HISTOCHEMICAL STUDIES
ON
ECHINOCOCCUS GRANULOSUS

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ABSTRACT

The object of this investigation was to examine certain biochemical constituents of the parasite through all its stages and to demonstrate the relation of these substances to the organs and cellular elements in which they occur, as well as to clarify the structure of the hydatid.

The cementing substance between the germinal and laminated layer of the hydatid cyst wall was found to dissolve rapidly in acetic acid, Carnoy's fixative, and to a lesser degree in alcohols. It was best fixed by formalin and Zenker's fluid.

Using various staining procedures, the germinal membrane, apart from small cells, showed the presence of an extensive excretory system composed of canals, canalicules, and flame cells.

The hydatid cyst and scolices were found to be devoid of acid and alkaline phosphatases, while the adult showed a strong, alkaline phosphatase in its cuticle and subcuticular cells.

The PAS reaction with saliva digested controls indicated that the germinal membrane and larval scolices were rich in glycogen, while the laminated layer did not seem to contain any. Variation in the amount of glycogen was observed between adult worms coming from different hosts. It was demonstrated that the worms stored large amounts of glycogen in the parenchyma, especially in the medullary region adjacent

to osmoregulatory canals and the reproductive organs. The mature sperms and ova also gave a positive reaction. Except the vitelluria, the reproductive organs were free of glycogen.

The laminated layer of the cyst wall was found free of lipids while the germinal membrane showed heavy deposits. Few droplets of fat were also seen in the excretory canals.

Three types of nuclei containing different amounts of DNA were observed on the germinal membrane, while the laminated layer showed no Feulgen positive material. The nuclei of both scolices and adult worms gave a positive reaction. In the cyst wall, like DNA ribose nucleic acid also was confined to the cells of the germinal membrane. In the adult worm the parenchyma was poor in RNA, while the subcuticular cells, reproductive organs, and the ova showed fair amounts.

The cyst wall did not show any connective tissue fibers. In the adult worm the basement membrane of the cuticle and the lining membranes of the ovary, testes, yolk gland, cirrus sac, and sperm duct gave a dark blue reaction with Mallory's triple stain suggesting a collagenous nature.

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INTRODUCTION

Echinococcus granulosus Batch 1786 is the smallest medically important cestode parasite of canines. It is better known for its larval form, the hydatid, that occurs in a wide range of mammalian hosts and causes hydatid disease in man. In spite of its medical and economic importance in many parts of the world, many of the essential facts about the physiology of this parasite remain unknown.

Of the several methods available for the study of physiological processes in cestodes, the histochemical method has been chosen for this study. Although this method may not be as precise or quantitative as chemical ones, it does permit a more exact localization of the chemical substances involved in physiological processes. In addition to determining the chemical composition of the studied tissues, histochemistry also reveals their structural details. Since the histology of the hydatid is not very well defined, this method becomes more advantageous in correlating structure with function.

As the broad definition of parasitism implies, the habitat of a parasite is some part of the body of another living organism that has its own physiological properties and is in constant interaction with the parasite. The need for an intermediate host for the completion of the development of the parasite means a basic change in its physiological requirements. Therefore, all stages of the changing parasite must

be considered if we are to understand the metabolism of the total organism.

The object of this investigation, then, is to examine certain biochemical constituents of the parasite on a comparative basis, from the primordial ovum to the fully mature adult, and to demonstrate the relation of these substances to the organs and cellular elements in which they occur.

Life Cycle

As so often happens in the discovery and description of parasites, the larval form of Echinococcus granulosus was noticed and named before the adult worm. Reference to large "water bladders" both in humans and animals were made from ancient times. However, the first accurate description of the hydatid cyst was made by Georze in 1782 who named it Taenia visceralis socialis granulosa. The adult form was recorded later as Taenia echinococcus by Siebold, and various aspects of the life cycle were subsequently elaborated by Siebold, Leuckart, and Heubner (Dévé, 1949).

The life cycle of E. granulosus is a relatively simple one involving only two hosts. The adult worm occurs in the small intestine of dogs, wolves, jackals, coyotes, and jaguars (Dévé, 1949; Dew, 1928; Rausch, 1956). The dog is the most common definitive host and the chief reservoir of the infection for man and domestic animals. Although the fox has very often been mentioned as a definitive host (Wardle and McLeod, 1952), it has been shown experimentally that it is an unsuitable host; the life span of E. granulosus in this host is very short, and most of the worms do not mature (Matoff and Jantscheff, 1954; Bronzini and Bertolino, 1954). Contrary findings were reported because it was not realized that there exists a second species, Echinococcus multilocularis, which causes alveolar hydatid disease and for which the fox is a natural definitive host (Rausch, 1956). For many years the identity of the cestode

causing alveolar hydatid disease had been a matter of controversy (Dew, 1953). Some investigators have had the opinion that under certain conditions the larva of E. granulosus develops abnormally and produces the alveolar form; others have believed that a distinct species of Echinoccocus is involved. The truth of the latter view has been proved by the works of Rausch (1953, 1956), Rausch and Schiller (1954, 1956), and Vogel (1955), and the difference in host species occurence and in the morphology of the adult and larval forms of the two species has been clarified.

Similar to the case of foxes, reports of immature worms in cats have given rise to dispute about the suitability of the domestic cat as a definitive host of E. granulosus. There is enough experimental evidence to show that the cat is an abnormal host (Dévé, 1949; Berberian, 1936). Berberian (1936) has tried unsuccessfully to infect kittens, rats, and rabbits by feeding them with large quantities of scolices. After trying the action of gastric and intestinal juices of various animals on scolices, he concluded that those animals which have an intestinal juice capable of digesting scolices are immune to infection with E. granulosus and those which have an inactive intestinal juice are either susceptible, as the dog, or abnormal hosts permitting only partial development of the worm, as the cat.

Unlike the adult worm, the larval E. granulosus is neither very host specific nor organ specific. It can occur in a wide range of mammals and is able to develop almost in

any part of the host body. Artiodactyls and perissodactyls form the main groups of intermediate host (Rausch, 1953). However, the cyst has also been reported from rodents, monkeys, and canines (Brumpt, 1936). Man is only an accidental host and a dead end for the life cycle of the parasite since human carcasses are not normally available to carnivores. The sheep occupies the same important place in the intermediate host group as does the dog among the definitive hosts, and for this reason hydatid disease is most commonly associated with sheep-raising areas as Australia, New Zealand, Iceland, South America and parts of north and south Africa (Pipkin, Risk, and Balikian, 1951.

The adult parasite is remarkably small when compared with the larval stage; its length may range from one and a half to six mm. The body consists of a scolex and two to four segments (Plate I). The scolex is provided with an eversible rostellum armed with a double row of hooks, 32-40 in number and varying in shape depending on geographical distribution and the age of the parasite. The first one or two segments following the scolex are not well differentiated but contain newly developing sex organs. The next segment contains only a gravid uterus filled with embryophores or eggs (Rausch, 1953).

Egg production by the adult worm begins six to seven weeks after the infection of the final host. As the terminal segments become gravid, they are shed and excreted with the feces of the host. The segments then disintegrate rapidly and free the eggs that are the source of infection for the various

intermediate host species. The eggs consist of an onchosphere or hexacanth embryo possessing six hooks and surrounded by a radiated shell (Plate II, Fig. 2.)

When the eggs are ingested by a suitable intermediate host, the egg-shell ruptures under the influence of the digestive processes of the host, liberating the onchosphere. The free and actively motile onchosphere enters the blood stream through the intestinal wall. Because of the arrangement of the visceral circulation, the onchospheres are first carried to the liver where most of them settle. If an onchosphere passes through the liver, it develops in the lungs, the next most frequently diseased organ. Onchospheres that succeed in returning to the heart from the lungs pass into the systemic circulation, and these account for cysts occuring in such remote parts of the body as brain, kidney, spleen, peritoneum, bone marrow, heart, etc.

After the onchosphere localizes in a given organ, it grows rapidly and develops into the larval hydatid cyst. A fully developed hydatid cyst is a spherical sac filled with a clear liquid, the hydatid fluid. It is attached to the host by an adventitious membrane, the "pericyst," which is an inflammatory product of host irritation. The cyst wall proper is composed of two layers: an outer "ectocyst" or laminated layer and an inner "endocyst" or germinal membrane which is the vital part of the cyst (Plate II, Figure 3). In most cysts, after a period of development the germinal membrane gives rise to "brood capsules." These arise as a mass of cells in which

a cavity develops transforming them into hollow spheres that are attached to the germinal membrane by a stalk (Plate II, Figure 4). The wall of the brood capsule is formed of a single layer of cells and is thought to be of the same nature as the germinal membrane (Coutelen, 1931a). Brood capsules do not develop evenly over the whole germinal membrane but tend to aggregate in particular areas. Rarely, cysts fail to produce any brood capsules; Dew (1928) thinks the major cause of such sterility is some abnormal local condition rather than inherent inability of the cyst to reproduce. The brood capsules give rise to the scolices which are thought to be unable to arise directly from the germinal membrane (Dew, 1928). The number of scolices inside a single brood capsule may range from 2 to 60 or even 120 (Dévé, 1949). Ripe brood capsules readily become detached and fall into the hydatid fluid,and some of them rupture freeing the scolices.

The scolices are the source of infection for the definitive host; on ingestion by a suitable host, they pass to the small intestine where they develop into the adult tapeworm (Plate II, Figure 1).

Apart from the ability to develop into adults, the scolices have another very interesting potentiality; namely, they can develop into miniature replicas of the mother cyst, thus forming "endogenous daughter cysts." Like the mother cyst, these can give rise to brood capsules, scolices, and even granddaughter cysts. Still another element of the hydatid is the "exogenous daughter cyst." The origin of this type of daughter

cyst is quite different from that of the endogenous daughter cyst; they arise from islets of germinal tissue imprisoned between the lamellae of the mother cyst wall. These germinal inclusions develop their own lamellae and progressively move to the outside surface of the mother cyst.

When a cyst ruptures in the intermediate host and the scolices are dispersed into the surrounding host tissue, each develops into an individual cyst, thus multiplying in a geometrical fashion the severity of the infection. This aspect of the life cycle was clarified by the work of Dévé (1946) who termed it 'secondary echinococcosis.' Experimentally, secondary echinococcosis has been produced in mice and rats (Wuele and Cooman, 1938; Coutelen et. al., 1939).

Very few attempts have been made towards culturing E. granulosus in vitro. Dévé (1926, 1928) and Coutelen (1927a, 1927b) were the only workers who tried to culture the scolices. Successful cultures would clarify the metabolic requirements of the parasite and the factors that determine whether a scolex will produce an adult or a cyst.

The Chemistry and Physiology of the Parasite

Due to its very small size comparatively few studies have been reported on the adult E. granulosus. Brault and Loeper (1904b) studied the distribution of fat. Their report of fat in the excretory system of the cestode was suggested by von Brand (1952) to be an end product of carbohydrate metabolism.

The hydatid fluid has been studied by several workers. It is thought to be the result of the metabolic end products of the various cyst elements and the osmoregularity capacity of the germinal membrane (Schwabe, 1959). Published data refer mainly to the chemical composition of the cyst fluid without much reference to the significance of these constituents. Hydatid fluid is a clear, colorless liquid, generally alkaline to litmus, with a specific gravity of 1.008-1.005. It has been found to contain K, Mg., Ca, Cl, S, P, traces of Fe and Si, lactic, succinic and higher fatty acids, lecithin, urea, uric acid, creatinine, betaine, sugar, glycogen, and very little protein (Mazzocco, 1923; Lemaire and Ribere, 1935; Flossner, 1924, 1925; Codounis and Polydorides, 1936; Wenicker and Savino, 1923). Lalif and El Kordy (1946), studying the vitamin content of the cyst fluid, found thiamin, nicotinic acid, and ascorbic acid to be present.

In spite of the several studies done on the cyst wall, neither its chemical nature nor its structure is well defined. The whitish, elastic, thick laminated membrane is formed of several regular layers of lamellae, that increase in number

with age and may reach up to a thickness of half a centimeter. Guiart (1930) considered this membrane to be the result of a hyaline secretion of the developing vesicle. Coutelen (1938), on the other hand, suggested it to be formed by successive pushes of spherical granules of mitochondrial nature extruded most probably by certain elements of the germinal membrane. The lamellated appearance was said to be due to the variation in the numerical density of these granules. Schwabe (1959), unlike the previous workers, suggested the laminated layer to be the result of a precipitin reaction between metabolic products of the germinal membrane or scolices , acting as antigens, and antibodies produced by the host.

The laminated layer has a certain degree of rigidity that enables it to act as a support and protective layer for the thinner, more delicate germinal membrane that lines it. Chauffard and Widal (Brumpt, 1936) showed its impermeability to microorganisms. From his permeability studies, Schwabe (1959) concluded that the laminated layer acts primarily as a filter which is able to retain large molecules. It is the inner germinal membrane that is chiefly responsible for permeability control and osmoregulation.

This thin nucleated membrane, 10-25·u in thickness, is the vital part of the cyst. Brault and Loeper (1904a) were the first workers to notice the abundance of glycogen in this membrane. The membrane has always been described as a fine, granular protoplasmic syncytium sprinkled with nuclei. It was Coutelen (1931a) who showed that the membrane was not a syncytium

but contained differentiated cellular elements. He (1938) described three distinct types of cells: young, small(5-10 u) polygonal, and glycogenated cells; larger cells (10-30 u) containing fat and glycogen; and bi-or tripolar cells. Watching the development of these cells, Coutelen has seen some of them rounding up and falling into the hydatid fluid or rupturing and freeing their contents. These works of Coutelen are the only studies on the nature of the germinal membrane.

Studies on the chemical composition of the cyst wall have shown it to contain carbohydrates, fats, proteins, as well as inorganic salts. Schmeideberg (1920) has reported the laminated membrane to consist mainly of "hyaloidin," a carbohydrate-protein complex. Porzecanski (1941) also found the cyst wall to be a glycoprotein. Cmelik (1952a) isolated an antigenic polysaccharide consisting mainly of aldohexoses and glucosamine from cyst membranes. Glucosamine in the cyst wall has also been reported by Flössner (1925) and Cmelik and Briski (1953). Another antigenic polysaccharide was isolated by Pirosky et al. (1949) Using histochemical methods, Lorvik and Moriconi (1956) characterized the membrane as a neutral mucopolysaccharide.

Unlike the polysaccharide, the lipid fraction of the cyst wall has been found to be quantitavely low. Cmelik (1952b) found the average fat content to be 1.25% of the dry weight of the cyst wall, of which 22.5% was cholesterol.

Grana and Oehninger (1944) isolated an insoluble protein that provoked eosinophilia in rabbits. A nucleoprotein, the

nucleic acid of which contained neither ribose nor desoxyribose but a hexose, most likely glucose or galactose, was isolated by Cmelik and Briski (1953). Cameron (1923) and Cameron and Fitzpatrick (1925) have studied the chemistry of the cyst wall using histochemical methods. They found the laminated layer rich in K, Na, Ca, and poor in Fe, I_2, phosphates, and chlorides, while the germinal membrane showed well-marked reactions for all of the above substances. They found glycogen and fat to be distributed throughout both membranes. Contrary to their findings, Brault and Leoper (1904a) and Lorwic and Moriconi (1956) have found the glycogen limited to the germinal membrane. Cameron and Fitzpatrick (1925) observed that as the hydatid grows older, the Na and K salts decrease while Ca salts and fats increase. They found that in general the ectocyst stains with acid dyes while the endocyst shows affinity to basic dyes. In all their studies the reaction of the scolices were very much like those of the germinal membrane.

The scolices are oval in shape, containing a central retracted rostellum with four suckers and a crown of hooks (Plate II, Figure 4). They contain many calcareous corpuscles. Some recent studies have been reported on the metabolism of scolices. They have been found to contain large amounts of protein, a small amount of lipid, glycogen and a polysaccharide containing galactose and glusosamine. The aerobic respiration was found to be cyanide-and fluoroacetate-sensitive but was not inhibited by malonate (Agosin et al., 1957). Their glycogen

was found to disappear upon incubation under both aerobic and and anaerobic conditions giving rise to a mixture of lactic, acetic, succinic, and pyruvic acids, and ethyl alcohol under aerobic and to a mixture of lactic, succinic and acetic acids and ethyl alcohol under anaerobic conditions (Agosin, 1957).

Very little work has been done on the enzyme systems of the hydatid. In the examination of hydatid cysts and fluid, Cameron (1926) found certain enzymes present which were confined to the cyst wall. He found diastatic, starch, and glycogen-splitting enzymes to be most prominent. Certain fat-splitting and proteolytic enzymes acting mostly on milk and slightly on gelatin were also present. The reactions of oxidases were very slight. De Ley and Vercruysse (1955) showed the absence of glucose-6-phosphate dehydrogenanse and gluconate-6-phosphate dehydrogenase in homogenates of the cyst wall, thus eliminating the hexose-monophosphate oxidative route.

From the foregoing brief review of the published data on the chemistry and physiology of hydatid tissues, it is clearly evident that many lacuna exist in our knowledge of the metabolism of this parasite.

MATERIALS AND METHODS

A. Origin and Preparation of Tissues.

Since E. granulosus can not be cultivated in vitro to provide all stages, the materials for this study have been obtained both from the natural definitive and intermediate hosts; namely, dogs and cattle, respectively.

The adult E. granulosus were obtained from the intestines of experimentally and naturally infected dogs soon after autopsy. Since it was very difficult to pick up the whole worms with a pair of forceps without breaking a proglottid or the scolex, the mucosa of small pieces of infected intestines were scraped off, together with the worms, into petri dishes. To free them from intestinal debris, the worms were transferred through several dishes of tap water by a Pasteur pipette. The clean worms were fixed immediately in the appropriate fixatives.

The hydatid cysts were obtained from cattle slaughtered at the Beirut Municipality Slaughter House. The infected lungs and livers were brought to the laboratory within a few hours after the slaughter of the animals. The hydatid fluid was drawn from intact cysts with a syringe, the cysts were cut open, and the cyst wall and, if present, daughter cysts removed. Pieces of non-infected white mother and daughter cyst membranes were washed in distilled water and fixed immediately.

The fluid drawn from fertile cysts was collected in conical concentration flasks to permit settling of the hydatid sand. The scolices and brood capsules were picked from the

bottom of the flask with a wide mouthed pipette, placed in very small stender dishes, washed with distilled water, and fixed.

The various solutions through which the adults and brood capsules with scolices were carried during dehydration and clearing were added and then pipetted off. When the tissues were ready to be embedded, the entire dish of paraffin containing the materials was emptied into a paper boat. Before the paraffin solidified, most of the worms or scolices settled to the bottom.

The adult worms were sectioned longitudinally to allow a greated variety of tissues to be tested per section. All paraffin sections were cut at a thickness of 7 u and fixed on slides with egg albumin.

Flat preparations of the germinal membrane were made subsequent to fixing and washing the cyst wall. These pieces of membrane were mounted on slides without any egg albumin and immediately after drying were carried through the various procedures. When cyst membranes were put in Carnoy's fixative (absolute alcohol, chloroform, acetic acid - 6:3:1) the germinal membrane separated very easily. These were mounted on slides within 5 minutes before membranes became brittle. The mounted sections dried in a few minutes and were immediately immersed in alcohol for at least half an hour to complete fixation.

B. Histochemical Procedures

1. Acid and Alkaline Phosphatase

For the study of acid and alkaline phosphatases, materials were fixed in 80% alcohol and acetone, dehydrated by alcohols and acetone, respectively, and cleared in petroleum ether. All three steps were performed at icebox temperature (5° C). Cleared materials were carried through two changes of paraffin, fifteen minutes each, not exceeding a temperature of 58°C. The second change of paraffin was performed under partial vacuum using a vacuum oven. Paraffin sections were dried at room temperature for 24 hours. Flat preparations of fixed germinal membranes and also some fixed scolices were hydrated instead of dehydration and carried through the procedure. The scolices were carried through the various solutions in centrifuge tubes, using centrifugation at low speed and decantation.

The histochemical calcium-cobalt method of Gomori as given in Glick (1949) was followed for the alkaline phosphatase, and the lead method of Gomori as given in Gomori (1952) for the acid phosphatase.

The substrate used in testing for alkaline phosphatase consisted of sodium glycerophosphatase, calcium chloride, magnesium sulphate as an activator and sodium barbital; the final pH of the solution was 9.2 ± 1.

The phosphatase, when present, liberates phosphate ions from the sodium glycerophosphate which, in turn, react with the calcium ions forming insoluble calcium phosphate. The site of

phosphatase activity is finally revealed, after the treatment with cobalt solution and ammonium sulfide, as a black precipitate of cobalt sulfide. To prevent false positives due to pre-formed calcium salts, the sections were treated with citrate buffer of pH 4-4.5 for 15 minutes previous to incubation in the substrate.

The acid phosphatase technique differs essentially from the alkaline procedure by the substitution of lead nitrate for calcium chloride and subsequent conversion of lead phosphate to black lead sulfide. The pH of the medium was 5 ± 1.

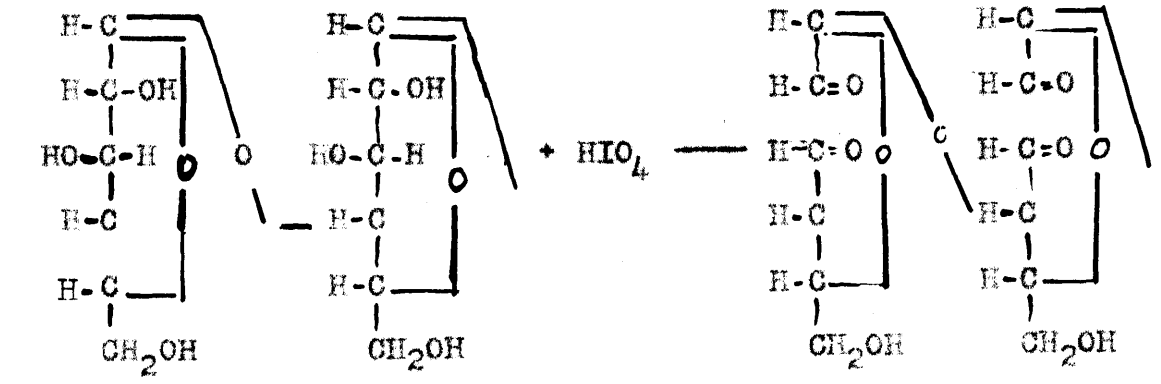
Control sections for both phosphatases were of two kinds. In one the enzyme was inactivated by placing the slides face down on boiling H_2O for 10 minutes. The second control consisted of sections incubated in mixtures in which the glycerophosphate solution had been replaced by distilled water.

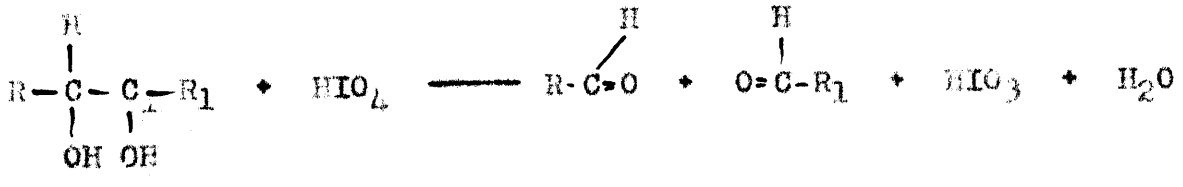
2. Glycogen

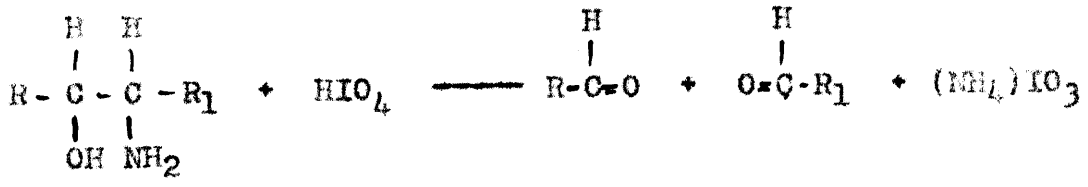
To determine the distribution of glycogen, materials fixed at ice box temperature in 80% alcohol, alcohol-formalin (absolute alcohol, 40% formaldehyde 9:1) or Carnoy's fixative were used. Sections were stained by the periodic acid Schiff method (PAS) using the "traditional" method as given in Lillie (1954). Control sections were digested in several changed of saliva for one hour at room temperature or in filtered 1% malt diastase solution for one hour at 37° C. These were carried through the staining procedure along with the extracted sections

The method is based on the reaction between the Schiff

reagent, fuchsin-sulfurous acid, and the aldehyde groups produced by the oxidizing agent, the periodic acid. The mechanism of the reaction has been clerified by Wieland and Schening (1921). In an acid solution fuchsin is transformed by an excess of sulfide into a colorless N-sulfinic acid which forms highly colored addition complexes with aldehydes. Periodic acid will produce aldehyde groups not only from glycogen or polysaccharides but also from any compound possessing vicinal free-OH or OH and NH_2 groups, according to the following schemes:







Saliva digestion differentiates glycogen from other PAS positive compounds. Some saliva treated sections were also incubated for 3 hours at 37° C in 1% hyaluronidase solution in 0.85% NaCl and then stained with Schiff's reagent to detect the

presence of hyaluronic acid.

3. Nucleic Acids

The Feulgen reaction using the "cold Schiff" reagent as given in Lillie (1954) was used to determine the localization of desoxyribonucleic acid (DNA). The principle of the reaction is the same as that of the PAS. Hydrolysis of the nucleic acid with N HCl at 60° C for 12 minutes liberates the purine and pyrimidine from the pentose phosphoric acid complex leaving a reactive aldehyde group to form the colored complex with the Schiff reagent. Non-hydrolized sections concomitantly stained with hydrolized ones were used as controls. The materials used were fixed in various fixatives, such as neutral 10% formalin, 80% alcohol, or Zenker's fixative.

Several basic stains were used to determine the distribution of ribosenucleic acid (RNA). The Methyl green-Pyronin method as given by Kurnick (1952) was followed, using 02% acqueous solutions of chloroform-extracted stains. Sections were also stained overnight in 1% Toluidine blue, and 20-30 seconds in 0.1% Thionin in 0.05M citrate buffer of pH 4. Control sections were extracted by 0.1 N KOH solution for one hour as suggested by Sulkin (1946).

4. Lipids

For the study of lipids, materials were fixed in 10% formalin containing 1% $CaCl_2$. Frozen sections were cut on the freezing microtome at a thickness of 20 u. They were floated on distilled water on slides without any fixative and allowed to

dry at room temperature. They were stained in a freshly filtered, saturated solution of Sudan Black B in 70% alcohol (Gomori, 1952), and mounted in glycerine jelly. This method is based on a physical reaction rather than a chemical one; it depends on the high solubility of the stain in lipids.

5. Fibrous Constituents

To study the distribution of various types of fibers, Zenker-fixed materials were stained with Mallory's Triple stain following the procedure given in Guyer (1953). Few sections of the hydatid membrane and flat germinal membrane were also stained with Orcein (Lillie, 1954) for elastic fibers.

All the chemicals used for the various procedures were of reagent grade.

RESULTS

A. Observation on the Structure of the Germinal Membrane

Early attempts at separating the germinal membrane from the laminated layer of fresh pieces of hydatid cyst wall were unsuccessful. Later, it was noticed that when a piece of cyst wall was dropped in Carnoy's fixing fluid, a considerable quantity of whitish cloudy substances diffused from the membrane into the fixative, and in a few minutes the germinal membrane started forming little baloon-like bubbles. With very little effort the whole membrane could be peeled from the laminated layer like a coat. This fixative was later used as a tool to separate the two membranes. To discover which component of the fixative was responsible for this type of action, pieces from a single cyst wall were placed in acetic acid, chloroform and absolute alcohol, respectively. Acetic acid was found to act exactly like Carnoy's fluid; chloroform and absolute alcohol also facilitated the separation, but to a lesser degree. In trying the effect of other fixatives, it was found that a lower concentration of alcohol, such as 70%, favored the separation of the two membranes, while formalin and Zenker's fluid acted as the best fixatives for the cementing substance between the two membranes. The older the cyst, the looser the attachment of the two membranes.seemed to be. In very young daughter cysts, however, even Carnoy's fluid did not always cause separation. Also fertile germinal membranes could be peeled off more easily than nonfertile ones.

Flat preparations of the germinal membrane were stained with a variety of stains with the main objective of locating the various types of cells that Coutelen (1938) had described. However, none of his large cells loaded with glycogen and fat could be found. This could be due to the fact that all studies were done on fixed membranes and those cells which Coutelen said have the ability of becoming free could have been lost during the fixing process. Also Coutelen's observations were done mostly on fresh brood capsule membranes; the cysts used in this study might be too old in relation to brood capsules to retain those cellular elements.

Hematoxylin-eosin stained membranes showed a very faint eosinophilic ground substance sprinkled with numerous nuclei as the membrane has always been described in the past. They showed also patchy pink staining materials superimposed on the fainter background; these were later found to be metabolic products. The majority of the nuclei were perfectly round ranging from 2 to 4.2 u in diameter; they showed a very conspicuous nucleolus and several chromatin dots (Plate III, Figure 1). In addition to these usual nuclei, two other types of nuclei were also observed which will be described later under Feulgen reaction.

One preparation of germinal membrane overstained with Heidenhain's hematoxylin revealed a dense framework of branching "fibers" extending throughout the membrane (Plate III, Figure 2). They seemed much more rigid than what Coutelen (1938) had

described as cytoplasmic extensions joining two cells. At the edges of parts of the membrane they were seen sticking out of the cytoplasmic ground substance (Plate IV, Figure 1). Several of these "fibers" were observed running from the wall of brood capsules into the surrounding mother germinal membrane (Plate IV, Figure 2). On closer examination, the thicker branches that measure 1.2 u in diameter showed a definite lumen; also some of them were seen to arise from certain cells that seemed to correspond to what Coutelen (1938) had described as bi or tripolar cells. The "fibers" were concluded to be excretory canals and canalicules; and the cells to which they were connected, flame cells. Similar flame cells were also seen in the scolices and brood capsule membrane.

To make sure that all the fiber-like structures of various thickness were part of this excretory system, the membranes were stained for various connective tissue fibers. After formalin fixative, orcein was used to detect elastic fibers; Widar stain, reticular fibers; Mallory's triple stain after Zenker's fixative, collagen fibers. All the stains gave a negative reaction.

Coutelen (1931b), studying the excretory system of hydatids, found the germinal membrane devoid of excretory elements. He cited three previous workers, Huxley, Wagener, and Leuckart, who had reported some canals and "cilia" on the germinal membrane and wall of brood capsules. Judging from their drawings and the very large measurements of "cilia"

reported, Coutelen found their work undependable. From his studies he concluded that flame cells were limited to scolices, and excretory canals were present both in scolices and brood capsules. Only once did he see a flame cell on the wall of a fresh brood capsule, and he thought it was an abnormality. In contrast, Schwabe and Schinazi (1958) saw several actively contracting flame cells on brood capsules that seemed to be in close association with the excretory canals described by Coutelen. They did not see any flame cells on the few mother cysts that they examined.

The flame cells observed in the present study were mostly elongated and sometimes spherical, ranging from 8 to 9.5 u in length including the flame (Plate V, Figures 1 and 2). They consisted of a cell body and a "flame." The cell body consisted of a nucleus with a little bit of cytoplasm surrounding it. The nuclei were not any different from the other typical nuclei of the membrane, except that of the elongated cells were more oval rather than spherical. The spherical flame cells seemed to contain more cytoplasm around their nuclei and seemed to be placed at the junction of three canalicules. Though sometimes it was very hard to decide if the two projections that were opposite the flame of the cell were truly canalicules or just cytoplasmic tails. The elongated flame cells were at the junction of 2 canalicules. The "flames"of these cells appeared as transparent cones, 3-4.2 u in length. They seemed to be attached to the nuclei of the cells at their

base by a darker staining basal plate and continued by a canaliculе at their pointed end. The "flames" of the elongated cells seemed more conspicuous and longer than those of the round cells. Some of the canalicules showed nuclei in their lumen or lying tangentially outside their wall and attached to the sides by small cytoplasmic projections. These could be the canalicular cells.

Apart from these, there were some other cells in the germinal membrane that consisted mostly of a typical nucleus with a very little bit of cytoplasm around them either in a ring form or in little projections. A few cells with prominent cytoplasmic extensions looked very much like the round flame cells except for their lack of the flame. Sometimes it was very hard to decide if they were flame cells or not. It was impossible to see any connection between these cells and the flame cells or canalicules.

From the above observations it was concluded that besides its bare nuclei the germinal membrane did have some small cells. It also has a very involved system of excretory canals and canalicules connected with those of the brood capsule, and that apart from performing excretory function, it seems to provide a perfect framework for the other more delicate components of the membrane.

B. The Distribution of Acid and Alkaline Phosphatases

Cyst - In spite of repeated efforts, all attempts at the

demonstration of both acid and alkaline phosphatases in the germinal membrane, laminated layer and scolices were unsuccessful. In a few cases the calcareous corpuscles in unsectioned scolices on flat preparations of the germinal membrane showed some black precipitate. However, these were concluded to be due to the incomplete removal of preformed calcium by the citrate buffer since some control sections also showed a similar reaction.

Adult Tapeworm - Acid phosphatase tests were all negative except for one worm that after 20 hours of incubation showed a patchy reaction in the inner layer of the cuticle of its mature proglottid only. Other worms or the same slide did not show any reaction. Control sections of the same worm were also negative.

An intense alkaline phosphatase activity was observed in the cuticle of the adult after one hour of incubation with a minor reaction in the subcuticular muscle fibers and subcuticular cell nuclei (Plate VI, Figure 1). The reaction was most pronounced in the cuticle of the gravid proglottid, diminishing gradually towards the scolex where only a very slight reaction was evident.

The staining of the subcuticular cell nuclei became more pronounced with prolonged incubation (Plate VI, Figure 2). This could be partly the result of diffusion since worms that had lost their cuticle during the various embedding procedures showed very slight activity in these cells.

None of the internal organs gave any positive reaction. The ciliary region of the vagina showed some browning; this happened also in the control section. A very few mature ova in some of the gravid proglottids showed an intense black precipitate; these did not seem to contain healthy embryos.

C. The Distribution of Glycogen

Cyst - The laminated layer gave a very strong PAS reaction which was not extractable by saliva. After saliva, treatment with hyaluronidase also did not deminish the intensity of the reaction. The germinal membrane both in cross section and in flat preparations showed glycogen in globules of various sizes distributed irregularly. The droplets did not seem to be arranged in any particular order in relation to the nuclei. In flat membranes, at the junction of brood capsules and the membrane, there seemed to be more glycogen present. This effect could be the result of the superimposed brood capsule membrane that also has some glycogen.

There was no noticeable constant difference in the amount of glycogen in membranes from lung or liver cysts. Some variation in staining occured in membranes from various cysts derived from either lungs or livers, and even in the different portions of the same membrane.

Scolices and Brood Capsules - The parenchyma of healthy scolices and the muscles of their suckers showed heavy deposits of glycogen (Plate VII, Figure 1). After saliva extraction, the cuticle of the scolices, specially at the base of the hooks, remained PAS positive. Incubation with Hyaluronidase did not

alter the reaction. A similar positive reaction after both saliva and hyaluronidase digestion was given by some smaller unhealthy looking scolices, which Dévé (1949) calls "ortho" scolices,and also by the substance found in between the scolices in intact brood capsules (Plate VII, Figure 2). This could only be the brood capsule fluid that had been fixed. The brood capsule walls also showed the presence of glycogen; because of their thinness, no idea could be obtained as to their precise localization.

Adult Tapeworm - There was considerable variation in the amount of glycogen present in the worms collected from different dogs. The worms collected from the same host seemed to have nearly equal amounts of glycogen. The pattern of distribution of glycogen seemed to be the same irrespective of some variation in amount, except for the one interesting difference that was observed in worms that had greatly reduced amounts of glycogen in the gravid and mature proglottids. The scolex and the neck region did not seem to have lost their glycogen (Plate VIII, Figure 1). The following results were observed in worms that had a fairly good amount of glycogen:

Glycogen was scattered throughout the whole scolex with heavier deposits occuring in the sucker-cups, especially at the base of the radial muscles (Plate VIII, Figure 2). In the immature proglottid, posterior to the scolex, heavy deposits of glycogen occured in the medullary region. The area from the cuticle to the base of the subcuticular cells contained little

glycogen and in finer droplets. This pattern was the same throughout the whole worm, becoming more emphasized in the mature and gravid proglottids where the glycogen was most concentrated around the reproductive organs and osmoregulatory canals.

In the immature proglottid, the primordia of reproductive organs did not show any glycogen, while the parenchyma around them had heavy deposits (Plate IX, Figure 1). In the mature proglottid (Plate IX, Figure 2) the testes, the ovary, and Mehlis gland contained no glycogen; however, the walls surrounding the testes and ovary and some parenchyma between the lobes of the ovary gave positive reactions. PAS positive material that was not extractable by saliva remained in the wall of the ootype and in between the cells of the Mehlis gland. Some glycogen was present in the terminal coiled portion of the sperm duct and also in the seminal receptacle. Very probably this glycogen was in the sperms, but it could not be definitely seen. Heavy deposits of glycogen were observed in what were thought to be vitelline glands.

Some glycogen was also present in the uterus containing immature ova, most of which seemed to be extracellular (Plate X, Figure 1). Apart from glycogen globules, there were several pink staining round bodies in the uterus that did not show a nucleus clearly and remained pink after saliva extraction. Well differentiated ova that had not yet formed a complete shell showed very little glycogen, but there were heavy deposits in the tissue surrounding them. In the gravid uterus the mature

ova contained more glycogen than the young ones, while the tissues around them had very little (Plate X, Figure 2). Apart from glycogen, the mature ova also showed the presence of a second polysaccharide, non-extractable with saliva.

D. The Distribution of Lipids

Cyst - The laminated layer was found to be free of sudanophilic material, while the germinal membrane was very rich (Plate XI, Figures 1 and 2). No difference seemed to occur between cysts of various ages or between cysts coming from lung or liver. As was seen in the distribution of glycogen, different areas on the same piece of germinal membrane showed different concentrations of fat. The fats occur in irregular shapes in the matrix of the germinal membrane and in perfectly spherical globules of various sizes on its surface. These globules are very loose because both in fresh membranes and in stained preparations several of them floated off the membrane into the surrounding fluid medium. The excretory tubules appeared as colorless tubes. In some of them a few fat droplets were seen.

The walls of the brood capsules showed very small droplets of fat as compared with the large ones of the germinal membrane.

Because of their minute size no successful thin frozen sections could be made either of the scolices or the adults.

E. The Distribution of Nucleic Acid

1. Desocyribonucleic Acid

Cyst - As may have been expected from its staining reaction with acid stain, the laminated layer did not give any positive Feulgen reaction.

All of the chromatin granules of the nuclei in the germinal membrane proved to be DNA. The nucleoli did not show any positive reaction. Aside from the typical vesicular nuclei of the germinal membrane, two other types of nuclei were observed (Plate XII, Figures 1 and 2). The first type was again vesicular with scattered chromatin granules but oval in shape and much larger in size. They measured 9-12 u in length and 4.5-7 u in width. In spite of their large size, no mitotic figures could be observed. The other type was round and as large as the normal nuclei, 2-5 u in diameter, but was packed with DNA.

Scolices and Brood Capsules - No large nuclei were found in any of the sections of scolices and brood capsule membranes examined. Most of their nuclei were of the small vesicular type and a few of the compact type. The "ortho" scolices also showed a few nuclei containing DNA.

Adult - The nuclei of the adult did not differ in shape or in DNA content from those of the cyst. Both small vesicular and compact nuclei were widely distributed in the parenchyma and various organs. There was a distinct row of vesicular nuclei lining the cuticle, while the organ primordia and developing ova contained only compact nuclei in large numbers.

The sperms, both in the sperm duct and in the seminal receptacle, showed an interesting distribution of DNA. It was not possible to decide as to what made part of a single nucleus, but there were compact dots and wavy threads of DNA.

2. Ribosenucleic Acid

Cyst - The laminated layer seemed to be devoid of this nucleic acid also. The basophilia of the nucleoli and the cytoplasm of the cells on the germinal membrane was found to be due to RNA. As discussed previously, the cells of the germinal membrane showed very small amounts of cytoplasm; therefore, the RNA content was understandably low. In comparison to these cells, the flame cells showed a considerable amount of RNA. In the elongated flame cells the greatest amount of RNA was localized in a triangular mass at the opposite side of the flame; the basal plate, the tip of the flame, and a few dots on its sides gave a positive reaction thus actually outlining the whole cell. In the round flame cells again the basal plate and all the star-shaped cytoplasmic extensions gave a positive reaction.

Scolices and Brood Capsules - In methyl green-pyronin stained preparations some cells on brood capsule walls that had remained on the mother germinal membrane showed several cells close together with much more cytoplasm than those of the germinal membrane; thus, brood capsules seemed to contain more RNA. Most of the RNA of scolices was localized in cells lining the cuticle. The more central parenchyma seemed to be poor in RNA. The primordia of scolices also were rich in RNA (Plate XIII, Figure 1).

Adult - Only a few adult worms were studied for the distribution of RNA. Like the scolices, the cells lining the cuticle seemed to be rich in RNA. A high concentration was also seen in the primordia of reproductive organs, the cells surrounding the testes, the ovary, the viteline glands, and particularly the cells of the immature and mature ova (Plate XIII, Figure 2).

F. The Distribution of Connective Tissue Fibers

Mallory's triple connective tissue stain, apart from staining collagen fibers dark blue and elastic fibers pink to yellow, also stains mucus amyloid and other hyaline substances in various shades of blue. Nuclei, cytoplasm, fibrin, axis cylinders, neuroglia and fibroglia fibers stain red, and myelin, yellow. Though the latter colors are not specific for a single substance, they give an idea of the general nature of stained materials.

Cyst - The germinal membrane did not show the presence of any connective tissue fibers. The whole membrane seemed to be covered by a superficial layer of red to orange staining granules that were thought to be the various metabolic end products. Only in certain areas, where this top layer was missing or lost, could the basal layer be seen (Plate IV, Figure 1). The excretory canalicules stained in a greyish-blue shade and stood out prominently.

The laminated layer showed an inconsistent but

interesting staining reaction. The lamellae usually stained grey to various light shades of blue. The layers nearer the germinal membrane were more "hyaline" and greyish than the more distal bluish layers. Sometimes rows of small vacuoles were seen between various layers of lamellae making a distinct layer themselves. Sections from a few pieces of cyst membrane (and sometimes only one part of a section) showed this row of vacuoles filled with granular substances of various nature, and staining blue, red or yellow (Plate XIV, Figure 1). Another peculiar layer that stood out from all the rest by staining clearly yellow or a definite blue appeared in some sections. At certain points this band changed its straight path and crossed the other lamellae obliquely or in a transverse line (Plate XIV, Figure 2). This lamella showed a darker eosinophilic reaction than the other layers when stained with hematoxylin-eosin.

Scolices and Brood Capsules - The walls of the brood capsules did not stain any differently from the germinal membrane. In a few sections some red nuclei on a patchy blue background were noticed. The scolices did not show any staining reaction typical of collagen or elastic fibers except the dark blue cuticle and its basement membrane as well as the blue membrane lining the sucker-cups. The cuticle of the invaginated part of the scolex showed an additional outer red layer. The muscle cells of the suckers stained very bright red; whereas, the parenchyma cells were pale blue with red nuclei. The hooks appeared yellow in color.

Adult Tapeworm - The cuticle of the adult, similar to that of the invaginated portion of the larval scolex, showed a thin outermost red and an inner dull blue layer with a very dark blue basement membrane. The outermost layer of the cuticle appeared to be serrated. However, this could be an artifact. It was observed that all basement membranes or cuticular linings such as those of the ovary, testes, yolk gland, and cirrus sac, gave a similar dark blue staining reaction. As in the larval scolex, the parenchyma of the adult stained light blue and the muscle fibers a bright red. Unlike the parenchyma, the cells of the various organs, such as the ovary, testes, and vitelline gland, stained with the red fuchsin. The cell walls of the Mehlis gland stained a deep blue, and demarcated this organ from the surrounding parenchyma. The shell of the mature ovum within the gravid uterus stained a bright yellow, whereas the inner vitelline membrane was red. The embryo itself contained red and blue granules.

DISCUSSION

A. Phosphatases

Both acid and alkaline phosphatases have been reported from larval cestodes. Erasmus (1957a, 1957b) was unable to demonstrate any phosphatase activity in the cysticercus of Taenia pisiformis and Cysticercus tenuicollis by using histochemistry. However, by biochemical means, he demonstrated both acid and very slight alkaline phosphatase activity in both cysticerci. Therefore, the negative results in the hydatid cyst observed here may not necessarily reflect the complete absence of the enzyme. There may be an initially low enzyme concentration that did not survive fixation and dehydration procedures, or the phosphatase present may have a different pH optimum than that of the incubation mixture used.

It is interesting to note that the larval scolices did not show a histochemically demonstrable enzyme activity while the scolices of the adult tapeworm that do not seem much different in structure gave a positive alkaline phosphatase reaction. A change in the predominant phosphatase with change of stage was observed in other cestodes by Erasmus (1957a,1957b). This indicates a change in the physiological requirements of the organism as it grows and changes its host.

Alkaline phosphatase was demonstrated in the cuticle of Moniezia expansa both by Rogers (1947) and Erasmus (1957b). Erasmus (1957a) also demonstrated enzyme activity in the cuticle

of Taenia pisiformis. It is significant that a similar cuticular location of alkaline phosphatase has been demonstrated in several species of Acanthocephala (Bullock, 1949, 1958), that, like tapeworms, lack mouth and anus as well as an alimentary canal. Therefore, all metabolites of the worm pass through the alkaline phosphatase-rich cuticle. Interestingly enough, Ascaris, which does have an alimentary canal, did not show any enzyme in its cuticle but was shown to contain phosphatase in its intestinal cells through which the metabolites pass (Bullock, 1949). Several functions have been suggested for the alkaline phosphatase in vertebrates. This enzyme has usually been associated with processes of absorption and secretion, formation of fibrous proteins, and calcification. In addition, a close association between alkaline phosphatase and nucleic acids has frequently been observed in vertebrates animals. (Roche , , 1950). Because of its cuticular position, the alkaline phosphatase of helminths, as the intestinal phosphatase of vertebrates, has been associated by all workers with the first of these possible functions. The phosphatase may help the phosphorylated passage of food substances, especially sugars, either by providing the phosphate ion from other esters or by dephosphorylating incoming phosphorylated substances. The subcuticular cells in the adult E. granulosus, apart from showing phosphatase activity, were also found to be strongly pyroninophilic, or rich in ribose nucleic acid. The presence of phosphate radicals in RNA suggest either their

hydrolysis by alkaline phosphatase or contribution to their syntheses by providing phosphate groups (Borghese, 1957).

The site of enzyme activity has been one of the important factors leading to the conclusion that alkaline phosphatase is important in absorptive processes. The cuticular phosphatase activity reported in the present study for E. granulosus gives further supporting evidence for this assumption.

B. Glycogen

Glycogen is the most commonly found polysaccharide in all parasitic helminths. Its distribution has been studied in several larval and adult tapeworms (von Brand, 1952). Heyneman and Voge (1957), studying three hymenolepidid cysticercoids, found glycogen localized primarily in the parenchyma of their tail. Smyth (1949) also found glycogen in the parenchyma of the plerocercoid of Ligula intestinalis. The germinal membrane of the hydatid cyst seems to fulfill the function performed by the parenchyma of these other types of cestode larvae. Cameron (1923) and Cameron and Fitzpatrick (1925) found glycogen not only in the germinal membrane but also in the laminated layer. The results obtained in this study agree with those of Brault and Loeper (1904a) and Horvik and Moriconi (1956) who also found glycogen limited to the germinal membrane.

Agosin et al. (1957), using chemical and electrophoretic

methods, found the hydatid scolices to contain, in addition to glycogen, another polysaccharide composed of glucoseamine and galactose. In the present study only the cuticle of the healthy scolices showed the presence of PAS positive material after saliva digestion. In addition, the "ortho" scolices and the fluid in the intact brood capsules contained a non-saliva digestible polysaccharide which apart from the cuticle, could be the source of Agosin's second compound.

Hedrick and Daugherty (1957) studied the distribution of glycogen in the adult Raillietina cesticillus and Hymenolepis diminuta and found heaviest deposits in the medullary parenchyma. The vitellaria, sperm, and ova were also found to contain some glycogen. The adult E. granulosus showed generally similar results. The most important storage place for glycogen was the medullary parenchyma. No other organ except the mature vitellaria seemed to contain any demonstrable polysaccharide. Like Hymenopelis, E. granulosus differed from Raillietina in lacking other non-glycogen polysaccharides in the parenchyma and musculature. The glycogen stores of the sperm are probably used in the production of energy for its motion. It was interesting to note that with the development of the ova their glycogen reserves increased; also, the fully mature eggs showed some other polysaccharide. This may be a preparation of the ovum for the independent free life that will follow until its settlement in a suitable intermediate host.

The type of host diet and the time of removal of the parasite after feeding of the host has been found to have a definite effect on the glycogen content of cestodes (Reid, 1942; Read, 1949, 1956). Therefore, the variation in the glycogen content of the different batches of worms is expected. No comment can be made on the nutritional condition of the host since most of the worms were from naturally infected stray dogs and were killed at different times of the day. The worms obtained from a laboratory-infected dog showed very little glycogen. This could have been due to the great number of worms present in this animal which extended from duodenum to the large intestine. It would be interesting to check in such extensive infections if the glycogen content of the small intestine, where most of the food absorbtion occurs, differs from that of worms at the end of the intestine.

Some worms that showed negligible amounts of glycogen in the last two proglottids were found to have a moderate deposit in the scolex and the following proglottid. Daugherty and Taylor (1956) studying the regional distribution of glycogen in Hymenolepis diminuta found the highest concentration in the first 10 cm. section of the worm. However, after the starvation of the host this same region showed the maximum drop in the amount of glycogen. On the other hand, Reid (1942) reported that, in the case of Raillietina cesticillus worms removed from the host at the end of feeding period showed a higher glycogen level at the anterior end than

in the posterior. The above mentioned E. granulosus adults could have been collected at the end of the feeding period of a previously starved host.

C. Lipid

Sudan Black B does not stain all kinds of lipid. It is primarily soluble in neutral fats and phospholipids (Gomori, 1952). The germinal membrane showed an extensive sudanophilic reaction. Using chemical methods, Cmelik (1950 found the cyst membrane poor in lipids. However, the germinal membrane represents only a small fraction in the total cyst membrane.

In cestodes, lipids have very often been considered as metabolic end products rather than reserve food (von Brand, 1952). Smyth (1949) observed that the plerocercoid larva of Ligula intestinalis produces large mounts of cytoplasmic fat during starvation under aseptic conditions. Reid (1942) noted that the fat content of Raillietina cesticillus was the same from worms taken from fed and starved chickens although the parasites occuring in the latter had lost most of their glycogen. In this connection, the fat deposits observed in the excretory system of the adult E. granulosus are of interest (Brault and Leoper, 1904b). A similar result has been observed in Moniezia expansa (von Brand, 1933). Some droplets of fat were also seen in the extensive excretory system of the hydatid germinal membrane in this

study. The rest of the fat deposits of the germinal membrane that are so easily detached may be metabolic wastes unexcreted through the excretory system but freed directly into the hydatid fluid which has been shown to contain some fat (Mazzocco, 1923; Lemaire and Ribère, 1935).

D. Nucleic Acids

Cmelik and Briski (1953) studying the protein fraction of the hydatid cyst wall reported the absence of both ribose- and desoxyribose nucleic acids from the cyst membranes. However, in the present study, the germinal membrane showed the presence of both nucleic acids though in very small amounts, when stained with the Feulgen and Methyl green-pyronin stains. The negative chemical results are not surprising when we consider the minute amounts of the substances seen on the germinal membrane, and when we recall that the whole germinal membrane itself is only a small fraction of the complete cyst wall. If chemical methods are to be used, the germinal membrane must be collected in sufficient quantity to make accurate determinations. The difficulty in collecting this material, however, makes this method unpractical.

It was interesting to see different types of nuclei on the germinal membrane. Since the compact nuclei did not show much difference in size from the ordinary vesicular nuclei, they might be of those same nuclei in a different

physiological state - containing a large quantity of DNA. However, the large nuclei seem to be of a different nature. The fact that they are fewer in number and found only in groups on certain areas of the germinal membrane suggests very strongly their being germinal nuclei that will give rise to the brood capsules. The brood capsules, like these nuclei, tend to aggregate in certain areas of the germinal membrane.

No studies seem to have been done on the nucleic acids of other adult tapeworms. The mesenchyme being more of a storage tissue seems to be free of RNA except in the immature proglottid where the reproductive organ primordia are developing.

A high concentration of RNA has been observed in embryonic tissue of vertebrates (Burghese, 1957). Similarly, the ova of E. granulosus showed a high pyroninophilia. The subcuticular cells which are always associated with the secretion of the cuticle showed a considerable amount of RNA. The true nature of cestode cuticle is not yet known. However, Mallory's triple stain showed a dark blue basement membrane that could be collagenous in nature. This association seems interesting because both in vertebrates and invertebrates RNA has been found to be plentiful in cells associated with protein secretion (Davidson, 1949).

E. Connective Tissue

In spite of the fact that Mallory's triple stain did

not contribute appreciably in clarifying the chemical nature of the parasite, it was very helpful in understanding the various structures of the worm. Very little work has been done on the connective tissue of other flatworms. Smorodincen and Pawlowa (1936) reported the presence of collagen, elastin, reticulin in Taenia saginata. In E. granulosus the only structures that could be collagenous in nature are the basement membrane of the cuticle of both scolices and the adult, the lining of the ovary, testes, yolk gland, cerrus sac and sperm duct. Though the laminated layer of the cyst wall showed some yellow lamellae and granules, when tested with orcein for elastic fibers, no positive results were obtained. The different kinds of staining reactions given by the various layers of the laminated membrane suggest that they may not be exactly of the same chemical nature, and may reflect the variation in metabolic products excreted by the parasite as its physiological state changes.

CONCLUSION

The object of this investigation was to examine certain biochemical constituents of E. granulosus through all its stages of development and to demonstrate the relation of these substances to the organs and cellular elements in which they occur, as well as to make observations which may contribute to our knowledge concerning the structure of the hydatid cyst.

The two layers of the hydatid cyst wall showed dissimilar results for all the constituents studied, with the germinal membrane proving to be the probable site of all metabolic activivities. Unlike the laminated layer, the germinal membrane showed heavy deposits of glycogen and fat. Different types of nuclei containing DNA and the small cells rich in RNA were also limited to the germinal membrane. These stores of substances intimately concerned with metabolic processes offer presumptive evidence of an actively metabolizing tissue.

The observations reported in this study reveal more clearly the nature of the excretory system in the germinal membrane, the presence of which was reported by some early workers and refuted later by others. This extensive excretory system consisting of flame cells and interconnected cannalicules that, in turn, are continuous with the excretory canals of the brood capsules, would seem to be an important corrolary to our concept of the germinal membrane as the site of physiological activity in the hydatid cyst wall.

The larval scolex resembles the adult worm in most of the reactions studied here. The parenchyma, like that of

the adult, stores large amounts of glycogen. The nature of its cuticle as shown by Mallory's triple stain is similar to the adult cuticle. Both the larval scolex and adult worms show a similar localization of RNA in the subcuticular cells. The only observed major difference is the absence of histochemically demonstrable alkaline phosphatase in the cuticle of the larval scolex in contrast to the strong activity in the adult cuticle. This indicates a significant physiological change in the parasite during the course of development. It should be remembered also that this developmental change is accompanied by a change from intermediate to definitive host.

The ova, as may be expected of all embryonic tissues, are rich in both RNA and DNA. As they mature, they accumulate not only glycogen but also some other polysaccharide before the start of their independent existence outside the host.

The difference in the amount of glycogen in the adult worms, thought to be a result of host diet, the nature of the polysaccharide in the ortho scolices, the fat deposits as an excretory product, and the connection of the excretory system to the cellular elements of the germinal membrane, raise new questions for further study.

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Plate I

Adult *E. granulosus*

Plate II

Fig. 1. Adult E. granulosus in dog intestine.

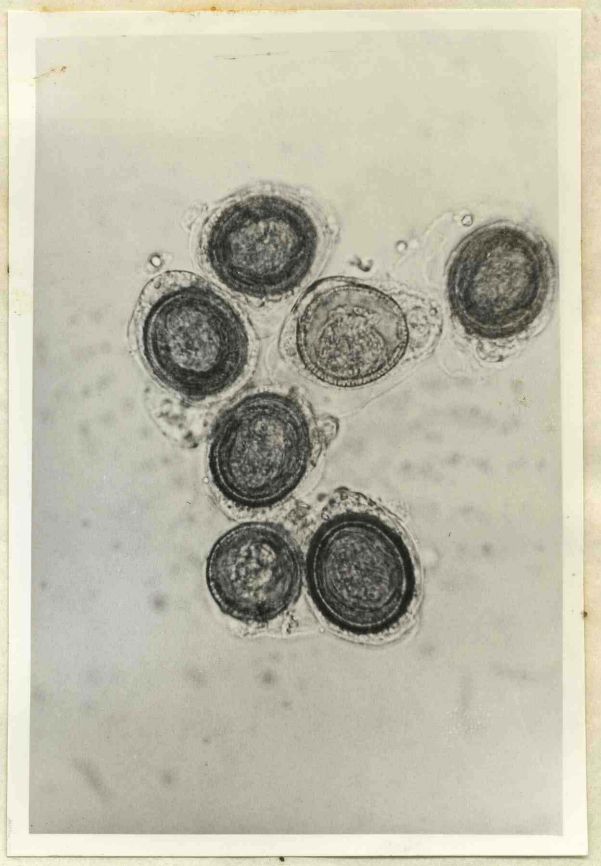

Fig. 2. Ova. 430x.

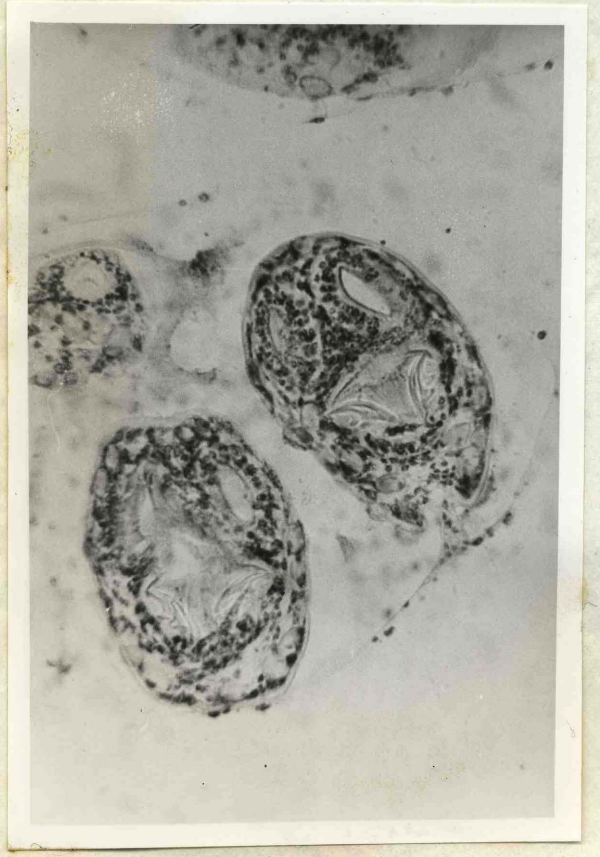

Fig. 4. Scolices in brood capsule. 430x.

Fig. 3. Section of cyst wall. 100x.

Plate III

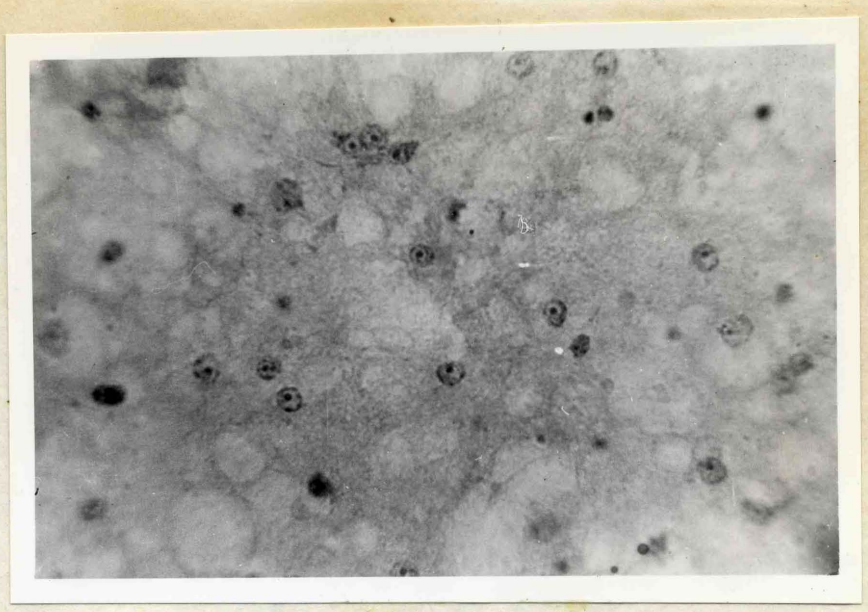

Fig. 1. Flat preparation of germinal membrane showing nuclei. H-E x 970.

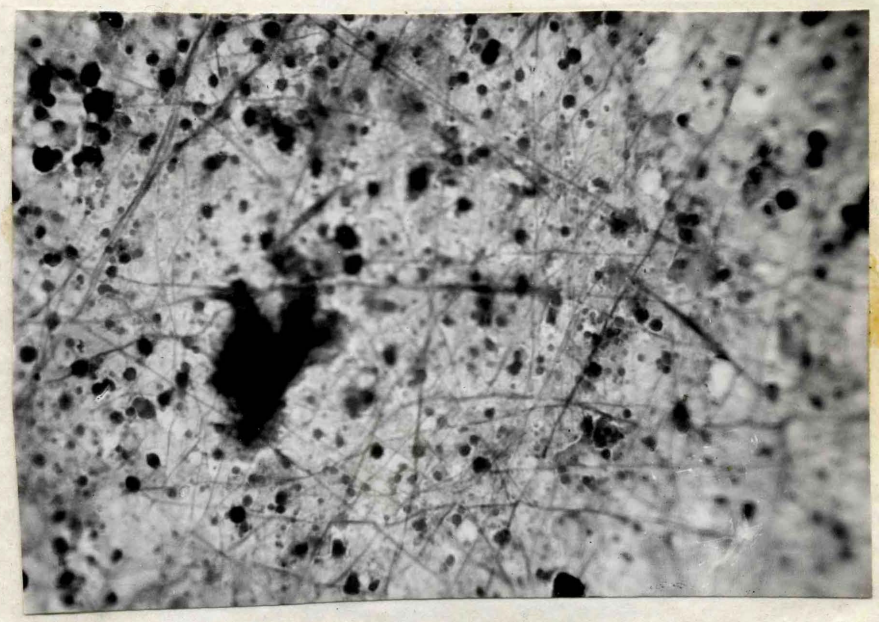

Fig. 2. Flat preparation of germinal membrane showing branching excretory canals. Heidenhain's Hematoxylin.

Plate IV

Fig. 1. Flat preparation of germinal membrane showing excretory fibers sticking out of cytoplasmic ground substance. Mallory's triple stain. 100x.

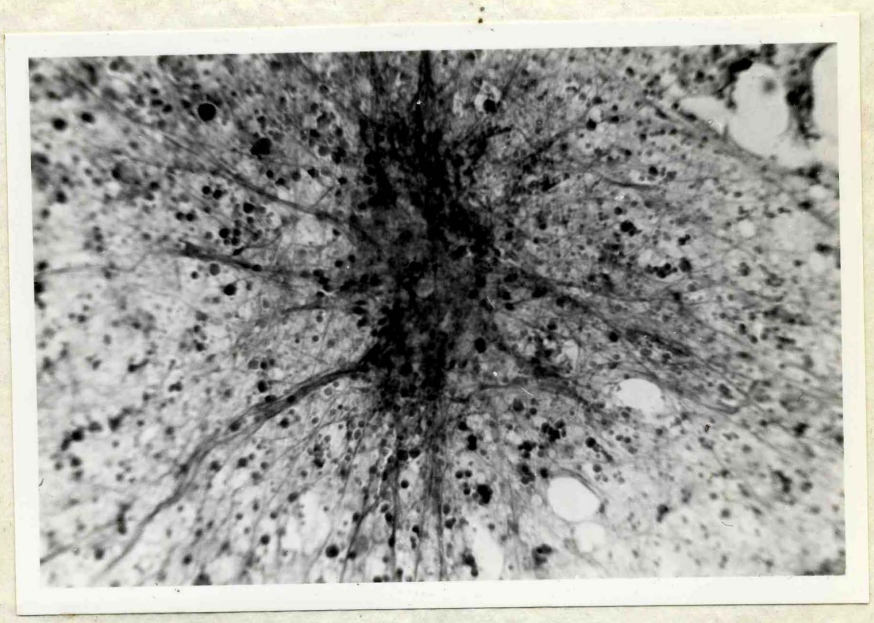

Fig. 2. Flat preparation of germinal membrane showing excretory fibers of brood capsule running into mother germinal membrane. Heidenhain's hematoxylin. 100x.

Plate V

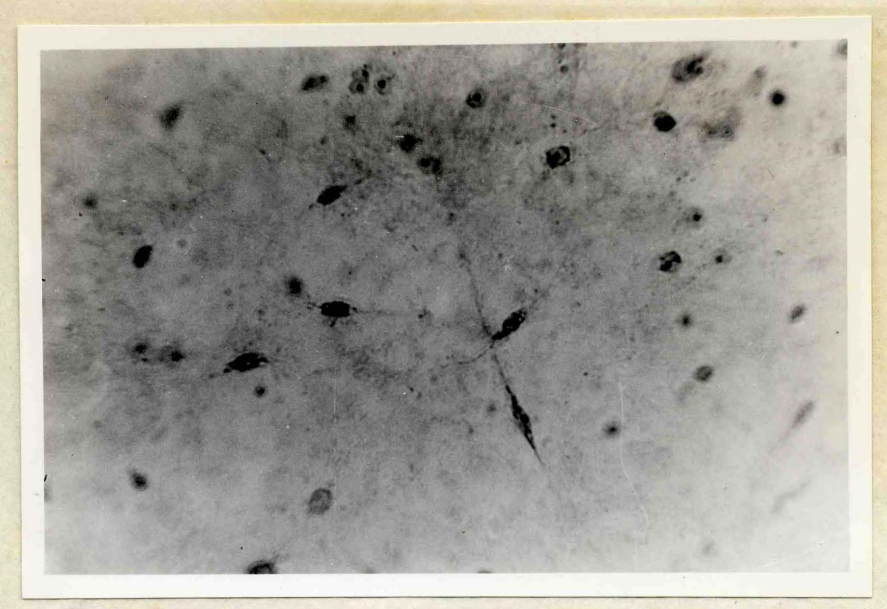

Fig. 1. Flat preparation of germinal membrane showing elongated flame cells. Thionin. 970x.

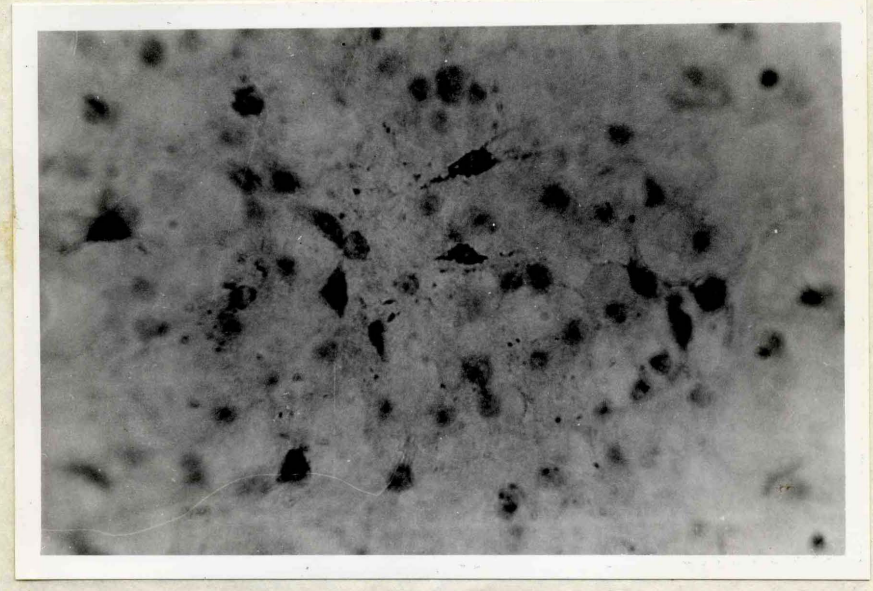

Fig. 2. Flat preparation of germinal membrane showing spherical flame cells. Thionin. 970x.

Plate VI

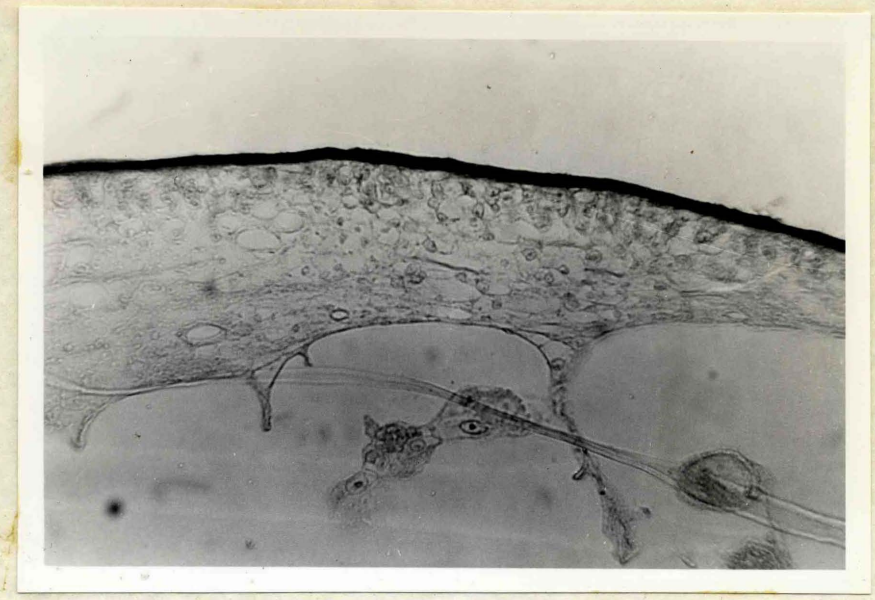

Fig. 1. Alkaline phosphatase reaction in the cuticle and subcuticular cell nuclei in horizontal section of gravid proglottid of E. granulosus. Incubation, 1 hour. Section, unstained. 32x.

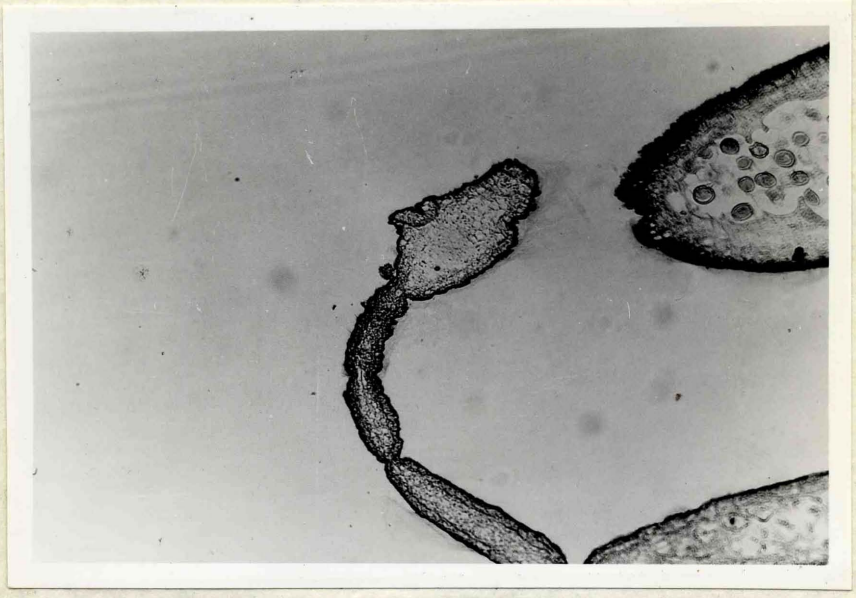

Fig. 2. Alkaline phosphatase reaction in the cuticle and subcuticular cells in horizontal section of E. granulosus. Incubation 4 hours. Section unstained. 32x.

Plate VII

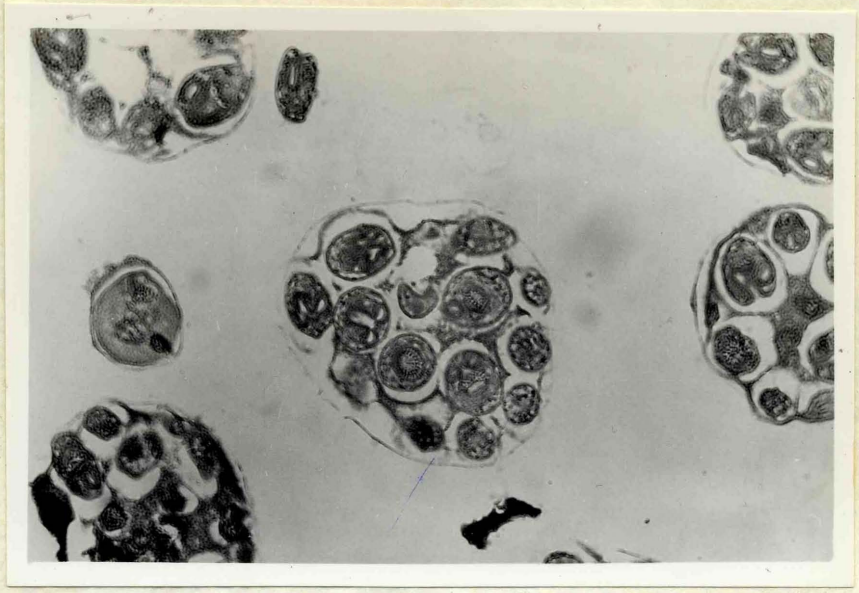

Fig. 1. A section through brood capsules showing heavy deposits of glycogen in the scolices. PAS. 100x.

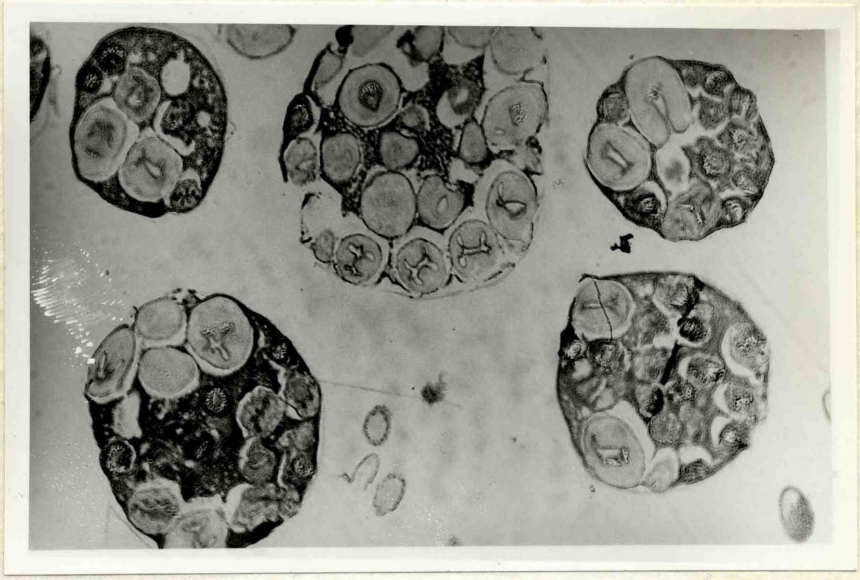

Fig. 2. A section through brood capsules showing saliva non-digestible polysaccharide in "ortho" scolices and brood capsule fluid. PAS after saliva digestion. 100x.

Plate VIII

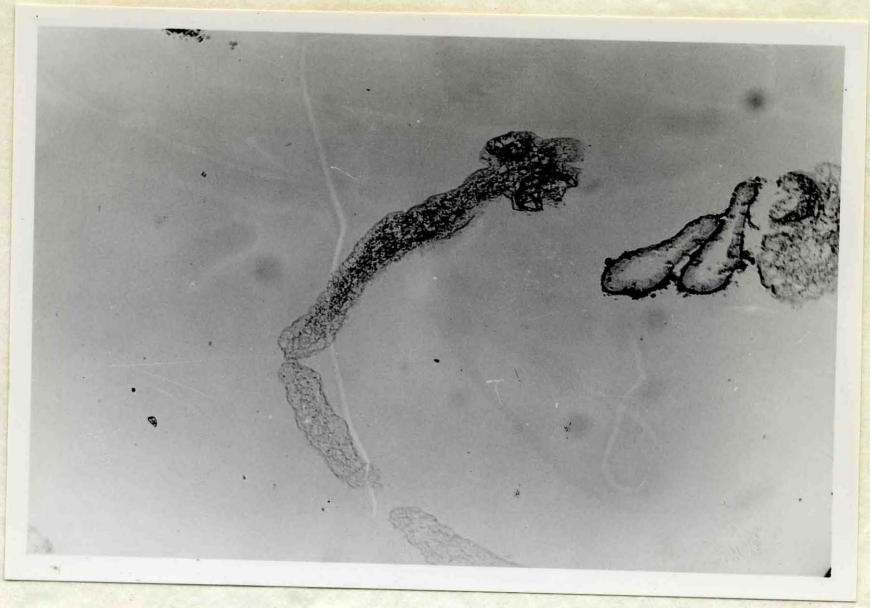

Fig. 1. Longitudinal section of adult E. granulosus showing heavy deposits of glycogen in scolex and following proglottid and none in the mature and gravid proglottids. PAS. 32x.

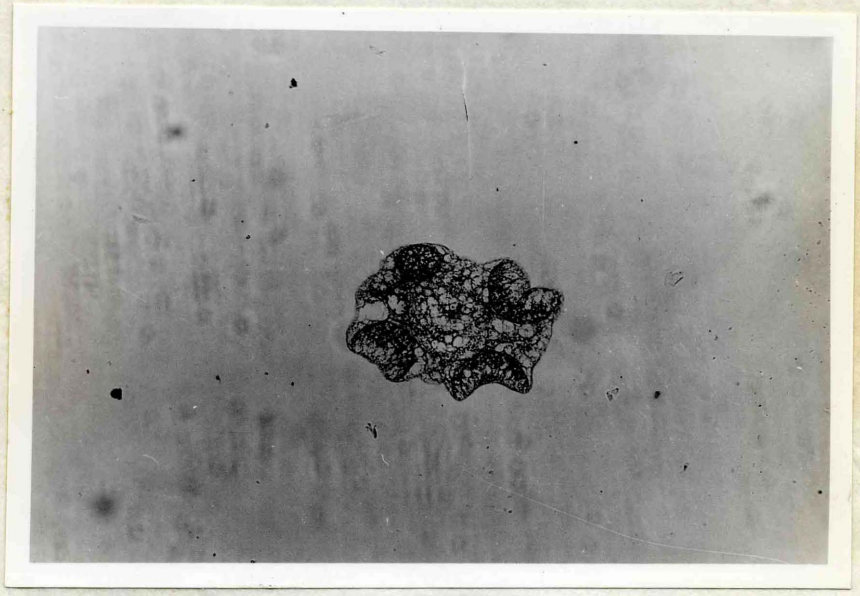

Fig. 2. Cross section through adult scolex showing distribution of glycogen with heavy deposits in sucker-cups. PAS. 100x.

Plate IX

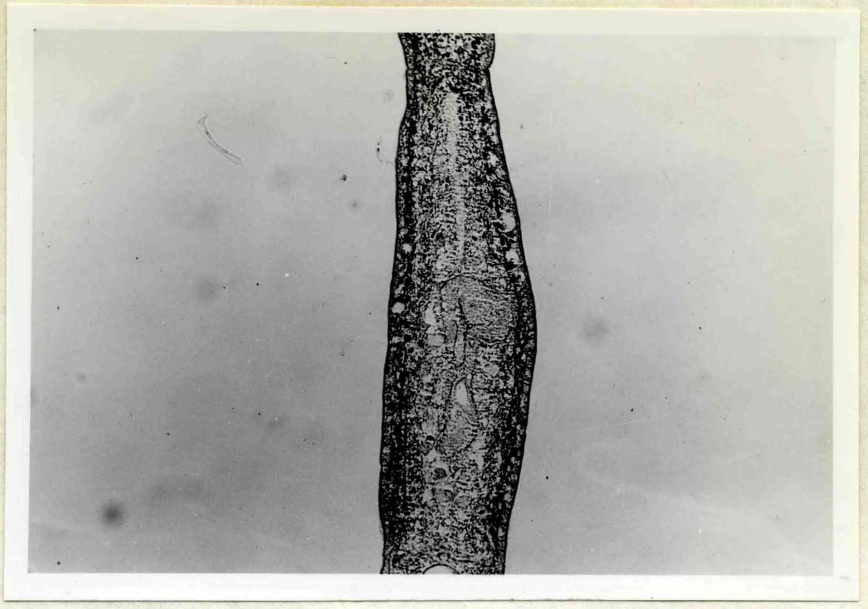

Fig. 1. Longitudinal section through an immature proglottid showing heavy deposits of glycogen in the parenchyma surrounding the organ primordia. PAS-H. 100x.

Fig. 2. Longitudinal section through a mature proglottid showing heavy deposits of glycogen in the parenchyma and vitelline glands. PAS-H. 100x.

Plate X

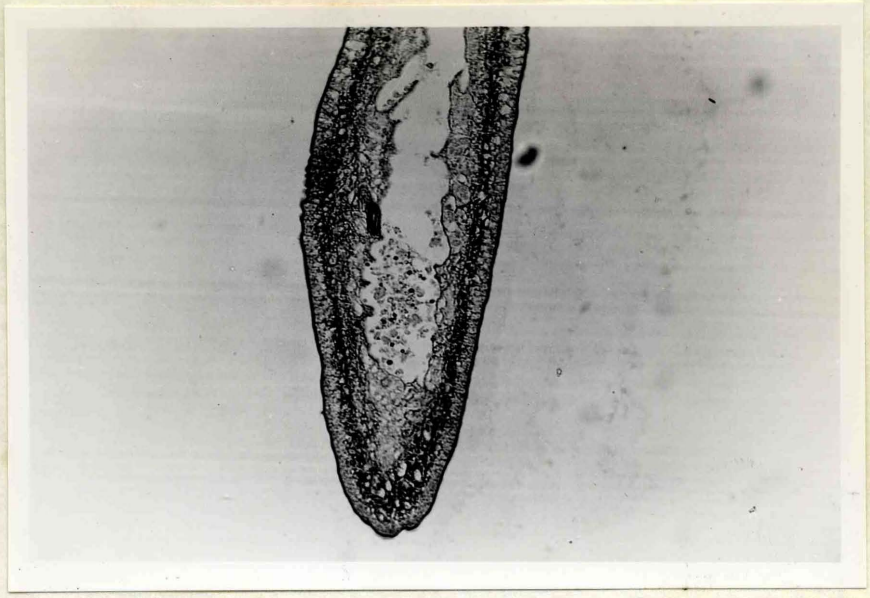

Fig. 1. Longitudinal section through a very mature proglottid showing heavy deposits of glycogen in the parenchyma and some extracellular glycogen in the uterus. PAS-H. 100x.

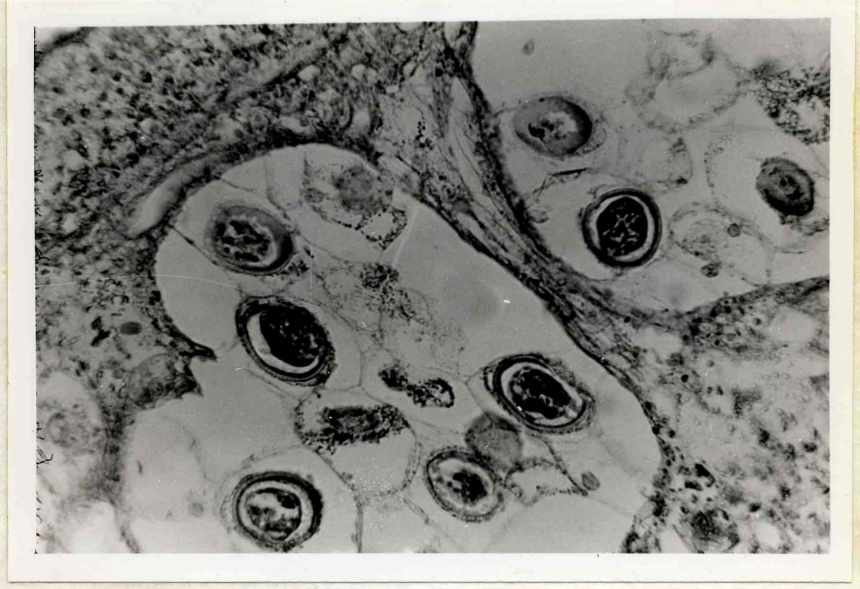

Fig. 2. Cross section through gravid proglottid showing heavy deposits of glycogen in ova. PAS. 430x.

Plate XI

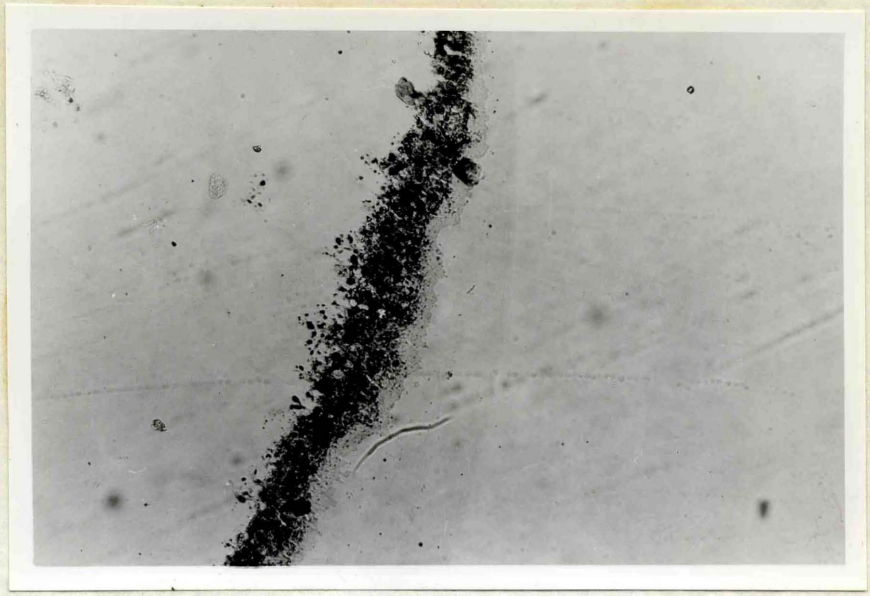

Fig. 1. Transverse section through hydatid cyst wall showing heavy deposits of fat in the germinal membrane and no fat in the laminated layer. (L.M.) Sudan Black - B. 430x.

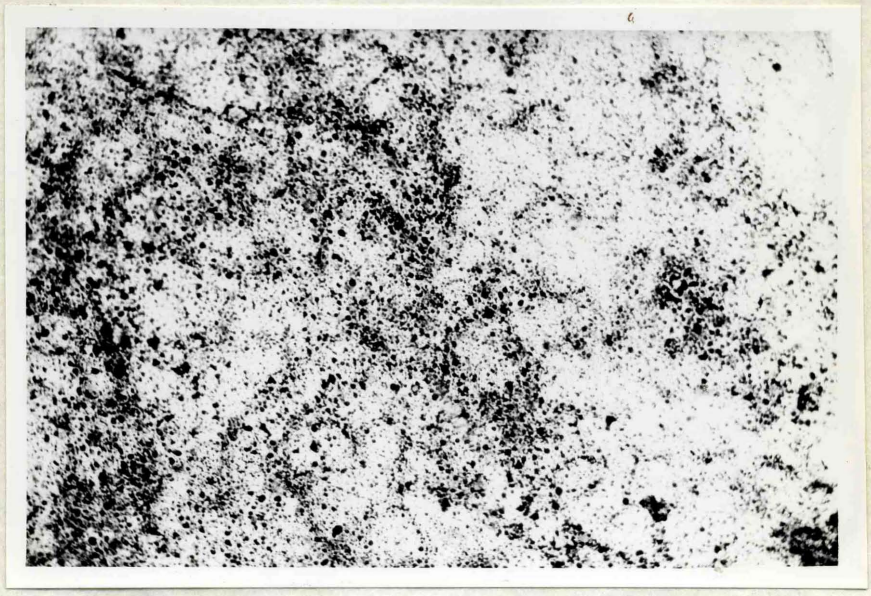

Fig. 2. Flat preparation of germinal membrane showing distribution of fat. Sudan Black - B. 100x.

Plate XII

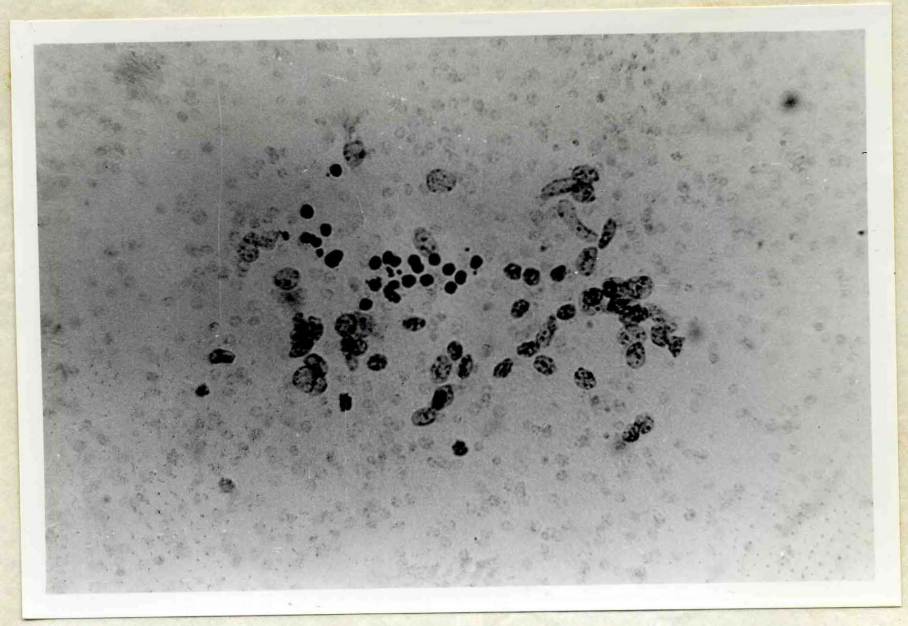

Fig. 1. Flat preparation of germinal membrane showing the three types of nuclei. Feulgen. 430x.

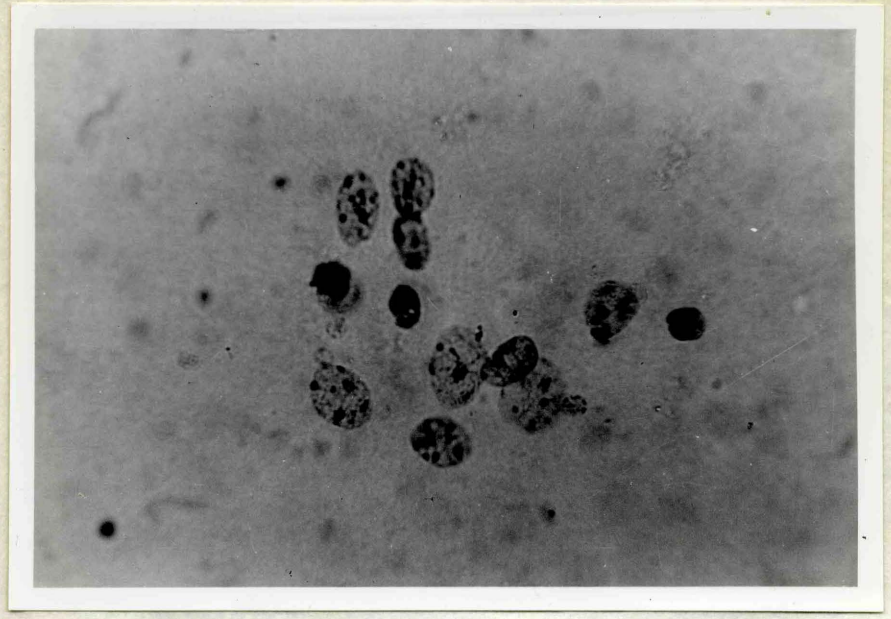

Fig. 2. As Fig. 1. 970x.

Plate XIII

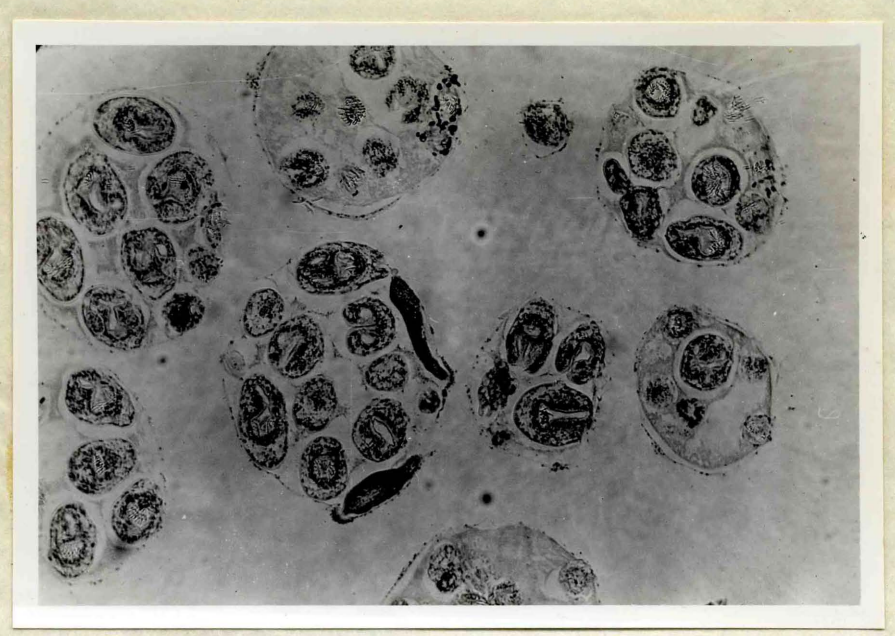

Fig. 1. Section through brood capsule showing high concentration of RNA in the primordia of scolices and in cells lining the cuticle of mature scolices. Toluidine blue. 100x.

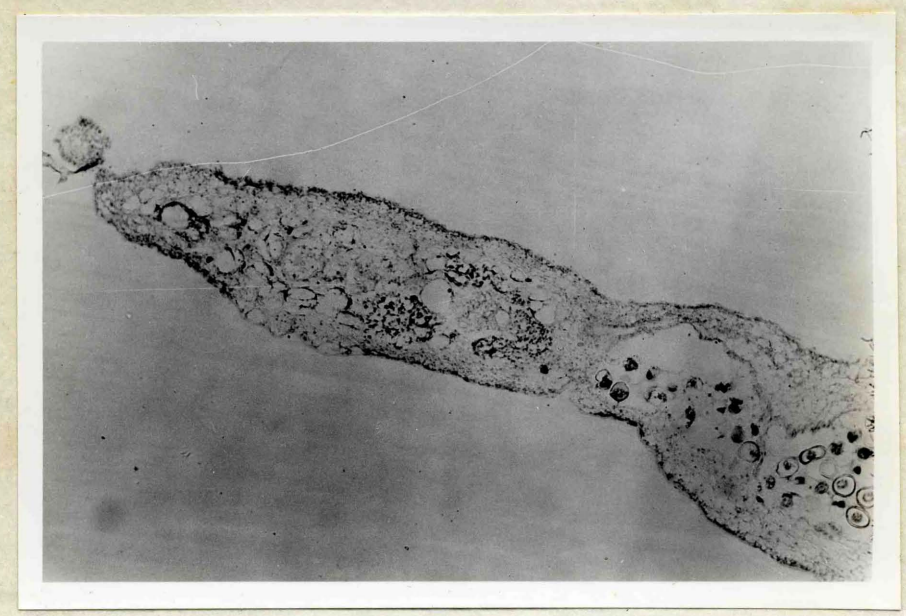

Fig. 2. Longitudinal section through mature and gravid proglottids showing high concentration of RNA in the subcuticular cells, in the ovary, vitelaria and ova. Methyl green-pyronin. 32x.

Plate XIV

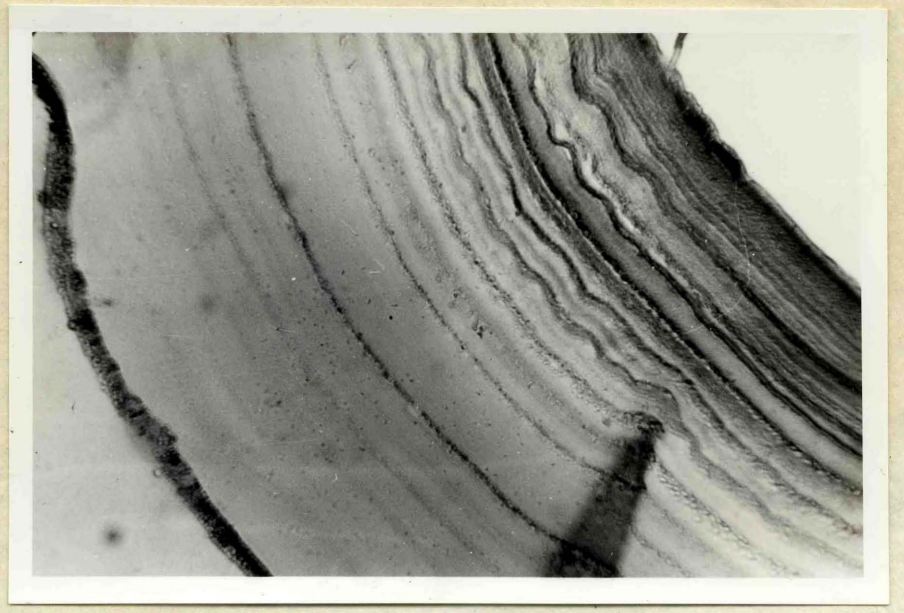

Fig. 1. Cross section through cyst wall showing the differential staining of the different layers of lamellae. Mallory's triple stain. 430x.

Fig. 2. Cross section through cyst wall showing the single outstanding layer. Mallory's triple stain. 100x.