ULTRASTRUCTURAL CHARACTERISTICS OF YOLK IN THE CHICKEN EGG. MECHANISMS FOR YOLK ABSORPTION AND ITS DIGESTION BY THE YOLK SAC AT DIFFERENT STAGES OF EMBRYONIC DEVELOPMENT

by

PAULA M. E. ALLAN-WOJTAS, BSc. Hon.

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Department of Anatomy and Neurobiology
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Abstract

An ultrastructural study using electron microscopy was undertaken of yolk and yolk sac to elucidate digestive mechanisms and to gather evidence to further substantiate the belief that intracellular digestion of yolk takes place in yolk sac epithelial cells during incubation.

Initial work focused on the ultrastructure yolk in unincubated eggs using transmission electron microscopy of thin sections of yolk which had been fixed using conventional and novel fixatives. These experiments confirmed earlier results by other workers that yolk is composed of granules (which have a sub-granular structure and lack a boundary membrane) and matrix. The matrix contains matrix particles which consist of low-density lipoprotein particles. The next phase of the study involved the ultrastructural comparison of granules from sub-blastodermic fluid from 9 day old embryos, and extracellular yolk attached to the yolk sac of 15 day old embryos with the granules from yolk of unincubated eggs. Granules in the yolk from incubated eggs were identical to those from unincubated eggs, and showed no ultrastructural changes to indicate that digestion had taken place outside of the cell.

The third phase of the study, after establishing optimal fixation conditions, and using half-strength Karnovsky's fixative, was the ultrastructural description of yolk sacs at 3 ages (3 days, 8 days and 15 days of incubation). Scanning electron microscopy provided details of the age-related folding of the yolk sac which substantiated and extended macroscopic and light microscopic observations published in the classical literature. Epithelial cells were studied systematically from apex to base. Uptake structures associated with the process of endocytosis were observed at the apical portions of the cells. These appeared as microplicae (phagocytosis) and coated pits (receptor-mediated endocytosis) by transmission electron microscopy, and as fossae of different sizes by scanning electron microscopy of the apical surface of the epithelial layer of the yolk sac. The fossae, not previously described in the literature, represented a 3 dimensional view of structures observed in sections viewed by light microscopy and transmission electron microscopy. In addition to the organelles regularly present in most cells (mitochondria,

golgi apparati, endoplasmic reticulum, etc.) we observed numerous organelles, usually forming part of an active intracellular digestion system as it is presently known. These included phagosomes, endosomes and secondary lysosomes. The identification of these organelles was confirmed by Acid Phosphatase histochemistry and cytochemistry. The results of these individual experiments were used to propose a scheme of yolk digestion based on progressive ultrastructural changes of intracellular yolk. The blood vessels of the yolk sac, incompletely described in the literature, were also studied, and were observed to take the form of large blood sinuses, the endothelium of which shares some of the structural characteristics described in adult liver sinusoids and bone marrow sinuses.

A transport system for yolk lipid and its digestion products to the embryo was demonstrated ultrastructurally using the Imidazole-buffered Osmium Tetroxide protocol of Angermuller and Fahimi (1982) which enhanced lipid staining. This transport system shared ultrastructural characteristics with that reported for lipid transport in intestinal cells, especially with respect to the formation and transport of chylomicron-like particles. In yolk sac, these particles were observed to move into the intracellular spaces and were assumed to move directly into the embryonic bloodstream, through the open fenestrae of the endothelium of the sinuses.

We observed no ultrastructural changes in extracellular yolk granules and matrix particles at any age studied. Granules and matrix particles are taken up by endocytosis, and ultrastructural changes of yolk granules occur in epithelial cells, in acid phosphatase-containing vacuoles (which we have identified as secondary lysosomes), which are part of the functioning intracellular digestive system. We have ultrastructurally demonstrated the transport of lipids, which becomes more evident in 13 day old embryos. On the basis of the above evidence we conclude that yolk is digested in epithelial cells of the yolk sac, and the digestion products enter the blood sinuses and travel in the bloodstream to deliver nutrients to the developing embryo.

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List of Abbreviations

Symbol	Definition
A	Apex of epithelial cell
AM	Apical membrane
bl	basal lamina
bv	blood vessel
C	developing blood vessel
c	blood cells
Ca	calcium spherule
cf	collagen fibrils
cv	coated vesicles
d	desmosome
En	endothelia cell
Ep	epithelial cell
F	fenestrations
f	fossa
G	Golgi apparatus
g	yolk granule
gl	glycogen
IBO	Imidazole-buffered osmium tetroxide treatment of Angermuller and Fahimi (1982)
ics	intracellular space
if	intermediate filaments
jc	junctional complex
L	lateral membrane of epithelial cell
LDL	low-density lipoprotein
LM	light microscopy
l	lipid droplet
ll	lipid-like inclusion
M	mitochondrion
mes	mesenchyme
mf	microfilaments
mp	microplica
mt	microtubules

Symbol	Definition
my	myelin figures
n	nucleus
nm	nuclear membrane
p	matrix particles of yolk
R	apical surface of yolk sac epithelium
RT	room temperature
rER	rough endoplasmic reticulum
S	blood sinus
SEM	scanning electron microscopy
s	secondary fold of yolk sac
TEM	transmission electron microscopy
VYS	visceral yolk sac
v	vacuole
ve	vesicle
vp	particles in vesicle
YS	yolk sac

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1. Introduction

1.1. Overview

An interesting topic in the field of embryology is to establish how the nutrients required by the developing embryo are transferred to it in a usable form. In the case of birds, the egg is a closed system where all nutrients for embryo growth and development are packaged with it in a shell. Because nutrients must be absorbed by the yolk sac (which holds the yolk mass) to become available to the embryo, its study, with emphasis on its epithelial cells, can give important information about nutrient transfer from the source to the embryo.

Yolk utilization by the chick embryo has been studied using techniques which can be classified as structural, biochemical, or some combination of the two. The use of structural information to explain biochemical changes, and vice versa, the search of structural expression for known biochemical events, has supplied a comprehensive body of information on the events connected with yolk utilization in the avian egg.

In reviewing the literature, it can be seen that histochemistry and cytochemistry, structural methods which can track biochemical reactions *in situ*, were considered, at one time, to be the most direct ways of combining these two basic techniques. However, information obtained from using these techniques was limited in that histochemistry is limited itself by the resolution of the light microscope, and both histochemistry and cytochemistry are limited by the reduced number of chemical reactions which can yield a reaction product detectable by microscopical examination.

Extensive information has, however, been acquired from viewing biochemically extracted samples using transmission electron microscopy (TEM). Many of the recent advances in Cell Biology have resulted from the integration and correlation of structural and functional information. While structural studies have shown that yolk sac epithelial cells have the morphology of absorptive cells, having structures and showing morphological changes indicative of cells which undertake intracellular digestion, there is still

incomplete agreement between structural and biochemical data with regards to functions attributed to the yolk sac.

Even today, the relative importance of intracellular versus extracellular digestion of yolk has still to be firmly established. The basis will be laid for the discussion of this problem by a literature review of biochemical and structural information. After a critical evaluation of the literature is handled, and some of its shortcomings noted, the direction for the experiments presented in this work can be more clearly stated.

1.2. Yolk

The name of yolk (vitellus) is given to a complex variety of macromolecules stored in the eggs of most animals and subsequently used for the nutrition of the developing embryo. During the process of oogenesis, oocytes store in their cytoplasm variable amounts of yolk which remains as inactive cytoplasmic inclusions (yolk granules or platelets) until after fertilization. The eggs of birds (as well as those of fishes, reptiles and some lower mammals) are very large and contain huge amounts of yolk which occupies most of the egg (Romanoff, 1960). The cytoplasm with the nucleus and all organelles is reduced to a pole (animal pole). The plasma membrane of the oocyte is surrounded by an acellular membrane known as the vitelline membrane. After fertilization, only the cytoplasm undergoes cleavage (cell division) resulting in a thin multicellular disc (blastoderm or blastodisc). Because cleavage does not affect the huge mass of yolk, this mass becomes from now on extracellular: it will be only surrounded by the vitelline membrane since the plasma membrane disappears. In the following description the word yolk will be used to refer to extracellular yolk except when preceded by the word intracellular (Romanoff, 1960).

Fertilization of the chicken egg occurs at the hen's ovary and cleavage proceeds before the egg is laid. While development of the embryo continues, the egg descends through the oviduct and uterus; these organs secrete the secondary envelopes of the egg

(albumen, shell membranes and shell). Thus, if the egg has been fertilized, the newly laid egg contains an embryo at the stage of blastoderm and a large mass of extracellular yolk. Both are separated by the vitelline membrane from the albumen. The descent in temperature which occurs after laying stops embryonic development at the stage of blastoderm; development resumes only if the egg is incubated at higher temperatures (Romanoff, 1960). If this occurs, the embryo will transport ions and water from the albumen to the yolk. The yolk which underlies the embryo will be diluted by this input of water and ions and this diluted yolk is known as sub-embryonic fluid (Freeman and Vince, 1974).

1.2.1. Structure

Macroscopic characteristics

Macroscopically, the yolk appears to be homogeneous, unless special precautions have been taken (such as cooking, freezing or chemical fixation) to stabilize the whole yolk while still contained by the vitelline membrane, before study (Romanoff and Romanoff, 1949; Schlesinger, 1958; Bain and Hall, 1970; Perry and Gilbert, 1985). If the yolk is prepared in this way, and sliced through the diameter, concentric bands of yellow yolk (which is deeply pigmented), and white yolk (which is paler and more fluid) are observed. The white yolk is also found beneath the blastodisc and is connected to the centre of the yolk, the latebra (Schlesinger, 1958).

Light microscopic characteristics

Romanoff and Romanoff (1949) described large particles, which they called spherules, in smears of fresh yolk; they were able to differentiate spherules from yellow and white yolk. Grodzinski (1951) studied these structures in more detail, renamed them as yolk spheres, and hypothesized that because they responded to osmotic changes, they are bound by a semipermeable membrane. Bellairs (1964) used polarizing light microscopy to show that the spheres were bound by a laminated capsule-like structure.

Bellairs (1961), Bain and Hall (1970) and Bellairs et al. (1972) characterized with light microscopy the particles which make up yellow and white yolk. They classified the yolk spheres (50 to 100 μm in diameter) according to their origin in the yolk, and reported that yellow yolk spheres were packed with many small particles which were assumed to be LDL, while white yolk spheres contained, instead, 1 or 2 large droplets, which appeared to consist of lipid. Bain and Hall (1970) further characterized the white yolk spheres into types A, B or C on the basis of number and size of droplets contained within them. Fujii et al. (1977) used light microscopy of yolk smears to study yellow and white yolk spheres without the detrimental effects of embedding, and found that their structure was very sensitive to preparation conditions.

Yolk granules, of submicroscopic size, can be discerned only as components of the yolk spheres and as a granular background in light microscopy of whole yolk smears (Burley and Vadehra, 1989).

Juurlink and Gibson (1973) and Cheville and Coignoul (1984) found focal areas of yolk, adjacent to the luminal surface of yolk sac epithelial cells, which stained heavily with the von Kossa method for calcium. These areas were observed, late in incubation, in both chicken and turkey embryos, respectively.

Ultrastructural characteristics

Ultrastructural studies have further identified the structure of yolk spheres and granules and, in addition, have detected submicroscopic particles in the yolk matrix, which with light microscopy, appears as homogeneous.

Bellairs (1961) showed that there is no unit membrane around the yolk spheres. Bain and Hall (1970) and Bellairs et al. (1972) used TEM to extend their LM observations on yellow and white yolk. Bain and Hall (1970) further characterized the white yolk spheres into types A, B or C on the basis of number and size of droplets contained within them. Bellairs et al. (1972) confirmed the impression of light microscopists who had suggested that the matrix contained a large number of particles of very small size (approximately

25 nm); furthermore, they proposed that these particles are formed by low density lipoproteins (LDL).

Perry and Gilbert (1985) used TEM to study yellow spheres in yolk from both oocytes and laid eggs, which had been prepared using a number of different fixatives. In all samples, they described the yolk mass as consisting of discrete polygonal units, approximately 140 µm in diameter, which were less closely packed toward the periphery of the yolk. The yolk spheres were bounded by a 7 nm thick membrane, and contained granules, fine particles and lamellar bodies. Fujii et al. (1973) used SEM of yolk smears to study the structure of yellow and white yolk spheres without the detrimental effects of embedding, and found that their structure was very sensitive to preparation conditions. The hypothesis of Fujii et al. (1973) that yolk spheres may be the remnants of the polyhedral compartments which exist in intact yolk is supported by this work by Perry and Gilbert (1985).

Another type of large particle (0.125-0.250 µm in diameter), morphologically described as having a thick shell with a featureless interior, was resistant to treatments which disrupted other yolk components. These were called insoluble yolk globules and described by Vadehra et al. (1977).

Yolk granules, have been identified in the TEM by their distinct electron-dense appearance due to intense osmiophilia because of the phospholipid moiety in the lipovitellin component (Bernardi and Cook, 1960) and/or the electron density of the bulk minerals, such as iron and copper they contain (Table 23 in Romanoff, 1967), in their phosvitin component (Lange et al., 1983). TEM of thin sections of resin-embedded samples have shown that granules are roughly spherical, and that the diameter is approximately 1.6 µm (Chang et al., 1977).

There is no general agreement with respect to the inner structure of granules or on the presence or absence of a membrane surrounding them.

The presence of low density lipoprotein particles, invisible at the level of the light microscope was confirmed by TEM. They appear as spherical particles of varying size (Nichols et al., 1969), bound by an osmiophilic monolayer (Evans et al., 1974; Fawcett, 1986) which Bellairs et al. (1972) had described as rings, and Perry and Gilbert (1985) had described as "particulate ground plasm".

1.2.2. Biochemistry of the yolk

Compositional changes in yolk during incubation of a fertilized egg can be evaluated by comparison with yolk of an infertile, unincubated egg. The newly laid chicken egg contains about 30-40% yolk, which ranges from yellow to orange in color due to carotenoid pigments (Romanoff and Romanoff, 1949). Romanoff and Romanoff (1949), Romanoff (1967) and Vadehra et al. (1977) showed that yolk is an oil-in-water emulsion consisting of 50% water, 35% lipid, 15% protein and 1% carbohydrates. Cook (1968) isolated 3 fractions from yolk using centrifugation - granular, water soluble (livetins - including α-livetin, the plasma albumin; β-livetin, the α_2–glycoprotein; and γ-livetin, the plasma γ-globulin) and low-density or floating fraction. Most of the lipid is part of the soluble low-density lipoprotein (LDL) fraction, which itself is 12 - 15% protein (the apoprotein component), and is the main carrier of cholesterol in vertebrates (Rawn, 1989). The rest of the lipid is found in the aqueous medium, forming a third of the yolk granules. Yolk lipids occur mostly in a non-covalent combination with proteins and lipoproteins and also as membrane and globule components.

Yolk granules are the most plentiful, the smallest and the most stable particles in the yolk, and have been studied in great detail biochemically (Burley and Vadehra, 1989). Burley and Cook (1961) described 3 main components of the granules. They are lipovitellin, a globular protein (Evans et al., 1968) characterized by Chang et al. (1977) as a high density lipoprotein (HDL), which functions as an important carrier of phospholipids in living systems (Rawn, 1989); phosvitin, the major phosphoprotein component of

the yolk, which was shown by Richards and Steele (1987) to bind ferric and copper ions, and by Burley and Cook (1961) to bind calcium; and low density lipoprotein (LDL) (Saari et al., 1964), an important cholesterol carrier in living systems (Darnell et al., 1986). Carbohydrates, minerals (listed in Table 23 in Romanoff, 1967) and yolk pigments (Bellairs, 1964) are also found in the granules.

An exhaustive list of enzymes have been biochemically isolated from egg yolk. A summary of early work identifying enzymes found in the yolk of fertilized eggs appears as Table 27 in Romanoff (1967) and includes phosphatases, proteases, and esterases. More recently, these and additional enzymes have also been isolated from yolk from fertilized or unfertilized eggs, or eggs whose status was not reported, and are reported in Table A3 in Burley and Vadehra (1989). Moors and Stockx (1972) isolated and characterized phosphoesterases from fertilized and unfertilized egg yolks; RNase and β-glucopyranosidase from fertilized and unfertilized egg yolks by Willems et al. (1976); and alkaline phosphatase in unfertilized egg yolks by Debruyne and Stockx (1979). In addition, acid phosphatases from unfertilized egg yolks have been purified and studied in detail by Debruyne (1983), and acid proteases, identified as Cathepsin D, were purified and isolated from egg yolks of unknown status by Wouters et al. (1985).

1.2.3. Integration of structural and biochemical characteristics of yolk

Bellairs et al. (1972), in a combined structural and biochemical study, correlated biochemical data with distinct structures visible in egg yolk by TEM. The free-floating lipid droplets and contents of the yolk spheres described by Bellairs (1961) were shown to correspond to the granule fraction which is rich in phosvitin and lipovitellin. The continuous phase fluid described morphologically was correlated to the supernatant, or floating fraction, which is rich in LDL. One important observation made during this study was that only a few types of structures were detected in the same yolk from which many biochemical compounds had been isolated.

Because we are interested in granules and LDL in this study, we will confine the remainder of this section to these two yolk components.

Much information on granule structure has been obtained by subjecting them to physicochemical treatments which were then evaluated by thin-section TEM. Using this approach, Willems et al. (1976) proposed that granules consisted of "a core of lipovitellin surrounded by phosvitin and an outer layer of LDL, in close association with a few enzymes". Chang et al. (1977) gave a TEM description of yolk granule structure based on sequential extraction with increasing concentrations of NaCl. Partial disruption in 0.34M (1.9%) NaCl resulted in the visualization of "distinct electron dense subunits distributed throughout this loosely-structured granule without any definite pattern". The outside of the granule was "fluffy, suggesting the presence of thin fibres". The granules were completely disrupted when exposed to 1.71M (9.8%) NaCl, releasing myelin figures and highly electron-dense masses. These were associated with large globules (which they hypothesized were lipovitellins, supporting the work of Evans et al. (1968) on *in vitro* enzymatic digestion of lipovitellin), and small globules (falling in the size range reported by Sugano and Watanabe (1961) and Martin et al. (1964) for LDL). Chang et al. (1977) hypothesized that these components were clustered together as slightly electron-dense masses. On the basis of these observations, they described the granules as being composed of "electron-dense microparticles, hair-like strands, small and large globules and myelin figures". Myelin figures have been observed by other workers which include Bellairs (1964), and Garland and Powrie (1978a and b). Chang et al. (1977) concluded that the microparticles were lipovitellins, strongly attached to the hair-like, strands which they suggested were the phosvitin molecules. They proposed that the sub-granules were "constructed of one large globule around which numerous strands with absorbed microparticles are intertwined. The subunits appear to be dispersed in a matrix consisting of small globules". More recently, Richards and Steele (1987), in agreement with Bellairs et

al. (1972), described the yolk granules simply as phosvitin-lipovitellin-metal associations which are packaged as calcium complexes.

Using discontinuous centrifugation followed by electrophoresis, Willems and Stockx (1973) demonstrated the existence of 2 types of granules in yolk from unfertilized eggs. The G1 type of granule was found to contain most of the iron and ribonuclease, while G2 contained most of the acid phosphatase, and more LDL than G1.

1.2.4. Changes of structural and biochemical characteristics of yolk during development

Biochemical analysis of yolk during embryonic development has shown that its composition is continuously changing. One of the earliest changes is the formation of the sub-blastodermic fluid (also called fluid yolk) beneath the embryo from day 2 of incubation reaching a maximum volume at about the seventh day. It occupies the yolk sac nearest to the embryo. Schlesinger (1958) described how the internal structure of the yolk was destroyed by the formation of this fluid. Howard (1957) proposed that this fluid was formed by the movement of sodium ions from the albumen into the yolk sac, with the accompanying movement of chloride ions. This fluid accumulates on top of the rest of the yolk in the yolk sac because of the difference in density. This hypothesis has been supported by recent studies of Stern and MacKenzie (1983) and Deeming (1989).

Bellairs (1964) suggested that the change in consistency of the yolk was due to "enzyme activity and uptake of water from the egg white". Generally speaking, the major changes taking place in the egg are due to a redistribution of substances from outside the embryo to the embryo proper, and the production of energy for this distribution. Compositional changes result from such processes as the breakdown of high molecular weight proteins, the dephosphorylation of phosphoproteins (Martin and Saito, 1967; Debruyne and Stockx, 1981) and the movement of albumen and metal-carrying proteins into the yolk around day 11 of incubation (Richards and Steele, 1987). Movement of

calcium into the yolk mass from the shell occurs around 13 days of incubation, due to the action of the chorioallantoic membrane. This calcium is then stored in a non-toxic form in the yolk mass, in the form of spherules (Cheville and Coignoul, 1984; Threadgold, 1976). Burley and Vadehra (1989) have summarized the major changes in yolk during development: 1) an increase in free amino acids; and 2) a breakdown in yolk structure, representing alteration of granule structure and compositional changes in the lipids. Noble (1987) reported, based on biochemical analysis, the degradation and resynthesis of yolk lipids by the yolk sac during the massive relocation of lipid from yolk to embryo in the last week of incubation.

With regards to the yolk granules themselves, Debruyne and Stockx (1981) found that the number of granules steadily decreases during incubation. Granules are removed and processed rather than the LDL in the first half of incubation (Saito et al, 1965), and Cook (1968) found that some granules remained even at the end of incubation. Sugimoto and Yamada (1985) used biochemical analysis including electrophoresis to investigate changes in the water-soluble fraction and granule fraction of the chicken egg yolk during development. They demonstrated that the activity of benzoyl-L-tyrosine ethyl ester hydrolase in yolk sac epithelium was at its maximum from days 14 to 20 of incubation (Sugimoto and Yamada, 1986), suggesting that some yolk proteins are degraded during the last half of incubation. Phosphorus is more rapidly liberated from the granules than from the phospholipids early in development, suggesting that this form of phosphorus is more readily accessible to the embryo. However, this work of Sugimoto and Yamada (1985, 1986) demonstrates that certain phosphoproteins are taken up from the yolk from approximately Day 15 of incubation to hatching. McIndoe (1960) had discussed the importance of the uptake and processing of phosphoproteins in providing a source of inorganic phosphate required by the embryo for the production of ATP. He proposed that the phosphate is first incorporated into an acid soluble organic fraction and then converted to inorganic phosphate.

The yolk and yolk sac undergo many changes in composition during incubation which are known to be due to enzyme action, but the location of the action is not clear. Bellairs (1964) hypothesized that during incubation, enzymes act on yolk to break it down, suggesting that some enzymes were synthesized by the developing yolk sac while others were preformed in the yolk. She felt (as did Romanoff, 1960) that some of this activity was extracellular in nature. She noted, however, that earlier investigators had not made the distinction between yolk and yolk sac in their biochemical enzyme studies, and that it was difficult to rule out the extracellular action of these enzymes. Since the enzymes listed earlier in Section 1.2.2. can be demonstrated in both yolk (Table 27 in Romanoff, 1967, and Table A3 in Burley and Vadehra, 1989) and yolk sac (Table 22 in Romanoff, 1967, and Kusuhara and Ishida, 1974), their origin is not clear.

Holdsworth and Wilson (1967) demonstrated that segments of yolk sac at all ages studied were able to take up glycine from culture media. They hypothesized that the presence of such a transport system for amino acids could be correlated with the appearance, early in incubation, of proteolytic enzymes (to degrade proteins to amino acids in the yolk mass) reported by Emanuelsson (1955) and Taussig (1965). Holdsworth and Wilson (1967) believed that this correlation provided evidence that intracellular digestion of yolk at early ages may be largely replaced by extracellular digestion and absorption of soluble products as incubation progressed. Sugimoto and Yamada (1985) found by electrophoresis and measurement of enzyme activities that protease and hydrolase activity was higher in the yolk sac than the yolk. They were able to determine that endogenous proteolytic activity was greatly increased in the yolk at acid pH, and concluded that endogenous yolk sac proteinases play an important role in yolk degradation.

In the most recent discussion of this problem of intracellular versus extracellular digestion of yolk, Burley and Vadehra (1989) summarized the subtleties of the dispute in three hypotheses, which do not exclude each other. 1) Enzymes originate from the yolk sac and are secreted from them into the yolk; 2) Cofactors in the yolk sac are released

which activate yolk enzymes [Burley and Vadehra note that it would be difficult to differentiate between 1 and 2j; and 3) The site of enzyme action is the cells, and some products return to the yolk. According to Burley and Vadehra (1989), this last hypothesis is supported by the work of Noble et al. (1984) on the re-esterification of cholesterol by the yolk sac, but his work actually gives no details on a possible return mechanism.

The above hypotheses are based mainly on biochemical data, indicating changes in amounts of components, without pinpointing the location of these changes. Morphological studies in conjunction with histochemistry and cytochemistry can potentially show where these changes occur, as changes in morphology and enzyme localization; However, these studies (including Mobbs and McMillan, 1979, 1981) were done over a decade ago, with inconclusive results.

1.3. The yolk sac

Classical knowledge on the yolk sac is extensively analyzed in various reviews (Hamilton, 1952; Romanoff, 1960; Freeman and Vince, 1974). The following description of the macroscopic and histological aspects originates mostly from these sources.

1.3.1. Structure

Macroscopical characteristics of the yolk sac

The chicken egg is fertilized and starts to develop before the egg is laid. At the time of lay the embryo has already progressed and it is constituted by a multicellular disc situated at the embryonic pole of the egg and visible with the naked eye as a rounded whitish spot; this is known as blastoderm or blastodisc. As incubation progresses this disc starts to expand very rapidly and begins to surround the yolk. As this growth progresses three concentric zones become clearly visible in the blastoderm when seen from above. The central area of the blastodisc remains more transparent and is known as the area pellucida. It is in this zone where the embryo proper appears. The intermediate area

surrounding the area pellucida becomes rapidly vascularized and is known as the area vasculosa; a thin peripheral zone, remains unvascularized and is known as the area vitellina. As the embryo in the zona pellucida differentiates and grows, the non-embryonic portion of the blastoderm (areas vasculosa and vitellina) continues to expand and surround the yolk; it is the area vasculosa which becomes larger and larger, while the area vitellina constitutes the advancing edge. Both areas can be seen with the naked eye by transparency and easily dissected; they are known as the yolk sac. By the fifth day of incubation half of the yolk has thus been enclosed and by the 10th day the yolk is totally surrounded except for a very small pole that remains permanently open and communicates with the albumen (albumen umbilicus). The yolk is originally surrounded by an acellular membrane known as the vitelline membrane. As the yolk sac expands and surrounds the yolk the vitelline membrane is seen to retract towards the lower pole. When the yolk sac is opened and its internal surface is observed, one can see that numerous folds are formed; these increase in size, length and complexity with age, and provide an increasing surface area in contact with the yolk.

Bellairs (1964) described how the folding of the yolk sac depends upon the development of the blood vessels in the following way: "the main folds radiate from the embryo and the principal omphalo-mesenteric vessels run along these ridges. As development proceeds, and the ridges branch and become more complex and some places they fuse together so that the cavity of the yolk sac becomes divided into a series of chambers. The ridges are only found in the vascularized regions."

Histological characteristics of the yolk sac

The blastoderm at the time the egg is laid is formed by a double layer of undifferentiated cells. During the first hours of development the blastoderm is seen to organize itself into 3 distinct layers (ectoderm, mesoderm and endoderm). In the area pellucida a primitive streak and later a neural plate are formed and constitute the axis of the future

embryo. Both the embryo itself and the peripheral zone of the blastoderm are now formed by three layers of cells. The central layer, mesoderm, becomes rapidly split into two layers separated by a cavity, the extraembryonic coelom. Thus, one of the layers remains attached to the endoderm, both forming the splanchnopleura, while the other layer remains attached to the ectoderm forming the somatopleura. It is the mesoderm of the splanchnopleura which contains most of the blood islands and newly formed blood vessels. While the somatopleura will contribute to form the amnion and the chorion, the two-layered extraembryonic splanchnopleura will form the yolk sac. Thus, the yolk sac is a bi-layered structure constituted by the inner endoderm which is facing the yolk and which we shall call from now on the yolk sac epithelium and by the mesoderm which will form the mesenchyme or connective tissue of the yolk sac and will contain all its blood vessels and blood islands or hematopoietic islands. As folds are formed they are also formed by both layers: a core of mesenchyme covered by epithelium.

The epithelium was described as a simple, columnar epithelium, whose cells have a vacuolated cytoplasm and a small, basal nucleus (Romanoff, 1960; Juurlink and Gibson, 1973). According to Mobbs and McMillan (1979), most of the cytoplasm is occupied by large intracellular yolk drops, and the rest of the cytoplasm was found at the apical and vascular poles of the cell, and in between the intracellular yolk drops.

The splanchnic mesoderm, in close contact with the epithelial layer, differentiates into blood vessels, blood cells and mesenchymal cells. As development progresses the mesenchymal layer differentiates. Some mesenchymal cells become fibroblasts and produce a modest number of thin collagen fibres. The layer can thus be called loose connective tissue. At regular intervals other mesenchymal cells proliferate and form groups of cells separated by very little intercellular space. These are called blood islands and their evolution will be discussed in the next section. In the external wall of the yolk sac the connective tissue is usually formed by 2-3 layers of fibroblasts. As folds are formed, the connective tissue forms their core.

Blood vessels in the yolk sac

Detailed descriptions of the hematopoiesis in the mesenchymal layer are available (Romanoff, 1960; Small and Davies, 1972; Cuardros et al., 1992). A discussion of this subject is out of the scope of this Introduction. I shall only summarize location and timing of the process.

The extraembryonic (or vitelline) blood vessels (Patten and Carlson, 1974) start as blood islands in the area vasculosa of the chick embryo at the beginning of day 2 of incubation. The blood islands form from mesoderm, as small clusters of cells in intimate contact with the endodermal layer of the yolk sac. As time goes on, some of the mesodermal cells flatten around the outside of each blood island, and separate from the rest of the cells inside. The external, flattened cells remain in contact with each other, and completely enclose the cells inside (Gonzalez-Crussi, 1971). These boundary cells become the endothelial cells of the primitive blood vessels. A series of communicating vessels results from the union and growth of the blood islands in this way (Patten and Carlson, 1974; Fawcett, 1986). Meanwhile, the cells inside these vessels have differentiated into blood cells. At about 3 days of incubation, this network of blood vessels communicates with the heart and circulation becomes established.

Classical histological studies have failed to disclose the presence of a lymphatic circulation in the yolk sac (Romanoff, 1960).

Histochemical characteristics of the yolk sac

Moog (1944) was the first to histochemically study the progression of alkaline and acid phosphatase activity in the embryo and yolk sac during incubation. Using an azo-dye method to study the presence of esterases and their location in the early chick embryo, Zacks (1954) found that their activity in the extraembryonic membranes develops concurrently with the vascular system. Medda and Bose (1967) confirmed the presence of alkaline phosphatase and glycogen in the yolk sac epithelium throughout incubation. Chapeville and Fromageot (1967) and Bennett (1973) used the histochemical localization

(by a lead capture method) of cysteine lyase (an enzyme which catalyzes the substitution of the cysteine thiol group to produce cysteic acid, a step in the specific enzyme system allowing the reduction of oxidized inorganic sulfur and its utilization for the synthesis of organic-sulfur containing compounds) to study differentiation in the yolk sac epithelium. They established a relationship between the morphological specialization (differentiation) and organization of epithelial cells, their loss of mitotic activity and the increase in cysteine lyase activity in yolk sac epithelium. Willier (1968) described a gradient of Nile Blue sulfate staining, which reflected changes in acidity of fats (i.e., ranging from neutral lipids to phospholipids and fatty acids) from apex to base of yolk sac epithelial cells as yolk was digested; the emphasis of this work was on glycogen metabolism in the yolk sac epithelium. Juurlink and Gibson (1973) histochemically localized protein (by detection of the terminal α-amino groups), acid mucopolysaccharides (glycosaminoglycans), carbohydrates, RNA, alkaline phosphatase, acid phosphatase and lipid in the epithelial cells, connective tissue and blood vessels of the chick yolk sac at different stages of development. They found a similar staining pattern of the yolk granules and epithelial cell "vesicles" during development to that described by Willier (1968), which they believed reflected the intracellular digestion of yolk nutrients.

Cheville and Coignoul (1984) studied turkey yolk sac epithelium, and found that similar to chick yolk sac epithelium, they contained massive amounts of neutral lipids, phospholipids, glycolipids and glycogen, and showed a high concentration of alkaline phosphatase at the luminal surface. In addition, they found that late in incubation there was a heavy cytoplasmic staining of the epithelium, when using the von Kossa or alizarin red S stains for calcium.

Biochemical analysis of yolk sac has found it to contain many enzyme species with activities which reach their maxima at different times throughout development, which could be involved directly or indirectly in the digestion of yolk components. Romanoff (1967, Table 22) has summarized a list of yolk sac enzymes, as discussed earlier, in

Section 1.2.2. More recently, Kushuhara and Ishida (1974) histochemically demonstrated the presence and constant high activity of the glycolytic enzymes glucose-6-phosphatase, acid phosphatase, lactate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase in yolk sac epithelium incubated for 12 to 18 days.

In this study, the presence of acid phosphatase (that is, the group of general phosphatases active at acid pH) in yolk sac is of interest, because it can also signify the presence of lysosomal activity in cells (Schellens et al., 1977). Juurlink and Gibson (1973) found in their histochemical study (using the Gomori method) that acid phosphatase was present in the epithelial cells of the yolk sac, in vesicles which contained yolk granules, with its maximum activity occurring between 9 and 12 days of incubation. However, this study was inconclusive in confirming the presence of lysosomes and their associated structures in yolk sac epithelial cells. Kusuhara and Ishida (1974) used the Azo-dye method to histologically localize acid phosphatase activity in the apices of the yolk sac epithelial cells, and found its activity increased around the time of hatching.

Ultrastructure of the yolk sac

Epithelium

Lambson (1970) was the first to give a detailed ultrastructural account of the chick yolk sac, throughout incubation, using TEM. He observed that the cells of the epithelial layer were distinctly polarized, contained basal nuclei, mitochondria, some rough endoplasmic reticulum, and glycogen granules. The apical surface was described as specialized, having short microvilli and coated pits, and the apical cytoplasm just beneath the membrane exhibited absorptive features such as canaliculi, coated vesicles and other membrane-bound apical vesicles. A collection of large, heterogeneous vacuoles were present in the apical cytoplasm, and junctional complexes were observed at the lateral membrane. Juurlink and Gibson (1973) described the large basolateral intercellular spaces between epithelial cells at early ages.

Mobbs and McMillan (1979) used TEM and SEM to study the structure of yolk sac epithelial cells before and after vascularization to observe whether the cellular structure and mode of nutrient transfer changed. They showed that "granular" intracellular yolk drops resulted from engulfment of extracellular yolk. The "pleiomorphic" intracellular drops which occupied most of the cell and gave it a vacuolated appearance (as previously described by Bellairs, 1964) were more complicated in structure, and contained material of a varied appearance. Mobbs and McMillan (1979) also showed that the non-vascular area pellucida was comprised of small, squamous epithelial cells, containing mostly homogeneous yolk drops, while the vascularized area vasculosa consisted of tall, columnar epithelial cells which contained both homogeneous and pleiomorphic yolk drops. Regarding transfer of digested substances from the epithelial cells to the embryo, they believed that the absence of a basal lamina at the young ages that they studied (approximately days 2 to 7 of incubation) would allow nutrients to diffuse or be pumped across the basal membrane and into the blood vessels. Cheville and Coignoul (1984) examined turkey yolk sacs throughout incubation using TEM techniques. They provided a detailed description of the epithelial cells, which are very similar to those of the chick (described above) and emphasized the ultrastructural aspects of the movement of calcium through these cells.

Nature of intracellular digestion

As discussed previously, most authors studying the electron microscopy of yolk agreed that an active process of uptake of yolk takes place at the apical end of the cell.

There has been disagreement in the description and in the nomenclature given to the various vacuolar inclusions which occupy a large proportion of the epithelial cells.

It is however evident that the structures known as intracellular yolk drops (types A, B, and complex; Bellairs, 1958, 1963), yolk spheres (types L1 to L5; Willier, 1968), intracellular yolk drops or inclusions (Lambson, 1970), yolk granules, lipid droplets and

myelin-like configurations (Juurlink and Gibson, 1973), apical vacuoles and intracellular yolk drops (granular, pleiomorphic and homogeneous; Mobbs and McMillan, 1979, 1981) and granules, lipid droplets and lysosomes (Cheville and Coignoul, 1984) all constitute different stages in the processing of yolk constituents. There is no clear indication as to what stage in the process do primary lysosomes become attached to the endocytosed material. It is not clear, either, at what stage the digestion products of this degradative process leave the lysosomes and are either stored by the cell or are transported.

Epithelial storage and transport of carbohydrates, protein and lipids

For many years the general idea of transfer of substances to the developing embryo was the very obvious one of yolk to yolk sac membrane to the vitelline circulation, to the embryo, to be used for energy production and growth (Romanoff, 1960). With time, and more information obtained from many different types of experiments, a better understanding of the mechanisms associated with this process was gleaned. The most recent viewpoint is that digestion and transport exist in intimate association depending upon the nutrient (protein, carbohydrate, lipid, metals), duration of incubation and other unknown factors. Biochemical and structural work is in agreement in this theory. Literature published over the past three decades indicates that the transfer of nutrients from yolk to embryo has been defined as one process which includes uptake, digestion and transport of digested substances, the steps of which are intimately related, as discussed in the previous section.

Embryologists believed that structural differentiation of embryonic cells were concurrent with their functional differentiation, and vice versa. They had found, from culturing experiments, that the avian yolk sac assumed certain functions during incubation, which would eventually be taken over once the organ responsible for this function in the developing embryo was well enough differentiated. This line of thought guided the research of those who compared functional development (development of

enzyme systems) between yolk sac and liver or intestine. Holdsworth and Wilson (1967) investigated transport of sugar in yolk sac epithelium and intestine with kinetic studies and isotopes and concluded that transport of sugar in yolk sac epithelium was similar to that in intestine. They also concluded that yolk sac epithelium acquired the ability to transport sugar into the cells against a concentration gradient as incubation progressed. Willier (1968) studied the morphological manifestations of carbohydrate metabolism and glycogen storage in the yolk sac epithelium and demonstrated that the yolk sac epithelium and liver function in cooperation during embryonic development, especially as sites for glycogenesis.

Holdsworth and Wilson (1967) also hypothesized that the yolk sac epithelium possesses a transport system for amino acids by the sixth day of incubation, based on their kinetic studies and use of isotopes. They found that this system is active throughout incubation, but its activity decreases at the time of hatching.

Lambson (1970) was the first researcher to use TEM to study the ultrastructural basis for the various and changing transport processes in yolk sac epithelium. He sought morphological manifestations of protein transport in yolk sac epithelial cells, and could find little evidence of such processes at this level. This was surprising, since Brierly and Hemmings (1956), who injected "immune serum" into the yolk held in the yolk sac, found that some specific proteins, namely antibodies, could be transported from the yolk through the yolk sac epithelium without being broken down. The process of selective transport of antibodies from the chick yolk sac to the embryonic circulation was discussed in detail by Woods and Roth (1983), and was attributed to the presence of specific receptors on the yolk sac epithelial cells.

Mobbs and McMillan (1981) investigated the transport process in the yolk sac before and after vascularization, using the tracer proteins horseradish peroxidase and ferritin, as well as latex spheres. On the basis of this work, they proposed separate routes for uptake of large and small particles by the epithelial cells, which they believed represented the

pathways for yolk granules and LDL particles, respectively. In addition, Mobbs and McMillan (1981) hypothesized that the products of intracellular digestion reach the yolk sac circulation by transport or diffusion through the plasma membrane rather than by exocytosis. This hypothesis was based on the morphological characteristics of the vascular surface of the epithelial cells which they observed.

The role of the yolk sac in the lipid metabolism of the embryonic chick has been studied for many years. Lambson (1970) was the first to describe chylomicron-like particles in epithelial cells of the chick yolk sac during the last week of incubation. These components had been detected biochemically after ultracentrifugation of blood taken from the yolk sac (Schjeide et al., 1963). Biochemical studies have demonstrated that a massive transport of lipid from the yolk to the embryo occurs from days 14 to 21 (Noble and Moore, 1964), is accompanied by extensive and unique changes in lipid composition and metabolism, and is mediated by the yolk sac (Noble, 1987).

Because no lymphatic system has been demonstrated in the yolk sac (Romanoff, 1960), lipid is assumed to be transported in the blood circulation to the embryo. This is the route for absorption of dietary fats even in the adult chicken because the lymphatic system is not as well developed in birds as it is in mammals (Bensadoun and Rothfeld, 1972). The yolk sac is believed to resynthesize unsaturated fatty acids (especially arachidonic acid) from the highly saturated ones present in the yolk. Noble (1987) suggested this process goes on because the embryo needs much material for the assembly of membranes during its growth, and that the types of fatty acids needed are not available as digestion products of the processed yolk.

Mesenchyme and blood vessels

Mobbs and McMillan (1979) described the mesenchymal layer as being composed of one or two layers of loose, stellate mesenchymal cells with overlapping processes. Coated pits were observed on their plasma membranes, and their cytoplasm contained

rER, free ribosomes, lipid droplets and a central nucleus with a nucleolus. Microfilament tracts and Golgi stacks were also observed in the cytoplasm. The processes of the mesenchymal cells extended to the basal lamina of the epithelial layer and endothelium of the blood vessels. Desmosomes were observed where mesenchyme cells came in contact with each other. During incubation, some of the mesenchymal cells became organized into blood vessel walls, while others remained stellate in shape and were distributed randomly throughout the mesenchyme. Later in incubation, these differentiate into fibroblasts, which produce collagen fibres and flocculent material observed in the intercellular spaces.

Mobbs and McMillan (1979) also described the blood vessels found throughout the mesenchymal layer, and indicated that arteries could not be differentiated from veins in the early stages. The endothelial cells which comprised the blood vessels contained the usual compliment of maintenance organelles and were described as being "arranged as a discontinuous layer", but the true nature of their association cannot be discerned from either the micrographs or their description. The junctional associations between the cells varied. Mobbs and McMillan (1979) observed a discontinuous basal lamina associated with the endothelium.

1.3.2. Biochemistry of the yolk sac

Biochemical analyses of the yolk sac (as compiled by Romanoff, (1967)) have shown that up until the 15th day of incubation, much of the yolk that the yolk sac takes up is used for its own growth, and in the early stages, comprises the bulk of living matter in the egg. It has a distinct chemical composition, and consists of 90% water. The total solids are 75% protein and about 25% lipid. Toward the end of incubation, the protein content of total solids drops, and although the lipid concentration remains constant, there is a drastic increase in neutral lipids and cholesterol. Generally speaking, the absolute values of the chemicals (excluding vitamins) rises then until approximately day 15 of

incubation, when the yolk sac is at its maximum size, and falls after that as the yolk sac begins to decline in size and weight.

Many enzymes have been biochemically isolated from the yolk sac. Grillo and Grillo (1966) isolated a phosphorylase, whose activity rose and fell periodically during incubation, possibly in response to glucagon. Pons et al. (1986) studied the ontogeny of amino acid metabolism (nitrogen metabolism) in liver, intestine and yolk sac during incubation of chick embryos. They isolated and studied the activities of the enzymes aspartate and alanine transaminase, glutamate dehydrogenase, adenylate deaminase, glutamine synthetase and xanthine dehydrogenase. Pons et al. (1986) found that these tissues paralleled each other in the pattern of enzyme development and initial levels of enzymes during incubation. After hatching, amino acid metabolism was found to be enhanced in yolk sac and intestine, which they hypothesized was comparable to changes observed in mammalian tissues at the onset of weaning.

Sugimoto and Yamada (1985) purified and characterized benzoyl-L-tyrosine ethyl ester hydrolase found in chick yolk sac as a protease with an activity similar to chymotrypsin. They then studied the activity of this enzyme in yolk sac homogenates and found that its activity increases from day 5 until the end of incubation (Sugimoto and Yamada, 1986). Wouters et al. (1985) isolated acid proteases in yolk sac at 12 days of incubation which they identified using biochemical and physicochemical techniques as cathepsin D, which were found to hydrolyze endogenous proteins and enzymes.

1.4. Rationale for experiments

From previous studies on the structure, function and biochemistry of the yolk and yolk sac, the major aspect on which there is no total agreement is to what extent the yolk is digested intracellularly. The most recent studies on chick yolk sac were done over 10 years ago (Mobbs and McMillan, 1979, 1981).

In order to investigate this aspect, and making use of new information on conventional and novel fixation procedures, I will attempt to further clarify the ultrastructure of the yolk during development in order to test the hypothesis that a certain amount of extracellular digestion of yolk occurs.

1) It is still not established whether yolk granules are membrane-bound, or whether this confusion results from discrepancies in the literature in the names and definitions of the structural components of yolk used by different authors. By using TEM to study yolk granule structure under different conditions of fixation and incubation, we can investigate whether granules are in fact membrane-bound, and how their structure changes during digestion. With this information I hope to provide a clearer picture of the site of yolk digestion (i.e., intracellular or extracellular).

2) As discussed previously, important aspects of intracellular digestion remain to be clarified. I shall attempt to demonstrate the presence, in the epithelial cells, of structures specialized for uptake, digestion and transport of yolk and digestion products. Acid phosphatase histochemistry and cytochemistry will be used to identify structures as lysosomes. This would signify the presence of intracellular digestion, the progress of which may be dependent on the age of the embryo.

3) As mentioned earlier, the ultrastructural characteristics of blood vessels in the yolk sac have been incompletely described in detail previously (see section 1.3.1). Since the wall of the blood vessel in the yolk sac must allow penetration of blood cells (which are formed outside of it), and, probably, macromolecules, one would want to establish if the endothelium has similar characteristics to those in the adult which allow this type of transport. For this reason, in our ultrastructural study, we shall pay special attention to the characteristics of the endothelial cells and the vascular basal lamina.

If the above objectives are met, it will be possible to reevaluate certain aspects of the old literature, to interpret any new results, and to establish whether a better agreement between structure and biochemistry can be made. The ultimate goal is to have a better understanding of the digestion of yolk in the yolk sac of the chick embryo.

2. Materials and Methods

2.1. Incubation of eggs

Eggs were obtained from White Leghorn hens, from either the Greenbelt Farm of Agriculture Canada or from a commercial source. Infertile eggs were collected from the hens and were used within 24 hours, without incubation. Fertilized eggs were incubated in an automatic incubator (Petersime 1) which maintained constant temperature (37°C) and relative humidity (60%), and automatically turned the eggs every three hours. Fertilized eggs were removed for study as required, age being counted after hour 0 of incubation.

2.2. Fixation of yolk samples

2.2.1. Yolk from unincubated eggs

In the case of unincubated eggs, the yolk was removed by cracking the egg shell, the albumen being separated from the yolk manually; the yolk was rolled on paper towels to free it of adhering albumen. The yolk was then placed into a regular kitchen plastic egg separator as a support to hold the yolk during sampling. After puncturing the vitelline membrane, the yolk was aspirated, using a 10 mL disposable syringe with a 21 gauge hypodermic needle, without dilution or separation of components. Droplets of yolk, 1-3 mm in diameter, were extruded from a 10 mL disposable syringe through a 21 gauge hypodermic needle directly into the fixative solutions, while holding the tip of the needle underneath the surface. The surfaces of the droplets solidified when they came into contact with the fixative. However, it took considerably longer for the interior of these droplets to attain the same consistency as the exterior, due to the slow penetration of the fixative solutions. Five droplets of yolk were put into each vial (which held about 10 mL of fixative). Seven different fixation procedures were used as described separately (1-7 in section 2.4). After 2h fixation at RT, droplets were cut in half with a blade and

allowed to sit in fixative overnight. They were postfixed and washed according to the different protocols, and then processed routinely for TEM.

2.2.2. Yolk from eggs incubated for 9 days

In the case of eggs incubated for 9 days, yolk was removed from the interior of the yolk sac using a 10 mL disposable syringe fitted with a 21 gauge hypodermic needle to draw up the viscous fluid. The fluid was decanted into 1.5 mL Eppendorf tubes and centrifuged in an Eppendorf Microcentrifuge 5415 at 14,000 rpm or decanted into standard centrifuge tubes, and centrifuged in a laboratory centrifuge (International Equipment Company) at 6000 rpm. In either case, the resulting pellets, which consisted of yolk granules, were processed whole for TEM.

2.2.3. Yolk from 15 day old embryos

Yolk from 15 day old embryos was not sampled directly. Instead the yolk adhered to the yolk sac from embryos of this age was studied.

2.3. Fixation of yolk sacs

Yolk sacs were studied at 3 ages, in order to observe morphological manifestations of the following stages in embryonic development (Romanoff, 1960):

Days 2-3 of Incubation - The embryo is young, and has no significant accumulation of lipid in its epithelial cells. There is early vascularization of the yolk sac. The yolk sac is only slightly folded at this early stage, without secondary folding.

Days 7-9 of incubation - At this stage, there is active uptake of yolk by the yolk sac (yolk reserves have been used up) and the yolk sac is extensively folded.

Days 13-15 of incubation - At this age, the yolk sac is at its maximum size; the embryo has now changed over from the yolk granules to the shell and chorioallantoic (CAM) system as the calcium source. The yolk sac is very heavily folded.

Incubated eggs were carefully cracked into a bowl containing cold isotonic saline, and the midregion of the area vasculosa of the yolk sac was securely held using forceps and was excised using sharp scissors. The excised piece of yolk sac was held with forceps and gently but thoroughly freed from adhering yolk in chilled isotonic saline before being quickly but gently transferred and submerged into chilled fixative. Samples were trimmed with a scalpel, and the edges which were trimmed off were discarded. The remaining tissue was minced into pieces approximately 2 mm x 3 mm in size and prepared for LM and SEM, or pieces 1 mm x 2 mm in size, to be prepared for TEM.

Samples of yolk sac were placed into a large volume of fresh cold half-strength Karnovsky's fixative, fixed on ice for at least 3 hours, and stored in the refrigerator (0^{o} - 8^{o}C) to continue the fixation overnight, approximately 15 hours. After a short wash the next morning in 0.1 M sodium cacodylate buffer at pH 7.4 containing 0.2 M sucrose, the samples were placed directly into 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4 for 2 hours at room temperature (approximately 24^{o}C).

Additionally, samples of yolk sacs from all ages were also fixed following the imidazole-buffered osmium tetroxide (IBO) procedure as described in Section 2.4.

2.4. Details on fixation protocols

1) Karnovsky's protocol (Karnovsky, 1965).

Primary fixation in Karnovsky's fixative, containing 4.0% paraformaldehyde (Sigma), 5.0% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 with 2 mM calcium chloride, for 2 hours at room temperature;

Wash in 0.1 M sodium cacodylate buffer, pH 7.2, overnight, in the refrigerator;

Postfixation in 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 hours at room temperature.

2) Half-Strength Karnovsky's protocol (modified from Karnovsky, 1965).

Primary fixation in half-strength Karnovsky's fixative, containing 2.0% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 with 2 mM calcium chloride, for 2 hours at room temperature;

Wash in 0.1 M sodium cacodylate buffer, pH 7.2, overnight, in the refrigerator;

Postfixation in 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 hours at room temperature.

3) Tannic acid protocol (modified from Perry and Gilbert , 1985).

Primary fixation in half-strength Karnovsky's fixative;

Wash in 0.1 M sodium cacodylate buffer, pH 7.2, overnight, in the refrigerator;

Postfixation in 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 hours at room temperature;

Tannic acid treatment (1.0% tannic acid (JBEM Services, Dorval, Quebec) in 0.1M sodium cacodylate buffer, pH 7.2, 18 hours in the refrigerator);

Wash in 0.1M sodium cacodylate buffer, pH 7.2, for 1 hour at room temperature;

Uranyl acetate treatment (0.5% uranyl acetate in veronal acetate buffer, pH 5.0, for 1 hour at room temperature);

Wash with veronal acetate buffer, pH 5.0.

4) Malachite green protocol (modified from Lawton, 1989).

Primary fixation in fixative containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, with 0.1% malachite green (Sigma) for 2 hours at room temperature;

Wash in 0.1 M sodium cacodylate buffer, pH 7.2, overnight, in the refrigerator;

Postfixation in 1.0% osmium tetroxide in 0.1M sodium cacodylate buffer, pH 7.2 for 2 hours at room temperature;

Wash in 0.1 M sodium cacodylate buffer pH 7.2, overnight, in the refrigerator;

Uranyl acetate treatment (2.0% uranyl acetate in water for 1 hour at room temperature);

Wash in distilled water, overnight, in the refrigerator.

5) Potassium ferricyanide protocol (Hayat, 1989).

Primary fixation in fixative containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, with 5 mM calcium chloride for 2 hours at room temperature;

Wash in 0.1M sodium cacodylate buffer, pH 7.2, with 5 mM calcium chloride, overnight, in the refrigerator;

Postfixation in 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 with 0.84% potassium ferricyanide (Electron Microscopy Services, Ft. Washington, PA), for 2 hours at room temperature;

Wash in 0.1M sodium cacodylate buffer pH 7.2, overnight, in the refrigerator.

6) Imidazole-buffered osmium tetroxide protocol (modified from Allan-Wojtas and Kalab, 1984).

Primary fixation in fixative containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, with 5 mM calcium chloride and 4.0% polyvinylpyrrolidone 40 (PVP 40) (Sigma) for 2 hours at room temperature;

Wash in 0.1 M sodium cacodylate buffer, pH 7.2, overnight, in the refrigerator;

Postfixation in 1.0% osmium tetroxide in 0.028 M veronal acetate buffer, pH 7.2 with 0.2 M imidazole, for 2 hours at room temperature;

Wash in 0.028M veronal acetate buffer pH 7.2, overnight, in the refrigerator.

7) Imidazole-buffered osmium tetroxide (IBO) protocol (Angermüller and Fahimi, 1982).

Primary fixation in fixative containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, with 5 mM calcium chloride and 4.0% polyvinylpyrrolidone 40 (PVP 40) (Sigma) for 2 hours at room temperature;

Wash in 0.1M sodium cacodylate buffer, pH 7.2, overnight, in the refrigerator;

Postfixation in 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 with 0.2 M imidazole, for 2 hours at room temperature;

Wash in 0.1M sodium cacodylate buffer pH 7.2, overnight, in the refrigerator.

2.5. Final preparation for light and electron microscopy

Samples were dehydrated in a graded ethanol series and infiltrated with mixtures of propylene oxide and Araldite 502 resin, embedded in fresh Araldite 502 resin in flat embedding molds, and polymerized in an oven at 60°C overnight (Hayat, 1989).

For light microscopy, thick sections (2.0 to 2.5 μm thick) were cut with glass knives using an LKB Pyramitome. Sections were placed on glass slides on a drop of distilled water, and affixed to the slides using heat. They were stained for 30 to 60 seconds on a hotplate using toluidine blue in borax, rinsed under a stream of distilled water and allowed to dry on a hotplate. Slides were then viewed under the light microscope (Olympus BH-2) without a coverglass, or with a cover glass mounted with Permount. Images were recorded on either Kodak TMax or Plus-X 35 mm film.

For electron microscopy, silver-gold or grey-silver sections were cut from blocks using a Reichert OM-U2 ultramicrotome equipped with a diamond knife, and collected on either carbon-stabilized formvar-coated or naked 300 mesh copper grids. The sections were stained with uranyl acetate and/or lead citrate and viewed in a Philips 300 Transmission Electron Microscope (either at Agriculture Canada, or the University of Ottawa Health Sciences Facility) operated at 60 kV. Images were recorded on Kodak Electron Microscope Film (sheet film, Estar thick base, #4489).

2.6. Scanning electron microscopy (SEM)

For SEM studies samples fixed with half-strength Karnovsky's fixative were critical point dried from carbon dioxide using a Polaron E3000 critical point drier. Dried samples were mounted epithelium side up on aluminum stubs using silver paint. After gold-coating using a Technics Hummer-II sputter coater, they were viewed either in a Philips 505 SEM operated at 15 kV and images recorded on Plus-X 120 mm film, or an ISI-DS 130 SEM operated at 20 kV and images were recorded on Plus-X 35 mm film.

2.7. Acid phosphatase histochemistry and cytochemistry

2.7.1. Azo-dye histochemistry

The Azo-dye method (Barka and Anderson, 1965) for histological localization of acid phosphatase activity was utilized because it is not affected by the presence of endogenous phosphate. This is possible because in this method, as opposed to the Gomori-type methods, the organic portion of the hydrolyzed substrate is captured, rather than the evolved phosphate group. Hydrolysis of substrates (such as α- and β-naphthyl phosphates, and substituted naphthol phosphates such as naphthol AS-BI) yields naphthol or substituted naphthol. This reaction product immediately combines with a diazonium salt such as hexazonium pararosanilin (which is formed from pararosanilin which is diazotized just before use). The final reaction product is a substituted naphthol-diazotized pararosanilin complex, which is a highly-colored, insoluble reaction product (Bittensky and Chayen, 1977).

Fresh yolk sac samples were obtained from 3, 8, and 15 day old embryos. Unfixed samples were frozen in isopentane cooled with liquid nitrogen and sections (16 μm thick) were cut. These were collected on alcohol-cleaned glass slides, allowed to dry at room temperature and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 15 minutes at room temperature. Alternatively, whole pieces of yolk sac were fixed in the same fixative for 3 hours on ice. Cryosections were incubated according to the method of Burstone (1958), as modified by Barka and Anderson (1965). The following solutions were used (from Barka and Anderson,1965):

Stock solutions:

A) Pararosanilin-HCl stock solution: 2g pararosanilin-HCl is added to 50 mL 2N HCl and heated. After cooling, the solution is filtered.

B) 4% sodium nitrite in distilled water.

C) Michaelis veronal acetate stock solution: (9.714 g crystalline sodium acetate + 14.714 g sodium barbiturate in distilled water, made up to 500 mL).

D) Substrate solution: 100 mg naphthol AS-BI phosphate is dissolved in 10 mL N,N-dimethyl formamide and stored in the cold.

Working solution:

The stock solutions were combined in the following order and proportions:

5 mL of Solution C was diluted with 12 mL distilled water. To this was added 1 mL of Solution D.

0.8 mL Solution A was mixed with 0.8 mL of Solution B. This was added to the solution above.

The pH of the working solution was adjusted to pH 5.0 with NaOH.

Control solution:

The solution was prepared as above, with the omission of Solution D, the substrate solution.

An intense red reaction product formed in the samples incubated in the medium containing the substrate Naphthol AS-BI phosphate. Photomicrographs were taken of these sections using either a Zeiss light microscope at the University of Ottawa Health Sciences Centre, or an Olympus BH-II light microscope at Agriculture Canada. Images were recorded on either Kodak TMax or Plus-X 35 mm film.

2.7.2. Gomori lead-capture cytochemistry

In the Gomori method for localization of acid phosphatase activity (Barka and Anderson, 1965 as modified by Lewis, 1977), the incubation medium contains a buffer, a phosphate substrate (a phosphomonoester, such as β-glycerophosphate) and a high concentration of lead ions. The phosphate released at the sites of phosphatase activity in the tissue is immediately precipitated as an insoluble, electron-opaque lead phosphate.

Small blocks of tissue (0.5 x 2.0 mm) were cut by hand from yolk sac which had been fixed with half-strength Karnovsky's fixative for 3 hours on ice and stored in cacodylate buffer at 4°C. Acid phosphatase activity was determined according to the method of Barka and Anderson (1962) modified by Lewis (1977). The following solutions were used (from Lewis, 1977):

Stock solutions:

A) 0.2M Tris/maleate buffer, pH 5.2

B) 0.1M sodium β-glycerophosphate

C) 0.02M lead nitrate

Working solution:

10 mL Solution A + 4 mL Solution B + 6 mL Solution C + 30 mL distilled water + 4.2 g sucrose.

Control Solution:

The control solution was prepared as above, with the omission of Solution B, the substrate solution.

After incubation of the yolk sac samples in control and substrate (containing β-glycerophosphate) media for 1 or 2 hours at room temperature, they were then washed for 1 hour at room temperature in the following solution:

Wash solution:

12.5 mL Solution A + 37.5 mL distilled water + 4.2 g sucrose.

After washing, the reacted material was stored in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.2 M sucrose in the refrigerator overnight. After the usual postfixation with OsO_4, the samples were processed for TEM routinely. Silver sections were cut and collected on naked grids; the sections were viewed without poststaining.

Because this method is based on capture of released phosphate by lead, the interference of endogenous phosphate (DNA, yolk, etc.) is unavoidable.

2.8. Measurements of selected structures

Sampling

In this descriptive study of yolk and yolk sac, we were interested in quantifying certain aspects of some structural components. Therefore, in consultation with Dr. Brian Thompson, Statistician, Agriculture Canada, measurements of selected structures were carried out as outlined below. Structures were measured directly from micrographs which were taken over the course of the study. In each case, 20 structures were measured unless otherwise specified, and standard deviation was calculated according to standard methods.

Due to the location of the structures with the regard to the cells, random sampling (i.e., sampling from random electron micrographs) could not be done. In a situation such as this, random sampling is difficult when the structure of interest is small in relation to the rest of the cell, and/or is found only in certain parts of the cell. Micrographs were instead selected which contained the structure to be measured, and care was taken to measure the structure only once. This approach was taken by Mori and Christensen (1980) in their morphometric analysis of Leydig cells in the normal rat testis. Williams (1977) had earlier described such an approach as "biased sampling", and believed that it allows information to be obtained which could not be obtained using a more formal approach.

Measurement of structures

The diameters of structures which were measured were assumed to be spherical. This approach results in a rather simple approximation of structure size which is sufficient for our purpose of providing a first attempt at measuring structures in yolk and yolk sac epithelial cells. When measuring structures in thin sections, this approach is sound if it is

assumed that the structures measured are large in comparison to section thickness; the structures can then be considered to be 2 dimensional (i.e., a section of negligible thickness) (Bozzola and Russell, 1992). These 2 dimensional measurements can then be used without interpretation as a 3 dimensional sphere, when only measurements of a preliminary nature are sought (Rosenfeld and Kak (1982), volume 2, pages 340 and 341). Weibel (1979) reasoned that crude approximations resulting from this type of approach are useful in initial attempts to quantify morphology, and yield results which permit new speculations about cell and tissue function. In such situations, Weibel (1979) believes that the well-known statistical methods to estimate spherical volumes from diameters will yield more information than is required, and so it is reasonable to use the simple approximations in cases such as the one we encountered during the course of this study. Details of the well-known methods for estimation of spherical volume from diameter measurements in micrographs can be found in stereology texts such as Williams (1977), Weibel (1979) and Rosenfeld and Kak (1982).

Additional assumptions

Granules: Thin sections of granules were photographed in the TEM as described above, and granules were measured from the resulting micrographs. Because the measurements were made from thin sections, only the granules having the largest diameters were measured, assuming that the section cut the granules at the equator, where the diameter would be the largest.

Dense bodies (lysosomes): Thin sections of dense bodies were measured as outlined for granules. An additional assumption was made that structures in the low end of the size range represented single dense bodies before fusion with each other. Therefore the small, individual structures were those that were measured.

Fossae: Fossae observed in the apical membrane of yolk sac epithelium cells were measured directly from SEM micrographs. The maximum diameter of fossae was measured in each case.

3. Results

3.1. Ultrastructure of yolk - comparison of the effects of conventional and novel fixatives.

3.1.1. Yolk from infertile, unincubated eggs

In my present work, I have concentrated on the study of the ultrastructural characteristics of yolk granules and yolk matrix, disregarding other structures which constitute only a small proportion of the yolk (membranes, globules, spheres, etc.). Figures 1 to 8 show micrographs of undiluted yolk prepared for TEM using 6 different fixation protocols. With all fixations, thin sections showed well preserved, electron dense, roughly spherical granules (diameter = 1.6 ± 0.1 μm) suspended in a moderately electron-dense matrix; occasionally, bilobed or "double" granules were seen. Figure 2 illustrates that due to the great electron density of the granules, grey-silver (75 nm thick) sections provided a better image of their inner structure than those of the silver-gold (120 nm thick) sections originally cut, shown in figure 3. In figures 2 to 8, which show the granules at a magnification higher than that in figure 1, they were seen to be devoid of a membrane, and to have an uneven outer boundary. Treatments used in figures 4 to 6 reveal that the granules were composed of close-packed spherical "subunits", approximately 36 nm in diameter. The periphery of the "subunits" was of higher electron density than their centre. The matrix was composed of tightly packed particles (20-30 nm), which completely filled the intergranular spaces. Table 2 summarizes the slight variations observed in granule and matrix particle structure among the 6 fixation protocols, and notes some minor differences. No fixative was able to demonstrate a boundary membrane around the outside of each yolk granule. Figure 5 shows that yolk fixed with malachite green provided slightly more detail in granule structure than the other treatments, while figure 6 shows that yolk fixed with tannic acid provided slightly more detail in matrix

structure. Figure 8, shows that yolk fixed with the IBO protocol gave an image different from all the others, since matrix particles stained more densely than the granules themselves. This is consistent with the fact that lipid content in the matrix is much higher than in the granules.

3.1.2. Yolk from fertilized, incubated eggs

Yolk at 9 days of incubation

Sub-embryonic fluid is known to consist of regular yolk diluted by large amounts of water and ions pumped from the albumen reserve; this dilution does not affect the ultra-structural characteristics of the yolk granules. For this reason, in 9 day old embryos, yolk granules in sub-embryonic fluid were studied rather than those in undiluted yolk. Plastic thin sections of granules were obtained from low-speed centrifugation of the fluid in both Eppendorf tubes and standard centrifuge tubes, and processing the resulting pellets for TEM. These sections showed that the granules had not been affected by the incubation process; they were circular, electron-dense granules with an uneven outline and no membrane (Figures 9 and 10). They were also formed by subunits similar to those in yolk from unincubated eggs. There appeared to be no stratification of components in the pellet.

Yolk at 13-15 days of incubation.

In the case of 13 to 15 day embryos, a substantial amount of yolk remained adhered to the yolk sac during fixation. Observations were therefore restricted to this adhered yolk rather than sampling the fluid yolk directly. In figure 11, the yolk which adhered to the apex of the epithelial cells at 15 days of incubation can be observed. The structure of the granules at this age was identical to the structure of unincubated yolk. In addition to the granules, calcium deposits in the form of laminar spherules shown in figure 12 (6 - 8 μm diameter) composed of light and dark concentric rings, were sometimes seen in the extra-cellular yolk mass of 13 - 15 day yolk sac.

3.2. The yolk sac

3.2.1. Quality of fixation

Preliminary work showed that the fixation quality of epithelial cells was extremely variable, and was affected by fixation temperature, time, and composition; age of the material, and the presence and amount of extraneous yolk adhering to the cells. While preservation was adequate for light microscopical and SEM observations, during TEM observations, zones of inadequate fixation were found. These consisted mainly of loss of the typical granular aspect of cytoplasmic matrix, loss of glycogen particles, incomplete preservation of membranes, etc.. Because these alterations were patchy, only descriptions of zones, which by all criteria summarized in Table 1 were well preserved, is presented here. Variation in fixation quality was observed within individual pieces of yolk sac and within individual cells themselves.

The IBO protocol gave the best results for visualization of lipids in yolk sac samples, and was used to study lipid transport.

3.2.2. General description of yolk sac. Light microscopy and SEM observations.

Figures 13 to 15 show light microscopy of 2 μm thick plastic sections stained with toluidine blue. The yolk sac is observed to be composed of a single layer of tall (approximately 60 μm in height) columnar epithelial cells, and a layer of mesenchyme (connective tissue) with stellate cells (fibroblasts) separated by an abundant matrix. At all ages studied, this mesenchymal layer contained numerous blood vessels and foci of hematopoiesis. The apex of each epithelial cell was in direct contact with the yolk mass, and the basal part of each cell was in close proximity to the mesenchymal layer containing the embryonic circulatory system, illustrated in figures 13 to 15. Most of the cytoplasm of epithelial cells was occupied by large vacuoles with contents which varied in their density of staining. Figures 13 to 15 show that fine strings of cytoplasm were

interspersed between these vacuoles, and that those structures associated with the uptake of yolk were located at the apex (Figure 15). The nucleus, visible in some of the cells in figures 13 to 15, was small and triangular in shape (approximately 10 μm on its longest side). It was always located at the basal pole of the cell.

Studies with the SEM were conducted to investigate how the general architecture of the yolk sac changed with age. With increasing age, the yolk sac became more folded, and this represented an enormous increase in the surface area for maximum exposure of the epithelial cells to yolk. At the low magnification shown in figure 16, 3-day yolk sac was seen to be fairly simple in architecture, without any significant folding, although the surface was undulated. By 8 days of incubation, the surface of the yolk sac was covered with large and small secondary folds (Figure 17). By 15 days of incubation, the yolk sac was highly convoluted, as seen in figure 18, and consisted mostly of secondary folds. Because of the high degree of folding of the yolk sac and higher density of the yolk at this age, yolk became trapped during specimen preparation, and this affected the quality of fixation. Within each age, regional variations were seen in amount of folding, which appeared to depend on distance from the embryo (degree of folding increased with distance from the embryo).

3.2.3. Ultrastructural studies on the yolk sac

The epithelium

The yolk sac epithelium is a simple columnar epithelium. The basic structure was similar at all ages examined, so I will present a general description which fits all ages, and will indicate differences only when necessary. Figure 19 shows a low power TEM composite micrograph of a single epithelial cell, illustrating its general characteristics. The cytoplasm in these cells was seen to be very complex because of the presence of numerous inclusions, thus confirming observations made with the light microscope. Because most of the epithelial cell was full of inclusions, the relatively small amount of

cytoplasm and its organelles was distributed between the inclusions and against the boundary membranes of the cell.

Figure 20 shows that adjacent epithelial cells share a junctional complex on the lateral membrane, at their apices. This consisted of a zonula occludens and a zonula adherens. Desmosomes, also shown in figure 20, were observed at a more basal position. The lateral membranes were thrown into folds which interdigitated with equivalent folds of the neighbouring cells.

At 3 days of incubation, there were large intercellular spaces, which were much reduced at 8 days. The spaces were further reduced at 15 days, except at the level of the nucleus, and the area basal to it, where the spaces remained distended (Figure 19).

Filamentous structures were observed throughout the cytoplasm, but were more concentrated in the apical and lateral regions, as shown in figure 19. For the sake of simplicity, we have assigned the name "microfilaments" to these structures, with the understanding that some of these may, in fact, be intermediate filaments. Measurement and comparison of the filamentous cytoskeletal components of the yolk sac epithelial cells was beyond the scope of this work. Figures 19, 25, 26 and 27 showed the presence of many microfilaments in bundles, parallel to the surface and in close proximity to apical vesicles, while microtubules, illustrated by figures 20 and 24, were observed close to the membrane-bound vacuoles in the apical cap of cytoplasm of the cells. Because of their close proximity to both the apical membrane and the vacuoles in the apical cytoplasm these cytoskeletal elements might contribute to the formation and movement of these vacuoles, although the tracts of microfilaments were not a constant feature, and not seen in all cells.

Glycogen deposits were also found in the cytoplasm throughout the cell, and probably served as storage depots for nutrients liberated from the yolk (Figure 27). Figures 20 and 21 show that the cytoplasm also contained many scattered areas of rough endoplasmic reticulum. The Golgi apparati (Figure 52) consisted of a number of small stacks of cisternae surrounded by vesicles. Some of the vesicles had a dense content and could be either primary lysosomes (Figure 21) or, in the case of those Golgi apparati located close to the lateral membrane, chylomicrons (Figures 48 and 50). Oval and elongated mitochondria, with electron-dense matrices, were observed throughout the cytoplasm (Figure

23). Figure 22 shows the typical triangular nucleus, with one or more prominent nucleoli; it was always located in a basolateral position.

Morphology of yolk uptake

As previously described for light microscopy (Section 2.2), the apical contour became increasingly convex with increasing age. Microvilli (in which core filaments were not visualized) were observed on the apical membrane, and are shown in figures 19, 25 and 28; they increase its surface area, and this increase in surface area is probably necessary for the absorption of water, small molecules and ions. Microplicae and coated pits, also present on the apical membrane, and shown in figures 19, 24, 26, 30 and 32 were probably responsible for uptake of the granules and matrix particles, respectively. The microplicae (folds) were comparable to those found in most cells during phagocytosis and were seen to take up single granules. Figures 30 and 31 allowed the identification of three main stages in the uptake of granules - capture (1), engulfment (2) and internalization (3). Note in figure 30 that the granule which has been internalized (3) is completely surrounded by cytoplasmic processes containing microfilaments. This probably shows the role of cytoskeleton in uptake of granules. SEM of yolk sac material gave a 3-dimensional view of the cytoplasmic extensions which extended from the apices of epithelial cells and engulfed granules as seen in TEM. In figures 33 to 38, the progression of the process of yolk uptake was followed through its different stages at 3 ages as described above for TEM - capture (1) from the yolk reserves outside of the cell, uptake (2) into the cell, and finally, fusion of the cytoplasmic arms so that only a protrusion was seen on the surface (internalization - (3)), which finally disappeared further into the cell. In some cases, as illustrated by figures 34 to 36, the granules had been removed during processing for microscopy and fossae remained (2 - 20 μm in diameter), which were studied to give yet another view of the process. Active phagocytosis of yolk probably does not occur in all cells to the same degree since zones without microplicae (TEM) or fossae (SEM) were frequently found. SEM showed that a large variation existed within

the tissue with increasing age; cells with abundant microvilli were seen directly next to cells which had few or no microvilli but had smooth apical surfaces; this probably indicates that cells might follow different phases in the process of phagocytosis and intracellular digestion.

In samples fixed using the IBO protocol which stains the lipids in yolk matrix (see Discussion, section 4.3.1.) it was possible to observe the matrix entering the epithelial cells along with the granules (Figure 29). In samples fixed using half-strength Karnovsky's fixative, the matrix particles were also seen to enter the cells in coated pits found between the microvilli and microplicae, and at their bases, as shown in figures 30 to 32. Coated vesicles found under the apical plasma membrane also contained these matrix particles (Figure 31).

In figure 27, TEM shows that upon entering the cell completely, the granules and matrix particles remain bound by the membrane with which they enter the cell. Granules and matrix particles retain their integrity and their original appearance at this stage. There had been no significant morphological changes in these structures to indicate that digestion had taken place.

As a result of this active process of uptake, the subapical zone had numerous "phagosomes" and "endosomes" containing either granules alone, granules and yolk matrix or matrix alone. It also can be seen that in the cytoplasm just basal to this area, these structures appear to be fusing with each other (Figure 27). As a result of these fusions, the structures became larger, and were observed to contain both granules and matrix particles.

Apart from these phagosomes and endosomes in the subapical cytoplasm, and the larger structures resulting from the fusions of these, the rest of the cytoplasm was filled with inclusions, which appeared to result from the digestion of the phagosomes. We shall identify these inclusions as vacuoles and they will be described below.

Morphology of yolk digestion - vacuoles

In addition to the above described inclusions ("phagosomes" and "endosomes", glycogen granules and typical lipid droplets) the epithelial cells contained many large inclusions to which I refer as "vacuoles" (Figures 19, 28, 39 and 40). I could not demonstrate a limiting membrane around most of these vacuoles, especially the larger ones. These vacuoles were observed to be membrane-bound in the most apical region, but a boundary membrane was not observed in the more basal regions. The vacuoles were much larger than the endosomes and phagosomes, and measured up to 35 μm in diameter. A survey of thin plastic sections from this area gave the impression that these heterogeneous vacuoles may have been connected.

The contents of the vacuoles was extremely variable (Figures 19, 39, 41 and 42) and included typical lipid droplets, dense bodies of variable shape, zones of very low electron density, vesicles, granular material and myelin-like bodies. The use of the IBO protocol demonstrated that some of the rounded spaces which appeared empty with regular fixation (Figures 39 and 41) were in reality due to lipids which had been dissolved during the process of fixation (see Figure 42 and compare with Figures 39 and 40).

Some of the vacuoles (Figures 39 and 40) contained yolk which had been altered morphologically; these appeared as round, electron-dense areas whose morphology ranged from compact, where no internal structure is observed, to loose, where it was possible to observe a substructure; the boundaries of these dense areas ranged from smooth to spikey. These appeared to correspond to partially digested granules, shown in figures 41 and 42. Other dense bodies (Figure 28) were larger than yolk granules (2.7 ± 0.6 μm, n=20), and, being of rounded or oval shapes, appeared to be similar to secondary lysosomes as described by other authors. Although some of the vacuoles had roughly the same dimensions as the phagosomes and endosomes described above, others were two to three times as large. The vacuoles were distinguished from the endosomes and phagosomes described above because they were generally more electron-dense, and each

vacuole contained areas which varied in electron density from moderate to high. The moderately electron-dense areas were more electron dense than matrix particles, but contained particles which resembled them in terms of size. The high density areas were generally two to three times larger than the average diameter of yolk granules, and ranged from one to approximately 5 in number. The high density areas existed either as distinct spherical areas, located away from the boundary, or as irregular or semi-circular areas located at the boundary of the vacuole. A membrane bounded these vacuoles. The size and of content of these vacuoles did not vary between adjacent cells, or among the ages studied.

The above mentioned types of contents of vacuoles were associated in different combinations and it was not found useful to attempt to further classify the vacuoles according to content.

It was also difficult to demonstrate a consistent correlation between type of vacuolar content and the position of the vacuole in the cell or with the age of the embryo from which the sample had been obtained, and no quantitative evaluation was attempted. Subjective evaluation appeared, however, to suggest that: a) vacuoles containing elements which could be interpreted as granules altered in structure by digestion, were in a subapical position, just basal to the endosomes. b) free lipid droplets were more frequent at the basal part of the cell of the yolk sacs from older embryos; c) vacuoles in cells from yolk sacs from earlier ages appeared to contain a larger number of lipid droplets than those from older embryos; d) vacuoles in cells from yolk sacs from older ages appeared to contain a higher heterogeneity of vacuolar contents, especially with respect to myelin figures e) some single, membrane-bound yolk granules were observed in the basolateral regions of the epithelial cells, but both granule and membrane retained their integrity, and at no time were granules ever seen leaving the base of the cell in that form.

The basal zone

Figure 43 illustrates that the basal zone of the epithelial cells were filled mainly with accumulations of lipid droplets of moderate electron-density, which figure 44 shows are not membrane-bound. Differences in accumulation of stored lipid were observed which will be discussed in Section 3.3, Morphology of lipid transport.

The basal membrane, illustrated in Figure 45, contained coated pits and vesicles, which may have a transport function, most likely, import of substances from the blood. The thin basal lamina was continuous at all ages examined.

3.2.4. Blood vessels in the yolk sac

Apart from the larger blood vessels, most of which can be visualized with the naked eye, the light microscope observations had shown that the circulatory system of the yolk sac is comprised of large blood sinuses (up to 100 μm in diameter) which have thin walls, rather than regular capillaries. These sinuses were always close to the yolk sac epithelium. Features of the blood sinuses, which can be observed by TEM are best illustrated in the yolk sac epithelium by figure 46. This figure shows that the endothelium was composed of cells with highly attenuated processes, and was devoid of a basal lamina. Neighbouring endothelial cells overlapped and simple tight junctions joined the processes. Large fenestrations were observed in the walls of the sinuses. Most of these sinuses contained a large number of red and white blood cells, an indication of their blood rather than lymphatic nature.

3.3. Morphology of lipid transport

Because of the osmiophilic nature of the lipids observed by TEM, this study was done on well-fixed epithelial cells prepared using the conditions established in the initial phase of this work.

I have referred above to the morphological indications of lipid transport by the epithelial cells of the yolk sac. I thus indicated that some of the vacuoles contained lipid

droplets and other myelin-like bodies. These probably are demonstrations of the dissociation of neutral lipids and phospholipids from the lipoproteins in the absorbed yolk, shown in figures 39 - 42, as a result of its digestion in the cell. In addition, I found numerous isolated lipid droplets independent of the vacuoles; they were similar to the droplets which in most cells are considered to represent storage. Finally, in the epithelial cells of 13 day yolk sacs, in the vicinity of the Golgi apparati, especially of those located close to the lateral membrane, I observed vesicles with dense content which were similar to those found in cells secreting lipoproteins (25 - 40 nm in diameter, Figures 47, 49 and 50). In closer examination of this region in the cell (Figures 47 and 49), Golgi apparati at the level of the nucleus were observed to have dilated cisternae which were filled with these particles. In the vicinity, vesicles full of particles appeared to bud off from the cisternae, to fuse with the lateral plasma membrane, and figure 48 showed that the contents emptied into the intercellular spaces.

Consistent with the above, in the intercellular spaces separating the epithelial cells in 13 day yolk sacs, it was possible to demonstrate with the IBO protocol (figures 47, 48 and 50), accumulations of small osmiophilic particles (approximately 100 nm in diameter), in closely packed groups, from the level of the nucleus to the base of the cell. These characteristics are consistent with those described for the chylomicrons produced by intestinal absorptive cells. No structures were seen in these spaces when the cells were fixed using half-strength Karnovsky's fixative, as shown in figure 51.

There appeared to be a correlation between the age of the yolk sac, the amount of lipid accumulated in the basal part of the epithelial cells and the mode of transport of lipid to the embryo. At 2 days of incubation, very little lipid was observed in the base of the epithelial cells. At 3 days of incubation, a small amount of lipid was observed in the base of the epithelial cells. By 8 days of incubation, there was a significant accumulation of lipid in the basal region of the epithelial cells, the intercellular spaces had dilated greatly. By 13 days of incubation, the basal part of the epithelial cells had huge lipid

accumulations, Golgi cisternae were dilated and contained particles, shown in figure 52, and the intercellular spaces appeared to be less dilated than they were at 8 days of incubation.

3.4. Acid phosphatase histochemistry and cytochemistry

Acid phosphatase was successfully localized in the yolk sacs from embryos of 3 ages prepared using the Azo-dye method and observed with light microscopy. An intensely red reaction product was formed in the samples incubated with substrate. This reaction product was localized in the same areas in each of the 3 ages studied, that is, in the cytoplasm which separates the numerous inclusions and was more concentrated the apical part of the cell (Figures 53, 54 and 55). Figure 52, which shows a control sample incubated without substrate and did not exhibit any of the reaction product, was considered to be negative.

Yolk smears from the free yolk contained in 17 day embryos were reacted for acid phosphatase using the Azo-dye method and gave negative results.

Preliminary experiments attempting to localize acid phosphatase with TEM using a modification of the lead capture method failed to further clarify the location of the enzyme. Yolk sacs from 3 day old embryos were incubated *en bloc*; this limited the penetration of the components of the incubation media so that the localization was only obtained in apical regions of the cells. Probably also because of the thickness of the incubated tissue, endogenous substrates failed to diffuse out so that I was unable to obtain negative controls (with medium lacking substrate). Despite these limitations, the positive localizations corresponded to relatively large (2.5 ± 1.1 μm, n=8 in diameter) rounded, oval or irregularly shaped dense bodies (Figure 56). These characteristics are consistent with their identification as secondary lysosomes. New experiments using more appropriate techniques are required in order to establish the role of lysosomes in yolk intracellular digestion.

Table 1: Criteria for good fixation of epithelial cells of the yolk sac

Cell structure	Good fixation	Poor fixation
Plasma membrane	single and intact (Hayat, 1989) coated pits and vesicles present in apical membrane (Mobbs and McMillan, 1979)	blisters (Bowers and Maser, 1988)
Microvilli		blisters (Hayat, 1989; Bowers and Maser, 1988)
Internal membranes	membranes continuous without distortion or break (Hayat, 1989; Glauert, 1974; Bowers and Maser, 1988)	
Vacuoles	smooth and circular (Bowers and Maser, 1988) not empty (Glauert, 1974; Bowers and Maser, 1988)	shrunken and distorted, resulting in buckling of surrounding membrane (Bowers and Maser, 1988)
Cytoplasmic ground substance	fine, granular, showing no empty spaces (Hayat, 1989; Glauert, 1974)	
Rough endoplasmic reticulum	flattened cisternae with attached ribosomes, usually arranged in long profiles (Hayat, 1989; Mobbs and McMillan, 1979)	swelling/vesicularization (Bowers and Maser, 1988)
Golgi apparatus	intact membranes (Hayat, 1989); present in small stacks of flattened cisternae close to the nucleus (Mobbs and McMillan, 1979; Holtzman, 1989)	
Primary lysosomes	small granules consisting of a dense matrix bound by an unbroken single membrane (Hayat, 1989); in the vicinity of phagosomes, endosomes, Golgi apparatus or endoplasmic reticulum (Holtzman, 1989)	
Lipid droplets	uniformly dark (Hayat, 1989)	myelin figures (Bowers and Maser, 1988)
Phospholipids		artifactual precipitation of lead over insufficiently fixed phospholipids (usually membranes) (Mobbs and McMillan, 1979; Hayat, 1989; Bell, 1988)
Glycogen	dark, individual granules or clumps (Hayat, 1989)	
Mitochondria	outer double membrane and cristae intact, dense matrix (Hayat, 1989) disruption of matrix and loss of granules; appearance of irregular profiles (Bowers and Maser, 1988)	swollen or shrunken (Hayat, 1989)
Cytoskeleton	microfilaments and microtubules present and visible in apical and lateral cytoplasm (Holtzman, 1989; Mobbs and McMillan, 1979)	
All structures	same appearance of the structure with all fixations (Glauert, 1974) final appearance similar to living organism viewed by LM, including the observation of thick sections (Glauert, 1974) subjectively speaking, the image is distinct, orderly and overall grayish in electron opacity, especially mitochondria and the lumina of blood and lymph vessels; every component should possess a certain degree of opacity (Bowers and Maser, 1988)	

Table 2: Effects of various fixatives on the ultrastructure of yolk

	Granules			Matrix particles
Fixative	**boundary membrane demonstrated**	**increase of contrast**	**internal structure seen**	**increase of contrast**
Karnovsky's type (control)	No	No	No	No
Potassium ferricyanide	No	No	Yes	No
Tannic acid	No	No	Yes	Some
Malachite green	No	No	Yes *	Some
Imidazole (Allan-Wojtas and Kalab, 1984)	No	Yes *	No	No
Imidazole (Angermüller and Fahimi, 1982)	No	No	No	Yes *

* = best in category

FIGURES

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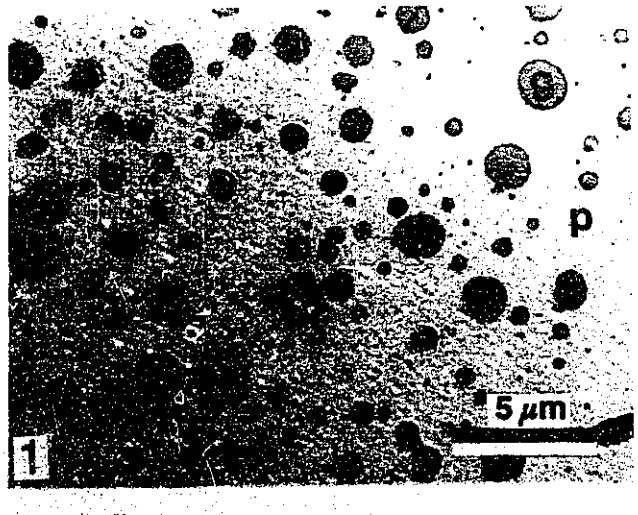
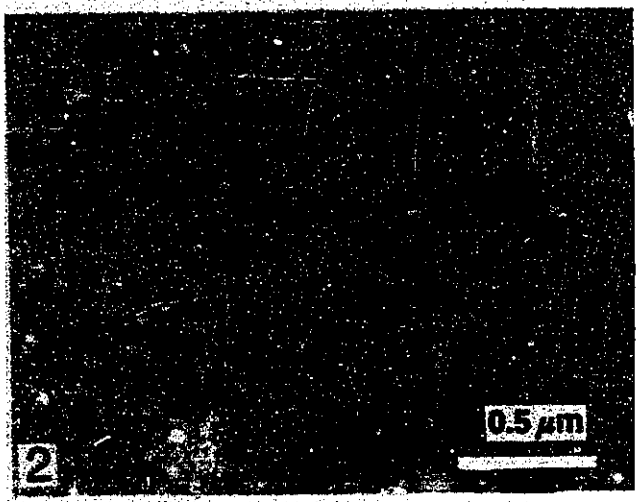
Figure 1. Granules (g) (electron-dense spheres) and matrix (p) (grey background) of yolk.

Figure 2. Higher magnification of a single granule (g) and particles (p) making up the matrix.

Figure 3. Similar details as seen when using a somewhat thicker section.


p
5 μm
1


0.5 μm
2

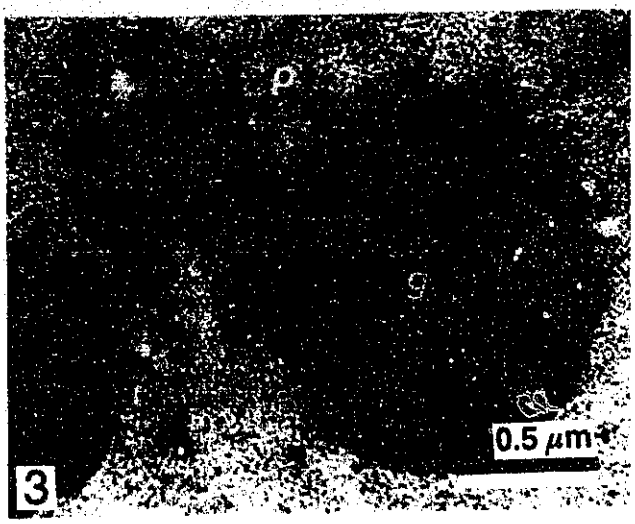

p
0.5 μm
3

Figure 4. Yolk granule (g) and particles (p) of the matrix after fixation with potassium ferricyanide.

Figure 5. Fixation with malachite green.

Figure 6. Fixation with tannic acid.

Figure 7. Fixation with the Imidazole-buffered osmium tetroxide protocol of Allan-Wojtas and Kalab (1984).

Figure 8. Fixation with the Imidazole-buffered osmium tetroxide protocol of Angermuller and Fahimi (1982) (IBO).

Bar (0.5 μm) applies to all figures in this plate.

4
5
6
7
8
0.5 μm

Figure 9. Distribution of the granules (g) in a pellet of subembryonic fluid from a 9-day embryo. The granules appear to be closely packed due to centrifugation.

Figure 10. High magnification of a granule (g) from the subembryonic fluid from a 9-day embryo. The granules have retained their integrity during incubation, and look very similar to those from unincubated yolk.

Figure 11. Granules (g) in the yolk from an egg incubated 15 days. The granules have retained their integrity, although they are reduced in number. Matrix particles (p) are also observed.

Figure 12. Yolk from an egg incubated for 15 days. Deposits of calcium in the form of spherules (Ca) are observed in the yolk mass.

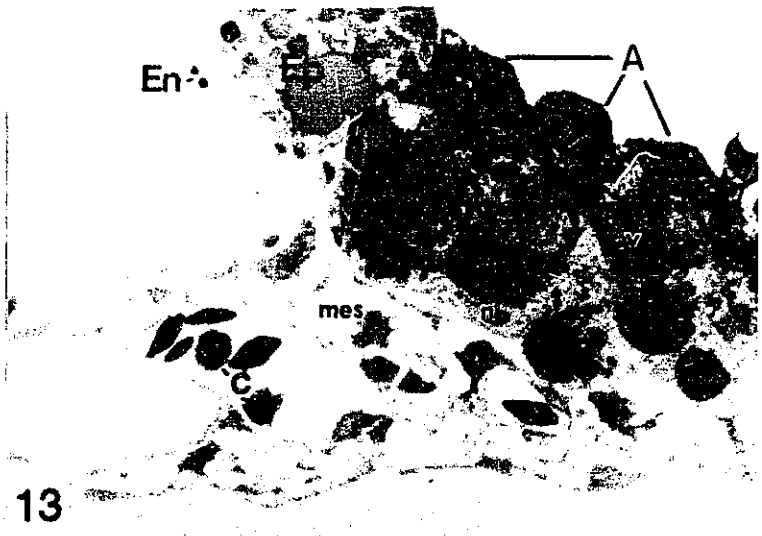

0.5 μm
10
0.5 μm
11
5 μm
12

Figure 13. Three days of incubation. The cell layers of the yolk sac can be discerned. Epithelial (Ep) cells; mesenchyme cells, (m), stellate and undifferentiated; endothelial cells (En). The epithelial cells display a prominent basal nucleus (n), and vacuoles (v) which varied in their intensity of staining. The apices (A) of the epithelial cells are normally in contact with the yolk mass. Blood cells (both erythrocytes and leucocytes) (c) can be seen in the lumen of the forming blood vessels.

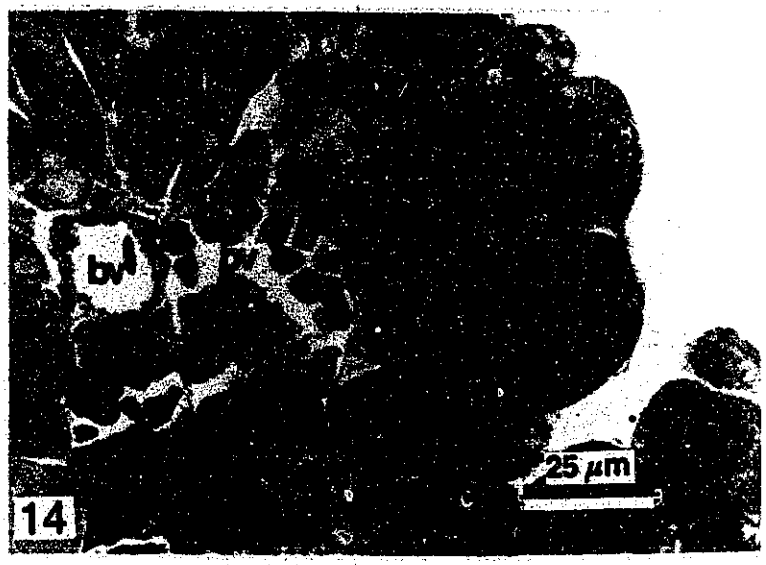
Figure 14. Eight days of incubation. Epithelial cells (Ep) are in close proximity to the blood vessels (bv), and have basal nuclei (n). Their cytoplasm is filled with vacuoles (v).

Figure 15. Fifteen days of incubation. The apices (A) of the epithelial cells (Ep) have become increasingly convex. The cytoplasm is packed with vacuoles (v) throughout. n = nucleus

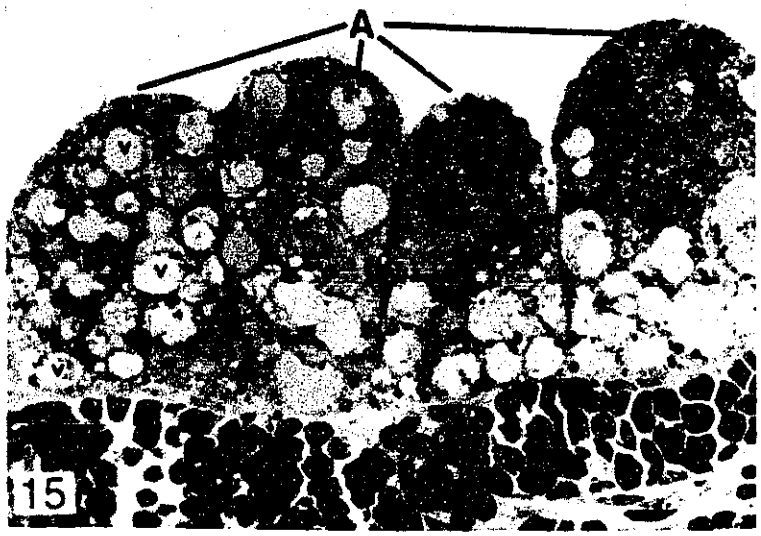
Bar (25 μm) applies to all figures in this plate.



13



14



15

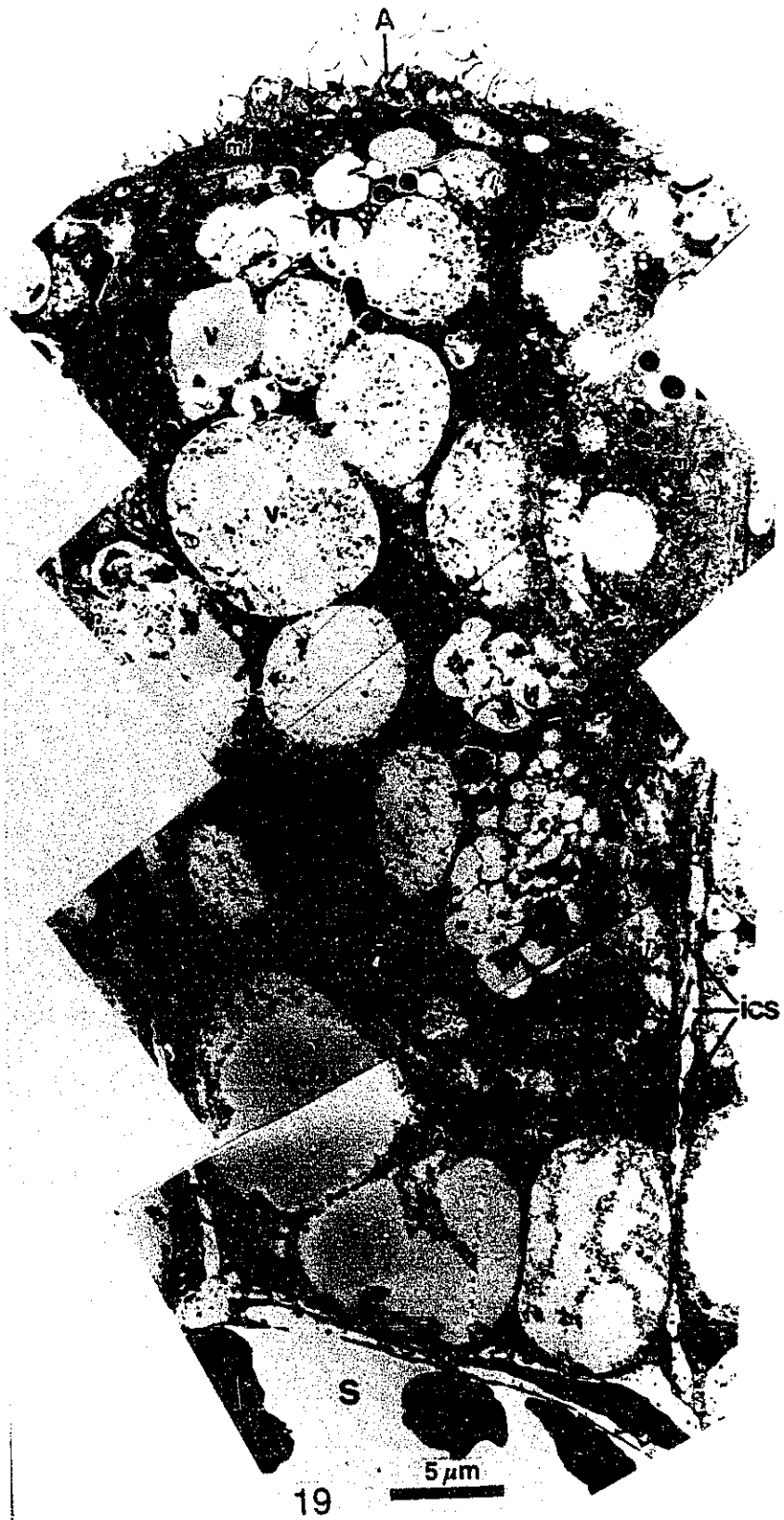
Figure 16. Three days of incubation showing the apical surface (R). At this age, the yolk sac is simple in structure, with no folds. The apical surface of the epithelium has a cobblestone appearance.

Figure 17. Eight days of incubation. At this age, secondary folds (S) are developing. The folds have greatly increased the surface area of the yolk sac in contact with the yolk as compared to three days of incubation.

Figure 18. Fifteen days of incubation. The yolk sac consists mostly of secondary folds (S). These folds increase enormously the surface area in contact with yolk as compared to the 8 day samples.


100 µm
16
500 µm
17
500 µm
18

Figure 19. Composite electron micrograph of a "typical" epithelial cell from the yolk sac from a 15 day old embryo. It illustrates the general features shared by the cells at all ages. These include abundance of vacuoles (v) and phagocytotic and endocytotic structures at the apex (A). Mitochondria (M) and microfilaments (mf) are found throughout. A blood sinus (s) close to the base of the cells. ics = intercellular space.



19

Figure 20. Electron micrograph of the yolk sac epithelium of a 3 day old embryo. Apical region of 2 apposed cells. The cells share a junctional complex (jc) which includes a desmosome (d). Vacuoles (v), mitochondria (M), rER and microtubules (mt) are clearly observed. Half-strength Karnovsky's fixative.


V
0.5 μm
20

Figure 21. Yolk sac epithelium from a 3 day old embryo. The vesicles with an electron-dense content (ve) might be either lysosomes, or most probably chylomicrons, about to be secreted into the intercellular space (ics). Half-strength Karnovsky's fixative.

Figure 22. Yolk sac epithelium from a 3 day old embryo. l = lipid droplets; mes = mesenchyme; n = nucleus. Half-strength Karnovsky's fixative.

Figure 23. Yolk sac epithelium from an 8 day old embryo. ics = intercellular space; M = mitochondria. Half-strength Karnovsky's fixative.

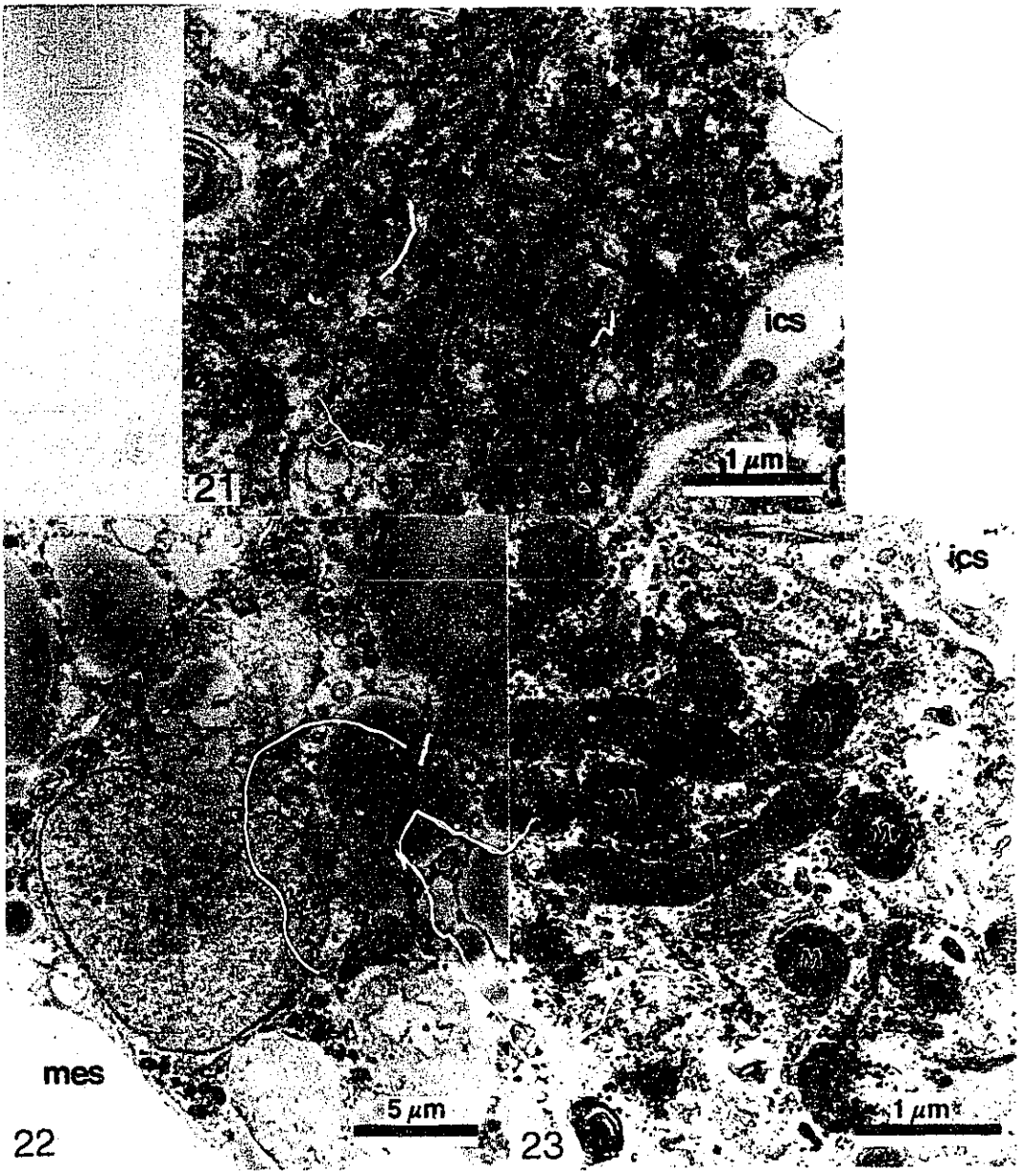

ics
1 µm
21
ics
mes
5 µm
22
1 µm
23

Figure 24. Yolk sac epithelium from a 3 day old embryo. Apical zone. Microtubules (mt) exist in random arrangement just below the apical membrane (AM). Half-strength Karnovsky's fixative.

Figure 25. Yolk sac epithelium from a 15 day old embryo. Apical zone. Microfilaments (mf) are also seen just below the apical membrane (AM). Half-strength Karnovsky's fixative.

Figure 26. Microfilaments (mf) exist in oriented groups in close proximity to membrane-bound vacuoles (v) Half-strength Karnovsky's fixative.

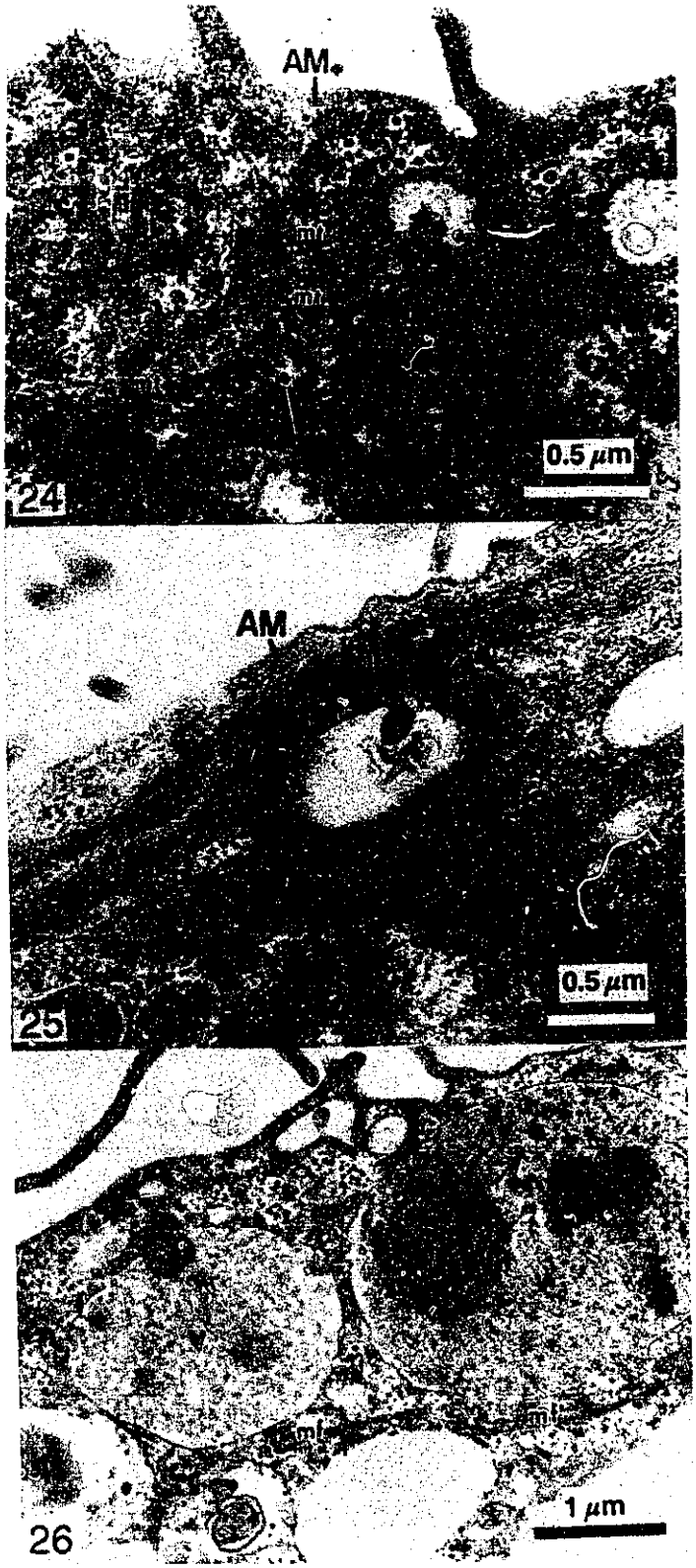
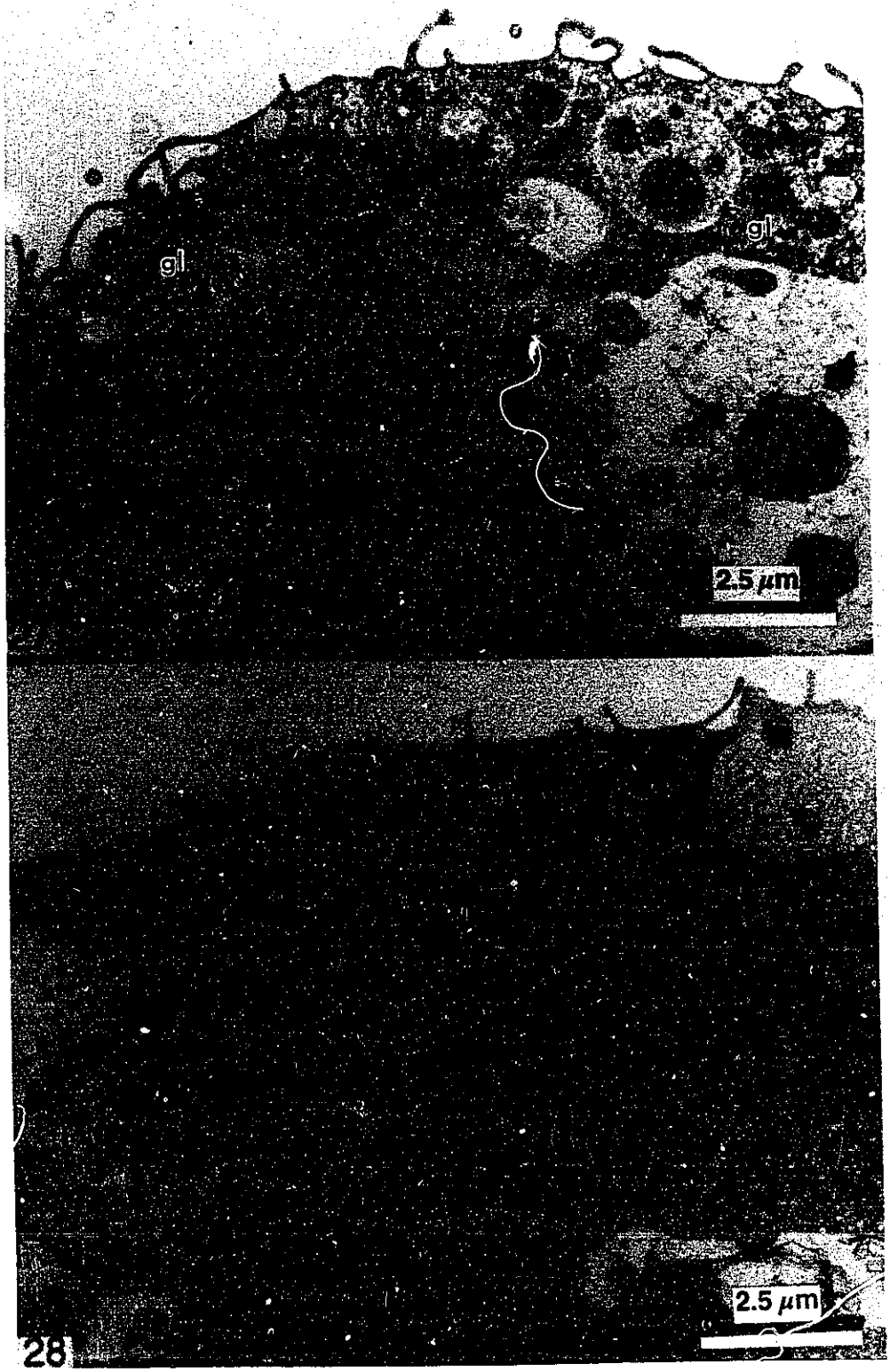

AM
0.5 μm
24
AM
0.5 μm
25
1 μm
26

Figure 27. Yolk sac epithelium from an 8 day old embryo. Apical zone. Endosomes containing different types of content are shown: single yolk granule (1); matrix particles (2); mixture of granules and matrix particles (3). Glycogen deposits (gl) are also shown. Half-strength Karnovsky's fixative.

Figure 28. Yolk sac epithelium from an 8 day old embryo. Apical zone. The presence of "dense bodies" (arrows) is shown. These were called vacuoles in the text. Half-strength Karnovsky's fixative.



28

Figure 29. Yolk sac epithelium from a 15 day old embryo. Apical zone. Yolk granules (g) and matrix particles (p) can be taken into the cell together. IBO fixative.

Figure 30. Yolk sac epithelium from a 15 day old embryo. Note stages in the uptake of yolk granules. (Capture (1), engulfment (2) and internalization (3)). Microfilaments (mf) in the cytoplasmic extensions encircle the internalized granule. cp = coated pit. Half-strength Karnovsky's fixative.

Figure 31. Yolk sac epithelium from an 8 day old embryo. Stages in yolk uptake (1,2, and 3, as above) can be observed. Half-strength Karnovsky's fixative.

Figure 32. Yolk sac epithelium of a 15 day old embryo. A coated vesicle (cv) is about to pinch off from the apical membrane. Half-strength Karnovsky's fixative.

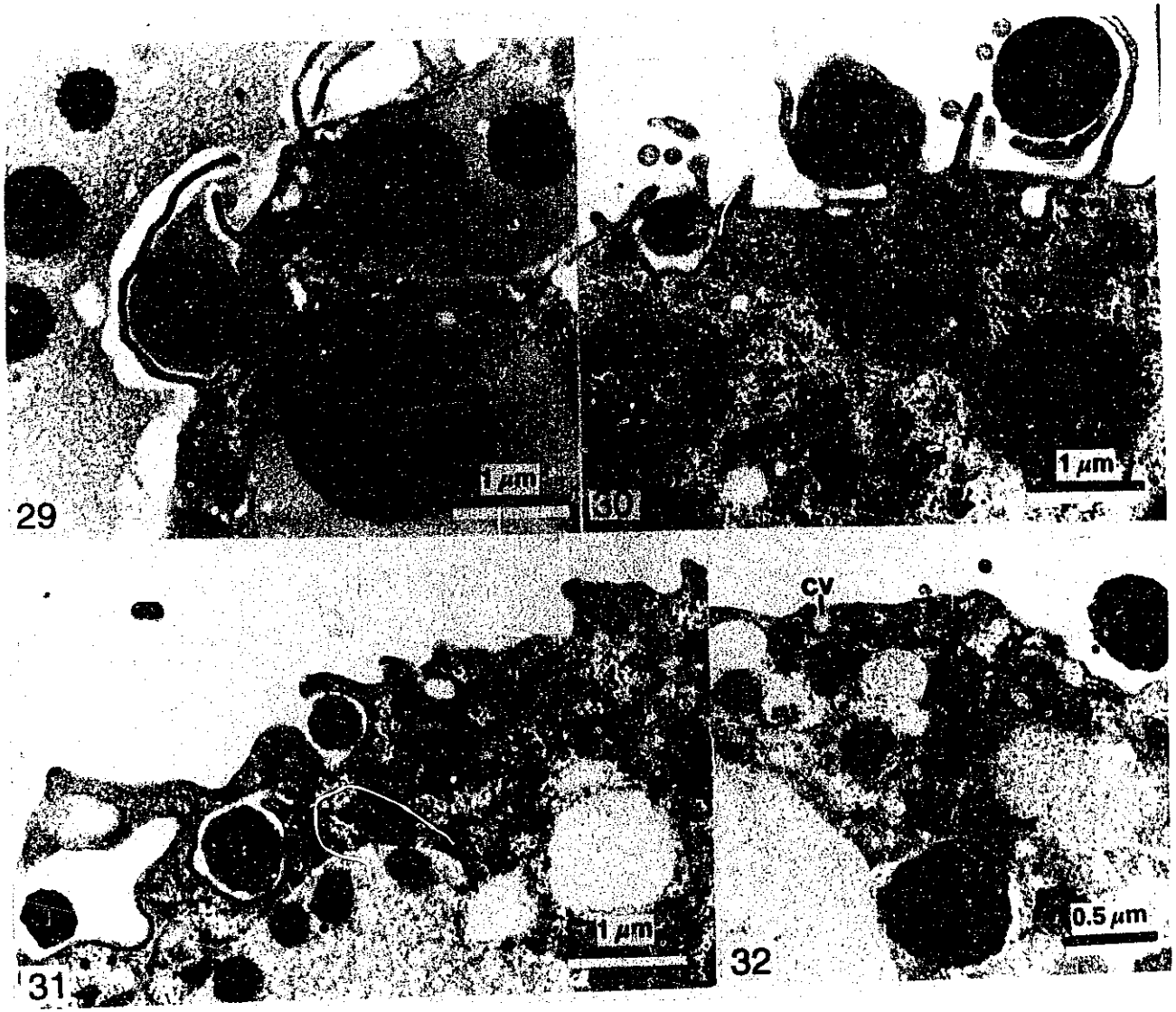

1 μm
29
1 μm
30
1 μm
31
cv
0.5 μm
32

Figure 33. SEM of the inner surface of a yolk sac from a 3 day old embryo showing different stages of granule entry into the epithelial cells. Engulfment (1), capture (2), and internalization (3). Half-strength Karnovsky's fixative.

Figure 34. Apex of an epithelial cell from a 3 day old embryo. Some of the granules in the process of being taken up have been washed away during specimen preparation, leaving fossae (f), which are the approximate diameters of granules. The cell surface is relatively smooth, except for occasional microplicae (mp). Half-strength Karnovsky's fixative.

Figure 35. Inner surface of the yolk sac from an 8 day embryo showing how the apex of the epithelial cells bulges into the yolk mass. Occasional fossae (f) are seen. Numerous microplicae (mp) are shown. Half-strength Karnovsky's fixative.

Figure 36. Yolk sac epithelium from an 8 day embryo showing fossae of different sizes at the cell apex. Compare (a) and (b). Half-strength Karnovsky's fixative.

Figure 37. Yolk sac epithelium from a 15 day old embryo. The cells show more microplicae (mp) than those observed in samples from 3 and 8 day old embryos. Half-strength Karnovsky's fixative.

Figure 38. Similar sample as in Figure 37 but with larger magnification. Half-strength Karnovsky's fixative.

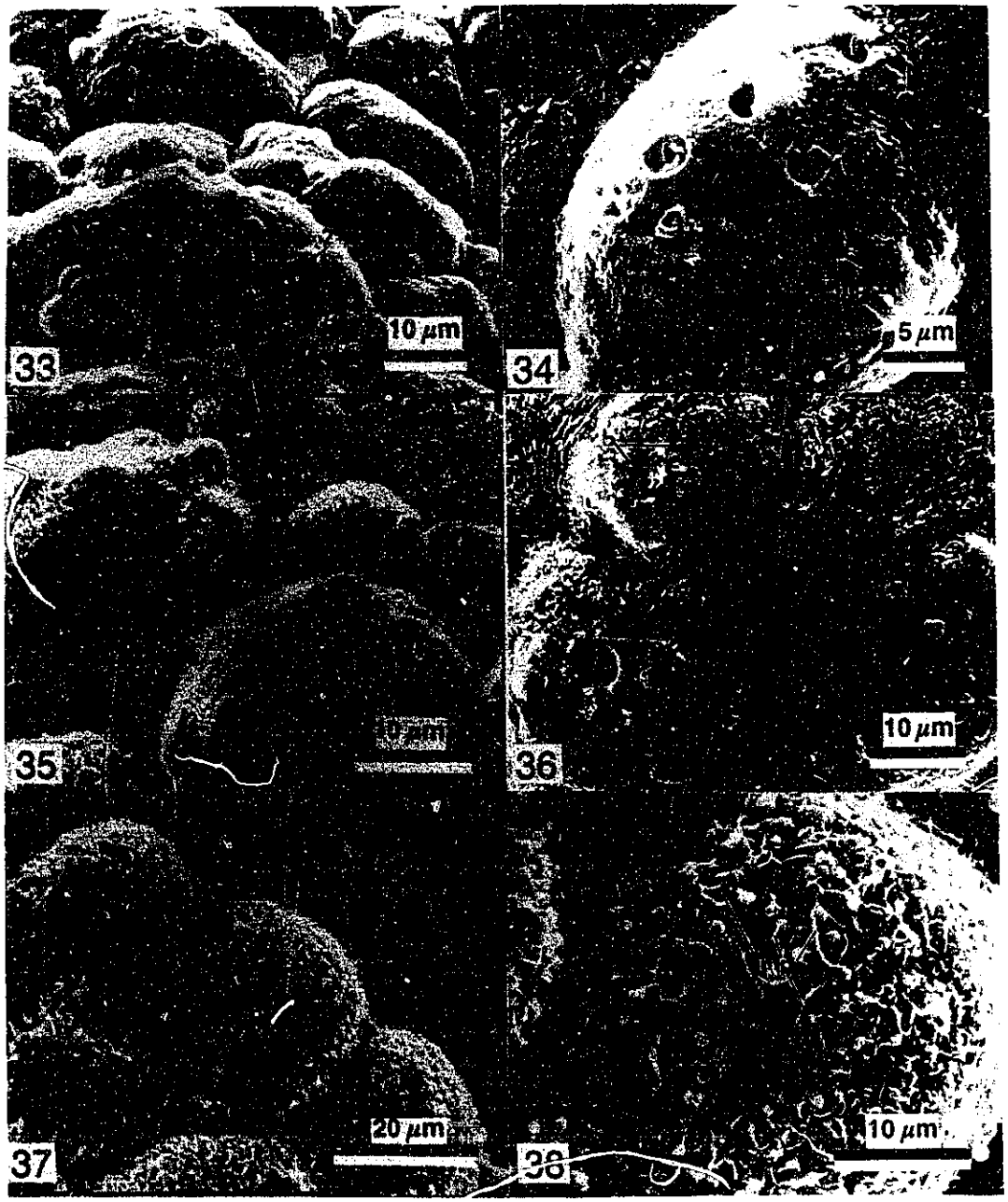

10 μm
33
34
5 μm
35
36
10 μm
37
20 μm
38
10 μm

Figure 39. Yolk sac epithelium from a 3 day old embryo. A single large vacuole (v) containing mainly electron-dense granule-like structures and membranes comprises most of the middle zone of the cell. Half-strength Karnovsky's fixation.

Figure 40. Yolk sac epithelium from an 8 day old embryo. Vacuoles (v) in the middle zone which contain myelin figures (my) and lipid-like inclusions (ll). Half-strength Karnovsky's fixative.

Figure 41. Yolk sac epithelium from a 15 day old embryo. Higher magnification of an area similar to the one shown in Figure 39. The granules (g) have begun to lose their integrity, and an internal structure can be seen. Half-strength Karnovsky's fixative.

Figure 42. Yolk sac epithelium from a 15 day old embryo. An area similar to the one shown in Figure 41, but after fixation using the IBO protocol. Granules (g) are observed, and lipid (l) has been retained *in situ.*

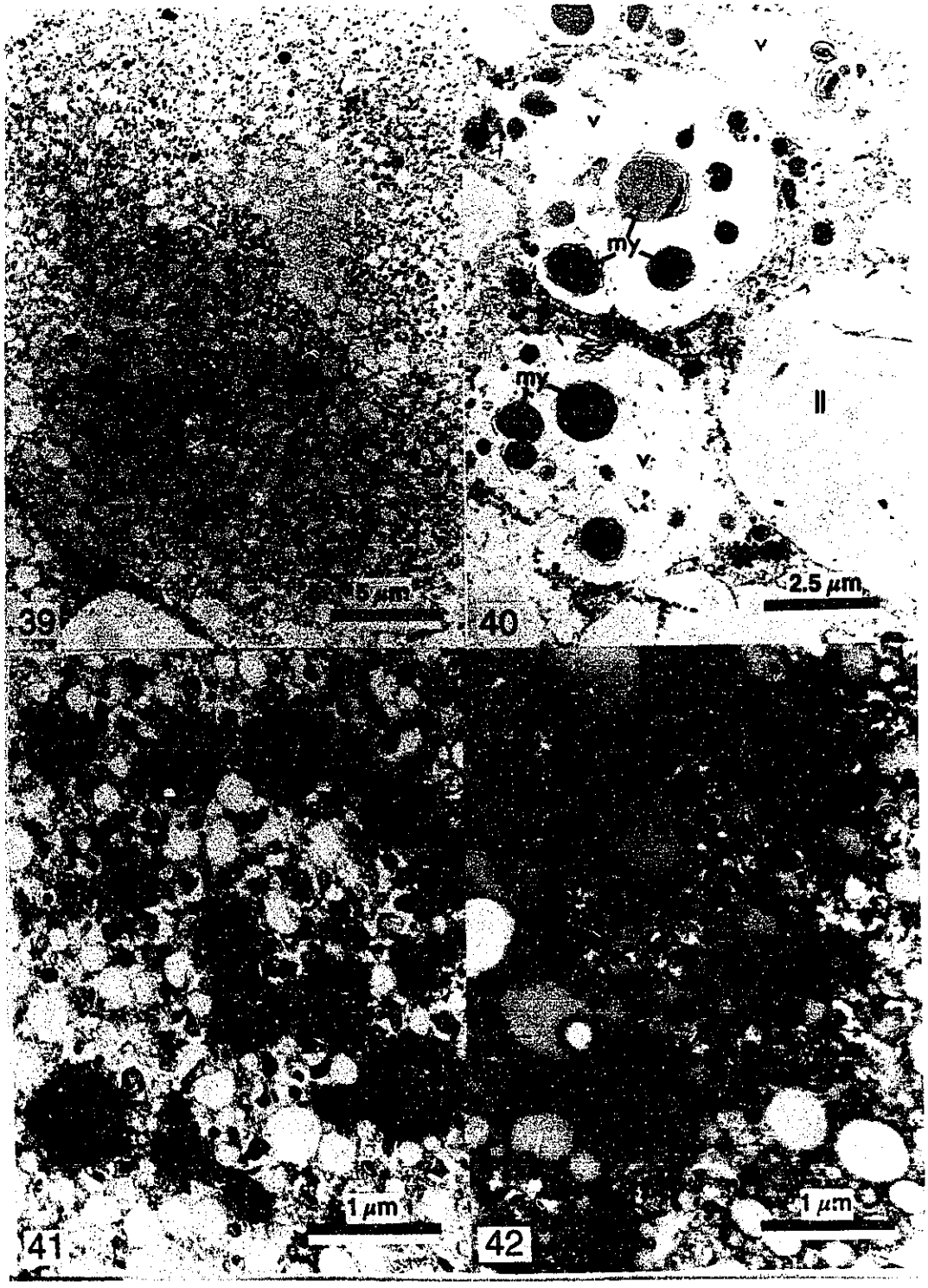

39
1 µm
40
my
my
v
v
v
II
2.5 µm
41
1 µm
42
1 µm

Figure 43. Cross section of the basal zone, as indicated by the presence of the nucleus (n) in this section, to show the accumulation of lipid droplets (l).

Figure 44. Higher magnification of Figure 43 to show lipid accumulation and nucleus. The nuclear membrane (nm) is clearly visible.

Figure 45. Longitudinal section of the basal membrane of the cell. Coated pits (cp) are observed. The basal lamina (bl) is continuous.

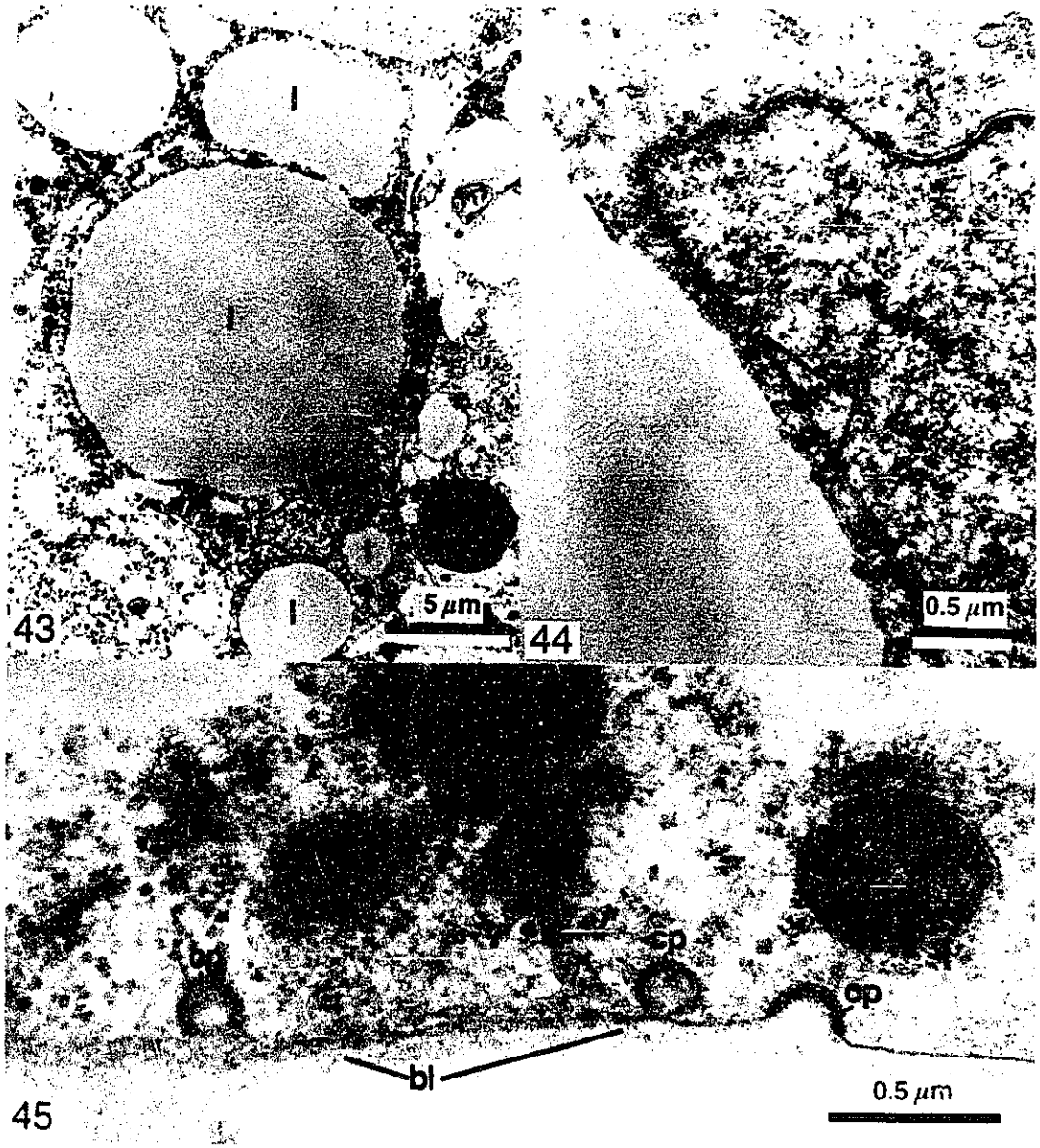

I
I
I
5 μm
43
44
0.5 μm
bp
cp
cp
bl
0.5 μm
45

Figure 46. Blood sinus from the yolk sac of a 15 day old embryo. Extreme basal zone of the epithelial cells (Ep) and the developing blood vessel (C). The epithelial cells, blood vessels and blood cells are closely associated. The presence of fenestrations (F) having a wide range of diameters are present. Collagen fibrils (cf) are numerous, both in cross section and longitudinal section.

46

Figure 47. Many vesicles (ve) with electron-dense contents can be seen throughout the lateral cytoplasm. A Golgi apparatus (G) can be seen with electron-dense vesicles budding from it. Intercellular spaces (ics) are filled with electron-dense particles of various sizes.

Figure 48. Intercellular spaces (ics) are full of electron-dense particles. Microfilaments (mf) are observed in the lateral cytoplasm, in close proximity to vesicles. Coated vesicles (cv) are seen in the lateral membrane.


47
1 μm
48
1 μm

Figure 49. Composite electron micrograph of the lateral cytoplasm. A Golgi stack (G), and associated vesicles (ve) carrying electron-dense particles, are observed. Microfilaments (mf) are observed running parallel with the lateral membranes (L) of adjacent cells. IBO fixative.

Figure 50. An intercellular space (ics) seen with higher magnification to show that the containing particles of various sizes and electron density. Vesicles (ve), full of particles (vp), and microtubules (mt) are also visible in the lateral cytoplasm. IBO fixative.

Figure 51. A Golgi apparatus (G) filled with particles (vp), fixed using half-strength Karnovsky's fixative. A comparison with Figure 50 shows that the IBO protocol can be a valuable tool for visualizing structures involved in lipid transport in the yolk sac epithelium.


49
0.5 μm
50
0.25 μm
51
0.25 μm

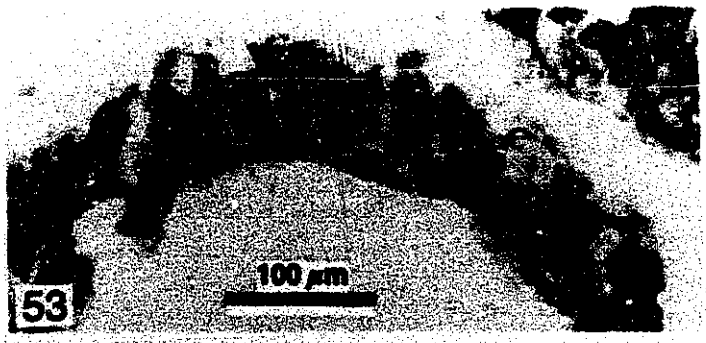
Figure 52. Control (without substrate). No histochemical reaction was observed.

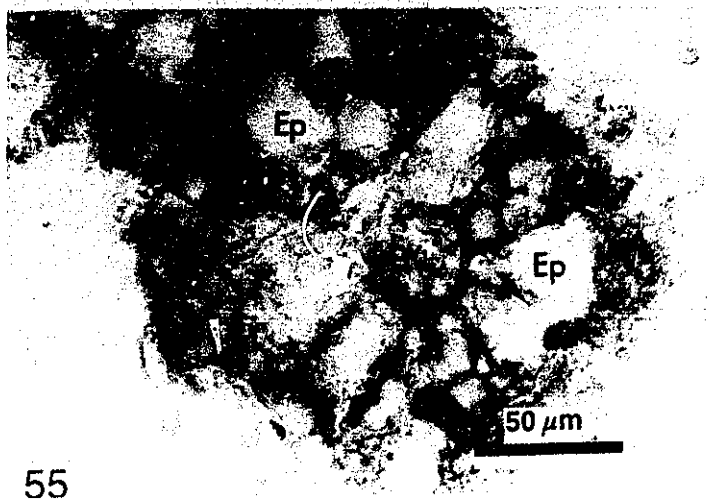
Figure 53. Experimental sample. Localization was observed outside of the middle zone.

Figure 54. Same sample as in Figure 53. Localization was observed outside of the vacuoles.

Figure 55. Cross section of a blood vessel (bv), showing the arrangement of epithelial cells (Ep) around it. The histochemical reaction is located outside of the vacuoles of the middle zone.

52





55

Figure 56. Acid phosphatase cytochemistry in yolk sac epithelium from a 3 day embryo. The locations and sizes of the lead deposits (arrows) corresponds to the dense bodies observed in our ultrastructural studies. Half-strength Karnovsky's fixative.


2.5 μm
56

4. Discussion

4.1. Ultrastructure of yolk - granule structure

4.1.1. Yolk from infertile, unincubated eggs

Yolk from infertile, unincubated eggs prepared using 6 fixation protocols showed granules to be electron-dense spheres with a diameter of approximately 1.6 ± 0.1 μm, n=20, in agreement with the work of Chang et al. (1977), with an uneven, membraneless outer boundary. The lack of a membrane boundary is in agreement with an earlier observation using TEM that in the laid egg, intracellular yolk granules were membrane-bound and extracellular granules were not (Bellairs, 1963, 1964). Willems et al. (1976) provided evidence that a boundary membrane did not exist by using TEM on granules which had been treated with a dilute solution of NaCl, and concluded that "surface components were detached", which would not happen if the granules were membrane-bound. The granules are, instead, held together by the affinity of their components, lipovitellin and phosvitin (Burley and Cook, 1961; Chang et al., 1977). Some of 6 fixation protocols enhanced subunit visualization to permit study of granule substructure (Table 1), possibly due to extraction of some components by fixatives (Hayat, 1989). The inherent high electron-density of the granules hampered the study of the internal structure of the granules and the distribution of their electron-dense subunits. I was able to observe granule substructure more clearly by studying thinner plastic sections. Chang et al. (1977) approached the same problem by doing a partial disruption of the granules using a dilute solution of NaCl before TEM. My approach had the advantage of allowing the granule substructure to be observed without perturbation of native structure. Granule substructure, which consisted of spherical units with electron-dense peripheries, agrees with that described by other authors as consisting of electron-dense "microparticles" and high and low-density patches (Bellairs, 1961; Chang et al, 1977).

The fact that there was good agreement of yolk granule structure among treatments indicates that the images produced give an accurate representation of granule structure after chemical fixation, since all fixatives yielded the same results (see Table 1).

The matrix in which the granules sit consists mainly of densely packed spherical particles 20 - 30 nm in diameter, with electron-dense peripheries. They have been characterized by a number of authors (Holdsworth and Finnean, 1972; Garland and Powrie, 1978 a and b; Fawcett, 1986) as LDL particles on the basis of density, size range and staining characteristics. Bellairs et al. (1972) described their structure, and equated them to the LDL particles described by the biochemists. In TEM, matrix particles become visible due to the crosslinking action of the fixative (Hayat, 1989), and due to the osmiophilic nature of the lipoprotein component. Perry and Gilbert (1979), using tannic acid fixation to study chicken egg yolk, showed lipoprotein in tightly packed arrays of particles where material of low electron-density was enclosed in a shell of greater electron density. Their protocol was one of those used in my study, and enhanced visualization of matrix particle structure. The IBO protocol produced matrix particles of extremely high contrast, but the structure in these was not as clear as when the tannic acid protocol was used (see Table 1).

The presence of membranous stacks in the matrix was observed only rarely, and the intact large yellow and white yolk spheres as described by Bellairs (1964) and Fujii et al., (1973) were never observed. It is possible that the method of sampling of the yolk used in this study, that is, by aspirating this viscous sample through a small gauge needle, and then expressing it into fixative, could have destroyed large, fragile structures such as the yolk spheres, leaving fragments as stacks of membranes. This observation would agree with the comments of Bain and Hall (1970) and Fujii et al. (1973) regarding the fragile nature of the yolk spheres.

Separate studies of granules from yellow and white yolk spheres were not done; no attempt was made to separate the white and yellow yolk during preparation of samples for TEM.

4.1.2. Yolk from fertilized, incubated eggs

Granules were sampled from fertilized eggs at different ages and under different conditions to detect whether any morphological changes had occurred. Bellairs (1963) used morphological alterations as an indicator of granule digestion, assuming that unaltered granules had not been digested. Lemanski and Aldoroty (1977) have also assessed the digestion of yolk platelets in amphibian embryos in this way. In our work, yolk granules taken from the subembryonic fluid from 9 day chick embryos had the same appearance as those from infertile, unincubated eggs, as did granules which adhered to 15 day yolk sac samples. The absence of any observed morphological change indicated that extracellular digestion of granules had not occurred during 15 days of incubation, and does not support the presence of extracellular digestion of yolk granules by the embryo. This disagrees with the interpretation of Lambson (1970) and Mobbs and McMillan (1979, 1981) who argued that although intracellular digestion of yolk was occurring in the yolk sac epithelium, extracellular digestion of yolk in the yolk mass was not ruled out.

Although no morphological changes were observed in the granules in the yolk held by the yolk sac over time, a gross change in the yolk was observed. From 13 days of incubation, large spherical bodies having concentric rings of varying electron-densities were observed in extracellular yolk by TEM. Similar structures were observed in 11 - 13 day old chick embryos by Juurlink and Gibson (1973), who histochemically showed that they contained calcium, and called them calcium spherules. Calcium spherules were studied histochemically by Cheville and Coignoul (1984) in turkey embryos, and they observed the ultrastructure and formation of calcium spherules using TEM. They also observed that calcium spherules formed in response to the exhaustion of calcium reserve in the

yolk. During the last half of incubation, the embryo switches over to the chorioallantoic membrane (CAM) and shell for its calcium supply, and the calcium derived from this new source is stored in the extracellular yolk as calcium spherules. My observations of similar structures found in 15 day yolk sac is in accordance with this hypothesis.

4.2. The yolk sac

4.2.1. Quality of fixation

In all trials, fixation quality was determined to be acceptable at the LM level in that cells had complete boundary membranes, and the cells did not appear swollen, broken, or otherwise deformed. Poor preservation was detectable only at the ultrastructural level using the criteria defined in Table 2. At the ultrastructural level, the variation in quality of fixation was affected by temperature, time, composition of fixative, age of sample, and the presence of yolk adhering to cells. Ultrastructural studies were carried out only on cells which met the criteria listed in Table 2. One criterion which was not satisfied was the preservation of an intact membrane surrounding the large inclusions which comprised most of the middle zone of the epithelial cells.

In this study, the use of thick plastic sections of yolk sac samples fixed using EM fixatives (including the lipid fixative OsO_4) allowed better visualization of lipid than that observed by other authors using yolk sac samples fixed with LM fixatives (and not exposed to OsO_4) and embedded in wax (Willier, 1968; Juurlink and Gibson, 1973). This advantage was the most obvious in the appearance of the lipid-rich inclusions in the middle zone of the cell which appeared to be full, while in micrographs of wax samples they appear to be highly vacuolated and empty.

4.2.2. General description of the yolk sac. Light microscopy and SEM observations.

My observations using light microscopy confirmed that the yolk sac is composed by a single layer of tall columnar epithelial cells and a layer of connective tissue with

and undifferentiated stellate cells. The layer of connective tissue has an abundant matrix, and contains blood vessels and foci of hematopoiesis. The apices of the epithelial cells are in contact with the yolk, while the bases are in close contact with the circulatory system. The cytoplasm of the epithelial cells was occupied by large vacuoles whose contents varied in staining density. Fine strings of cytoplasm were observed between the vacuoles. Uptake structures were situated at the apex, while at the base was found a small, triangular nucleus. These results are in agreement with those reported by Juurlink and Gibson (1973) for wax-embedded samples and Mobbs and McMillan (1979) for plastic sections.

Age-related architectural changes were studied in yolk sac samples at 3 ages using SEM. They were manifest as an increase in degree and complexity of folding with regional differences. Although no other reports exist in the literature of SEM studies of folding of yolk sac during incubation, these results are in agreement with and support the gross observations of Romanoff (1960) and the LM observations of Juurlink and Gibson (1973), who described the increase in folding and complexity of the yolk sac with age.

4.2.3. Ultrastructural studies on the yolk sac

The epithelium

TEM studies confirmed light microscopy observations that the yolk sac epithelium consisted a single layer of tall, columnar epithelial cells, whose apex was convex, and whose base was separated by a thin basal lamina from the underlying blood vessels.

The complexity of cytoplasm of the columnar epithelium observed in the LM was found to be due to the presence of numerous inclusions and vacuoles, and shared this feature with Kupffer cells in liver tissue, described by Wisse (1977). The rest of the organelles in yolk sac epithelial cells were found between the vacuoles and toward sides of cell, with the exception of the nucleus, which was always found in the base. Bundles of filamentous structures, which could be microfilaments or intermediate filaments, were observed parallel to the surface and to the apical vacuoles, while microtubules were found close to membrane-bound vacuoles. Rough endoplasmic reti-

lum was distributed throughout the cytoplasm, as were mitochondria. Small stacks of Golgi were observed, with vesicles which may be primary lysosomes or chylomicrons in close proximity. Glycogen deposits were found throughout the cell, in agreement with the observations of Willier (1968). The folded lateral membranes contained junctional complexes at the apices, and desmosomes along their length. Intercellular spaces were reduced in size as incubation proceeded. This description agrees with that of Lambson (1970), Mobbs and McMillan (1979) and Cheville and Coignoul (1984).

The above observations on eggs studied at three broad ages of incubation indicated that the cell ultrastructure is basically the same throughout incubation. Our interpretation is that the content of the inclusions, rather than the organelle complement of the cell changes with age (except at later ages, during lipid transport). Some confusion exists in the literature as to whether it is cell structure or the content of the inclusions which change during incubation. Romanoff (1960) found that epithelial cells vary greatly in appearance over incubation, and that yolk spheres show a progressive modification in appearance from distal to proximal part of the yolk sac. He classified the epithelial cells by the content of their inclusions rather than by their basic organelle complement, and did not consider inclusions to be cytoplasmic organelles. On the other hand, our interpretation of cell structure agrees with that of Bellairs (1958), Lambson (1970) and Juurlink and Gibson (1973) who believe that it is the content of the inclusions which changes with time. Many authors have gone one step further and classified inclusions on the basis of position in the cell, position in the yolk sac and age of the yolk sac. On the basis of TEM work, Bellairs (1958) observed that morphological changes in yolk inclusions in epithelial cells were not confined to a single stage of embryonic development. Lambson (1970) observed that the only significant change in structure of the yolk sac epithelial cells is during the last week of incubation, during the massive transport of lipid which occurs at this time. Juurlink and Gibson (1973) have classified cells on the basis of content, and age of the yolk sac and the position of the cell in it, where older cells appear "vacuolar"

with most fixation protocols, and younger cells contain more granules and are described as "yolk-laden".

Mobbs and McMillan (1979) differentiated cells on the basis of their position in the early yolk sac, and the content of their inclusions, while Cheville and Coignoul (1984) described the epithelium of the yolk sac in general terms, and noted few ultrastructural changes throughout incubation. Our interpretation of structure is in the best agreement with this work of Cheville and Coignoul (1984).

Morphology of yolk uptake

Uptake structures

We observed by TEM that at all stages studied, microplicae were present on the apical membrane, which took up single granules by the process of phagocytosis, and look like microplicae in other cells with the same function, such as Kupffer cells, described by Wisse (1977). Silverstein et al. (1977) used the term "pseudopodia" to describe such structures. Anderson (1991) went one step further and used the name "lamellopodia" to describe such structures, because they arise at the site of active membrane ruffling where the membrane is arranged into wavy sheet-like processes, or thin membranous pseudopodia.

Three main stages of granule uptake were observed: capture, engulfment and internalization. At no time were yolk spheres observed being endocytosed. This observation agrees with Lambson (1970), Juurlink and Gibson (1973), and Mobbs and McMillan (1979, 1981) who described the uptake of yolk granules and matrix, but disagrees with the observations of Bellairs (1963, 1964) and Willier (1968), who hypothesized that yolk was taken up in the form of spheres.

Matrix particles were observed to be taken up in coated pits on the apical membrane, in the process known as receptor-mediated endocytosis (Goldstein et al., 1985). Our observations support those of Willier (1968), Lambson (1970) and Mobbs and McMillan (1979, 1981), who all agreed that matrix particles were taken up in a system different

from the one used for taking up granules. We observed, however, using the IBO protocol, that matrix particles were also taken up during phagocytosis of granules. This observation is not in agreement with those reported in the literature cited above regarding the uptake of LDL particles by structures associated with the process of receptor-mediated endocytosis. This observation could explain, however, endosomes which contain both granules and matrix particles, and also suggests that the IBO protocol could be useful in studying, in more detail, the process of uptake materials having a high content of lipid. There are no other reports in the literature of observations of this type.

Cytoskeletal involvement in uptake of yolk

We found that by TEM, cells became increasingly convex with time, with a resultant increase in surface area. Madison et al. (1979), using TEM and SEM had indicated that changes in the topography of the cell surface of mouse peritoneal cells was due to the involvement of the cytoskeleton. Pasternak et al. (1989) expanded on this hypothesis by showing that the cortical tension in *Dictyostelium* cells was determined by an actin/myosin system. In yolk sac epithelial cells observed by TEM, we did not observe core filaments in the microvilli. King (1983) observed the organization of the actin filaments in microvilli of guinea pig yolk sac epithelial cells by using specialized techniques, which included a myosin subfragment binding technique to decorate actin filaments. However, in the yolk sac epithelial cells, we occasionally observed microfilaments in the microplicae which took up granules. Both Silverstein et al. (1977) and, more recently, Anderson et al. (1991) have discussed cytoskeletal involvement during phagocytosis, in their reviews of endocytosis. They have summarized morphological changes associated with phagocytosis, such as aggregations of actin-like filaments localized to the area of cytoplasm directly beneath the particle being phagocytosed (Silverstein et al., 1977), and the formation of projections to enclose particles. They cite evidence that the process consumes ATP and is inhibited by microfilament inhibitors such as cytochalasin A. Our observation of microfilament tracts

throughout the apical cytoplasm, in close proximity to both the microplicae and membrane-bound inclusions in the apex is in agreement with their hypothesis of direct involvement of microfilaments in the process of phagocytosis. King (1983) also observed microfilaments in the apical cytoplasm of guinea pig yolk sac epithelial cells, close to the apical membrane, which he concluded comprised a poorly organized terminal web.

The presence of microfilament tracts just under the apical membrane also indicates that they may have some significance in the uptake of matrix particles during the process of receptor-mediated endocytosis. Although microfilaments do not appear to have a direct role in endocytosis like they do in phagocytosis, the microfilaments must be rearranged to allow invagination to proceed. In addition, Salisbury et al (1980), working on cultured B lymphoblastoid cells, have presented both direct and indirect experimental evidence for the association of microfilaments with clustered ligand-receptor complexes in the apical cell membrane. Pasternak et al. (1989) studying the effects of concanavalin A in *Dictyostelium* cells, demonstrated the involvement of apical microfilaments in capping of receptors for LDL in the apical plasma membrane. Our observations support these of Salisbury et al. (1980) and Pasternak et al., (1989).

We also observed microtubules in the apical cytoplasm, arranged in a criss-cross meshwork. This observation is in agreement with their possible participation in long-distance translocation of endosomes and lysosomes in the cell, and are in agreement with the work of Matteoni and Kreis (1987) (see below).

SEM of yolk uptake

In my studies, three-dimensional information about the uptake process in epithelial cells of the yolk sac was obtained using SEM. Ishii and Sakurai (1991) used SEM to better image the three-dimensional aspects of endocytosis in their study of phagocytosis in planarian intestinal cells. We share their view, that structural observations from previous LM and TEM studies were incomplete "due to technical limitations in viewing sections". In accordance with this reasoning, and, using SEM, we observed that the

surface of the cells was covered with microplicae, which became more numerous throughout incubation. This observation is in agreement with work summarized by Anderson (1991) describing microplicae (lamellopodia) scattered on the surface of cells engaged in active membrane ruffling, and work cited by Silverstein et al. (1977), summarizing SEM observations of morphological stages of phagocytosis. Our SEM results are in agreement with those summarized by Silverstein et al. (1977).

In my study, two sizes of fossae were observed in the cell apices. The larger ones were roughly the same size as granules (diameter = 3.6 $\pm$ 0.9 μm, n=20), while the smaller ones (20 - 30 nm in diameter) were about the same size as the matrix particles. At 3 days of incubation, only large fossae were apparent; both sizes were observed at 7 days, but only small fossae were observed at 15 days of incubation. We interpret these observations to indicate the existence of 2 types of yolk uptake mechanism which are structurally manifest as 2 size ranges of fossae. We also believe that the apparent shift in the sizes of the fossae observed during incubation may reflect the shift in emphasis of what is being taken up at different times during incubation. This would agree with the TEM observations of Lambson (1970) and the biochemical observations of Noble (1987) regarding the massive lipid transport which occurs toward the end of incubation.

The fact that we observed only 2 sizes of fossae using SEM, none as large as yolk spheres, also adds to the evidence that yolk is not taken up in the form of spheres. These interpretations also agree with the TEM tracer studies of Lambson (1970) and Mobbs and McMillan (1981) who were able to demonstrate uptake and processing paths for particles of 2 sizes.

Calcium spherules were never observed being endocytosed, by either TEM or SEM.

Early Intracellular Depots of Yolk

We observed that the yolk components were membrane-bound, once inside the cell, regardless of how they were taken up. Courtoy (1991) argues that the passage of uptaken substances through endosomes occurs irrespective of the mode or port of entry, the

nature of the macromolecule or its final destination. Juurlink and Gibson (1973) observed that the membrane is retained from the uptake structures, and Mobbs and McMillan (1979) called these organelles "apical vacuoles". The current names for such organelles are phagosomes and endosomes; in chick yolk sac epithelial cells, they contain either granules alone, granules and matrix, or matrix alone, which have retained their morphological integrity.

Our observation that phagosomes and endosomes were located in the apex of the cell, just under the apical membrane, supports the view of Courtoy (1991), who hypothesized that endosomes are not randomly distributed in the cell. Early endosomes are concentrated at the cell periphery, and the late endosomes at the cell centre near the centrioles and Golgi complex. Phagosomes and endosomes do not show acid phosphatase activity, and usually have an electron-lucent content except when they contain lipoproteins. Late endosomes appear "condensed", in comparison to early endosomes, with an increase of electron density of contents.

In his review, Courtoy (1991) discusses that the presence of phagosomes and endosomes, both structurally and functionally distinct from lysosomes, demonstrates that a period of time exists before uptaken materials are extensively degraded. Both Helenius et al. (1983) and Courtoy (1991) prefer to call these structures by the generic name endosomes, because it does not assume specific compositional features, or emphasize a single function.

Silverstein et al. (1977) concluded, in their review of endocytosis, that the size and shape of a phagosome corresponds to that of the uptaken particle. Anderson (1991) hypothesized that membrane-bound structures in the range of 1-2 μm resulted from uptake during phagocytosis because they are too large to have resulted from uptake in coated pits. Our observations are in agreement with this hypothesis, because we found the yolk granules that were taken up in microplicae fell into this size range (approximately 1.6 μm in diameter).

Fusions of phagosomes and endosomes and cytoskeletal involvement

Just basal to the area which contained the phagosomes and endosomes was an area where these structures appeared to be fusing with each other. These vacuoles are larger, and contain both granules and matrix. These observations agree with those of Lambson, (1970) who hypothesized that even though different yolk constituents were absorbed separately in the epithelial cells, the presence of all these materials within a single cytoplasmic membrane inclusion suggested that different intracellular absorption vacuoles often fused with one another.

More recent work provides convincing evidence for the fusion of phagosomes and endosomes which we have observed. Mayorga et al. (1988) and Diaz et al. (1991) used probes for examination of fusion between early endosomes in a cell free system. Using sequentially labelled cells, split into 2 groups, they compared mixing of contents *in vivo* and *in vitro*. Their results, showing a shift in the buoyant density of the endosomes, suggested that early endosomes fused more frequently than did late endosomes. Salzman and Maxfield (1988) using polyclonal antibodies and Chinese hamster ovary (CHO) cells, showed that sequentially endocytosed probes mix intracellularly, thereby showing that endosomes fuse. Courtoy (1991) has summarized 3 dimensional reconstruction work in his review that demonstrates that although endosomes can fuse, they are distinct entities and do not have permanent connections to each other.

As mentioned in the section on yolk uptake, we observed both microfilaments and microtubules in the apices of yolk sac epithelial cells, which are responsible for movement of phagosomes and endosomes. In related work, Fernandez et al. (1988) treated chick neuroepithelial cells with the antimicrotubular agent colchicine and the antimicrofibril agent cytochalasin B to investigate the possible role played by the cytoskeleton in the maintenance of the cellular distribution of yolk droplets and lipid bodies during neurulation. They found that the distribution patterns of yolk droplets and lipid bodies were drastically changed by treatment with colchicine, implying a

relationship between microtubules and inclusions in these cells. In mouse macrophage cells, Toyohara and Inaba (1989) found that the transport of large phagosomes was inhibited by cytochalasin B (a microfilament inhibitor) but not vinblastine or podophyllotoxin (microtubule inhibitors), and that small phagosomes showed the reverse behaviour. Immunofluorescence microscopy showed that small phagosomes accumulated in the central region of the MT network, and that large phagosomes were surrounded by actin-rich cytoplasm and occasionally actin-filament-like structures could be recognized. They concluded that there are 2 different transport systems of phagosomes, depending on size. They also observed that large and small phagosomes could fuse with each other.

With regards to endosomes, Ostlund et al. (1979) did early work using colchicine and cytochalasin A for LDL processing in cultured human fibroblasts and aortic medial cells. More recently, Matteoni and Kreis (1987) have used video-enhanced fluorescent microscopy of living normal rat kidney cells labelled with acridine orange to study translocations and clustering of endosomes. They found that endosomes moved bidirectionally with respect to the microtubule organizing centre, and this behaviour ceased when microtubules were depolymerized by nocadazole treatment. Microfilament depolymerizers had no effect. they concluded that translocation of endosomes occurs along microtubules and is independent of both intermediate and microfilament networks.

The rest of the inclusions that we observed in yolk sac epithelial cells, appeared to result from the digestion of the phagosomes, and will be referred to as "vacuoles".

Morphology of yolk digestion - vacuoles

As explained in the preceding section, we have defined "vacuoles" as cell inclusions which do not include phagosomes, endosomes or typical lipid droplets. The vacuoles (diameter = 2.7 $\pm$ 0.6 μm, n=20) were larger than the endosomes and phagosomes, and gave the impression that they may be connected to each other. Vacuole contents were observed to be variable, and included typical lipid droplets, "dense bodies of variable

shape", zones of very low electron density, vesicles, granular material and myelin-like bodies.

Because the vacuoles contain yolk which has been altered morphologically, they can be considered to be secondary lysosomes, according to the broad definition based on this criterion as suggested by Bellairs (1963) for chick embryo yolk granules and Lemanski and Aldodoty (1977) for amphibian embryo yolk platelets. In our work, the smaller vacuoles, described earlier as dense bodies, just basal to the phagosomes and endosomes, can be considered to be early lysosomes based on this criterion. In addition, we observed that the overall density of the contents is more intense than the contents of other vacuoles, a criterion for lysosome identification according to Holtzman (1989). The large vacuoles in the area basal to these early lysosomes which contain lipid and myelin figures can be considered to be late secondary lysosomes. Little is left of the original structure of the yolk, and the contents of the vacuole is mainly lipid in nature, apparently due to the digestion of other components. Although Bellairs (1963, 1964) did not use the term "lysosomes" to describe such structures, she reported that the significant structural changes which yolk granules had undergone, which she had detected by LM and TEM, indicated the process of digestion of yolk granules took place in the epithelial layer of the yolk sac. In the case of the amphibian embryo, Lemanski and Aldoroty (1977) have correlated the progressive changes in structure, size and number of yolk platelets to digestion in the Mexican Axolotl. Bellairs (1963) observed that structurally altered (digested) granules and unaltered (undigested) granules could exist side by side in the cytoplasm of yolk sac epithelial cells. This agrees with our observation that unaltered yolk granules are sometimes observed in the base of the cells. Lemanski and Aldoroty (1977) have described a similar situation during yolk platelet breakdown in cells of Mexican Axolotl embryos, and have interpreted it as a staggered utilization of yolk, which may serve as a mechanism for controlling synthetic and energy-consuming functions.

The vacuoles that we have observed and described above were associated in different combinations, and it was not found useful to attempt to further classify or quantify the vacuoles according to content and position in the cell or age of the embryo. Some authors have attempted to classify the inclusions on the basis of content, position in the epithelial cell, position of the cell in the yolk sac, and age of the yolk sac, as discussed earlier. These reports are very detailed, and cannot be directly compared because vacuoles have been classified using different criteria in each report. Yolk digestion has also been described ultrastructurally in other chick embryo cells which contain yolk due to segmentation early in development. Because these cells contain a finite amount of yolk to be digested, stages of yolk digestion may be more easily seen in such cells than in yolk sac epithelial cells, and may be helpful in the interpretation of our results. Fernandez-Alvarez et al. (1987) used TEM and morphometry to describe yolk digestion in chick embryo neuroepithelial cells, and found that "yolk droplets and lipid bodies...accumulate in the apical zone", and concluded that the patterns of inclusion and organelle polarity may be related to active consumption of the reserves contained in inclusions during growth and development.

Most authors, however, described yolk digestion by yolk sac epithelium as an orderly process, i.e., according to some time sequence. Threadgold (1976) and his contemporaries attempted to define a temporal relationship between components of an intracellular digestion system in general terms, and separated structures associated with intracellular digestion into a limited number of broad groups. 1) prelysosomes (heterophagosomes); 2) primary lysosomes; secondary lysosomes (heterolysosomes); 3) post lysosomes (residual bodies). This is generally the system in use today.

Fernandez-Alvarez et al. (1987) related consumption of yolk reserves present in the apex of chick embryo nerve cells to the progress of differentiation of these cells during neurulation, therefore adding a temporal component to the digestive process.

Fusions and cytoskeletal involvement in yolk digestion

Authors generally agree that most of the epithelial cells are composed of inclusions containing yolk, which may be connected, as we have indicated. Lambson (1970) felt that the apparent fusion and engulfment of several types and sizes of vacuoles suggests that different intracellular membrane-bounded compartments frequently combine with one another, and hypothesized that this possibly indicated the presence of a "phagosome-lysosome system".

We observed that vacuoles from older samples showed more heterogeneity of contents, especially in their content of myelin figures. Although connections between the vacuoles was not studied in detail, such connections and fusions could explain the heterogeneity observed in vacuole contents. According to Dean and Barrett (1976), the fusions of secondary lysosomes with newly formed endocytotic bodies allows slowly digested substances to spread throughout the secondary lysosomes in a single cell. The age of secondary lysosomes (and so, of the cells which hold them) have been determined, using tracer experiments (such as those done by Mobbs and McMillan (1981)), by the heterogeneity of their contents, which increases with age. This probably accounts for our observation that vacuoles in cells after 15 days of incubation had more heterogeneous content than did vacuoles from cells at younger ages. These results are in agreement with those of Ferris et al. (1987), who employed cell fusion to address the question of whether macromolecules are rapidly exchanged between lysosomes in Chinese hamster ovary cells, using conjugated dextrans as markers. The results of the experiments suggested that *in vivo* the lysosome is a rapidly intermixing organellar compartment. In another system, Oates and Touster (1980) showed that cyclic nucleotides control rate and vacuole subpopulations control extent of fusions of phagolysosomes in *Acanthamoeba* homogenates.

We observed microtubules in the cell cytoplasm around the outside of lysosomes in the epithelial cells (micrographs not shown), which we believe are responsible for

movement of lysosomes during fusion events. This hypothesis is in agreement with the work of Matteoni and Kreis (1987) who used indirect immunofluorescent labelling of normal rat kidney cells with enzymes recognizing a lysosome glycoprotein to show that the movement of lysosomes to the region around the microtubule organizing centre depends on microtubules. In another experiment, they used video-enhanced fluorescence microscopy to study the behaviour of lysosomes in living cells labelled with acridine orange. On the basis of these experiments, they concluded that translocation of endosomes and lysosomes occur along microtubules and is independent of intermediate filament and microfilament networks.

Because we observed microtubules arranged around the outside of the lysosomes, we also believe that they may be responsible for maintaining the lysosome shape. This idea would agree with the work of Heuser (1989), who labelled the lysosomes in cultured chick fibroblasts and mouse macrophages with horseradish peroxidase, and followed the changes in lysosomal shape and distribution with pH changes and exposure to the microtubule depolymerizer, nocodazole. From this work, they concluded that in these cells, lysosomes are acted upon by both retrograde and anterograde microtubule motors, and that cytoplasmic pH affects fusion/fission properties of the lysosomes.

Delivery of hydrolases to lysosomes

We could not unequivocally identify, as primary lysosomes, structures which were morphologically similar to secretory granules. It is a well known fact that identification of primary lysosomes using morphology is not diagnostic; the identification of primary lysosomes is possible only at the level of the TEM by combining morphology and cytological tests for the presence of acid phosphatase (Schellens et al., 1977).

We never observed the fusion of primary lysosomes with endocytotic structures. We did observe, however, images of small, electron-dense bodies around the periphery of the vacuole, just inside the membrane. Threadgold (1976) explains that such an image can result when primary lysosomes remain in a condensed state for a period of time after

fusion with an endocytotic structure, and do not immediately diffuse throughout it. We observed such images in the sub-apical cytoplasm, just basal to the endosomes and phagosomes, and believe that they represent early secondary lysosomes because we detected slight morphological changes in the yolk they contained. Identification of such structures as early secondary lysosomes is in agreement with the work of Salzman and Maxfield (1989) who studied fusion accessibility of endocytic compartments along lysosomal pathway in intact cells of a Chinese hamster ovary cell line. Their work, based on fusion compatibility, has shown that newly synthesized lysosomal enzymes are delivered preferentially to recently formed endocytotic bodies such as endosomes rather than to older secondary lysosomes.

Content of lysosomes

The vacuoles we have described as secondary lysosomes can be separated into 2 groups, those containing morphologically altered granules, and those containing lipid and myelin figures, or a mixture of the two. The larger and more prominent of these is the second group, which is high in lipid content. We observed that these 2 groups of vacuoles appeared morphologically similar in all yolk sac epithelial cells.

We have classified the dense bodies as early secondary lysosomes, because their content is composed of granules which are starting to become altered, but are still recognizable as granules, and have a content which is condensed in comparison to the endosomes. We observed that the contents of late secondary lysosomes had some small electron-dense areas, but were mainly composed of lipid droplets and myelin figures. The IBO protocol showed that some of the areas which appeared empty with Karnovsky fixation were in fact filled with lipid. The persistence of structures high in lipid content in the lysosomes may be because the enzymes which digest lipids have difficulty in negotiating them in the organized structures, such as membranes, in which they are found. Devlin (1986) feels that, generally speaking, cells may have the same problems with digestion of lipid as is faced on a larger scale in the intestine. In the intestine, the lipids are relatively

inaccessible to enzymes in the aqueous phase, since even upon digestion, the products usually have a tendency to aggregate. In this form, they also have difficulty exiting through the lysosomal membrane.

Garland and Powrie (1978a and b), used TEM to study the structure of yolk granules which had been disrupted using NaCl solution, and using centrifugation, separated the components into 2 floating and 3 sedimenting fractions. The floating fraction FII was found to be particularly rich in myelin figures and LDL. The myelin figures they described resembled those present in the vacuoles which we have identified as late secondary lysosomes in the yolk sac epithelium. The myelin figures observed in the cells may therefore represent the lipid portion of digested yolk after the protein has been digested away. The myelin figures described by Garland and Powrie (1978 a and b) were similar to the ones we observed; the number of lamellae were variable, and consisted of phospholipids which were osmiophilic. The internal cores of their myelin figures were only weakly osmiophilic, suggesting the presence of neutral lipids, such as we have observed in our samples of yolk sac epithelium.

Vadehra et al. (1977) studied "insoluble yolk globules", which were isolated from yolk by gel filtration chromatography or centrifugation, were stable in solutions of NaCl and urea which dissolved or disrupted other yolk components, and had the same chemical components as LDL. Their ultrastructure and persistence after disruption of other yolk components resembles the lamellar structures we observed inside the large vacuoles after granule digestion that we have described as late secondary lysosomes. Burley et al. (1982) also described structures which they called "membranous globules", resulting from yolk lipoproteins subjected to biochemical procedures, which shared many similarities with the insoluble yolk globules described by Vadehra et al. (1977).

Kayahara (1983) has described digestion of yolk granules in chick notochord cells (which have a finite amount of yolk), and have related the structural changes occurring in granule breakdown to acid phosphatase activity. He observed that yolk persisted in these

cells until 2 days of incubation, and then disappeared by 3 days of incubation, indicating digestion and use of the stored materials. He describes yolk inclusions as being more similar to lipid inclusions in adipose cells than to yolk platelets in amphibian cells, and never observed "membranous delamination" in the digestion of yolk granules in chick cells up to 3 days of incubation. We observed what we believe is "membranous delamination", manifest as myelin figures, in yolk sac epithelium cells only at a much later time, at 8 days of incubation.

The phenomenon of "membranous delamination" was described earlier by Jurand and Selman (1964), in their work on the digestion of yolk platelets in newt embryo notochord cells, and the structural changes that the platelets undergo within the cell. Although yolk platelets are crystalline while chicken yolk granules are not, the end product of yolk digestion in either case is a collection of concentric lamella in the digestive vacuoles i.e., the evolution from a discrete compact structure to a loosely wound lamellar one (the myelin figures observed in the chick epithelial cells). Lemanski and Aldoroty (1977) have taken this work one step further, and have given a more in depth description of yolk digestion, using the Mexican Axolotl and the interrelationship between Acid phosphatase activity and the breakdown of yolk platelets in developing amphibian embryos. This system is similar to that found in the chick embryo because in the Mexican Axolotl, yolk is available to sustain the embryo through the first month of life. Initially the yolk platelets are the most prominent components of the cytoplasm of the embryonic cells. As development and differentiation proceeds, the platelets become smaller, fewer, and finally disappear. EM shows that the platelets are originally a crystalline central core surrounded by a peripheral granular layer, both contained within a limiting membrane. They describe how early studies of amphibian embryos showed a correlation between the utilization of platelets and the progressive level of differentiation of cells. Lemanski and Aldoroty (1977) also report enzyme histochemistry which relates acid phosphatase with yolk catabolism in Amphibians. They concluded that: 1) most platelet degradation is by

"membranous delamination" or unravelling at periphery. 2) lytic activity (acid phosphatase activity) is associated with this breakdown. 3) there is evidence of packaging of lytic enzymes by Golgi and transported to lysosomes containing platelets.

During the early stages of breakdown, little or no reaction product seen in platelets; as development progresses and the yolk is more rapidly utilized, acid phosphatase activity increases. Yolk platelets within a given cell show varied levels of acid phosphatase staining, ranging from no detectable activity at all to an intense reaction product. The highest activity is seen in those which have the most extensive membranous delamination which suggests lytic activity is associated with yolk platelet breakdown. Our observation of lysosomes having a high lipid content is in agreement with the work of Glaumann et al. (1975a and b) who studied degradation in lysosomes in Kupffer cells and observed "lipid-like droplets of various electron densities and pentalaminar structures".

Vacuole membranes in the medial zone of the cell

A complete membrane was never observed around the vacuoles in the medial part of the cells, even though all other criteria for good fixation were met. This phenomenon was also reported by Bellairs (1963) and Mobbs and McMillan (1979) who were not able to preserve membranes around these structures in yolk sac epithelial cells. Slade (1970) reported the presence of "broken vesicles" in his TEM study of protein transmission across the rabbit visceral yolk sac.

A discussion of the two interpretations of this observation have been made by Slade (1970). Either the fixation of the membranes was inadequate, or an incomplete membrane is the structure which really exists. Each of these interpretations has significant ramifications in the description of the intracellular digestive system in these cells, i.e, either these structures represent poorly preserved early secondary lysosomes, or they represent late secondary lysosomes or telolysosomes which are being subjected to lysosomal cathepsins to break down the membrane so that they can liberate substances to the cytoplasm.

Although Slade (1970) supports the second explanation, generally speaking, more authors seem to support the first choice. Threadgold (1976) explains that the membrane mosaic (after fusion of primary lysosomes, whose membrane originated from the Golgi, and phagosomes, whose membrane is derived from the plasma membrane but may differ from it in proportion of constituents - Courtoy, 1991) bounding the secondary lysosome is fragile just after formation because of the different origins of the regions. In addition, because they contain substances too large to pass through the membrane, Lloyd and Foster (1986) describe how lysosomes can behave as simple osmometers, which will rupture if exposed to hypertonic conditions. Because of this property, lysosomes can be difficult to fix for EM, since a solute (in the fixative solution) to which the cell is permeable will initially stabilize the lysosome, but as it penetrates the lysosome, a progressive osmotic imbalance develops and the lysosome swells and bursts. This hypothesis would explain the difficulties we encountered in fixation of the vacuole membranes. In light of this fact, we consider these vacuoles to be secondary lysosomes, which would probably be bounded by a complete membrane *in vivo*.

Lloyd and Foster (1986) and Arterburn et al. (1991) stress the importance of the membrane bounding the secondary lysosomes in intracellular digestion. Arterburn et al. (1991) in their review, discuss that 3 classes of molecules are, at present, recognized to be important in the biogenesis, structure and function of lysosomes. They are, the acid hydrolases, the mannose-6-phosphate receptors and the lysosomal membrane proteins. The last class is of interest to us, since we observed a "halo" around the inside of the lysosomal membrane in chick yolk sac epithelial cells. Using immunoelectron microscopy, Lewis et al. (1985) has shown that in purified rat liver lysosomal membranes, this halo consists of glycoproteins. Neiss (1984) used specific EM staining of rat kidney lysosomal membrane to observe the glycoprotein halo. Arterburn et al. (1991) have provided the following summary of the present information available on these glycoproteins. There are 20-30 extensively glycosylated integral membrane

proteins of the lysosomal membrane which contribute to its structure and function. They provide ion channels, proton pumps for acidification of lumen, selective transport of hydrolytic products, binding and recognition function for specificity in vesicular fusion events and protection of the organelle membrane against degradation by lysosomal proteases. Recently, Mittieux and Rousset (1989) have shown that an integral membrane protein in the lysosomal membrane in thyroid cells binds directly to MT in a process which can be regulated by ATP. This *in vitro* study showed that detergent-solubilized lysosomal membrane binds to preassembled MT. On the basis of this work, they proposed that this protein could account for the association of lysosomes to MT demonstrated in both *in vitro* and intact cells.

Forster and Lloyd (1988) have reviewed the present views of solute transport across mammalian lysosomal membranes. Lysosomal membranes allow free passage of small molecules (Iveson et al., 1989) such as pentose and hexose monosaccharides (Maguire et al. 1983), smaller carbohydrates and carbohydrate-based molecules (especially ones lacking ionizing groups) and l-amino acids (Bernar et al., 1986; Smith et al., 1987) after hydrolysis of peptides. Proteins, disaccharides and larger polysaccharides, glycosaminoglycans and polynucleotides cannot pass through the membrane, and so the presence of the intact membrane ensures exposure of the large molecules to the lysosomal enzymes so that digestion proceeds to the monomer stage.

I did not observe structures which could be identified as residual bodies (that is, have the same structure as residual bodies in other systems) although structures which we classified as late secondary lysosomes possessed many of the morpholgical criteria normally associated with residual bodies (electron-dense content, persistent structures due to the presence of undigestible or slowly digestible products, no acid phosphatase activity - Silverstein et al., 1977). No definite classification of structures as residual bodies was possible due to the inconclusive results we obtained from our work on acid phosphatase cytochemistry. Glaumann and Trump (1975) observed "lipid remnants" in Kupffer cell

residual bodies, after digestion of various substances, which appear to be structurally similar to the contents in structures we have identified as late lysosomes. The apparent lack of residual bodies we observed is consistent with the idea that rapidly growing tissues should have a small number of residual bodies, which would reflect an rapid and efficient breakdown of stored nutrients for utilization by the new living matter. In embryonic tissue such as yolk sac epithelium that we studied, this explanation is reasonable.

Basal zone

We observed that the basal zone of the epithelial cells was filled mainly with accumulations of lipid droplets of moderate electron density which lacked a membrane, with differences in accumulated lipid with age, discussed in more detail in the following section. We observed coated pits and vesicles within the basal membrane which may have a transport function. This supports the findings of Bellairs (1963) who reported pinocytotic indentations at the lower border of the epithelium, and Willier (1968) who had observed "micropinocytotic vesicles for transport of glucose to blood as well as an exchange of substances between epithelial cells and the embryo". Mobbs and McMillan (1979), on the other hand, had observed none of these structures, and instead felt that substances diffused actively or passively across the basal membrane.

We found a thin, continuous basal lamina at the vitelline surface of the epithelial cells in all ages studied. The products liberated through intracellular digestion of yolk in the lysosomes must negotiate this selective barrier which controls substances passing from epithelial cells to the connective tissue (Junqueira et al., 1989) before they can access the bloodstream and reach their final destination in the organism. Because it is thin but continuous, we would expect that it would exert effective control over transport of large molecules, but allow easy passage of small molecules, such as digestion products.

Some insights into the function of the basal lamina observed in chick yolk sac may be gleaned from the role of the basal lamina in transport of lipoproteins from the blood to

the granulosa cells of forming oocytes in the hen. Evans et al. (1979) found that the capillary basal lamina exerted a barrier to the passage of chylomicrons from the bloodstream. They observed large gaps in the capillary wall within the theca interna of the follicles, and found, in its basal lamina, many particles which they identified as plasma lipoproteins. They showed that the basal lamina is an electrostatic and mechanical barrier for the passage of materials, and that in this case, the high porosity of the basal lamina would allow unrestricted passage of large macromolecular complexes from the bloodstream towards the developing oocyte. They have summarized the importance of this work as follows: "morphological observations can contribute important evidence to distinguish between whether lipoprotein particles are transported intact or undergo a cycle of breakdown and reassembly of lipoprotein particles can be identified *in situ*".

Perry and Gilbert (1979) used tannic acid to study particles in the basal lamina, intercellular spaces and perivitelline space of the oocyte in the process of yolk deposition of the egg. In comparison to treatments without uranyl acid and tannic acid, the treatments which included these steps allowed the lipoprotein particles to be observed by stabilization of the phospholipids and phosphatidyl choline, respectively. Tannic acid was found to impart more contrast than uranyl acetate, probably due to some extraction of the sample. In addition, it selectively stained particle membranes. Perry and Gilbert (1979) also suggested potassium ferricyanide as a stain to be used for studies of lipoprotein particles.

Callebaut (1990) fixed quail oocyte follicle cells using a fixation protocol which included malachite green and studied them using TEM. He showed that the basal lamina prevented the passage of large very low density (VLD) lipoprotein particles and chylo-micron remnants through it; the particles accumulated at its outer boundary. He concluded that the basal lamina acts as a mechanical filter, allowing transit of molecules depending on macromolecular charge and pH. Transport of lipoprotein molecules

through a basal lamina will be discussed again in the section on morphology of lipid transport.

4.2.4. Blood vessels in the yolk sac

Large blood sinuses (up to 100 μm in diameter), rather than capillaries comprised the circulatory system within the yolk sac. The sinuses have thin walls, and are located close to base of epithelium. The endothelium cells comprising these sinuses have highly attenuated processes, and are joined with simple tight junctions. Neighbouring endothelial cells are observed to overlap. Large fenestrations of various sizes are observed in the walls. These characteristics allow the blood vessels found in the yolk sac to be identified as sinuses rather than capillaries (Fawcett, 1986). The lumen is filled with red and white blood cells, which is evidence that they are not lymphatic vessels. No basal lamina is present. Our observations support and extend those of Gonzalez-Crussi (1971) who described the circulatory system in the yolk sac up to 4 days of incubation as an "initial capillary network... sinusoidal in type. The major vitelline vessels acquire a more complex anatomy but the capillaries retain this simple pattern throughout the period studied." Mobbs and McMillan (1979) give only a brief description of blood vessels in the yolk sac. They hypothesize that two types of blood vessels exist; the vitelline vessels (which may correspond to the structures we have identified as sinuses) and "small, numerous vessels in the mesenchyme between the vascular surface of the endoderm and the established vitelline vessels" which may correspond to capillaries. Mobbs and McMillan (1979) do not provide detailed morphological descriptions of these vessels.

Our results are also in agreement with those of Kessel and Fabian (1985), who used SEM and LM to observe the morphogenesis of the chick yolk sac vascular system in blastoderms up to 3 days of incubation. They described the blood vessels as large and

sinusoidal, with fenestrations 0.2 - 1.4 μm in diameter in the ventral vessel walls where they were in proximity to the yolk sac epithelium.

Wisse (1977) has given an ultrastructural description of endothelial cells in the liver sinusoids, which includes a streamlined shape, flat processes which spread out to form the lining proper of the sinusoids; the processes are fenestrated, with a pore size of 0.1 μm. In the liver sinusoids, the fenestrations are, however, grouped together into sieve plates. Here, too, a basal lamina is lacking. The fenestrations are open spaces to the lumen and the spaces of Disse. This ultrastructural description is very similar to the one we have put forth for blood vessels of the chick yolk sac, and it can be seen that these tissues share a number of similarities.

The close proximity of the blood sinuses to epithelium in organs such as liver is due to their formation; Fawcett (1986) explains that sinuses develop during embryonic development by "an ingrowth of epithelial parenchyma into large, thin-walled embryonic blood vessels, which accounts for their close conformity to the geometry of the organ". This description is very similar to the one given by Romanoff (1960) regarding fold formation of the chick yolk sac during incubation, and is additional evidence for these vessels to be identified as sinuses. Junqueira et al. (1989) are of the opinion that blood vessels having these structural characteristics are found mainly in the liver and in hematopoietic organs such as bone marrow and the spleen. Fawcett (1986) provides a description of the progress of blood formation which interrelates these organs. Blood is first formed in the mesenchyme of the embryonic yolk sac, in aggregates (the blood islands). As development continues, blood-forming cells appear in the embryonic liver. Finally, during skeletal development, blood formation shifts to the primitive bone marrow. It can be seen, then, that the identification of blood vessels in the yolk sac as sinuses is therefore in agreement with its well-known function as the site of early hematopoiesis for the chick embryo. The presence of fenestrations in the sinuses of the yolk sac is also a structural characteristic of bone marrow, since the blood cells develop

outside of the vascular system, and must pass through the sinus walls into the bloodstream through the fenestrations (Fawcett, 1986). The variability in size of fenestrations that we observed in the blood sinuses of the yolk sac is a structural characteristic unique to sinuses found in the liver (Fawcett, 1986), and Junqueira et al. (1989) suggest that the structural characteristics of blood sinuses (discussed above) facilitate transport between blood and tissues. We would expect that in the case of the yolk sac, there would be easy movement of digestion products from the epithelium into the blood, and finally to the embryo.

4.3. Morphology of lipid transport

4.3.1. Use of the imidazole-buffered osmium tetroxide protocol of Angermüller and Fahimi (1982) (IBO) to study lipid transport.

We used this method, because in other systems we found that structures high in neutral lipids were better preserved than when conventional fixatives were used (Allan-Wojtas and Kalab, 1984). The problem of studying lipid processing in cells has been of interest for many years. Boyles (1984) reviewed the problem of staining neutral lipids (such as cholesterol or triglycerides), especially in chylomicrons in mammals, for ultrastructural study. The low number of unsaturated double bonds present which can react with osmium tetroxide results in poor contrast of the chylomicrons in the intestine, unless the animals have been fed additional oils high in polyunsaturated lipids, in an effort to increase the number of double bonds available to react with the osmium (Skipski, 1972; Jersild, 1966; Cardell et al, 1967). In the liver, newly synthesized lipids are in the form of saturated or monounsaturated lipids (in the form of very low density lipoproteins, VLDL), and the isolated organs have instead been perfused with polyunsaturated free fatty acids in an effort to increase contrast (Alexander et al., 1976).

A different approach to improving ultrastructural visualization of lipids in cells was pioneered by Angermüller and Fahimi (1982) by incorporating an imidazole buffer

"soak" step into a standard fixation protocol, which eliminates the necessity for feeding or perfusing the tissue with lipids, a preparatory step which was found to give unreliable results (Marenus and Sjostrand, 1982) The imidazole acts as a mordant for the osmium tetroxide, and selectively increases the osmiophilia of neutral lipids, although it does not affect the staining quality of the saturated ones. We selected this simple protocol to study lipid transport in the chick yolk sac, which has not been extensively documented in the literature. Recently, Franke et al. (1991) and Plonne et al. (1991) have used this technique to study the transport of lipoprotein particles in rat visceral yolk sac, a system similar to the one which we have studied.

4.3.2. Lipid transport in chick yolk sac epithelium

We observed that lipid found in yolk sac epithelium was in the form of droplets and myelin-like bodies in vacuoles. These structures probably represent morphological demonstrations of the dissociation of neutral lipids and phospholipids from the lipoproteins in the absorbed yolk, as a result of its digestion by the lysosomes in the cell. Isolated lipid droplets, independent of the vacuoles and lacking a membrane boundary, were also observed, which were similar to storage droplets found in other cells. In describing the structure of accumulations in cells which are active in the processing of large amounts of lipid, Threadgold (1976) explains that lipids and phospholipids evolved within the cell do not have limiting membranes. When lipids exist as anhydrous forms, they appear, using TEM, as amorphous areas of high or low-density. When they exist in a hydrated form, they appear as low density areas, with membrane arranged in a concentric manner, similar to myelin bodies. Phospholipids show an even more complex and variable morphology than lipids. These two forms may occur separately in the cytoplasm, or together within a membrane-limited, watery vacuole. The hydrated type forms caps, crescents or complete shells on or around the inner surface of the vacuole, or forms complex shapes within the vacuole. These descriptions are very similar to the lipid structures that we have observed in yolk sac epithelial cells.

We observed accumulations of lipid in the cell from day 2 of incubation which increased until about day 13 of incubation, when lipid starts to be transported in large quantities using the system described below. It is expected that accumulation of lipid droplets in the extralysosomal cytoplasm would be observed in cells which digest large quantities of lipid-rich substances such as yolk. Lipids, including cholesterol (Forster and Lloyd, 1988), move through the membrane of lysosomes as individual molecules, which will be modified or assembled into drops in the cytoplasm. The lipid droplets are used in metabolism, or transported, and eventually disappear, as described for chick neuroepithelial cells by Kayahara (1983).

During the same time, we observed that the intercellular spaces became less dilated from the level of the nucleus and basal to it.

In yolk sac epithelial cells at 13 days of incubation, we observed Golgi stacks close to lateral membrane, with cisternae and vesicles having dense content originating from them, which appear similar to those found in cells secreting lipoproteins. The vesicles, which appear to have budded off the cisternae, fused with lateral plasma membrane and appeared to be emptying theirs content into intracellular spaces. The IBO protocol enhanced the visualization of accumulations of small osmiophilic particles in intercellular space, from the level of the nucleus to the base. These particles were observed to be similar in morphology to chylomicrons produced by intestinal cells, and were not clearly observed when Karnovsky's fixative was used. The system that we have described for lipid transport on the basis of morphology agrees with Lambson (1970) who described an ultrastructural basis for a functioning lipid transport process in chick yolk sac epithelium, and is very similar to the lipid transport in adult intestinal cells.

We failed to detect a lymphatic system in the yolk sac, and we assume that these particles go directly into the bloodstream and are carried to the embryo. This assumption is in agreement with the work of Lambson (1970), who had, using TEM, observed the lipoprotein particles in endothelial cells of vitelline capillaries. He hypothesized that this was

the last step in a sequence of events in which the lipoprotein particles were released from epithelial cells into the extracellular space and then taken up by the vascular system for further transport. This observation is also supported by Bensadoun and Rothfeld (1972) who called these lipoprotein particles "portomicrons" because they travelled to the embryo liver through the portal blood system, rather than a lymphatic system. Freeman (1976) has also hypothesized that in adult chickens, absorbed fat is transported by the portal system as LDL.

Franke et al. (1992) have used EM and the IBO protocol to study the involvement of rat visceral yolk sac in lipid metabolism of the fetus, in a study similar to ours but extended to include immunocytochemistry. They observed particles located in the cisternal Golgi stacks, vesicles and secretory vesicles of cells in yolk sac epithelium as well as distended areas of intercellular space between adjacent epithelial cells. Using the protein A-gold method and anti-apoB antiserum, they achieved specific localization of immunogold over different compartments of the lipoprotein pathway (rough ER, Golgi complex, secretory vesicles) as well as over the distended intercellular spaces, confirming that these particles are lipoproteins. The combination of these results with those of culturing experiments showed that the feto-placental unit in the rat fetus can synthesize and secrete lipoproteins, and the epithelial cells of visceral yolk sac were shown to produce ApoB-containing particles. Although no migration of particles into the underlying mesenchymal layer towards the vitelline capillaries was evident, immuno-EM suggested the passage of lipoprotein-sized particles into the subepithelial space. An important point which they reported was the fact that the fixation required to retain antigenicity did not morphologically preserve lipoprotein particles (our results are in agreement with theirs on this topic), which were invisible by EM, but could be localized with the protein A gold probes. The data presented in the study by Franke et al., (1992) provides evidence that the rat visceral yolk sac epithelium produces ApoB-containing particles which might contribute to the supply of cholesterol to the growing organism in a stage of development when the liver is

still underdeveloped and the site of intensive erythropoiesis. The fetal membranes actively synthesize cholesterol on Day 17 of gestation. The visceral epithelium continuously loses its accumulated lipids after 16 days of gestation, which agrees with this conclusion. Biochemically, Noble (1987) has described a similar situation in the chick yolk sac during the last week of incubation, and our work suggests that possibly a similar process could take place in the chick yolk sac epithelium.

In similar work, Plonne et al., (1992) verified the mammalian fetoplacental unit of the as a significant source for LDL supply to fetus, and showed both biochemically and morphologically that the near-term rat visceral yolk sac is able to synthesize and secrete ApoB containing lipoprotein particles. It might be possible that these comments may be extended to explain a similar process in chick yolk sac.

The work of Franke et al. (1992) and Plonne et al. (1992), as well as the results we have presented in this work agree that the pathway of lipoprotein particles inside and outside of the absorptive yolk sac resembles that found in intestine of rat and man (Jersild, 1966; Cardell et al., 1967; Alexander et al., 1976; Mak and Trier, 1975, 1979).

Lambson (1970) concluded that the study of lipid utilization and transport with morphological techniques presents many problems of interpretation, but his work provided an insight at that time into some of the aspects of lipid transport which had been investigated with a variety of biochemical techniques. He described 2 apparently separate but possibly related manifestations of lipid transport by chick yolk sac described as : 1) phagocytosis of lipid droplets, whose fate cannot be followed (which our results do not support) and 2) spherules, similar to chylomicrons formed in the intestine and are transported into the vascular channels, as well as transport of LDL in the blood from the hepatocytes.

Noble (1985, 1987) has shown biochemically that in the last week of incubation that the chick yolk sac is the site of lipoprotein synthesis and assembly of absorbed yolk lipids, and also the major site of synthesis of cholesterol oleate which accumulates in the

liver. A function of the cholesterol esters is in the maintenance of particle stability for chylomicrons and large plasma lipoprotein complexes (Noble, 1987) in a process similar to that outlined recently by Franke et al. (1992) and Plonne et al. (1992), described above, for rat visceral yolk sac.

4.4. Acid phosphatase histochemistry and cytochemistry

We observed that acid phosphatase was localized in yolk sacs using the Azo-dye method, and was demonstrated in the cytoplasm that separates the inclusions in epithelial cells, mainly in the apical area. The mechanism of reaction product formation in the Azo-dye method is not affected by large amounts of endogenous phosphorous present in yolk. Control samples, incubated without substrate were negative, as were smears of yolk from yolk sacs at 17 days of incubation. This observation differs from results from biochemical analysis of other workers. Willems et al. (1976) showed that hen's egg yolk contains an acid phosphatase, which they showed biochemically to be equally distributed over supernatant and granules, and for which phosvitin is not a substrate.

Acid Phosphatase localization using the lead-capture (Gomori) method for TEM did not further localize the reaction. The cytochemical localization of acid phosphatase activity yielded results of interest, but of preliminary nature, which must be confirmed through the use of a larger number of samples and the introduction of additional control procedures.

Our results share some similarities with the ones reported by Kayahara (1983) in his TEM study, using the Gomori method, to detect acid phosphatase activity in yolk droplets of notochord cells of the developing chick up to 3 days of incubation. He observed reaction product around the peripheries of yolk droplets of various different sizes, which were often clustered together to form larger masses. Some of the droplets had an electron-dense matrix where the reaction product was scattered. These droplets occurred free in the cytoplasm. There was a large amount of reaction product in the large droplets

and small amounts in the small droplets. Our localizations of reaction product are very similar to those described above. In addition, Kayahara (1983) reported that rough endoplasmic reticulum and Golgi apparati also contained reaction product, and were found to be closely or directly associated with the yolk droplets and their masses. He interpreted these results to suggest that acid phosphatase is supplied by the rough endoplasmic reticulum and the Golgi apparati for the utilization of yolk in differentiating notochord cells. As discussed before, these cells have a finite amount of yolk, which is broken down and disappears from the cells during incubation, so in a system such as this one, it may be easier to see the interrelationship between yolk catabolism and acid phosphatase activity. Acid phosphatase activity in yolk droplets appears to be heterogeneous in these cells up to 2 days of incubation, and by 3 days of incubation, no large masses with acid phosphatase activity are left, indicating that they have been digested by 3 days of incubation. On the basis of this observation, he concluded that acid phosphatase was related to the breakdown of yolk. We were not able to localize acid phosphatase in either the rough endoplasmic reticulum or the Golgi apparatus, due to the reasons discussed below.

Lemanski and Aldoroty (1977) described the interrelationship between acid phosphatase activity as demonstrated using the Gomori method, and the breakdown of yolk platelets as followed by structural changes, in the developing Mexican axolotl. They observed that the activity increases from no activity in vacuoles containing unaltered yolk platelets to high levels in those vacuoles which have the most extensive platelet disintegration. They concluded that this lytic activity is associated with the breakdown of the yolk. They also presented evidence that the lytic enzymes are packaged by the Golgi apparati, and transported to the lysosomes containing the yolk.

Lead capture methods for various enzymes have, however, been plagued by difficulties (Pearse, 1968; Ganote et al., 1969; De Jong, 1982; Smith et al., 1990). This is especially true in the case of alkaline phosphatase and ATPase (Rosenthal et al., 1966),

because they work at high pH (Poelmann and Daems, 1973). In the case of acid phosphatase, the difficulties are reduced, and can be overcome with adequate controls (Pearse, 1968). De Jong et al. (1979) developed and used a model system to study 3 factors influencing metal-salt methods in enzyme cytochemistry a) enzyme activity; b) build up of primary (phosphate) product concentration at the enzymatic site; and c) trapping efficiency of the metal ions in the incubation medium. De Jong et al. (1979) and De Jong (1982) believe their model system simulates the situation at any enzymatic site and proved suitable for investigation of the influence of the composition of cytochemical media on the trapping reaction.

More specifically, the main difficulties reported in using the Gomori-type method for the localization of acid phosphatase activity have been:

a) Inability to obtain negative controls for a given structure which has the enzyme. It is believed that this is due to the fact that the same cellular structure which has the enzyme also has phosphate-containing compounds which are hydrolyzed, and yield positive results even if no substrate is present in the medium. De Jong et al. (1979) have investigated this problem using their model system. They found that phosphate groups in biopolymers such as RNA, histone, and casein had a nucleating effect and were able to precipitate lead after hydrolysis.

When this is the cause of the problem, additional controls must be planned in which not only substrate but also a specific inhibitor of the enzyme is included. The absence of reaction in these controls would then indicate that the precipitation observed in the experimental and false positive controls was due to the presence of enzyme in the structure. Conversely, if controls continue to be positive even in the presence of inhibitors, this means that we are observing totally unspecific, non-enzymatic precipitation, and the situation is recognized as the one described in b).

b) Inability to obtain negative controls for a given structure which has no enzyme. (Pearse, 1968; De Jong, 1982). It is interpreted that excessive concentrations of lead in

the medium or inadequate pH might spontaneously hydrolyze phosphate-containing compounds and liberate the phosphate which then precipitates *in situ*, yielding a positive reaction where no enzyme is present.

c) False negative results (structures which do have the enzyme yield negative results). (Schellens et al., 1977). The main reasons for these negative results are generally procedural errors such as inadequate fixation procedure, lack of penetration and inadequate pH (higher than adequate pH produces precipitation of lead in the incubation medium).

In the results of our acid phosphatase experiments, the inability to obtain negative controls in the case of structures which have the structural characteristics of secondary lysosomes is probably due to the situation described in a) (presence of phosphate-containing compounds in the same structure which has the enzyme). This should be confirmed by including enzyme inhibitors in the medium. The work done by De Jong et al. (1979) using their model system found that casein was a strong nucleator for non-enzymatic lead deposition, and concluded that this was due to the fact that casein had many phosphoserine residues which could be available to bind lead. In our system, phosvitin is also a phosphoprotein, with many long phosphoserine runs (Taborsky, 1980), so a) is a valid explanation for the inability of obtaining non-negative controls.

However, even before these controls are carried out, we can still conclude with a reasonable degree of conviction that the situation explained in a) is the situation which we are observing. More specifically described, the secondary lysosomes have acid phosphatase but the fact that they also contain phosphate-yielding compounds makes it impossible to obtain negative controls. This conclusion is based on the following arguments. The Azo-dye histochemical technique (Burstone's) proved without a doubt that there are structures with acid phosphatase in the same region in which structures with morphological characteristics similar to those of secondary lysosomes were observed with EM. The factors explained in b) cannot be the reason for lack of negative controls because yolk granules in the same sections do not show positive results (which they

would show if excess of lead or inadequate pH were the cause of falsely positive controls).

It is also legitimate to conclude that the negative results obtained in the large, lipid-rich vacuoles indicate that these structures do not have the enzyme. The conditions resulting in falsely negative results (summarized in c) cannot be invoked here because there are other structures in close proximity to the vacuoles which are positive. This excludes, as causes of false negative results, lack of penetration of the lead-containing medium, or precipitation of soluble lead in the medium due to the inadequate pH. It is thus probable that the lipid-rich vacuoles lack acid phosphatase activity.

Attempting to localize the enzyme by immunocytochemistry is a sound idea for the future. Before undertaking this new approach, however, the classical localization methods for acid phosphatase activity (Gomori and Azo-dye procedures) should be explored further. A larger number of trials and adequate controls (such as inclusion of inhibitors such as sodium fluoride in the reaction medium, and other control measures as discussed in Bittensky and Chayen (1977) and Pearse (1968) must be incorporated into these experiments so that readily interpreted results will be obtained. These are routine procedures which can be easily carried out.

4.5. Proposed schemes for intracellular digestion of yolk granules and matrix particles

On the basis of our morphological study, supplemented by information from other studies on related topics by other workers, we propose the following steps in the digestion of yolk granules and matrix particles by the epithelial cells of the chick yolk sac.

4.5.1. Granules

Granules are taken up by phagocytosis, either with or without matrix particles, into phagosomes. At some time, they acquire acid hydrolases, and become secondary lysosomes, or fuse with preexisting secondary lysosomes, and show morphological changes

in their contents. After digestion of protein portion of the granules, the lipid portion persists as typical lipid droplets and myelin figures, which also are digested. Individual molecules move out into the cytoplasm and accumulate up to day 13 of incubation as membraneless lipid droplets. They are then transported in a system similar to the one found in the intestine.

Our hypothesis that the protein component of the granules is digested first in the lysosomes is in agreement with the work of Wouters et al. (1985) who studied the catheptic activity of yolk by measuring its proteolytic activity at pH 3. They hypothesized that the action of the yolk cathepsins on possible native endogenous substrates (like ovalbumin, phosvitin, lysozyme and the vitelline membrane complex, which are best hydrolyzed by yolk cathepsin D2) was of particular interest with regards to yolk utilization during embryogenesis. Their work suggested that phosvitin is hydrolyzed in a very specific way, to mainly 3 degradation products, which they felt may be important in splitting native phosvitin into better substrates for the action of a phosphoprotein phosphatase present in yolk sac.

Other properties of phosvitin can also determine how it will be digested. After dissociation of yolk granules by NaCl solutions, phosvitin is water soluble. It is resistant to attack by pepsin and trypsin (Cook, 1968) or general phosphatases (Debruyne, 1983). Phosvitin has a stabilizing effect on yolk, by binding iron tightly, and protecting the otherwise labile fatty acids from oxidation. Lu and Baker (1986) hypothesized that once phospholipids are protected from oxidation, other classes of lipids will be presumed to be similarly protected.

Additional support for granule protein digestion being digested first is the work of Evans et al. (1968) on *in vitro* enzymatic digestion of the lipovitellin (lipoprotein) component of the yolk granules. They digested lipovitellin sequentially with trypsin, papain and pronase, after each digestion the lipids were extracted from the digest by ether, and the soluble peptides were separated from the insoluble residue by centrifugation. The

data obtained indicated that most of the lipid is held within the protein network of the lipovitellin molecule in such a way that it is not reached by ether during extraction. When part of the molecule is removed by proteolysis, or the molecule is opened up by disruption of hydrophobic bonding, ether can then extract part of the previously unextractable lipids and further proteolytic digestion exposes more lipid to ether extraction.

Another insight into the stepwise digestion of granules in the lysosomes comes from the biochemical work of Willems et al. (1976) treated granule suspensions with different concentrations of NaCl, the resulting fractions of phosvitin, lipovitellin and LDL were exposed to and more phosphate was evolved. This observation agreed with the one they obtained using isoelectric focussing.

They also set out a scheme for the stepwise liberation of granule components as a function of degradation, in this case, by exposure to increasing concentration of NaCl. Because phosvitin is richer in histidine and serine than lipovitellin and LDL, but is poorer in tyrosine and phenylalanine, at concentrations of 1-2% NaCl, phosvitin is set free into the supernatant and the supernatant is therefore high in histidine and serine and poor in tyrosine and phenylalanine. Lipovitellin is known to be high in methionine, leucine, valine and proline; the sediment is enriched in these up to 2.5% NaCl indicating the presence of lipovitellin in the sediment. The LDL fraction is rich in threonine and aspartic acid; the supernatant is enriched in these at 1% NaCl.

It would be useful if these results could be correlated with ultrastructure at each step of digestion in a combined structure and biochemical study.

Holtzman (1989) briefly summarizes the present-day view held with regards to yolk storage and digestion. Because the yolk itself has been found biochemically to contain acid phosphatases and other hydrolytic enzymes, the question which arises is why the yolk does not degrade itself. Substances which inhibit proteases have also been found in yolk, but features inherent to the yolk proteins themselves, such as the high number of

phosphate groups present in phosvitin, or other properties, such as the co-precipitation property of lipovitellin and phosvitin, may effectively control yolk breakdown outside of cells. It is possible that the stability of yolk is effected in a synergistic relationship of all of the above.

Once inside cells, disinhibition of the hydrolases, or alterations of the ionic environment and pH to levels favourable for the action of the hydrolases will initiate and perpetuate yolk digestion. As the yolk structure and composition change during digestion, yolk breakdown may be facilitated as groups which were inaccessible to enzyme action become free and available for attack by the same or other enzymes. Holtzman (1989) suggests that the removal of phosphate groups by acid phosphatase could possibly facilitate attack by other enzymes.

Wall and Meleka (1985) have discussed why in *Xenopus* yolk degradation occurs during embryogenesis and not in the oocyte. They have suggested that enzymes required for yolk degradation are missing in the oocyte and must be synthesized by the embryo. Levels of acid phosphatase, present in yolk of a number of organisms, are found to drastically increase during embryogenesis. Other explanations to answer the question of why yolk is not degraded in the oocyte include inactivation of lysosomal enzymes present in the yolk, or raising of the pH of the endosomes in the oocyte before or after fusion with the lysosomes. These explanations may also be applicable to the system we have studied in the yolk sac.

Barrett (1984), in his review of the lysosome and its membrane, have provided an in depth discussion of proteolytic and other metabolic pathways in lysosomes. By analyzing the mechanism of action of the enzymes present in lysosomes, he has hypothesized that these pathways are interdependent, and are very specific, since the digestion product in one pathway can be the substrate in at least one other pathway discussed digestion in lysosomes. It is likely that this is the case in the lysosomes in yolk sac epithelial cells.

4.5.2. Matrix particles (LDL)

According to our observations, the absorption and digestion of yolk matrix particles is very similar to the well known pathway of LDL metabolism in cells of many types, and shows morphological correlates which agree with the present description of other systems (for example, Brown et al., 1975, in humans).

LDL (low-density lipoprotein), a serum lipoprotein, is the main carrier of cholesterol and cholesterol esters in vertebrates (Rawn, 1989). Darnell et al. (1986) have described the structure of an LDL particle as a sphere, 20 - 25 nm in diameter, which is bound by a phospholipid monolayer in which is embedded Apoprotein B, which is hydrophobic. The apolar core consists of triacylglycerols and cholesterol esters. These particles are held together by non-covalent forces, and the components have no exact stoichiometry (Devlin, 1986). Fawcett (1986) explains that they are large enough to be visualized in the TEM as "spherical particulates of varying size".

The description of LDL metabolism given by Brown et al. (1975) and Brown and Goldstein (1986) shares many features with the work we present here. LDL's are taken up by the process of receptor-mediated endocytosis in LDL receptors in coated pits on the apical membrane. (As discussed above, we also observed LDL particles entering during phagocytosis of granules, and as granule components). In the process of receptor-mediated endocytosis, each receptor binds a single LDL molecule by recognition of the Apoprotein B component. Invagination of the coated pits forms coated vesicles, which lose their coating to become endosomes. The LDL becomes dissociated from its receptor in the compartment for uncoupling and recycling of ligands (CURL), and the receptors are recycled to the plasma membrane, while the LDL enters secondary lysosomes. In the secondary lysosomes, the particles are disrupted by acid lipases which deesterify cholesterol esters, hydrolyze triacylglycerols and other lipid components of LDL. Apoprotein B is degraded to free amino acids by protease, and free cholesterol is released by hydrolysis of cholesterol esters (fatty acids are split off) by lysosomal cholesterol esterase

(Darnell, 1986). The free cholesterol diffuses out of lysosome into the cytoplasm. Cholesterol is esterified for storage through the action of Acetyl Co-A cholesterol-acyl-transferase (ACAT), where a fatty acid group from fatty acyl CoA is transferred to cholesterol in an irreversible reaction (low-energy cholesterol esters have been made from energy-rich fatty acyl CoA). A large reserve of esterified cholesterol is packaged up as chylomicrons, which are not membrane-bound.

Chylomicrons, classified as HDL, act as the primary transport vehicles for triacyl-glycerols and cholesterol, resulting from digestion in the intestine, to other tissues including the liver (Rawn, 1989), and are removed from the circulation very quickly. The largest of the lipoproteins, having a diameter of 100 - 500 nm, they are visible by LM (Fawcett, 1986), and have a structure like that of LDL, including its ApoB component (it is important to note that ApoB is only synthesized by liver and intestine (Devlin, 1986). These particles are also taken up usually by hepatocytes using receptor-mediated endocytosis, similar to the process outlined for LDL particles.

4.5.3. Role of the yolk sac

From this structural study, and complimented from other structural and biochemical studies in the literature, we can make the following conclusions, structurally supporting the evolution of the yolk sac from a liver-like organ to a functional adult intestine.

a) The yolk sac behaves as a transitory liver. This is an old concept, where the yolk sac was believed to carry out some of the functions of the liver up until the liver was differentiated well enough to carry out these functions itself. From our structural study, we can see how the yolk sac epithelial cells early in development share their phagocytic function with Kupffer cells of the liver (Wisse, 1977); how the epithelial cells contain glycogen, and respond to glucagon like the liver (Thommes and Juste, 1964; the activity of phosphorylase and the effect of glucagon on the yolk sac, Grillo and Grillo, 1966); how the presence of glutamic dehydrogenase (Solomon, 1957) in extraembryonic tissues could provide a pathway for the production of glutamic acid from carbohydrate sources; how

the structure of the blood sinuses and their lining endothelial cells share many characteristics with the endothelial cells of the sinusoids in the liver.

b) With the establishment of the blood system, the yolk sac begins to function as a digestive organ (Mobbs & McMillan, 1979), and the site of blood formation shifts to the bone marrow (Fawcett, 1986). Our results support the idea that yolk is digested intracellularly in the yolk sac epithelial cells during embryonic life. Silverstein et al. (1977) believe that endocytosis is a primitive function in eukaryotic cells. Noaillac-Depeyre and Gas (1979) agree with this belief, and feel that "the ingestion of particulate materials by enterocytes in higher vertebrates may be a retained feature of a primitive mechanism where the glandular cells in the digestive tract have not matured". In embryonic mammals, intracellular digestion takes place in the visceral yolk sac until the chorioallantoic membrane is developed and functional to provide the embryo with nutrients (Freeman et al., 1981). King and Enders (1970) and King (1977) have concluded that the guinea pig visceral yolk sac continues to function in the uptake of iron and maternal antibodies once the chorioallantoic placenta is established, as do Haar and Ackerman (1971) for the mouse visceral yolk sac. (Moran (1985) believes that the differentiation of the embryonic chick intestine must be quite advanced, so that the IgG (passive immunity from the hen) which is deposited into the yolk during follicle development can be taken up by the yolk sac during resorption.) In the intestine of adult vertebrates, digestion is usually luminal, and during differentiation, the apex of the intestinal cells is organized into a brush border, with its associated enzymes (Overton and Shoup, 1964; Moog, 1950; Pons et al., 1986). In fact, the yolk sac epithelium, after hatching, becomes part of the adult intestine, and develops a brush border and its associated enzymes (Matsushita, 1986; Holdsworth and Wilson, 1967; Veini et al., 1986; Boorman, 1976).

Structural manifestations of lipid transport in yolk sac epithelium are very much like those which occur in mature intestine (including production of ApoB, as in the studies by Franke et al., 1992 and Plonne et al., 1992). Mak and Trier (1975) hypothesized that in

fetal rats, secretion of lipoproteins is the first sign of differentiation of intestinal cells, which was also supported by Levy et al. (1992). The ability to assemble and secrete chylomicron-like particles by yolk sac epithelium which is evolved in the last week of incubation may reflect the differentiation of these embryonic cells into functional adult intestine cells after hatching.

4.6. Concluding remarks

Our results support the intracellular digestion of yolk granules and matrix particles in lysosomes, in a scheme similar to that found in a variety of other cells. At approximately 13 days of incubation, lipid is transported from the epithelial cells using a system similar to that found in the mammalian intestine, except that the lipid travels in the blood vessels rather than in a lymphatic system. Our results do not support extracellular yolk digestion, or secretion of digestive enzymes by the epithelial cells into the yolk mass. The apparent changing composition of yolk in the yolk sac, which has been interpreted as extracellular digestion by some authors, is possibly merely an interaction between the yolk mass and substances moving into or out of it during incubation, i.e., formation of calcium spherules, subembryonic fluid, etc.. We believe that the overlap in enzymes observed between the yolk and yolk sac in biochemical studies in the literature may have been, in part, due to contamination of the yolk sac with yolk when samples were prepared and analyzed.

The importance of the results of structural studies following controlled granule degradation outside of the cell (Chang et al., 1977; and others) may help to explain the ultrastructural aspects of yolk granule degradation in yolk sac epithelial cells. Epithelial cells do not have the ultrastructural characteristics of protein-secreting cells as outlined by Goyal and William (1991), and do not secrete substances, in the strict sense. On the other hand, Young et al. (1980) and Kram and Klein (1976) have described the synthesis and "secretion" into culture medium of serum proteins by yolk sac epithelium. Other authors

have used the term "secrete" in connection with transport of lipoproteins and chylomicrons. It would be useful to have clearer definitions or separate terms to better describe the difference between the two processes.

4.7. Future work

My observations extend those of Bellairs (1958, 1961, 1963, 1964) and Lambson (1970) in that the morphological changes which take place in the yolk during uptake, digestion and transport, can be described as the formation and interaction of organelles which compose the intracellular digestive system. Our work also extends the work of Willier (1968) in providing another attempt at correlating structure and function, to better understand the ordered channelling and/or processing of yolk to and through epithelial cells to the blood and then the embryo.

New experiments are required in order to establish the role of lysosomes in yolk intracellular digestion. Immunoelectron microscopy, using the colloidal gold method for TEM would be useful to label lysosomal enzymes, such as specific phosphatases, or lysosomal membrane glycoproteins to give a more definite localization and identification of secondary lysosomes without the complicating effects of a high concentration of endogenous phosphorus found in yolk. The approach used by Franke et al., (1992) and Plonne et al., (1992) to study lipoprotein transport in the mammalian visceral yolk sac would potentially provide additional insight into the process as it occurs in the chick yolk sac epithelium, while avoiding problems inherent in working with samples having a high yolk content.

4.8. Conclusions

1) No unit membrane around granules in the laid egg.
2) Internal structure of granules becomes visible upon digestion.
3) Robustness and consistency of granule structure in extracellular milieu, throughout all ages studied indicates no extracellular digestion.

4) Yolk granules taken up singly, with or without matrix particles, by cytoplasmic extensions of the plasma membrane; matrix particles taken up in coated pits.

5) Intracellular digestion, detected as morphological changes in yolk, takes place in dense bodies in the apex of the yolk sac epithelial cells .

6) Histochemistry and cytochemistry confirms that the dense bodies are secondary lysosomes by their positive reaction for the marker enzyme, acid phosphatase.

7) Accumulations of glycogen and lipid throughout the epithelial cells, which indicate storage of digested nutrients.

8) Transport of lipid from yolk sac to embryo similar to that found in intestine, in blood sinuses similar to those found in the liver and bone marrow of the adult.

5. References

1. Alexander C.A., Hamilton R.L., Havel R.J. (1976). Subcellular localization of B apoprotein of plasma lipoproteins in rat liver. J. Cell Biol. 69: 241-263.

2. Allan-Wojtas P., Kalab M. (1984). Milk gel structure. XIV. Fixation of fat globules in whole milk yoghurt for electron microscopy. Milchwissenschaft 39: 323-327.

3. Anderson R.G.W. (1991). Molecular motors that shape endocytic membrane. in: Steer, C. J. and Hanover, J. A., eds. Intracellular Trafficking of Proteins. Cambridge, UK, Cambridge University Press. pp. 13-46.

4. Angermüller S., Fahimi H.D. (1982). Imidazole-buffered osmium tetroxide: an excellent stain for visualization of lipids in transmission electron microscopy. Histochem. J. 14: 823-835.

5. Arterburn L.M., McClellan D.A., August J.T. (1991). Biosynthesis and processing of lysosomal membrane proteins. in: Steer C.J. and Hanover J.A., eds. Intracellular Trafficking of Proteins. Cambridge, UK, Cambridge University Press. pp. 472-503.

6. Bain J.M., Hall J.M. (1970). The effects of dietary cyclopropene fatty acids on the structure of new-laid and stored hen's eggs: an optical and electron microscope study. Aust. J. Biol. Sci. 23: 657-672.

7. Barka T., Anderson P.J. (1965). Histochemistry. Theory, Practice and Bibliography. New York, Harper & Row, Publishers, Inc.

8. Barrett A.J. (1984). Proteolytic and other metabolic pathways in lysosomes. Biochem. Soc. Trans. 12: 899-902.

9. Bell M. (1988). Artifacts in staining procedures. in: Crang R.F.E., Klomparens K. L., eds. Artifacts in Biological Electron Microscopy. New York, Plenum Press. pp. 81-106.

10. Bellairs R. (1958). The conversion of yolk into cytoplasm in the chick blastoderm as shown by electron microscopy. J. Embryol. Exp. Morphol. 6: 149-161.

11. Bellairs R. (1961). The structure of the yolk of the hen's egg as studied by electron microscopy. 1. The yolk of the unincubated egg. J. Biophys. Biochem. Cytol. 11: 207-225.

12. Bellairs R. (1963). Differentiation of the yolk sac of the chick studied by electron microscopy. J. Embryol. Exp. Morphol. 11: 201-225.

13. Bellairs R. (1964). Biological aspects of the yolk of the hen's egg. Adv. Morphog. 4: 217-272.

14. Bellairs R., Backhouse M., Evans R.J. (1972). A correlated chemical and morphological study of egg yolk and its constituents. Micron 3: 328-346.

15. Bennett N. (1973). Study of yolk-sac endoderm organogenesis in the chick using a specific enzyme (cysteine lyase) as a marker of cell differentiation. J. Embryol. Exp. Morphol. 29: 159-174.

16. Bensadoun A., Rothfeld A. (1972). The form of absorption of lipids in the chicken, Gallus domesticus. Proc. Soc. Exp. Biol. Med. 141: 814-817.

17. Bernar J., Tietze F., Kohn L.D., Bernardini I., Harper G.S., Grollman E.F., Gahl W.A. (1986). Characteristics of a lysosomal membrane transport system for tyrosine and other neutral amino acids in rat thyroid cells. J. Biol. Chem. 261: 17107-17112.

18. Bernardi G., Cook W.H. (1960). Separation and characterization of the two high density lipoproteins of egg yolk, alpha and beta lipovitellin. Biochim. Biophys. Acta. 44: 96-105.

19. Bittensky L., Chayen J. (1977). Histochemical methods for the study of lysosomes. in J.T.Dingle, editor. Lysosomes: A Laboratory Handbook. Second edition. Oxford, Elsevier/North Holland Biomedical Press. pp. 209-243.

20. Boorman K.N. (1976). Digestion and absorption of protein. in: Digestion in the Fowl. Proc. 11th Poult. Sci. Symp., September 17-19th, 1975. Edinburgh. Boorman K.N., Freeman B.M. (eds.) Edinburgh, British Poultry Science Ltd., 27-61.

21. Bowers B., Maser M. (1988). Artifacts in fixation for transmission electron microscopy. in: Crang R.F.E., Klomparens K. L., eds. Artifacts In Biological Electron Microscopy. New York, Plenum Press. pp. 13-42.

22. Boyles J. (1984). The use of mordants and osmium to stain lipids. EMSA Bulletin 14: 73-76.

23. Bozzola J.J., Russell L.D. (1992). Electron Microscopy. Principles and Techniques for Biologists. Boston, Jones and Bartlett Publishers. pp.286-304.

24. Brierly J., Hemmings W.A. (1956). The selective transport of antibodies from the yolk sac to the circulation of the chick. J. Embryol. Exp. Morphol. 4: 34-41.

25. Brown M.S., Dana S.E., Goldstein J.L. (1975). Receptor- dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein. Proc. Nat. Acad. Sci. U.S.A. 72: 2925- 2929.

26. Brown M.S., Goldstein J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. Science 232: 34-47.

27. Burley R.W., Cook W.H. (1961). Isolation and composition of avian egg yolk granules and their constituent alpha- and beta- lipovitellins. Can. J. Biochem. Physiol. 39: 1295-1307.

28. Burley R.W., Sleigh R.W., Bain J. (1982). Conversion of a lipoprotein from egg yolk into a membranous form in the presence of yolk lipid. Aust. J. Biol. Sci. 35: 13-23.

29. Burley R.W., Vahehra D.V. (1989). The Avian Egg Chemistry and Biology. Toronto, John Wiley & Sons.

30. Burstone M.S. (1958). Histochemical demonstration of acid phosphatases with naphthol AS-phosphates. J. Natl. Cancer Inst. 21: 523-529.

31. Callebaut M. (1990). Improved visualization of ultrastructural components in the avian ovarian granulosa basement membrane. Cell Biol. Internat. Rep. 14: 653-658.

32. Cardell R.R., Jr., Badenhausen S., Porter K.R. (1967). Intestinal triglyceride absorption in the rat. An electron microscopical study. J. Cell Biol. 34: 123-155.

33. Chang C.M., Powrie W.D., Fennema O. (1977). Microstructure of egg yolk. J. Food. Sci. 42: 1193-1200.

34. Chapeville F., Fromageot P. (1967). "Vestigial" enzymes during embryonic development. Adv. Enzyme Regul. 5: 155-163.

35. Cheville N., Coignoul F. (1984). Calcium in the turkey yolk sac during late development and postembryonic involution. Am. J. Vet. Res. 45: 2458-2466.

36. Cook W.H. Macromolecular components of egg yolk. in: Egg Quality - A Study Of The Hen's Egg, 1968. Carter T.C. (ed.) Edinburgh, Oliver and Boyd, 109-132.

37. Courtoy P.J. (1991). Dissection of endosomes. in: Steer C.J. and Hanover J.A., eds. Intracellular Trafficking of Proteins. Cambridge, UK, Cambridge University Press. pp. 103-156.

38. Cuadros M.A., Coltey P., Nieto M.C., Martin C. (1992). Demonstration of a phagocytic cell system belonging to the hemopoietic lineage and originating from the yolk sac in the early avian embryo. Development 115: 157-168.

39. Darnell J., Lodish H., Baltimore D. (1986). Molecular Cell Biology. New York, W.H. Freeman and Company.

40. De Jong A.S.H. (1982). Mechanisms of metal-salt methods in enzyme cytochemistry with special reference to acid phosphatase. Histochem. J. 14: 1-33.

41. De Jong A.S.H., Hak T.J., Van Duijn P., Daems W.T. (1979). A new dynamic model system for the study of capture reactions for diffusible compounds in cytochemistry. III. Influence of the matrix composition on the lead phosphate precipitation process in acid phosphatase cytochemistry. Histochem. J. 11: 163-171.

42. Dean R.T., Barrett A.J. (1976). Lysosomes. Essays Biochem. 12: 1-40.

43. Debruyne I. (1983). Hen's egg yolk acid phosphatase: purification by hydrophobic chromatography; general characterization and kinetic properties. Int. J. Biochem. 15: 417-425.

44. Debruyne I., Stockx J. (1979). Alkaline phosphatases from the hen's egg yolk, liver, blood plasma and intestinal mucosa: affinity chromatographic purification and comparison. Int. J. Biochem. 10: 981-988.

45. Deeming D.C. (1989). Importance of sub-embryonic fluid and albumen in the embryo's response to turning of the egg during incubation. Br. Poult. Sci. 30: 591-606.

46. Devlin T.M. (1986). Textbook of Biochemistry with Clinical Correlations. Second Edition. New York, John Wiley & Sons.

47. Diaz R., Colombo M.I., Koval M., Mayorga L., Stahl P. (1991). Endosomal density shift is related to a decrease in fusion capacity. Eur. J. Cell Biol. 56: 223-232.

48. Emanuelsson H. (1951). Proteolytic activity in hen's egg prior to incubation. Nature (Lond) 168: 958-959.

49. Evans A.J., Perry M.A., Gilbert A.B. (1979). The demonstration of very low density lipoproteins in the basal lamina of the granulosa layer in the hen's ovarian follicle. Biochim. Biophys. Acta. 573: 184-195.

50. Evans R.J., Bandemer S.L., Heinlein K., Davidson J.A. (1968). Binding of lipid to protein in lipovitellin from the hen's egg. Biochemistry 7: 3095-3102.

51. Evans R.J., Bauer D.H., Flegal C.J. (1974). Lipovitellins in fresh and stored eggs. Poult. Sci. 53: 745-750.

52. Fawcett D.W. (1986). A Textbook of Histology. Eleventh edition. Philadelphia, W.B. Saunders Company.

53. Fernandez J.G., Chamorro C.A., de Paz P., Villar J.M. (1988). Effects of colchicine- and cytochalasin B-treatment on the intracellular distribution of yolk droplets, lipid bodies, and golgi apparatus of the chick neuroepithelial cells. J. Exp. Zool. 245: 17-23.

54. Fernandez-Alvarez J.G., de Paz P., Chamorro C.A., Fernandez- Caso M., Villar J.M. (1987). Intracellular distribution of yolk droplets, lipid bodies and Golgi apparatus in the chick neuroepithelial cells during neurulation. Cytobios 51: 25-33.

55. Ferris A.L., Brown J.C., Park R.D., Storrie B. (1987). Chinese hamster ovary cell lysosomes rapidly exchange contents. J. Cell Biol. 105: 2703-2712.

56. Forster S., Lloyd J.B. (1988). Solute translocation across the mammalian lysosome membrane. Biochim. Biophys. Acta 947: 465- 491.

57. Franke H., Plonne D., Winkler L., Dargel R. (1992). Synthesis, secretion and immunoelectron microscopic demonstration of apolipoprotein B-containing lipoprotein particles in the visceral rat yolk sac. Histochem. 97: 283-292.

58. Freeman C.P. (1976). Digestion and absorption of fat. in: Digestion In The Fowl. Proc. 11th Poult. Sci. Symp., September 17-19th, 1975. Edinburgh. Boorman K.N., Freeman B.M. (eds.) Edinburgh, British Poultry Science Ltd., 117-142.

59. Freeman S.J., Beck F., Lloyd J.B. (1981). The role of the visceral yolk sac in mediating protein utilization by rat embryos cultured in vitro. J. Embryol. Exp. Morph. 66: 223-234.

60. Freeman B.M., Vince M.A. (1974). Development of the Avian Embryo. London, Chapman and Hall, Ltd.

61. Fujii S., Tamura T., Okamoto T. (1973). Studies on yolk formation in hen's eggs.I. Light and scanning electron microscopy of the structure of yolk spheres. J. Fac. Fish. Anim. Husb. Hiroshima Univ. 12: 1-11.

62. Ganote C.E., Rosenthal A.S., Moses H.L., Tice, L. W. (1969). Lead and phosphate as sources of artifact in nucleoside phosphatase histochemistry. J. Histochem. Cytochem. 17: 641-650.

63. Garland T.D., Powrie W.D. (1978)a. Chemical characterization of egg yolk myelin figures and low-density lipoproteins isolated from egg yolk granules. J. Food Sci. 43: 1210-1214.

64. Garland T.D., Powrie W.D. (1978)b. Isolation of myelin figures and low-density lipoproteins from egg yolk granules. J. Food Sci. 43: 592-597.

65. Glauert A.M. (1974). Part 1. Fixation, Dehydration, and Embedding of Biological Specimens. in: Glauert, A. M., ed. Practical Methods in Electron Microscopy. Oxford, North Holland Publishing Company. 3: pp. 1-207.

66. Glaumann H., Berezesky I.K., Ericsson J.L.E., Trump B.F. (1975)a. Lysosomal degradation of cell organelles. I. Ultrastructural analysis of uptake and digestion of intravenously injected mitochondria by Kupffer cells. Lab. Invest. 33: 239-251.

67. Glaumann H., Berezesky I.K., Ericsson J.L.E., Trump B.F. (1975)b. Lysosomal degradation of cell organelles. II. Ultrastructural analysis of uptake and digestion of intravenously injected microsomes and ribosomes by Kupffer cells. Lab. Invest. 33: 252-261.

68. Glaumann H., Trump B.F. (1975). Lysosomal degradation of cell organelles. III. Uptake and disappearance in Kupffer Cells of intravenously injected isotope-labeled mitochondria and microsomes in vivo and in vitro. Lab. Invest. 33: 262-272.

69. Goldstein J.L., Brown M.S., Anderson R.G.W., Russel D.W., Schneider W.J. (1985). Receptor-mediated endocytosis:concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. 1: 1-39.

70. Gonzalez-Crussi F. (1971). Vasculogenesis in the chick embryo. An ultrastructural study. Am. J. Anat. 130: 441-460.

71. Goyal H.O., Williams C.S. (1991). Regional differences in the morphology of the goat epididymus: a light microscopic and ultrastructural study. Am. J. Anat. 190: 349-369.

72. Grillo T.A.I., Grillo D.L.B. (1966) The activity of phosphorylase and the effect of glucagon on the yolk sac of the chick embryo. Gen. Comp. Endocr. 7: 420-423.

73. Grodzinski Z. (1951). The yolk spheres of the hen's egg as osmometers. Biol. Rev. Camb. Philos. Soc. 26: 253-264.

74. Haar J.L., Ackerman G.A. (1971). Ultrastructural changes in mouse yolk sac associated with the initiation of vitelline circulation. Anat. Rec. 170: 437-456.

75. Hamilton H.L. (1952). Lillie's Development of the Chick. Third edition. New York, Holt, Reinholdt and Winston.

76. Hayat M.A. (1989). Principles and Techniques of Electron Microscopy. Biological Applications. 3rd edition. Boca Raton, Florida, CRC Press, Inc.

77. Helenius A., Mellman I., Wall D., Hubbard A. (1983). Endosomes. Trends Biochem. Sci. 8: 245-250.

78. Heuser J. (1989). Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. J. Cell Biol. 108: 855-864.

79. Holdsworth G., Finnean J.B. (1972). A structural study of the low-density lipoprotein fraction from hen's eggs and of an isolated surface component. Chem. Phys. Lipids 9: 217-229.

80. Holdsworth C.D., Wilson T.H. (1967). Development of active sugar and amino acid transport in the yolk sac and intestine of the chicken. Am. J. Physiol. 212: 233-240.

81. Holtzman E. (1989). Lysosomes. New York, Plenum Press.

82. Howard E. (1957). Ontogenetic changes in the freezing point and sodium and potassium content of the subgerminal fluid and blood plasma of the chick embryo. J. Cell Comp. Physiol. 50: 451- 470.

83. Ishii S., Sakurai T. (1991). Food ingestion by planarian intestinal phagocytic cells - a study by scanning electron microscopy. Hydrobiologia 227: 179-185.

84. Iveson G.P., Bird S.J., Lloyd J.B. (1989). Passive diffusion of non-electrolytes across the lysosome membrane. Biochem. J. 261: 451-456.

85. Jersild R.A. (1966). A time sequence study of fat absorption in the rat jejunum. Am. J. Anat. 118: 135-162.

86. Junqueira L.C., Carniero J., Kelly R.O. (1989). Basic Histology. Norwalk, Connecticut, Appleton & Lange.

87. Jurand A., Selman G.G. (1964). Yolk utilization in the notochord of the newt as studied by electron microscopy. J. Embryol. Exp. Morph. 12: 43-50.

88. Juurlink B.H., Gibson M.A. (1973). Histogensis of the yolk sac in the chick. Can. J. Zool. 51: 509-519.

89. Karnovsky M.J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27: 137A-138A.

90. Kayahara T. (1983). Acid phosphatase activity in yolk droplets of chick notochordal cells. Histochem. J. 15: 71-77.

91. Kessel J., Fabian B.C. (1985). Graded morphogenetic patterns during the development of the extraembryonic blood system and coelom of the chick blastoderm: a scanning electron microscope and light microscope study. Am. J. Anat. 173: 99-112.

92. King B.F. (1977). An electron microscopic study of absorption of peroxidase-conjugated immunoglobulin G by guinea pig visceral yolk sac in vitro. Am. J. Anat. 148: 447-456.

93. King B.F. (1983). The organization of actin filaments in the brush border of yolk sac epithelial cells. J. Ultrastruct. Res. 85: 329-337.

94. King B.F., Enders A.C. (1970). The fine structure of the guinea pig visceral yolk sac placenta. Am. J. Anat. 127: 397-414.

95. Kram D., Klein N.W. (1976). Serum protein synthesis in the early chick embryo. Dev. Biol. 52: 300-309.

96. Kushuhara S., Ishida K. (1974). Histochemical obvservations on the enzymes of chicken yolk sac membrane. Br. Poult. Sci. 15: 391-394.

97. Lambson R.O. (1970). An electron microscopic study of the entodermal cells of the yolk sac of the chick during incubation and after hatching. Am. J. Anat. 129: 1-19.

98. Lange R.H., Richter H., Riehl R., Zierold K., Trandaburu T., Magdowski G. (1983). Lipovitellin-phosvitin crystals with orthorhombic features: thin-section electron microscopy, gel electrophoresis, and microanalysis in teleost and amphibian yolk platelets and a comparison with other invertebrates. J. Ultrastruct. Res. 83: 122-140.

99. Lawton J.R. (1989). An investigation of the fixation and staining of lipids by a combination of malachite green or other triphenylmethane dyes with glutaraldehyde. J. Microsc. (Oxford) 154: 83-92.

100. Lemanski L.F., Aldoroty R. (1977). Role of acid phosphatase in the breakdown of yolk platelets in developing amphibian embryos. J. Morph. 153: 419-426.

101. Levy E., Thibault L., Menard D. (1992). Intestinal lipids and lipoproteins in the human fetus: modulation by epidermal growth factor. J. Lipid Res. 33: 1606-1617.

102. Lewis P.R. (1977). Metal precipitation methods for hydrolytic enzymes. in: Glauert A.M., ed. Practical Methods in Electron Microscopy. Oxford, North-Holland Publishing Company. 5: pp. 137-223.

103. Lewis V., Green S.A., Marsh M., Vihko P., Helenius A., Mellman I. Glycoproteins of the lysosomal membrane. J. Cell Biol. 100: 1839-1847.

104. Lloyd J.B., Forster S. (1986). The lysosome membrane. Trends Biochem. Sci. 11: 365-368.

105. Lu C.L., Baker R.C. (1986). Characteristics of egg yolk phosvitin as an antioxidant for inhibiting metal-catalyzed phospholipid oxidations. Poult. Sci. 65: 2065-2070.

106. Madison L.D., Bergstrom-Porter B., Torres A.R., Shelton E. (1979). Regulation of surface topography of mouse peritoneal cells. Formation of microvilli and vesiculated pits on omental mesothelial cells by serum and other proteins. J. Cell Biol. 82: 783-797.

107. Maguire G.A., Docherty K., Hales C.N. (1983). Sugar transport in rat liver lysosomes. Direct demonstration by using labelled sugars. Biochem. J. 212: 211-218.

108. Mak K.M., Trier J.S. (1975). Lipoprotein particles in the jejunal mucosa of fetal rats. Dev. Biol. 43: 204-211.

109. Mak K.M., Trier J.S. (1979). Lipoprotein particles in the jejunal mucosa of postnatal developing rats. Anat. Rec. 194: 491- 506.

110. Marenus K.D., Sjostrand F.S. (1982). The effects of different concentrations of administered fat on the structure of columnar cells in the small intestine. J. Ultrastruct. Res. 79: 110-120.

111. Martin W.G., Augustyniak J., Cook W.H. (1964). Fractionation and characterization of the low-density lipoproteins of hen's egg yolk. Biochim. Biophys. Acta 84: 714-720.

112. Martin W.G., Saito Z. (1967). Lipovitellin, phosvitin and other granule components in avian yolk during embryogenesis. Can. J. Biochem. 45: 493-501.

113. Matsushita S. (1986). Presence of sucrase in the yolk sac of the chick. J. Exp. Zool. 237: 339-346.

114. Matteoni R., Kreis T.E. (1987). Translocation and clustering of endosomes and lysosomes depends on microtubules. J. Cell Biol. 105: 1253-1265.

115. Mayorga L.S., Diaz R., Stahl P.D. (1988). Plasma membrane- derived vesicles containing receptor-ligand complexes are fusogenic with early endosomes in a cell-free system. J. Biol. Chem. 263: 17213-17216.

116. McIndoe W.H. (1960). Changes in protein content of yolk during chick embryogenesis. J. Embryol. Exp. Morphol. 8: 47-53.

117. Medda J., Bose A.K. (1967). A histochemical study in the yolk- and albumen-sac of chick embryo. Acta Histochem. 26: 311- 317.

118. Mithieux G., Rousset B. (1989). Identification of a lysosome membrane protein which could mediate ATP-dependent stable association of lysosomes to microtubules. J. Biol. Chem. 264: 4664-4668.

119. Mobbs I.B., McMillan D.B. (1979). Structure of the endodermal epithelium of the chick yolk sac during early stages of development. Am. J. Anat. 155: 287-310.

120. Mobbs I.G., McMillan D.B. (1981). Transport across endodermal cells of the chick yolk sac during early stages of development. Am. J. Anat. 160: 285-308.

121. Moog F. (1950). The functional differentiation of the small intestine. J. Exp. Zool. 15: 109-130.

122. Moog F. (1944). Localizations of alkaline and acid phosphatases in the early embryogenesis of the chick. Biol. Bull. 86: 51-80.

123. Moors A., Stockx J. (1972). Phosphoesterase activities in the hen's egg. I. Purification and characterization. Arch. Int. Physiol. Biochim. 80: 717-732.

124. Moran E.T., Jr. (1985). Digestion and absorption of carbohydrates in fowl and events through perinatal development. J. Nutrit. 115: 665-674.

125. Mori H., Christensen A.K. (1980). Morphometric analysis of Leydig cells in the normal rat testis. J. Cell Biol. 84: 340-354.

126. Neiss W.F. (1984). A coat of glycoconjugates on the inner surface of the lysosomal membrane in the rat kidney. Histochemistry 80: 603-608.

127. Nichols A.V., Forte G.M., Coggiola E.L. (1969). Electron microscopy of very low density lipoproteins from egg yolk using negative staining. Biochim. Biophys. Acta 175: 451-453.

128. Noaillac-Depeyre J., Gas N. (1979). Structure and function of the intestinal epithelial cells in the perch (Perca fluviatilis L.). Anat. Rec. 195: 621-639.

129. Noble, R. C., Shand, J. H. (1985). Unsaturated fatty acid compositional changes and desaturation during the embryonic development of the chicken (Gallus domesticus). Lipids 20: 278- 282.

130. Noble R.C. (1987). Lipid metabolism in the chick embryo: some recent ideas. J. Exp. Zool. Suppl. 1: 65-73.

131. Noble R.C., Connor K., Smith, W. K. (1984). The synthesis and accumulation of cholesteryl esters by the developing embryo of the domestic fowl. Poult. Sci. 63: 558-564.

132. Noble R.C., Moore J.H. (1964). Studies on the lipid metabolism of the chick embryo. Can. J. Biochem. 42: 1729-1745.

133. Oates P.J., Touster O. (1980). In vitro fusion of Acanthamoeba phagolysosomes. III. Evidence that cyclic nucleotides and vacuole subpopulations respectively control the rate and extent of vacuole fusion in Acanthamoeba homogenates. J. Cell Biol. 85: 804-810.

134. Ostlund R.E., Jr., Pfleger B., Schonfeld G. (1979). Role of microtubules in low density lipoprotein processing by cultured cells. Clin. Invest. 75-84.

135. Overton J., Shoup J. (1964). Fine structure of the cell surface specializations in the maturing duodenal mucosa of the chick. J. Cell Biol. 21: 75-85.

136. Pasternak C., Spudich J.A., Elson E.L. (1989). Capping of surface receptors and concomitant cortical tension are generated by conventional myosin. Nature 341: 549-551.

137. Patten B.M., Carlson B.M. (1974). Foundations of Embryology. Third Edition. New York, McGraw-Hill Book Company.

138. Pearse A.G.E. (1968). Histochemistry. Theoretical and Applied. Third Edition. Volume 1. London, J.& A. Churchill Ltd.

139. Perry M.M., Gilbert A.B. (1985). The structure of yellow yolk in the domestic fowl. J. Ultrastruct. Res. 90: 313-322.

140. Perry M.M., Gilbert A.B. (1979). Yolk transport in the ovarian follicle of the hen (Gallus domesticus): lipoprotein-like particles at the periphery of the oocyte in the rapid growth phase. J. Cell. Sci. 39: 257-272.

141. Plonne D., Winkler L., Franke H., Dargel R. (1992). The visceral yolk sac - an important site of synthesis and secretion of apolipoprotein B containing lipoproteins in the feto-placental unit of the rat. Biochim. Biophys. Acta 1127: 174-185.

142. Poelmann R.E., Daems, W. T. (1973). Problems associated with the demonstration by lead methods of adenosine triphosphatase activity in resident peritoneal macrophages and exudate monocytes of the guinea pig. J. Histochem. Cytochem. 21: 488-498.

143. Pons A., Garcia J., Palou A., Alemany M. (1986). Amino-acid metabolism enzyme activities in the liver,intestine and yolk sac membrane of developing domestic fowl. Arch. Int. Physiol. Biochim. 94: 219-226.

144. Rawn J.D. (1989). Proteins, Energy, and Metabolism. Burlington, North Carolina, Neil Patterson Publishers.

145. Richards M.P., Steele N.C. (1987). Trace element metabolism in the developing avian embryo:a review. J. Exp. Zool. Suppl. 1: 39-51.

146. Romanoff A.L. (1960). The Avian Embryo - Structural and Functional Development. New York, The Macmillan Company.

147. Romanoff A.L. (1967). Biochemistry Of The Avian Embryo - A Quantitative Analysis Of Prenatal Development. New York, John Wiley & Sons.

148. Romanoff A.L., Romanoff A.J. (1949). The Avian Egg. New York, John Wiley and Sons, Inc.

149. Rosenfeld, A., Kak, A. C. (1982). Digital Picture Processing. Second edition. Vols. 1 and 2. Orlando, Florida U.S.A., Academic Press, Inc.

150. Rosenthal A.S., Moses H.S., Beaver D.L., Schuffman S.S. (1966). Lead ion and phosphatase histochemistry. I. Nonenzymatic hydrolysis of nucleoside phosphates by lead ion. J. Histochem. Cytochem. 14: 698-710.

151. Saari A.L., Powrie W.D., Fennema O. (1964). Isolation and characterization of low-density lipoproteins in native egg yolk plasma. J. Food Sci. 29: 307-315.

152. Saito Z., Martin W.G., Cook W.H. (1965). Changes in the major macromolecular fractions of egg yolk during embryogenesis. Can. J. Biochem. 43: 1755-1770.

153. Salisbury J.L., Condeelis J.S., Satir P. (1980). Role of coated vesicles, microfilaments, and calmodulin in receptor- mediated endocytosis by cultured B lymphoblastoid cells. J. Cell Biol. 87: 132-140.

154. Salzman N.H., Maxfield F.R. (1989). Fusion accessibility of endocytic compartments along the recycling and lysosomal endocytic pathways in intact cells. J. Cell Biol. 109: 2097-2104.

155. Salzman N.H., Maxfield F.R. (1988). Intracellular fusion of sequentially formed endocytic compartments. J. Cell Biol. 106: 1083-1091.

156. Schellens J.P.M., Daems W.T., Emeis J.J., Brederoo P., De Bruijn W.C., Wisse E. (1977). Electron microscopical identification of lysosomes. in: Dingle J.T., ed. Lysosomes: A Laboratory Handbook. second edition. Oxford, Elsevier/North Holland Biomedical Press. pp. 147-208.

157. Schjeide O. (1963). Lipoproteins of the fowl-serum, egg and intracellular. in: Progress In The Chemistry Of Fats And Other Lipids. Oxford, Pergamon Press. 6: pp. 253-289.

158. Schlesinger A.B. (1958). The structural significance of the avian yolk in embryogenesis. J. Exp. Zool. 138: 223-258.

159. Silverstein, S. C., Steinman R.M., Cohn Z.A. (1977). Endocytosis. Ann. Rev. Biochem. 46: 669-722.

160. Skipski V.P. (1972). Lipid composition of lipoproteins in normal and diseased states. in: Nelson G.J., ed. Blood Lipids And Lipoproteins: Quantitation, Composition And Metabolism. New York, Wiley-Interscience. pp. 471-482.

161. Slade B.S. (1970). An attempt to visualize protein transmission across the rabbit visceral yolk sac. J. Anat. 107: 531-545.

162. Small J.V., Davies H.G. (1972). Erythropoiesis in the yolk sac of the early chick embryo: an electron microscope and microspectrophotometric study. Tiss. Cell 4: 341-378.

163. Smith C.E., Hermo L., Fazel A., Lalli M.F., Bergeron J.J.M. (1990). Ultrastructural distribution of NADPase within the golgi apparatus and lysosomes of mammalial cells. Progr. Histochem. Cytochem. 21: 1-120.

164. Smith M.L., Greene A.A., Potashnik R., Mendoza S.A., Schneider J.A. (1987). Lysosomal cystine transport. J. Biol. Chem. 262: 1244-1253.

165. Solomon J.B. (1957). Glutamic dehydrogenase in the developing chick embryo. Biochem. J. 66: 264-270.

166. Stern C.D., MacKenzie D.B. (1983). Sodium transport and the control of epiblast polarity in the early chick embryo. J. Embryol. Exp. Morphol. 77: 73-98.

167. Sugano H., Watanabe I. (1961). Isolation and some properties of native lipoproteins from egg yolk. J. Biochem. 50: 473-480.

168. Sugimoto Y., Yamada M. (1985). Changes in proteins from the yolk and in the activity of benzoyl-L-tyrosine ethyl ester hydrolase from the yolk sac membrane during embryonic development of the chicken. Poult. Sci. 65: 789-794.

169. Sugimoto Y., Yamada M. (1986). Purification and characterization of benzoyl-L-tyrosine ethyl ester hydrolase from the yolk sac membrane of chicken egg. Biochem. Cell Biol. 64: 543-547.

170. Taborsky G. (1980). Iron binding by phosvitin and its conformational sequences. J. Biol. Chem. 255: 2976-2985.

171. Taussig M.P. (1965). Incorporation of amino acids into chick proteins during embryonic growth. Can. J. Biochem. Physiol. 43: 1099-1110.

172. Thommes R.C., Just J.J. (1964). Endocrine control of yolk sac membrane glycogen in the developing chick embryo. 1. Glucagon. Gen. Comp. Endocrin. 4:

173. Threadgold L.T. (1976). The Ultrastructure of the Animal Cell. Oxford, Pergamon Press.

174. Toyohara A., Inaba K. (1989). Transport of phagosomes in mouse peritoneal macrophages. J. Cell Sci. 94: 143-153.

175. Vadehra D.V., Bain J.M., Burley R.W. (1977) Lipid-protein globules of avian egg yolk. Biochem. J. 166: 619-624.

176. Veini M., Tsitsiloni O.E., Martini S.M., Haritos A.A. (1986). Multiple molecular forms of soluble esterases in the digestive system of the developing chicken. Comp. Biochem. Physiol. 83B: 775-782.

177. Wall D.A., Meleka I. (1985). An unusual lysosome compartment involved in vitellogenin endocytosis by Xenopus oocytes. J. Cell Biol 101: 1651-1664.

178. Weibel, E. R. (1979). Stereological Methods. Vols. 1 and 2. London, U.K., Academic Press Inc.

179. Willems J., Teuchy H., Stockx J. (1976). Fractionation, structure and some properties of yolk granules from unfertilized hen eggs. Cytobios 17: 195-209.

180. Williams M.A. (1977). Quantitative Methods in Biology. in: Glauert A.M., editor. Practical Methods in Electron Microscopy. Amsterdam, North Holland Publishing Company. Volume 6, Part 2.

181. Willier B.H. (1968). Glycogen synthesis, storage and transport mechanisms in the yolk-sac membrane of the chick embryo. Wilhelm Roux's Arch. Dev. Biol. 161: 89-117.

182. Wisse E. (1977). Ultrastructure and function of Kupffer cells and other sinusoidal cells in the liver. in: Wisse E. and Knook D.L., eds. Kupffer Cells And Other Liver Sinusoidal Cells. Amsterdam, Elsevier/North Holland Biomedical Press. pp. 33-60.

183. Woods J.W., Roth T.F. (1983). Maternal-fetal protein transport. in: Receptor-Mediated Endocytosis. New York, Chapman and Hall. pp. 239-272.

184. Wouters J.M., Goethals M., Stockx J. (1985). Acid proteases from the yolk and the yolk-sac of the hen's egg. Purification, properties and identification as cathepsin D. Int. J. Biochem. 17: 405-413.

185. Young M.F., Minghetti P.P., Klein N.W. (1980). Yolk sac endoderm: exclusive site of serum protein synthesis in the early chick embryo. Dev. Biol. 75: 239-245.

186. Zacks S.I. (1954). Esterases in the early chick embryo. Anat. Rec. 118: 507-537.