from agarose after raising the yolk protein to pH 9 (Figure 12C) is a broad peak and elutes at about two times the void volume. This resembes the result obtained with the analytical ultracentrifuge at this pH (Figure 13C). In the centrifuge experiment, a longer time of centrifugation revealed heterogeneity (Figure 13D). Further analysis of the

fraction eluted from agarose at pH 9.0 or the 5-6S peak from urea might give similar results. Additional studies on the nature of the subunits formed at high pH and those found after treatment by 4 M urea are needed for complete understanding of the assembly of the yolk protein.

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