In Vitro Cultivation of *Echinococcus granulosus*

with Histochemical Observations

on

Glycogen Metabolism

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Echinococcus granulosus

A C K N O W L E D G E M E N T S

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A B S T R A C T

Echinococcus granulosus scolices were found to live longer in media containing rabbit serum than in purely synthetic media. A maximum survival of 84 days was obtained in Parker's TC medium 199 supplemented with inactivated rabbit serum and HeLa cells. Serum was found to retard vesiculation and evagination of scolices.

A histochemical study of the distribution and depletion of glycogen in fresh and cultured scolices in various media for different periods was carried out. No glycogen was detected in the cuticles of fresh or cultured scolices. Glycogen particles were found to occur mainly in the parenchyma and suckers in big or small diffuse particles.

A large amount of glycogen was noted in the parenchyma and suckers of scolices cultured in media containing glucose or galactose in Kreb-Ringer's solution. Parenchymal and, to a lesser extent, acetabular glycogen was found to be partially depleted in scolices cultured in media containing glucosamine in Kreb-Ringer's solution. Scolices starved in carbohydrate-free balanced salt solutions were observed to utilize glucose when added to the medium.

The effect of certain drugs and enzymes on scolices cultured *in vitro* was investigated. Physostigmine was found lethal, while ammonium molybdate was noted to initiate evagination and vesiculation. The enzyme hyaluronidase seemed to accelerate glycogenesis in all media.

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INTRODUCTION

In Vitro Cultivation of Cestodes

The ultimate purpose of in vitro cultivation of tapeworms is to find for each species a defined nutrient medium that resembles sufficiently the physical and chemical environment to which the parasite is normally exposed, so that the parasite can be induced to complete its life cycle in the absence of its host. Once the above conditions are known, control of nutritional and environmental factors is possible and individual variables can be investigated.

Various workers have attempted to culture larval and adult cestodes *in vitro* in media ranging from sea and tap water to synthetic media with or without such complex organic supplements as embryonic extracts, amniotic fluid, hydatid cyst fluid, ascitic fluid and various sera. Perhaps the earliest attempt to maintain a cestode in the absence of its host was that of Frisch who, in 1734, was able to maintain *Schistocephalus solidus* plerocercoids viable for two days in river water. Abildgaard, in 1793, kept plerocercoids of *Schistocephelus solidus* viable in fresh tap water six days longer than Frisch. A survival period of two weeks was attained by Fabricius in 1780 when he cultured another cestode *Proteocephalus percae* in sea water.

Attention was then directed to supplementing nutritive media with material of animal origin. Knock (1862) observed that holdfasts of bothriocephalid tapeworms from fish, probably Tetraphyllidean cestodes, would survive for eight days in an aqueous solution of egg albumin.

Similar early efforts at *in vitro* maintenance of various tapeworms also achieved brief periods of survival but without any development (Zschokke, 1888;

Ortaer-Schonbach, 1913). Then, in 1926, Devé succeeded in culturing Echinococcus granulosus scolices in hydatid cyst fluid and serum for 14 days, during which time scolices imbibed water, vesiculated and doubled or tripled in volume. Further efforts at the cultivations of Echinococcus spp. are given in detail below. Following this initial success of Devé, Coutelen (1929) observed that individual coenurids of Multiceps serialis also increased in volume and survived for twenty days in sterile saline and horse serum, renewed daily. Other workers, while still falling short of the goal of growth and development, were able, in some instances, to establish favorable chemical and physical limits for in vitro survival of several cestode species. Thus, Moniezia trisonophora, which died immediately in tap water at 37°C. lived at the same temperature for 2 days in distilled water and 12 days in M/10,000 NaOH in carbonate-free Ringer's solution (Cook and Sharman, 1930). Wardle, in 1932, found that plerocercoids of Diphyllobothrium latum and Triaenophorus tricuspidatus and adults of Bothriocephelus scorpini tolerated isotonic salt solutions better than hypotonic solutions. Two years later, Wardle (1934) cultured plerocerocercoids of Nybelinia in a range of saline nutrient media at pH 7-5 and temperatures of 18-24°C. They survived with no indication of larval growth or development for 456 hours in sterile double Locke's solution, 192 hours in serum plus saline gel, 200 hours in sterile Locke-bouillon and 408 hours in Locke-glucose solution. Mendelsohn (1936) maintained larvae of Taenia crassicollis at 37.5°C. for 35 days in a sterile medium with chick embryo extract and filtered horse serum in balanced saline. Evagination was noted , but there was no evidence of sucker formation.

The addition of vitamins to nutrient media was found by Markov (1938) to

increase the survival time of plerocercoids of Diphyllobothrium latum. They lived approximately twice as long when vitamins were added to the culture medium containing glucose. Markov also found that young plerocercoids, 1-2mm. long, lived only half as long in vitro as the older ones.

In a series of studies, Wilmoth (1938-1945) observed that larvae of Taenia taeniaeformis withstood gradual in vitro pH changes from 4.6-9.3 and abrupt changes from 6.5-6.8 in Tyrode's solution at 37°C. in the same medium at pH 7.4. Wilmoth also reported that anaerobiasis shortened the survival time of T. taeniaeformis larvae and led to a small decrease in the pH of the medium. The larvae survived for 14 days at 37°C. in sterile Tyrode's solution changed every 20 hours.

In all the above-mentioned experiments, with the exception of those on Echinococcus granulosus carried out by Devé (1926) and Coutelen (1929), no growth or development was noted. In addition the longevity attained was only a fraction of the life span in the normal host.

Stunkard, in 1932, demonstrated incomplete growth and segmentation of the terminal portion of the body of Crepidobothrium loennbergi in a veal broth medium at pH 7.3, but the proglottids were sterile and abnormal. There was, however, a 3-4 times increase in the length of the cultured worms in 32 days. The medium in these experiments was changed twice daily. Neither anaerobic conditions nor the addition of extracts of intestinal mucosa, pancreas and liver of Necturus, sterilized by filtration altered the rate of growth or the longevity of survival. Fresh serum from Necturus was found to be toxic (Stunkard, 1932).

The rat tapeworm Hymenolepis dinimuta was kept viable for a period of 20

days when cultured axenically in Baker's medium a period approximately equal to its life span in the natural definitive host (Green and Wardle, 1941).

In two studies, Joyeux and Baer (1938; 1942) described partial development of the plerocercoids of the pseudophyllidean tapeworm Ligula intestinalis in saline plus ascitic fluid, but development was abnormal in the presence of horse serum. Oviposition was noted when ascitic fluid was added to the medium, yet no spermatogenesis was noted. No further development was noted when testes, pituitary or Ligula extracts were added.

In a series of experiments, Smyth (1946-1959) studied the maturation and maintenance of the pseudophyllidean cestodes Ligula intestinalis and Schistocephilus solidus in vitro. Maturation of the plerocercoids was obtained in a semiaerobic axenic buffered horse serum-saline medium. Spermatogenesis, oogenesis, vitellogenesis and egg shell formation were reported in a balanced salt solution containing glucose and nutrient or peptone broth at 40°C. The medium was changed daily. The fertile eggs which were obtained hatched into normal coracidia. Smyth also cultured pieces of the plerocercoids of Diphyllobothrium dendriticum in chick embryo extract and balanced salt solution. Organogenesis, segmentation and development of the genitalia were observed by the twentieth day. However, little increase in size occurred and autolysis took place before ovulation. When these fragments were cultivated in a more concentrated embryo extract, proglottidization and the formation of a cirrus, cirrus sac, uterus, ovaries and testies were observed, but vitellaria and eggs were not noted.

A large number of eggs were obtained from Spirometra mansonoides, the eggs were hatched and coracidia collected (Mueller, 1959a). Copepods, the

intermediate hosts, were reared and procercoids were harvested aseptically (Mueller, 1959b). The procercoids were axenically cultured in a medium containing calf serum and embryo extract in Parker's TC medium 199. In a week, the procercoids developed to pleroceroids. The latter increased in length from 0.1-10mm. in one month, and to 20mm. in 20 months. When the cultured plerocercoids were fed to cats, adult worms developed (Mueller, 1959c).

Scolices with portions of the neck of Hymenolepis diminuta were cultured in roller tubes in 50 per cent horse serum and glucose in Parker's TC medium 199 (Taylor, 1961). Growth and strobilization occurred. Growth was stimulated when extract of cestode tissue was added to the culture medium. However, differentiation of the genitalia, increase of volume and strobilization were abnormal. Sixty per cent of the 11-day worms produced a new infection when implanted in rats (Schiller, Read and Rothman, 1959; Taylor, 1961).

Berntzen (1960-1962) devised the ingeneous continuous flow apparatus in which the medium was supplied drop by drop from an adjustable dropper. The container of the cultured material was tilted in such a way to permit the medium to have enough contact with the culture and then flow with the metabolic products to a collecting flask. The whole apparatus was enclosed in a container in which the gas phase and temperature were controlled. Berntzen used his apparatus to culture Hymenolepis diminuta and H. nana cysticercoids at 37°C. and pH 7.4-7.8 in a complex medium consisting of vitamins, amino acids, carbohydrates, coenzymes, yeast and chick embryo in Tyrode's balanced salt solution. Proglottidization as well as development of the oncospheres were described. Eggs recovered from adults grown in vitro developed into cysticercoids in eight days when fed to beetles. The cysticercoids developed to adult worms 14 days

after being fed to mice. As expected, H. nana eggs did not require an intermediate host to develop to adult worms.

In vitro cultivation of Echinococcus spp.

Following the initial success of Devé (1926) in culturing E. granulosus scolices in hydatid cyst fluid and serum, negative results were obtained by Coutelen (1927a;b) when scolices were cultivated in bacteriological media, mainly nutrient broth. When Coutelen cultured hydatid scolices from pig lung and liver cysts in hydatid cyst fluid, with ascitic fluid and organ extract, however, they survived 31 days, became vesicular and their volume increased 25-30 fold. The following year, Devé (1928) succeeded in obtaining somewhat greater longevity. He maintained viable scolices for 43 days in equal volumes of hydatid cyst fluid and horse serum at 37°C. and described the formation of a laminated membrane.

No further progress in the culture of Echinococcus spp. in vitro was made for the next thirty years. Then, Rausch and Jentoft (1957) reported that they had cultured undifferentiated fragments of Echinococcus multilocularis cyst material from voles in a basic medium of 40% ascitic fluid in Hank's balanced salt solution plus antibiotics and vole embryo extract with and without HeLa cells (human cervix carcinoma cells). The latter gave the best results, cells proliferated, vesicles were formed by the twenty-ninth day, in which scolices were detected by the fifty-fifth day and calcareous corpuscles by the thirtieth day. Vesicles reared in the basic medium without HeLa cells gave better results when introduced intraperitoneally into voles. Larval proliferation rate declined after attaining a peak, at which time the vesicles reached a maximum size. The experiment was terminated by the 134th day.

Cameron (1960) obtained fertile Echinococcus multilocularis secondary cysts from rodent primary cysts. Cultivated scolices vesiculated, early segmentation occurred and the formation of a well defined excretory system were observed, but technical details were not given. Then in 1963, Webster and Cameron described the formation of new vesicles in vitro from Echinococcus multilocularis with fertile and sterile cysts in Morgan's synthetic medium at pH 7.4. Glycogen was found to initiate vesiculation when substituted for glucose in the medium. Addition of serum to the medium and lowering the pH below 7 retarded vesiculation while sodium taurocholate was found to enhance segmentation.

New vesicles separated from the parent vesicles and produced new vesicles. Vesicle formation continued until the fourteenth day, when it ceased and degeneration started. Evagination of E. multilocularis scolices was observed within 4 to 6 hours in culture media devoid of serum, and vesiculation occurred in 12 hours-2 weeks, depending upon the culture medium. Webster and Cameron (1963) considered vesiculation to be a sign of malnutrition or undesirable environmental factors. They obtained lamination and the formation of brood-capsule primordia within 3 to 4 weeks. In another experiement, they reported a survival of 7 weeks, during which increase in size, formation of a strong scolex, and segmentation were observed. Complex media with amino acids, vitamins, coenzymes, bile salts, carbohydrates, bactopeptone, bactoyeast, lactalbumin hydrolysate, bovine serum, calf serum, insulin, meat digest and dog gut extract in Morgan's synthetic medium and Parker's synthetic medium CMRL 1066 were used at 37°C. and pH 7.4. The cultures made use of flasks placed on a slowly moving rocker. Webster and Cameron also reported growth and segmentation of Echinococcus granulosus scolices in all the media used

for cultivating E. multilocularis.

Schwabe et al. (1963) studied the in vitro survival of E. granulosus scolices in non-nutrient salt solution. Echinococcus granulosus scolices were found to favor isotonic salt solutions and aerobic conditions. Longevity of survival varied inversely with the rise in temperature (Schwabe et al., 1963). Survival time was less in media lacking $MgSO_4$ and KH_2PO_4. A maximum survival of 68 days was obtained in hydatid cyst fluid and sheep serum at 30°C. The respiratory rate of scolices was found to be higher in natural media than in balanced salt solution.

In an effort to explain host specificity of Echinococcus granulosus, bile from herbivores was found to cause cytoplasmic fat accumulation and lysis of the cuticle of the cultured scolices, while bile from carnivores did not produce this effect. Sodium salt of deoxycholic, glycodeoxycholic and taurodeoxycholic acids in ascending order of effectiveness, were found to lyse the cuticle. Herbivores' bile was reported rich in deoxycholic acid conjugated with glycine, while carnivores' bile was found relatively poor in deoxycholic acid, which in carnivores is conjugated with taurine, hence its ineffectiveness in lysing the cuticle. The explanation suggested for the lysis mechanism was that the cuticular mitochondria may be readily disintegrated by sodium deoxycholate and surface active agents (Berberian, 1936; Smyth, 1962).

Smyth (1961) described the formation of a thick laminated membrane about vesiculating E. granulosus scolices after 31 days of axenic cultivation in Parker's medium 199 and bovine amniotic fluid. These cultures were maintained at 38°C. in roller tubes or in tubes agitated in a water bath. The scolices remained viable for 100 days. Internal cellular accumulation, were reported

and thought to be the primordia of blood capsules. Smyth did not report any segmentation or proglottidization.

Yamashita et al. (1962) cultured Echinococcus multilocularis scolices obtained from trypsinized alveolar cysts of the liver of experimentally infected mice in a basic medium of 0.5 per cent lactalbumin in Hank's balanced salt solution and supplemented with bovine serum and liver extract. Vesiculation of the scolices was detected by the fifth day. Multilocular vesiculation was observed even in vesicles embedded in HeLa cells. Between them, Smyth (1961), Yamashita (1962) and Webster and Cameron (1963) described three forms of vesiculation, a globular vesiculation of the whole scolex, the formation of anterior or posterior enlargements and the formation of both.

Carbohydrate Metabolism in Cestodes

Cestodes, except for the egg stage, are completely endoparasitic throughout their life cycles. They lack a digestive system and nutrients enter the worms through their cuticles. Attempts to demonstrate the transport of nutrients through osmoregulatory canals have failed (MacPherson, 1958).

Electron microscopic studies of the cuticle of cestodes have revealed structures that help to explain the surface absorption phenomenon (Rothman, 1959, 1962). Rothman, reported, for example, that Hymenolepis diminuta cuticle have microvilli, called 'microtriches'. Read and Simmons (1963) demonstrated mitochondria in the cuticle of Hymenolepis diminuta, indicating utilization of energy, hence, metabolic activity in the cuticular region.

Tapeworms were found to possess certain mechanisms involved in active absorption of glucose and in the chemical specificity of the cuticle (Read

and Simmons, 1963; Phifer, 1960a;b;c). Rothman (1959, 1962) studied the kinetics involved in Hymenolepis diminuta absorption of glucose. He found that there was no direct linear relationship between the amount of absorbed glucose and its concentration in the culture medium, but that a certain adsorption mechanism was involved and glucose was absorved against a concentration difference. Prosser and Brown (1961) showed that glucose absorption did not directly involve phosphorylase, contrary to what was believed previously. Phlorizin inhibited fermentation of glucose from exogenous sources but had no effect on internal glucose fermentation (Hutchison, et al., 1960). Phifer (1969c) reported that phlorizin inhibited the absorption, 1-deoxyglucose, D-allose, 3-O-methylglucoside all competitively inhibited D-glucose absorption. On the other hand, L-fructose, 1.5-anhydromannitol, seorbitol, D-mannose, fructose, 2-deoxyglucose were without effect on glucose absorption. Reciprocally, glucose inhibited galactose absorption by various tapeworms. (Read, 1961; Crane, 1960).

Carbohydrate Utilization by Cestodes

Carbohydrates are utilized by cestodes mainly as an energy source. When its rat host is subjected to a carbohydrate deficient diet, there is a decrease in the size and number of Hymenolepis diminuta (Chandler, 1943; Read and Rothman, 1957a;b;c). The rate of reproduction of H. diminuta is also found to vary directly with the amount of carbohydrate ingested by the host (Read, 1959). To follow a normal growth pattern, Hymenolepis citelli and H. diminuta both require carbohydrates. H. nana, however, is affected by carbohydrate deficiency only in the early stages of its development in the rodent host (Read et al., 1958). This latter is probably due to the fact that the growth

of H. nana, unlike that of H. diminuta, ceases shortly after infecting the definitive host (Schiller, 1959).

Reid (1940, 1942) observed that Raillietina cesticillus strobila detach and are shed when its bird host is subjected to a carbohydrate-deficient diet. The glycogen content of the worm is reduced as well. Levine (1938) observed the same to be the case with other davaineid cestodes. On the other hand, the carbohydrate content of the host diet was found by Hopkins (1950; 1952) to have no effect on adult Schistocephalus solidus. He reported that the rate of glycogen depletion was the same in the gut of the bird host subjected to carbohydrate-deficient diets, as in the case of worms cultured in vitro in a balanced salt solution free of carbohydrates.

The rate of carbohydrate metabolism in cestodes is relatively high. Cestodes store carbohydrates, mostly in the form of glycogen (von Brand, 1950, 1952, 1960; Smorodintsev, 1935a;b). The rates of glycogenesis (Daugherty, 1956; Fairbairn et al., 1961; Markov, 1939; Read and Rothman, 1957), utilization of exogenous carbohydrates (Phiffer, 1960) and of endogenous carbohydrates and production of acid in vitro (Agosin et al., 1957 ; von Brand, 1933; von Brand et al., 1961; Ivanov, 1950; Read, 1956; Reid, 1942 and Smyth, 1947a) are all quite high.

Distribution of Endogenous Carbohydrates

Many investigators have studied the histochemical distribution of glycogen in different cestodes (Brault and Loeper, 1904a; b; Busch, 1905a; b; Ortner-Schonbach, 1913; Coutelen, 1931a; b; von Brand, 1933; Smorodintsev, 1936b; Smyth, 1947b, 1949; Yamao, 1952c; Hedrick and Daugherty, 1957; Rybicka, 1960;

Kilejian et al., 1961). Species investigated have included Anoplocephala plicata, A. perfoliata, A. mamillana, Caliobothrium coronatum, Caryophyllaeus mutabilis, Hymenolepis diminuta, Raillietina cesticillus and Echinococcus granulosus.

It is noteworthy that the distribution of glycogen in all species investigated followed a general pattern. No glycogen was detected in the cuticle and very little was found in the subcuticular layer. The parenchyma had most of the glycogen deposits. However, very little glycogen was detedted in the scolex parenchyma in each case. There were heavy glycogen deposits in the bothridia of Calliobothrium coronatum and in the suckers of Anophocephala plicata, A. perfoliata and A. mamillana. In Anoplocephala spp., glycogen deposits were found around the muscle bands, while the sucker muscles were devoid of glycogen. The reproductive, nervous and excretory systems were also devoid of glycogen. Cestode eggs sometimes contain small amounts of glycogen. The glycogen stored in Hymenolepis diminuta and Raillietina cesticillus was demonstrated to vary with the amount of carbohydrates ingested by the host (Read, 1949b; Reid, 1942). In the case of starvation, glycogen was depleted from the parasite to a large extent.

In well-fed birds, the mean weight of glycogen in Raillietina cesticillus was 4.6 per cent of the wet weight of the worm. In birds starved for 24 hours, however, the mean glycogen weight dropped to 0.25 per cent of the wet weight (Reid, 1940, 1942). A similar drop in glycogen reserve has been demonstrated in the case of Hymenolepis diminuta in starved rats (Goodchild, 1961a; Read 1949b). When carbohydrates are given orally to such rats, a high rate of glycogen resynthesis is noted (Read, 1955).

Glycogenesis has been detected *in vitro* in starved cestodes put in media containing monosaccharides (Daugherty, 1956, Fairbairn *et al.*, 1961; Markov, 1939; Read, 1956, 1957b). For example, in *Eubothrium rugosum* and *Triaenophorus nodulosus* adults and larvae of *Ligula* and *Dibothriocephalus latus* incubated in balanced salt solutions, a drop in the glycogen content was noted in all species. However, glycogenesis occurred in each of the worms when carbohydrates were added to the medium (Markov, 1939). The opposite, however, happens in the case of *Hymenolepis diminuta*. In this species, starvation seems to accelerate glycogenesis and glycogen deposits actually increase (Read and Rothman, 1957b). Glycogenesis takes place when *H. diminuta* is placed in a medium containing pyruvic acid (Daugherty, 1956). Carbon dioxide was found necessary for *in vitro* glycogenesis to take place in *H. diminuta* (Fairbairn *et al.*, 1961) and the nature of the buffer used also had an effect.

The glycogen content of the anterior portion of *Raillietina cesticillus* was found to differ from that of the posterior portion, (Reid, 1942) and glycogen content of *Hymenolepis diminuta* differs according to the number of worms, their locality and extent of growth (Fairbairn *et al.*, 1961; Roberts, 1961). The glycogen content of *H. diminuta* varied from segment to segment (Daugherty and Taylor, 1956; Read, 1956).

Cestode Utilization of Exogenous Carbohydrates

Read (1959) reported that tapeworms can utilize exogenous carbohydrates to a limited extent. At least nine species of cestodes, belonging to three different orders have been investigated as to the sugars they utilize. Eight of them were found to utilize significantly only galactose and glucose (Laurie, 1957; Read 1956). *Cittotaenia* sp. was observed to take up maltose

at a rate higher than that of glucose and galactose (Read and Rothman, 1958). The same was found in the case of Phyllobothrium foliatum (Laurie, 1961).

Cestodes so far investigated have not been shown to produce exogenous enzymes to take part in carbohydrate metabolism (Read and Simmons, 1963). They depend instead upon the absorbable monosaccharides present in the gut contents of their hosts.

The rate of fermentative metabolism in tapeworms has been found to be quite high. Fatty acids and lactic acid were detected in Moniezia expansa excretions when the worm was subjected to aerobic conditions and succinic acid, in addition, under anaerobic condition, (Alt and Tischer, 1931; von Brand, 1929, 1933). Taenia taeniaeformis larvae and adults have been shown to excrete fumaric, malic, acetic, succinic, lactic and pyruvic acids as well as glycerol and ethyl alcohol. The relative proportions of the secretions vary as to the stage of the life cycle, the species of the host, the media used, and the aerobic or anaerobic conditions to which the parasite is subjected (von Brand, 1961). Lactic acid, in relatively large amounts, has been detected in the excretions of Echinococcus granulosus, Oochoristica symmetrica and Hymenolepis diminuta. Excretions of lactic acid range from 37 to 98 per cent of its total acid excretions (Laurie, 1957). According to Fairbairn (1961), however, succinic and not lactic acid is the major acid excreted by Hymenolepis diminuta.

In six out of seven Tetraphyllidean species studied under aerobic conditions, lactic acid was found to be a minor product of carbohydrate metabolism. However, the contrary was noted for Phylloborthrium foliatum (Laurie, 1961).

Hymenolepis diminuta and Oochoristica symmetrica metabolism was inhibited at pH 7.0 but not at 7.4. Taenia taeniaeformis and Hymenolepis citelli metabolism was not influenced by bile salts at pH 7.0 or at 7.4. However, the latter stimulated endogenous sugar fermentation and acid excretion of Taenia crassiceps larvae (Rothman, 1959). Taenia taeniaformis carbohydrate metabolism in saline was found different from that in rat serum (von Brand, 1961).

Carbohydrate Intermediary Metabolism

Orembergen (1944) reported that glycerophosphatases were oxidized by Moniezia expansa. Hymenolepis diminuta was found to have enzymes associated with phosphorylative glycolysis, namely, hexokinase, aldolase, phosphohexomutase, phosphoglyceraldehyde dehydrogenase, lactic dehydrogenase, phosphorylase (Read, 1951c) and glycerophosphate dehydrogenase (Read, 1953). Inorganic phosphate was found associated with organic acid soluble elements of cestode tissue (Read and Simmons, 1963). Phosphorylative glycolysis took place when the co-factors, ATP, DPN and magnesium were supplied (Read, 1951b).

Read and Simmons (1953) reported that glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase were found in the cestodes Anoplocephala perfoliata, Moniezia benedeni, Dipylidium caninum, Taenia saginata and Taenia pisiformis. Ribose-5-phosphate and sedoheptulose formation was described in Hymenolepis diminuta homogenates (Rapport, personal communication, cited in Read and Simmons, 1963).

Phosphatase activity was demonstrated by histochemical methods. Quite an intense activity was demonstrated in association with the cuticle of a number of cestodes (Erasmus, 1957; Kilejian et al., 1961; Rogers, 1947; Yamao,

1952a, b). Phosphorylated substances were found to be hydrolysed by enzymes isolated from Moniezia expansa, Hymenolepis diminuta, Taenia taeniaeformis, Raillietina cesticillus, Echinococcus granulosus and Anoplocephela sp. (Agosin, 1959; Chowdhury et al., 1955; Erasmus, 1957a, b; Read, 1949a; Rogers, 1947; Yamao, 1952b, c, d).

Erasmus (1957b) associated phosphatase with sugar absorption. However, it was possible to inhibit phosphatase activity by molybdate ions, yet this did not affect glucose absorption. Phlorizin was found to inhibit glucose absorption without affecting phosphatase activity (Phifer, 1960c).

Oxidation of tricarboxylic cycle intermediate products was reported in several cestodes, succinate in Moniezia expansa (Grembergen, 1944), in Taenia pisiformis (Penniot et al., 1940), in Thysaniezia ovilla (Ivanov, 1945), in Hymenolepis nana (Goldberg, 1954) and in Hymenolepis diminuta (Read, 1952). Oxidation of fumerates and malates was demonstrated in homogenates of Hymenolepis diminuta (Read, 1953). Read (1963) found acetone extractable decarboxylase in H. diminuta. Malate was oxidized when minced with Moniezia expansa tissues (Grembergen, 1944). He found that malonate inhibits oxygen consumption of Moniezia expansa. On the other hand, Read (1956) observed that malonate stimulated oxygen consumption of H. diminuta. Heymeman and Voge (1960) reported succinic dehydrogenase in H. diminuta cysticercoids.

Carbohydrate Metabolism in Echinococcus granulosus

Agosin and Arvena (1959b) reported four hexokinases in Echinococcus granulosus scolices, specifically catalyzing glucose, fructose, mannose and glucosamine phosphorylation. They also demonstrated (1959a, 1960) several enzymes of the hexose monophosphate shunt in scolices cell-free extract and

observed that sedoheptulose was formed in the scolices. Deley and Vercruysse (1955) did not find glucose-6-phosphate dehydrogenase or gluconate-6-phosphate dehydrogenase in hydatid cyst fluid. Quite an intense phosphatase activity was associated histochemically with the cuticle of hydatid scolices (Kilejian et al., 1961). Agosin (1959) reported that phosphorylated substances are hydrolysed by enzyme isolated from Echinococcus granulosus scolices.

Schwabe et al. (1959) reported that the laminated membrane as well as the germinal membrane of the Echinococcus granulosus cyst are readily permeable to water, but permeability to non-electrolytes varied inversely as the molecular weight of the respective substance. He also demonstrated that hyaluronidase increased the permeability of the laminated membrane of the hydatid cyst wall to glucose and possibly mannital but not sucrose. Later, in 1961, Schwabe et al. showed that an acetylcholinesterase system is responsible at least in part, for the regulation of hydatid cyst wall permeability to glucose. More glucose was observed to pass out of the hydatid cyst into an isotonic Kreb-Ringer's solution when the parasite's acetylcholinesterase was inhibited by physostigmine or hexmethyl tetraphosphate. Malonate was found to inhibit oxygen consumption of E. granulosus scolices (Read and Simmons, 1963).

Several fatty acids, thought to be products of carbohydrate metabolism, were detected in hydatid cyst fluid (Coutelen, 1931c). However, no formic acid was detected in the cyst fluid. E. granulosus scolices were found to excrete pyruvic, lactic, succinic and acetic acid as well as small amounts of ethyl alcohol. Under anaerobic conditions, no pyruvic acid was excreted.

On the other hand, lactic acid was excreted in relatively large amounts in both aerobic and anaerobic conditions (Agosin, 1957).

It is clear from the above review that some successful *in vitro* culture work has been done on cestodes. Most significantly, adult *Hymenolepis diminuta* and *H. nana* have been reared from cysticercoids in complex nutritive media. Cysts with laminated membranes and primordia of brood capsules were reported when *Echinococcus granulosus* scolices were cultured in Parker's TC medium 199 plus bovine amniotic fluid. Segmentation and the formation of excretory system primordia were noted from other *in vitro* cultured *E. granulosus* scolices in complex media supplemented with dog intestinal extract.

Successful work has also been carried out on the absorption of nutrients by larval or adult worms. *In vitro* utilization of carbohydrates has also been investigated to a limited extent. The effect of enzymes and drugs on carbohydrate absorption had been partially studied also, for instance, phlorizin was found to inhibit glucose absorption by adult *Hymenolepis diminuta*. The distribution of glycogen in adult and larval *H. diminuta* and *E. granulosus* has also been studied to some degree.

In the current study, the effect of various factors on vesiculation, evagination and survival of cultured *E. granulosus* scolices was investigated. The distribution and depletion of glycogen in scolices cultured in various media for various periods was also studied, with or without different concentrations of various monosaccharides, sometimes in the presence of certain drugs or enzymes.

MATERIALS AND METHODS

There are three essentials for successful *in vitro* culture, namely, maintenance of sterile conditions throughout the investigation, use of a suitable medium and the periodic removal of metabolic waste products from the vicinity of the cultured organisms. Avoiding bacterial, viral or fungal contamination is a major issue in tissue culture work. They are detrimental to the cultured organisms through competition for vitamins and nutritive elements, through elaboration of toxic metabolic products or by lowering the pH of the medium to a lethal point.

Various attempts were made to mimic nutrient and environmental factors effecting growth and survival of *Echinococcus granulosus*, together with efforts to remove the metabolic products periodically.

The materials used in this study are listed below, followed by the design of each experiment.

Materials

A. Scolices were aseptically removed from sheep and cattle lung and liver hydatid cysts obtained from the Beirut and Tripoli abattoirs.

B. Glassware:

1. Screw-capped test tubes 16 x 150 mm (20ml) and 25 x 150 mm (50 ml) were used to collect hydatid cyst fluid with brood capsule and scolices and for *in vitro* culture purposes.
2. 15 ml. conical centrifuge tubes were used for embedding scolices.
3. 20 and 50 ml syringes, fitted with 15 or 16 gauge needles.

4. 200 ml, 160 x 150 mm square bottomed Kimax sampling screw capped bottles were used for culturing a monolayer of HeLa cells with scolices.

5. 4 ml Bijou, BTL, screw capped bottles were used for distribution of antibiotics.

6. 150, 500 and 1000 ml Corning pyrex filter flask, for Seitz filtration purposes, fitted with Seitz filters.

7. 200 and 500 ml staining dishes for carrying periodic acid Schiff staining with glass trays for carrying slides.

C. Chemicals:

1. Media

a. Parker's TC medium 199 was obtained from Difco Laboratories in dessicated 5 gm vials and in 10 times concentrated liquid in 5 ml and 50 ml vials.

b. Krebs-Ringer Solution: Prepared according to Umbreit, Burris and Stauffer (1957).

c. Hanks solution was prepared as described by Merchant et al., 1960.

d. Glucose, potassium chloride, sodium chloride (GKN), 10 times concentrated stock solution was prepared as follows: 20.0 gms glucose, 160.0 gms NaCl and 8.0 gms KCl were dissolved in 2000 ml of bidistilled water, then, 40 ml of 1% phenol red indicator were added. The solution was Seitz filtered stored at 4°C.

e. Rabbit serum: Blood was removed from the rabbit's heart. Six months old rabbits were used and 50 cc of blood were aseptically removed by 19 gauge needle from each rabbit's heart. The blood

was allowed to clot, the clot was broken by a sterile pipette, the serum was decanted and stored. 50 ml of rabbit blood rendered 20 ml of serum. The serum was inactivated at 60°C. for 35 minutes.

f. Growth medium was prepared as such: 65% by volume lactalbumin hydrolysate, 10% yeasteolate, 20% rabbit serum and 5% of 10% glucose were mixed and stored at - 20°C.

g. Lactalbumin hydrolysate was obtained from Difco Laboratories in powdered form.

h. Isotonic saline: 0.85% solution of NaCl was Seitz filtered and kept at 4°C. in screw capped sterile 200 ml sampling bottle.

i. HeLa cells were obtained frozen at -70°C. from the Naval Medical Research Unit (NAMRU-3) Cairo - U.A.R. through the Bacteriology and Virology Department, washed with alkaline GKN (pH 7.8) and were allowed to thaw, then 0.2 ml of 0.05% trypsin was added and the pH was adjusted to 7.8, the mixture was incubated at 37°C. for 10 minutes, then the cells were transferred to 200 ml, screw capped, square bottomed, sterile sampling bottles, 12 ml of growth medium was then added, the pH was adjusted to 7.4 and the bottles were incubated resting on the broadest side for 2-3 days until a monolayer was detected.

2. Reagents:

a. Phenol red indicator was obtained from Difco Laboratories in powdered form and used at a concentration of 10 mgm. per 1 ml of medium.

b. 2.8% $NaHCO_3$ was prepared in 5 ml amounts in screw capped test

tubes and was sterilized by autoclaving at 121°C. 15 lbs. pressure for 20 minutes.

c. Trypsin: 0.05% trypsin was prepared from the powdered, c.p. form obtained from Difco Laboratories.

3. Carbohydrates:

a. IM glucose, IM galactose and IM glucosamine were separately prepared and Seitz filtered, distributed each in 5 ml amounts in screw capped sterile 10 ml test tubes, and were stored at -20°C.

4. Enzymes and Chemicals:

a. ATP: Disodium salt (Na_2 H_2 ATP. 4 H_2O). Inorganic phosphate 0.04 micromols/mg. Organic phosphorous 99% or more as ATP. Processed for removal of inhibitors, obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

b. Hyaluronidase: Approximately 300 U.S.P. u/mgm obtained from Nutritional Biochemical Corporation, Cleveland, Ohio stored at 4°C. in a dry container with $CaCO_3$.

c. Malonic Acid: Disodium salt obtained from Eastman Organic Chemicals, Distillation Products Industries, New York, U.S.A.

d. Ammonium molybdate: from 'Analar',(R) Hopkin and Williams Ltd. Chadwell Health, Essex - England (NH_4) 6Mo7024, $4H_2O$ (mol. wt. 1236.0); Mo03 being 81.0-83.0%.

5. Physostigmine salicylate from May and Baker Ltd. Dagenham, England.

D. Staining Equipment:

1. Merck xylolum.

2. Absolute ethyl alcohol from Mack Laboratories.

3. 95% ethyl alcohol, from which 80%, 70% and 50% alcohol were prepared.

4. Schiff reagent was prepared after Ritter and Oleson (1950).

   a. Basic fuchsin in powdered form obtained from Spinco Division of Beckman Industries Inc. California, U.S.A.

   b. 2N-HCl.

   c. Potassium metabisulfite in powdered form.

   d. Powdered norit.

5. Powdered periodic acid was obtained from Hopkin and Williams Ltd. England, with 0.5% sulfated and 95% HIO_4. $2H_2O$ (mol. wt. 228.0).

6. Potassium metabisulfite rinse was prepared as follows: 5 ml of 10% potassium metabisulfite, 5 ml of IN HCl, and 100 ml distilled water were mixed and stored at 4°C.

7. Tissuemat(R) paraffin with melting point of 52.8°C. was obtained from Fisher Scientific Co. U.S.A.

8. Carnoy fixative was composed of 60% volume absolute ethyl alcohol, 30% Chloroform and 10% glacial acetic acid.

9. Meyer's egg albumin was prepared from egg albumin and glycerine in equal volumes.

10. Harleco synthetic resin was used for mounting.

11. Molt diastase in powdered form was obtained from Difco Laboratories.

E. Antibiotics:

   1. Streptomycin sulfate B.P. - 1 gram base, was obtained from Dista Products Limited, Liverpool, England.

   2. 'Solupin', Benzylpenicillin B.P. (Sodium salt), 1000,000 i.u. per vial was obtained from Dista Products Limited, Liverpool, England.

F. Marking apparatus: Mash 77 marking pen was used for labelling glassware.

G. Equipment:

1. Leybold vaccuum pump (1/4 h.p.) for Seitz filtration.
2. Seitz filter.
3. One direction shaker was obtained from A.S. Aloe and Co. with a regulator rheostat from the Supreme Electric Co. U.S.A.
4. The inverted microscope was obtained from Plankton Microscope Co.
5. Rotary Microtome (manufacturer unknown).
6. Counting Chamber (manufacturer unknown).

Methods

Sterile conditions were maintained throughout the experiments. Tissue culture tubes and bottles were thoroughly washed with detergent, soap and water, rinsed twice with distilled water, and sterilized by autoclaving at 121°C. and 15 lbs. pressure for 20 minutes.

Scolices were aseptically removed in the following manner: Cattle and sheep liver and lung cysts were washed with soap and rubbed with iodine. The hydatid cyst fluid with brood capsules and scolices was aspirated with 20 ml and 50 ml sterile syringes, fitted with 15 or 16 gauge sterile needles. A separate needle, syringe and 50 cc. sterile screw-capped tube were used for every cyst.

The number of scolices per millilitre was, then, determined in the following manner: The tube containing the scolices was shaken to obtain a homogeneous suspension and the number of scolices in a single drop was counted. The volume of the drop depended on the diameter of the lower opening of the pipette from which the drop was taken. The number of drops in a one ml. pipette was counted, and the volume of one drop was calculated. For example,

if the volume of the drop was found to be 0.05 ml., that is to day, 20 drops were counted out of the used 1 ml. pipette, then the number of scolices per drop was multiplied by 20, to determine the number of scolices in 1 ml. of scolex suspension.

The number of scolices per milliliter of scolex suspension was usually less than 1500. The hydatid cyst fluid was, hence, decanted in all cases to obtain the desired 1500 scolices per millilitre dilution. In all experiments, 4500 scolices were used in every culture tube or bottle. The supernatant hydatid cyst fluid was decanted in all tubes, except where scolices were cultured in it for control purposes.

Antibiotics were added to all cultures. Penicillin and streptomycin stocks were prepared as follows: One million international unit of Benzyl-penicillin powder were dissolved in 100 ml. of the desired medium and distributed in 2 ml. amounts in 4 ml. sterile screw-capped Bijou vials and stored in the deep freeze at -20°C.

Similarly 1 gram of streptomycin sulfate powder was dissolved in 100 ml. of the desired medium and distributed in 2 ml. amounts in the above mentioned vials, that is to say each vial had 5000 i.u. penicillin and 5 milligrams steptomycin per millilitre of stock medium. 200 i.u.' penicillin and 0.2 milligrams streptomycin were added to each millilitre of the culture medium, that is to say, one stock vial was added to 21 ml. of culture medium.

All culture tubes and bottles, except those containing HeLa cells were placed in a shaker adjusted at 80 agitations per minute. The culture and the tubes were incubated at 37°C. in a walk-in incubator.

Sterile, glass bidistilled water was used in all the experiments.

The pH of all culture media used was 7.4.

In experiments V and Vi, solutions containing drugs and enzymes were prepared immediately before starting the experiments.

The six experiments carried out were designed in the following manner:

EXPERIMENT I

In vitro maintenance and development of Echinococcus granulosus scolices.

Scolices were cultured in 5 ml. of each of the following media:

1. Parker's TC medium 199 (Parker et al., 1950).
2. Parker's TC medium 199 supplemented with rabbit serum in 4:1. proportion.
3. Parker's TC medium 199 supplemented with rabbit serum and HeLa cells.
4. 'Growth medium' supplemented with rabbit serum (see materials).
5. Hanks Balanced salt solution.

Each of the above media was changed daily. Percentage viability, evagination and vesiculation were calculated every 5 days, using an inverted microscope.

EXPERIMENT II

Glycogen distribution in fresh and cultured E. granulosus scolices:

Scolices were cultured in 5 ml. of each of the following media:

1. Hydatid cyst fluid.
2. Kreb-Ringer's solution (KR).

3. 0.154 M glucose in KR.
4. Parker's TC medium 199.
5. 'Growth medium' plus rabbit serum.
6. Lactalbumin hydrolysate.
7. Isotonic saline.
8. 0.154 M glucose in isotonic saline.

Scolices were fixed in Carnoy fixative when fresh, again after 5 days culture in media 1-8, after 10 days culture in media 1-5 and after 15 days culture in the first three media. Then, the fixative was decanted, absolute alcohol was changed twice, and the scolices were cleared thrice in xylol. Each of the above changes lasted 15 minutes. The cleared scolices were then infiltered by paraffin at 55°C for 2 hours and embedded in conical 16 ml. centrifuge tubes. The paraffin was allowed to solidify at room temperature. The test tubes were broken and the conical blocks were attached to 3 x 2 x 1 cm. labelled wooden cuboids.

The scolices embedded in paraffin were sectioned with a rotating microtome in 7 u sections. The sections were placed on Meyer's albumin-wetted slides, which were placed on a warm platform electrically adjusted at 52 to 54°C. for 2 hours. From each block, 2 slides were prepared, one of which was deparaffinized and then digested in 0.5% malt diastase for 18 hours at room temperature (19 to 22°C.) and the other was used as a control.

After deparaffinization in xylol and hydration, the experimental and control sections were placed in the same staining container. The staining sequence was: 0.5% periodic acid for 10 minutes, distilled water for 5 minutes, Schiff reagent for 30 minutes, followed by potassium metabisulfite and IN HCl

rinse for 10 minutes and running tap water for 15 minutes. The sections were, then, dehydrated in 50%, 70%, 80%, 95% and absolute alcohol, cleared thrice in xylol, and mounted in Harleco synthetic resin.

EXPERIMENT III

In vitro utilization of monosaccharides by E. granulosus scolices.

Scolices were cultured for 5 days in 5 ml. of each of the following media:

1. Hydatid cyst fluid.
2. Kreb-Ringer's solution (KR).
3. 0.154 M glucose in KR.
4. 0.154 M galactose in KR.
5. 0.154 M glucosamine in KR.
6. 0.154 M glucose and 0.154 M galactose in KR.
7. 0.154 M galactose and 0.154 M glucosamine in KR.

Fresh and 5-day cultured scolices in the above media were fixed in Carnoy fixative, sectioned and stained as described above in Experiment II.

EXPERIMENT IV

Utilization of different concentrations of glucose by starved scolices.

Scolices were cultured in 5 ml. of the following media:

1. Hydatid cyst fluid for 5 days.
2. Hydatid cyst fluid for 10 days.
3. Kreb-Ringer's solution (KR) for 5 days.
4. KR for 10 days.
5. KR for 5 days, then, this was decanted and replaced by 5 ml. of

0.04 M glucose in KR for 5 more days.

6. KR for 5 days, then, this was decanted and replaced by 5 ml. of 0.1 M glucose in KR for 5 more days.
7. KR for 5 days, then, this decanted and replaced by 5 ml. of 0.3 M glucose in KR for 5 more days.

Fresh, as well as, the above cultured scolices were placed in Carnoy fixative and stained as described in Experiment II.

EXPERIMENT V

The effect of certain enzymes and drugs on glycogen distribution and depletion in scolices cultured in Kreb-Ringer's solution (KR) with or without 0.154 glucose.

Scolices were cultured for 5 days in 5 ml. of each of the following media:

1. 0.5 mgm/ml. hyaluronidase in KR.
2. 0.5 mgm/ml. hyaluronidase in 0.154 M glucose in KR.
3. 5 mgm/ml. hyaluronidase in KR.
4. 5 mgm/ml. hyaluronidase in 0.154 M glucose in KR.
5. 5 mgm/ml. ATP in KR.
6. 5 mgm/ml. ATP in 0.154 M glucose in KR.
7. 25 mgm/ml. ATP in KR.
8. 25 mgm/ml. ATP in 0.154 M glucose in KR.
9. 5 mgm/ml. malonic acid in KR.
10. 5 mgm/ml. malonic acid in 0.154 M glucose in KR.
11. 25 mgm/ml. malonic acid in KR.

12. 25 mgm/ml. malonic acid in 0.154 glucose in KR.
13. 2 mgm/ml. physostigmine salicylate in KR.
14. 2 mgm/ml. physostigmine salicylate in 0.154 glucose in KR.
15. 10 mgm/ml. physostigmine salicylate in KR.
16. 10 mgm/ml. physostigmine salicylate in 0.154 M glucose in KR.
17. 5 mgm/ml. ammonium molybdate in KR.
18. 5 mgm/ml. ammonium molybdate in 0.154 glucose in KR.
19. 25 mgm/ml. ammonium molybdate in KR.
20. 25 mgm/ml. ammonium molybdate in 0.154 glucose in KR.
21. Hydatid cyst fluid (control).

Fresh and cultured scolices were fixed and stained as described in Experiment II.

EXPERIMENT VI

The effect of certain drugs and enzymes on glycogen distribution and depletion in glucose, KCl and NaCl medium (GKN).

Scolices were cultured for 5 days in the following media:

1. 5 mgm/ml. hyaluronidase in KCl and NaCl (KN).
2. 5 mgm/ml. hyaluronidase in GKN.
3. 25 mgm/ml. ATP in KN.
4. 25 mgm/ml. ATP in GKN.
5. 25 mgm/ml. malonic acid in KN.
6. 25 mgm/ml. malonic acid in GKN.
7. 10 mgm/ml. physostigmine salicylate in KN.
8. 10 mgm/ml. physostigmine salicylate in GKN.

9. 25 mgm/ml. ammonium molybdate in KN.

10. 25 mgm/ml. ammonium molybdate in GKN.

Fresh and cultured scolices were fixed and stained as in Experiment II.

RESULTS

In vitro Maintenance and Development of Echinococcus granulosus scolices

In vitro culture was carried out in duplicate in 5 different media, 3 of which contained serum, namely, Parker's TC medium 199 plus rabbit serum, Parker's TC medium 199 plus rabbit serum in the present of HeLa cells, and growth medium plus rabbit serum. The other two media were purely synthetic, namely Parker's TC medium 199 and Hank's solution.

A survival time of 30 to 35 days was observed in Hank's solution, 50 to 55 days in Parker's TC medium 199, 70 to 75 days in Parker's TC medium 199 plus rabbit serum, 84 days in Parker's TC medium 199 plus rabbit serum in the presence of HeLa cells, and 65 to 70 days in growth medium plus rabbit serum.

As shown in figure 3, Echinococcus granulosus scolices lived longer in each of the three media containing rabbit serum than in any other purely synthetic medium. A maximum survival time of 84 days was obtained in Parker's TC medium 199 supplemented with rabbit serum in the presence of HeLa cells.

Vesiculation of the scolices in the 5 media was also investigated. Five vesicular forms were noted, the vesiculation of the entire unevaginated scolex (fig. 13), the formation of an anterior vesicle (figs. 14, 15), the formation of a posterior vesicle (fig. 16), the formation of an anterior and a posterior vesicle with a constriction in between (fig. 17), and the vesiculation of the entire evaginated scolex.

As seen in figure 1, vesiculation of the viable scolices was invariably slower in each of the three media containing rabbit serum than in either of

the other two purely synthetic media. In the former, total vesiculation took place after 45 to 60 days, whereas in the synthetic media, total vesiculation was observed after 20 to 30 days.

Vesicle formation in the cuticle was clearly noted in the scolex in all media. This is shown in figures 9 to 12. Vesicle formation was noted previously by Webster and Cameron in 1963.

The gradients of the graphs representing percentage vesiculation in media containing serum are in each case, less than the gradients of the graphs representing percentage vesiculation in media without serum (fig. 1). It can thus be concluded that serum retards vesiculation.

The effect of serum on evagination was also investigated. As shown in figure 2, total evagination of scolices cultured in media with rabbit serum occurred after 45 to 55 days, while it was noted in less than half that period in synthetic media. Evaginated scolices, as clearly demonstrated in figure 19, underwent rapid and powerful contractions along their entire body, a phenomenon that might be associated with locomotion.

Scolices, hence, were found to live longer in the presence of serum and HeLa cells. Serum was found to inhibit vesiculation and evagination.

Glycogen distribution in fresh and cultured scolices

Scolices were cultured in various media for various periods, the distribution of glycogen was noted in the parenchyma and suckers of fresh and cultured scolices.

It was noted that scolices stored glycogen, presumably for nutritive

purposes. Glycogen reserves were unevenly distributed in the scolex with most being detected in the parenchyma and around the muscular bands of the suckers (table 1, figs. 24, 25).

It is clear from tables 1 to 5 that parenchymal and acetabular glycogen was most abundant in fresh scolices and in 5-day cultured scolices in glucose, galactose or glucose and galactose together in Kreb-Ringer's solution in glucose in isotonic saline, in Kreb-Ringer's solution alone, in Hank's solution, in Parker's TC medium 199, in GKN medium, in growth medium plus rabbit serum, in lactalbumin hydrolysate, in hydatid cyst fluid, or in 1% glucose. Parenchymal, and to a lesser extent, acetabular glycogen were partially depleted in 5-day cultured scolices in isotonic saline or in glucosamine in Kreb-Ringer's solution.

Parenchymal and acetabular glycogen reserves were abundant in 10-day cultured scolices in hydatid cyst fluid, in Hank's solution and in glucose in Kreb-Ringer's solution. Parenchymal glycogen was partially depleted in 10 and 15-day cultures scolices in Kreb-Ringer's solution, and in 15-day cultured scolices in hydatid cyst fluid or in Hank's solution.

Parenchymal glycogen was noted more than acetabular glycogen in 5-day cultured scolices in growth medium plus rabbit serum (figs. 20, 21). Glycogen particles in the parenchyma of 5-day cultured scolices in hydatid cyst fluid were quite large (figs. 26, 27, 28). While in 5-day cultured scolices in Parker's TC medium 199, it was noted that the parenchymal glycogen was finely dispersed (figs. 29, 30). Figures 35 and 36 showed that acetabular glycogen was more than parenchymal glycogen in 5-day cultured scolices in Kreb-Ringer's solution and in 0.154 M glucose in Kreb-Ringer's solution. In the latter,

however, parenchymal glycogen was more dispersed. Acetabular glycogen was found much more than the parenchymal glycogen in 5-day cultured scolices in 0.154 M galactose in Kreb-Ringer's solution (figs. 39, 40). Both parenchymal and acetabular glycogen were quite abundant in 5-day cultured scolices in 0.154 M galactose in Kreb-Ringer's solution, the parenchymal glycogen, however, was dispersed (figs. 43, 44). Figures 45 and 46 showed intense glycogen accumulations in the suckers and dispersed glycogen in the parenchymal of 5-day cultured scolices in GKN medium. A similar condition was seen in figures 47 and 48 in 5-day cultured scolices in lactalbumin hydrolysate, an evaginated scolex was demonstrated. In 5-day cultured scolices in 0.154 M glucose in isotonic saline, parenchymal glycogen was noted more than acetabular glycogen (figs. 51, 52). This was even more pronounced in figures 53 and 54, in 5-day cultured scolices in 1% glucose. In 10-day cultured scolices in Parker's TC medium 199, parenchymal glycogen appeared to be accumulated in larger particles than in the 5-day cultured scolices. Figures 22 and 23 demonstrated that parenchymal glycogen in 10-day cultured scolices in growth medium plus rabbit serum was substantially depleted when compared with 5-day cultures. Glycogen in the parenchyma of 10-day cultured scolices in 0.154 M glucose in Kreb-Ringer's solution appeared as small dispersed particles. The acetabular glycogen was partially depleted (figs. 37, 38).

In experiment IV, scolices were starved for 5 days in Kreb-Ringer's solution, and then transferred to media with 0.04 M, 0.1 M, 0.154 M and 0.3 M glucose in Kreb-Ringer's solution. Table 6 denoted that there was no appreciable difference in the parenchymal or acetabular glycogen reserves in all media. Control scolices held in Kreb-Ringer's solution for 10 days showed partial depletion of parenchymal glycogen.

In all experiments, the cuticle of fresh and cultured scolices contained PAS positive undigestible material in all media, suggesting that it was something other than glycogen.

The above results suggested that glycogenesis might be taking place in all media except in isotonic saline and in glucosamine in Kreb-Ringer's solution. It was clear that scolices were able to utilize various concentrations of glucose from the culture media.

The effect of certain drugs or enzymes on the viability, evagination, vesiculation and glycogen distribution in scolices

Scolices were exposed to 5 enzymes or drugs, namely, ammonium molybdate, physostigmine salicylate, malonic acid, ATP and hyaluronidase. The effect of each of them on viability, evagination, vesiculation and glycogen depletion was investigated in four media, namely, Kreb-Ringer's solution with or without glucose and NaCl-KCl medium with or without glucose.

Only 2 out of the 5 drugs and enzymes showed a marked effect on viability, evagination or vesiculation, the rest had no marked effect. Tables 8 and 9 demonstrated that physostigmine salicylate was lethal, only 3.5 to 12.5% of the scolices were viable by the fifth day. Ammonium molybdate however was found to accelerate vesiculation and evagination.

There was no histochemical evidence that the drugs and enzymes except hyaluronidase had any effect on glycogen distribution or depletion (tables 6, 7). Two observations were, however, noteworthy. The first was that scolices exposed to hyaluronidase had bigger glycogen particles in the parenchyma than any other cultured scolices. The second was that dead scolices in all

cultures had undigestible PAS positive material in their parenchyma.

Scolices cultured in GKN medium looked healthier than those cultured in NaCl-KCl solution. By the 5th day the percentage of the living scolices in the former was greater than that in the latter.

From the above results, it was clear that serum increased the survival time of scolices cultivated in vitro, but retarded evagination and vesiculation. It was also noted that scolices stored glycogen mostly in the parenchyma and suckers. No glycogen was detected in the cuticle. Scolices utilized glucose and galactose but not glucosamine.

TABLE I

THE DISTRIBUTION AND DEPLETION OF GLYCOGEN IN *ECHINOCOCCUS GRANULOSUS* SCOLICES CULTURED *IN VITRO* HYDATID CYST FLUID FOR VARIOUS PERIODS AT 37°C.

Culture Medium and Duration of Culture	CONTROL			DIGESTED			NET GLYCOGEN		
	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle
Fresh Scolices n=8	+++	+++	+++	-	-	+++	+++	+++	-
Scolices cultured 5 days in HCF n=3	+++	+++	+++	-	-	+++	+++	+++	-
Scolices cultured 10 days in HCF n=4	+++	+++	+++	-	-	+++	+++	+++	-
Scolices cultured 15 days in HCF n=3	+	++	+++	-	-	+++	+	++	-

* Key for tables 1-7

+++ Intense PAS Stain

++

\+ Weak PAS Stain

\- Negative PAS Stain

n number of repeated experiments

KR Kreb-Ringer's solution

HCF Hydatid Cyst Fluid

GKN Glucose-NaCl-KCl medium

TABLE 2

THE DISTRIBUTION AND DEPLETION OF GLYCOGEN IN ECHINOCOCCUS GRANULOSUS SCOLICES IN VARIOUS NUTRITIVE MEDIA AND BALANCED SALT SOLUTIONS AT pH. 7.4 AND 37°C.

Culture Medium and Duration of Culture	CONTROL			DIGESTED			NET GLYCOGEN		
	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle
Hanks solution for 5 days n=5	+++	+++	+++	-	-	+++	+++	+++	-
Hanks solution for 10 days n=10	++	+++	+++	-	-	+++	++	+++	-
Hanks solution for 15 days n=1	++	+++	+++	-	-	+++	++	+++	-
Growth medium for 5 days n=3	+++	+++	+++	-	-	+++	+++	+++	-
Growth medium for 10 days n=1	++	++	+++	-	-	+++	++	++	-
Parker's TC medium 199 for 5 days n=3	+++	+++	+++	-	-	+++	+++	+++	-
Parker's TC medium 199 for 10 days n=1	++	+++	+++	-	-	+++	++	+++	-
OKN for 5 days n=3	+++	+++	+++	-	-	+++	+++	+++	-
NaCl and KCl solution for 5 days n=2	++	+++	+++	-	-	+++	++	+++	-
Isotonic saline for 5 days n=5	+	++	+++	-	-	+++	+	++	-
Isotonic saline plus 0.154 M glucose for 5 days n=3	+++	+++	+++	-	-	+++	+++	+++	-
Lactalbumin hydrolysate for 5 days n=2	+++	+++	+++	-	-	+++	+++	+++	-
1% glucose for 5 days n=2	+++	+++	+++	-	-	+++	+++	+++	-

TABLE 3

THE DISTRIBUTION AND DEPLETION OF GLYCOGEN IN *ECHINOCOCCUS GRANULOSUS* SCOLICES CULTURED *IN VITRO* IN KREB-RINGER'S SOLUTION AT 37°C. AND pH. 7.4.

Culture Medium and Duration of Culture	CONTROL			DIGESTED			NET GLYCOGEN		
	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle
Scolices cultured 5 days in KR n=6	+++	+++	+++	-	-	+++	+++	+++	-
Scolices cultured 10 days in KR n=4	++	+++	+++	-	-	+++	++	+++	-
Scolices cultured 15 days in KR n=5	+	++	+++	-	-	+++	+	++	-
Scolices cultured 5 days in KR + 0.15% glucose n=2	+++	+++	+++	-	-	+++	+++	+++	-
Scolices cultured 10 days in KR glucose n=2	+++	+++	+++	-	-	+++	+++	+++	-
Scolices cultured 15 days in KR + glucose n=1	++	+++	+++	-	-	+++	++	+++	-

TABLE 4

THE DISTRIBUTION AND DEPLETION OF GLYCOGEN IN ECHINOCOCCUS GRANULOSUS SCOLICES CULTURED IN VITRO FOR 5 DAYS IN VARIOUS MONOSACCHARIDES.

Culture Media Duration of Culture	CONTROL			DIGESTED			NET GLYCOGEN		
	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle
0.154 M glucose in KR n=3	+++	+++	+++	-	-	+++	+++	+++	-
0.154 M galactose in KR n=3	+++	+++	+++	-	-	+++	+++	+++	-
0.154 M glucosamine in KR n=3	++	++	++	++	+	++	-	+	-
0.154 M glucose and 0.154 M galactose in KR n=3	+++	+++	+++	-	-	+++	+++	+++	-
0.154 M galactose and 0.154 M glucosamine in KR n=3	++	++	+++	+	+	+++	+	+	-

TABLE 5

THE DISTRIBUTION AND DEPLETION OF GLYCOGEN IN ECHINOCOCCUS GRANULOSUS SCOLICES STARVED FOR 5 DAYS IN KREB-RINGER'S SOLUTION THEN CULTURED IN DIFFERENT CONCENTRATIONS OF GLUCOSE IN KREBS RINGER SOLUTION AT 37°C. AND pH. 7.4.

Culture Medium and Duration of Culture	CONTROL			DIGESTED			NET GLYCOGEN		
	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle
Scolices were starved for 5 days in KR, then were cultured in each of the following media:									
5 days in 0.04 M glucose n=3	+++	+++	+++	-	-	+++	+++	+++	-
5 days in 0.1 M glucose n=2	+++	+++	+++	-	-	+++	+++	+++	-
5 days in 0.3 M glucose n=3	+++	+++	+++	-	-	+++	+++	+++	-

TABLE 6

EFFECT OF VARIOUS CONCENTRATIONS OF ENZYMES OR DRUGS ON THE DISTRIBUTION AND DEPLETION OF GLYCOGEN IN E. GRANULOSUS SCOLICES CULTURED IN VITRO IN KREBS RINGER SOLUTION WITH OR WITHOUT 0.15% GLUCOSE WITHIN FIVE DAYS AT pH. 7.4 AND 37°C.

Enzyme or Drug Used	CONTROL			DIGESTED			NET GLYCOGEN		
	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle
I. In KR plus:									
0.5mgm/ml hyaloronidase n=2	+++	+++	+++	-	-	+++	+++	+++	-
5mgm/ml hyaloronidase n=2	+++	+++	+++	-	-	+++	+++	+++	-
5mgm/ml ATP n=2	+++	+++	+++	-	-	+++	+++	+++	-
25mgm/ml ATP n=2	++	++	+++	-	-	+++	++	++	-
5mgm/ml malonic acid n=2	+++	+++	+++	-	-	+++	+++	+++	-
25mgm/ml malonic acid n=2	+++	+++	+++	-	-	+++	+++	+++	-
2mgm/ml physostigmine n=2	+++	?	+++	++	?	+++	+	?	-
10mgm/ml physostigmine n=2	+++	?	+++	++	?	+++	+	?	-
5mgm/ml ammonium molybdate n=2	+++	+++	+++	-	-	+++	+++	+++	-
25mgm/ml ammonium molybdate n=2	+	+++	+++	-	-	+++	+	+++	-
II. In KR plus 0.15% M glucose plus: 0.5mgm/ml hyaloronidase n=1	+++	+++	+++	-	-	+++	+++	+++	-
5mgm/ml hyaluronidase n=1	+++	+++	+++	-	-	+++	+++	+++	-
5mgm/ml ATP n=1	+++	+++	+++	-	-	+++	+++	+++	-
25mgm/ml ATP n=1	++	+++	+++	-	-	+++	++	+++	-
5mgm/ml malonic acid n=1	+++	+++	+++	-	-	+++	+++	+++	-
25mgm/ml malonic acid n=1	+++	+++	+++	-	-	+++	+++	+++	-
2mgm/ml physostigmine n=2	+++	?	+++	++	?	+++	+	?	-
10mgm/ml physostigmine n=2	+++	?	+++	++	?	+++	+	?	-
5mgm/ml ammonium molybdate n=2	+++	+++	+++	-	-	+++	+++	+++	-
25mgm/ml ammonium molybdate n=2	+++	+++	+++	-	-	+++	+++	+++	-

TABLE 7

THE EFFECT OF VARIOUS ENZYMES AND CHEMICALS ON THE DISTRIBUTION AND DEPLETION OF GLYCOGEN IN *E. GRANULOSUS* SCOLICES CULTURED *IN VITRO* IN GKN SOLUTION OR WITHOUT 0.15% GLUCOSE WITHIN 5 DAYS AT pH. 7.4 AND 37°C.

Enzyme or Drug Used	CONTROL			DIGESTED			NET GLYCOGEN		
	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle	Parenchyma	Sucker	Cuticle
25mgm/ml ammonium molybdate in:									
KR without glucose n=2	++	+++	+++	-	-	+++	++	+++	-
KR with glucose n=2	+++	+++	+++	-	-	+++	+++	+++	-
10mgm/ml physostigmine									
KR without glucose n=2	+++	+++	+++	+	+	+++	++	++	-
KR with glucose n=2	+++	+++	+++	+	+	+++	++	++	-
25mgm/ml malonic acid									
KR without glucose n=2	+++	+++	+++	-	-	+++	+++	+++	-
KR with glucose n=2	+++	+++	+++	-	-	+++	+++	+++	-
25mgm/ml hyaluronidase									
KR without glucose n=2	+++	+++	+++	-	-	+++	+++	+++	-
KR with glucose n=2	+++	+++	+++	-	-	+++	+++	+++	-

TABLE 8

THE EFFECT ON CERTAIN ENZYMES OR DRUGS ON THE VIABILITY EVAGINATION AND VESICULATION OF ECHINOCOCCUS GRANULOSUS SCOLICES CULTURED IN VITRO FOR 5 DAYS AT 37°C.

Enzyme or Drug	Per cent Viability	Per cent Evagination	Per cent Vesiculation
I. In Krebs Ringer plus:			
5mgm/ml ammonium molybdate n=2	95%	44.5%	25%
25mgm/ml ammonium molybdate n=2	95%	86%	80.5%
2mgm/ml physostigmine n=2	12%	?	21%
10mgm/ml physostigmine n=2	6%	?	24%
5mgm/ml malonic acid n=2	88.5%	45%	22.5%
25mgm/ml malonic acid n=2	95%	49%	31.5%
5mgm/ml ATP n=2	91.5%	46%	25%
25mgm/ml ATP n=2	72%	43.5%	20.5%
0.5mgm/ml Hyaluronidase n=2	95%	44.5%	23.5%
5mgm/ml Hyaluronidase n=2	95%	55.5%	28.5%
II. In Krebs Ringer solution plus: 0.154 M glucose plus:			
5mgm/ml ammonium molybdate n=2	91%	34%	29%
25mgm/ml ammonium molybdate n=2	91.5%	48%	31.5%
2mgm/ml physistigmine n=2	3.5%	?	19.5%
10mgm/ml physostigmine n=2	7.5%	?	32.5%
5mgm/ml malonic acid n=1	83%	38%	27%
25mgm/ml malonic acid n=1	90%	46%	25%
5mgm/ml ATP n=1	90%	37%	23%
25mgm/ml ATP n=1	76%	40%	23%
0.5mgm/ml hyaluronidase n=1	93%	37%	29%
5mgm/ml hyaluronidase n=1	95%	31%	28%
III. Controls			
5 days in Krebs Ringer n=1	93%	43%	21%
5 days in KR plus 0.154 M glucose n=2	95%	41%	22.5%

TABLE 9

THE EFFECT OF CERTAIN ENZYMES OR DRUGS ON THE VIABILITY, EVAGINATION AND VESICULATION OF *ECHINOCOCCUS GRANULOSUS* SCOLICES CULTURED *IN VITRO* IN NaCl AND KCl WITH OR WITHOUT 0.154 M GLUCOSE WITHIN 5 DAYS AT 37°C. AND pH. 7.4.

Enzymes and Drugs		Per cent Viability	Per cent Evagination	Per cent Vesiculation
I.	In NaCl + KCl			
	25mgm/ml ammonium molybdate n=2	49%	40.5%	34.5%
	10mgm/ml physostigmine n=2	4.5%	?	18.5%
	25mgm/ml malonic acid n=2	52.5%	32%	22%
	25mgm/ml ATP n=2	28.5%	40%	27%
	5mgm/ml hyaluronidase n=2	36.5%	49.5%	23.5%
II.	In GKN			
	25mgm/ml ammonium molybdate n=2	77%	85%	32.5%
	10mgm/ml physostigmine n=2	12.5%	?	27.5%
	25mgm/ml malonic acid n=2	79.5%	30.5%	23.5%
	25mgm/ml ATP n=2	55.5%	49%	20%
	5mgm/ml hyaluronidase n=2	85.5%	32.5%	28%
III.	Controls			
	In NaCl + KCl n=2	32.5%	35%	27%
	In GKN n=2	88.5%	40%	34.5%

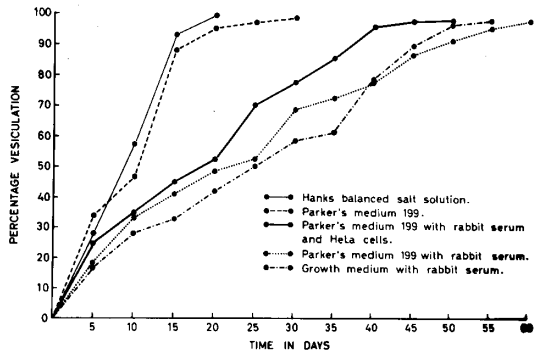

Fig. 1. THE EFFECT OF SERUM ON VESICULATION OF ECHINOCOCCUS GRANULOSUS SCOLICES CULTURED IN VITRO IN VARIOUS MEDIA WITH OR WITHOUT SERUM.

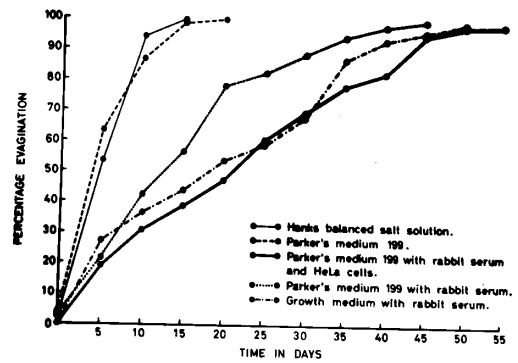

Fig. 2. PERCENTAGE EVAGINATION OF ECHINOCOCCUS GRANULOSUS SCOLICES CULTURED IN VITRO IN VARIOUS MEDIA WITH OR WITHOUT SERUM.

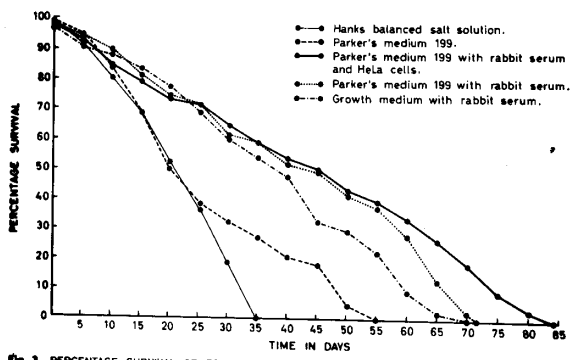

Fig. 3. PERCENTAGE SURVIVAL OF ECHINOCOCCUS GRANULOSUS SCOLICES CULTURED IN VITRO IN VARIOUS MEDIA WITH OR WITHOUT SERUM.

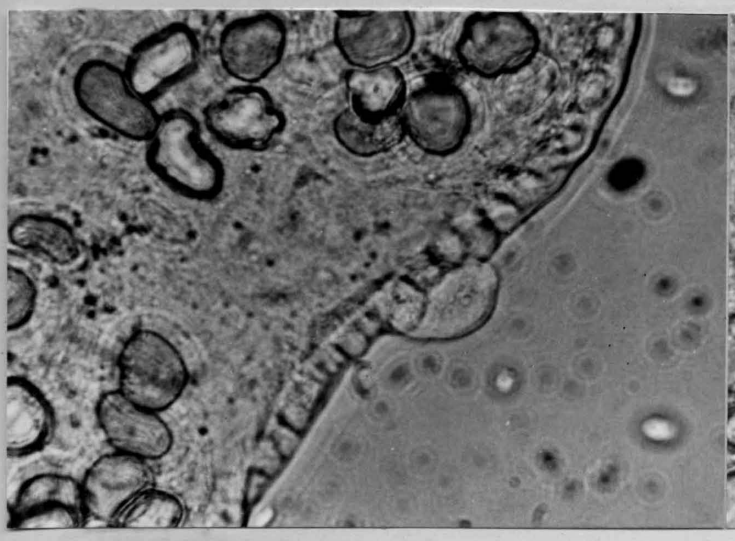

Fig. 4 (Mag. 1100x)

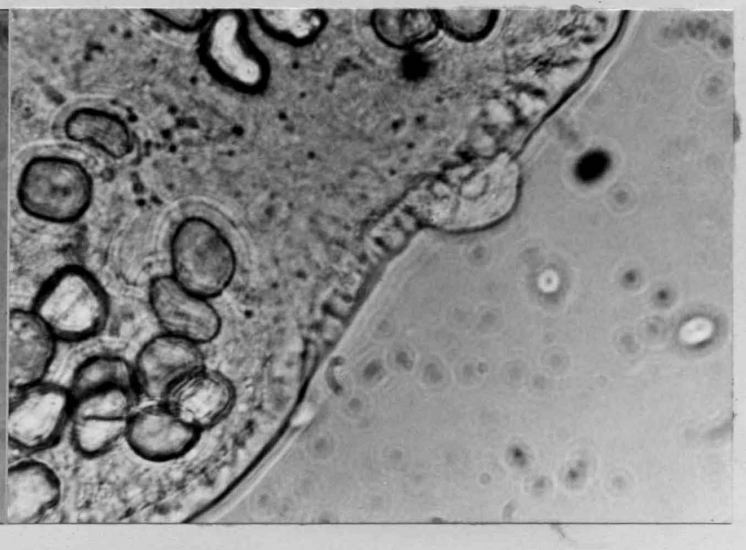

Fig. 5 (Mag. 1100x)

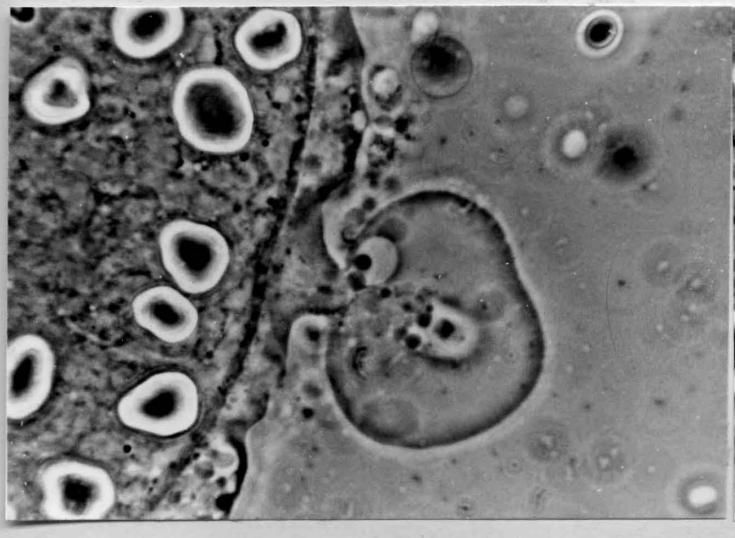

Fig. 6 (Mag. 1100x)

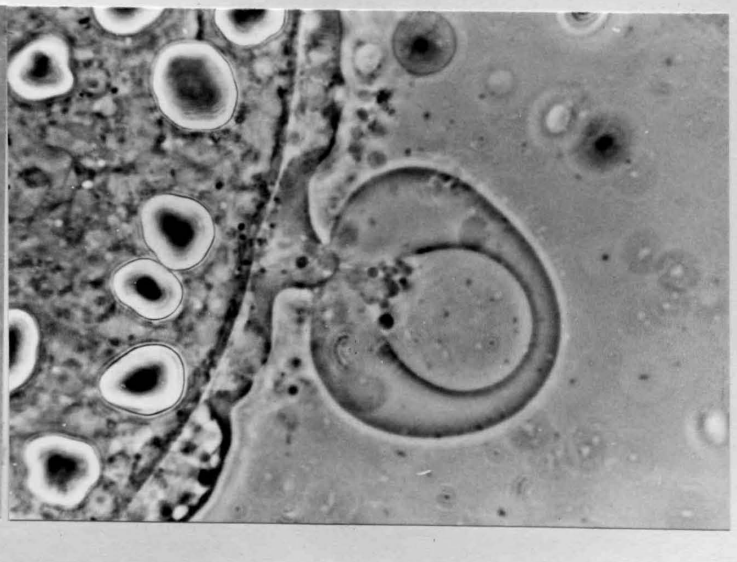

Fig. 7 (Mag. 1100x)

Figs. 4-7. Cuticular vesicle formation (5-day culture in TC medium 199).

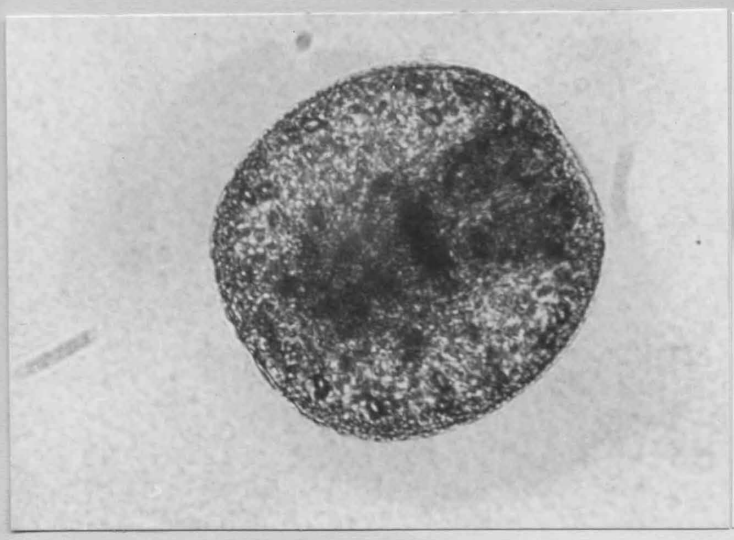

Fig. 8

The vesiculation of the entire unevaginated scolex. (15-day culture in medium 199). (Mag. 500x).

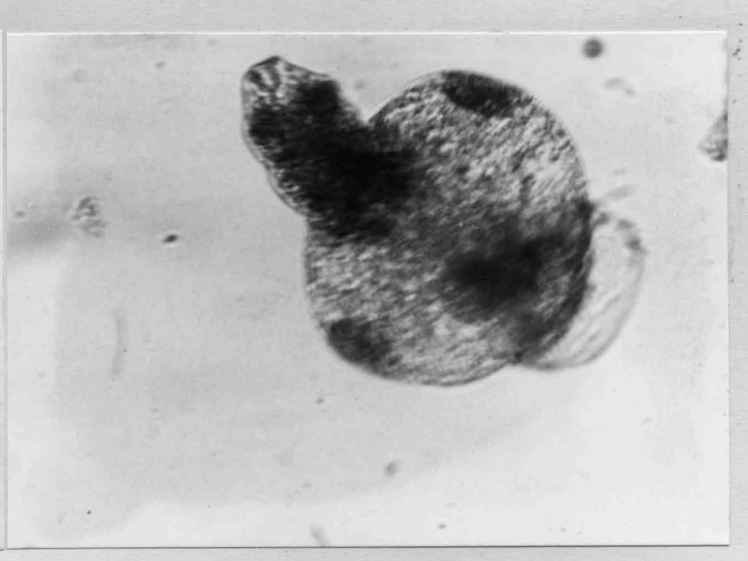

Fig. 9

The vesiculation of the anterior portion of the evaginated scolex. (35-day culture in medium 199). (Mag. 500x).

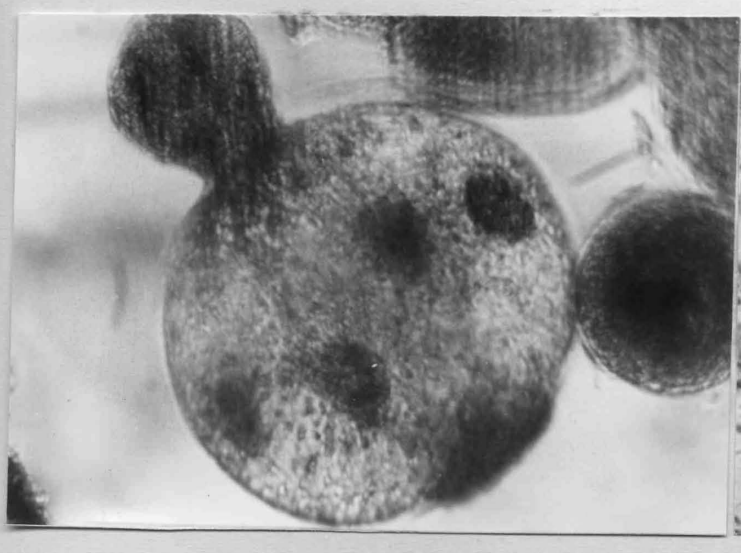

Fig. 10

Further vesiculation of the anterior portion of the evaginated scolex. (40-day culture in medium 199). (Mag. 500x).

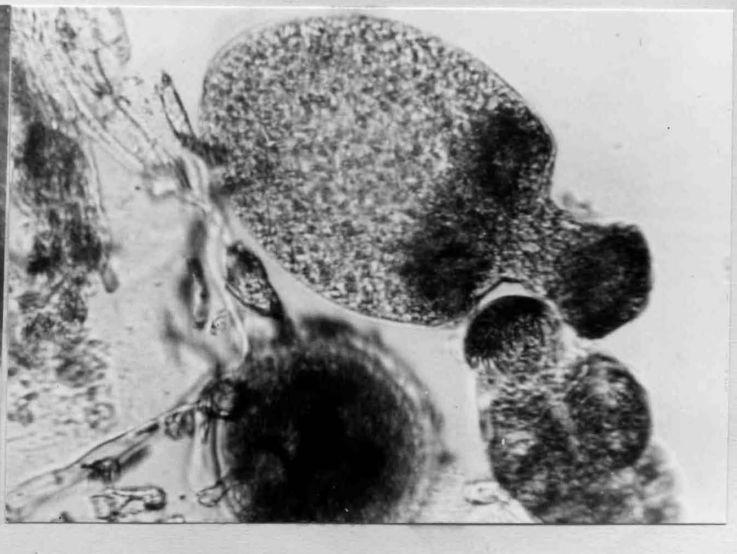

Fig. 11

The vesiculation of the posterior portion of the evaginated in medium 199). (Mag. 500x).

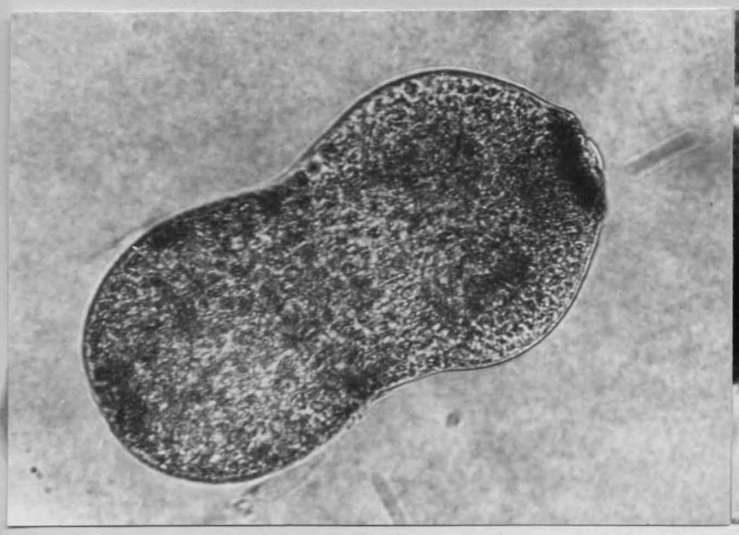

Fig. 12

The vesiculation of the entire evaginated scolex. (20-day culture in medium 199). (Mag. 500x).

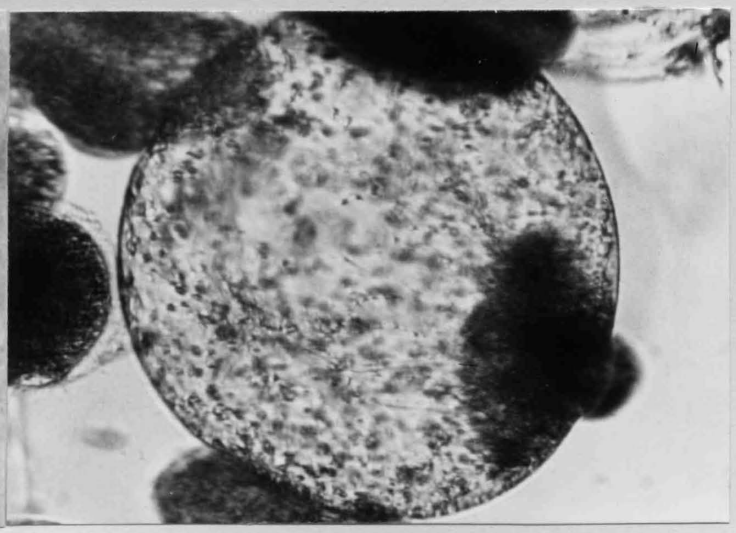

Fig. 13

Hydatid cyst formation. (45-day culture in medium 199). (Mag. 500x).

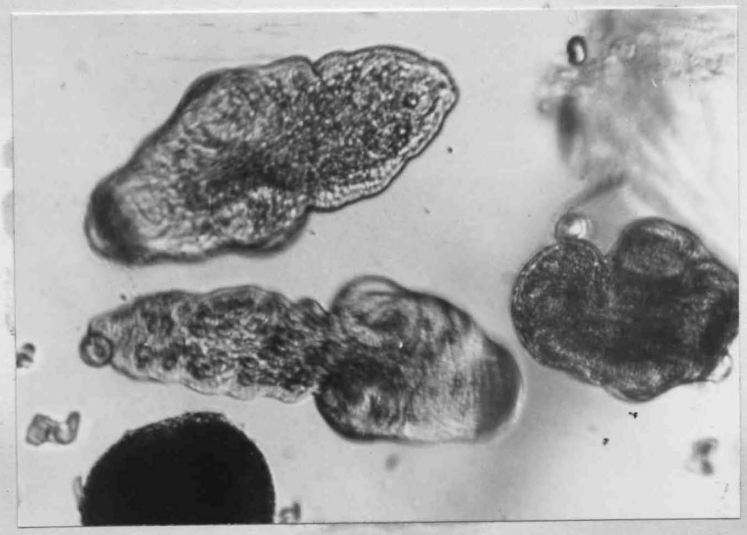

Fig. 14

Evaginated unvesiculated scolices. (15-day culture in medium 199). (Mag. 500x).

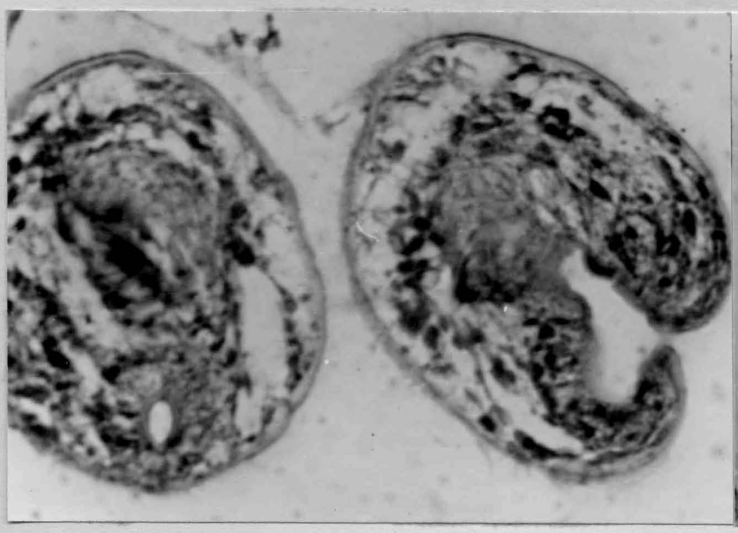

Fig. 15

Undigested sections
stained with PAS.
(Mag. 420x).

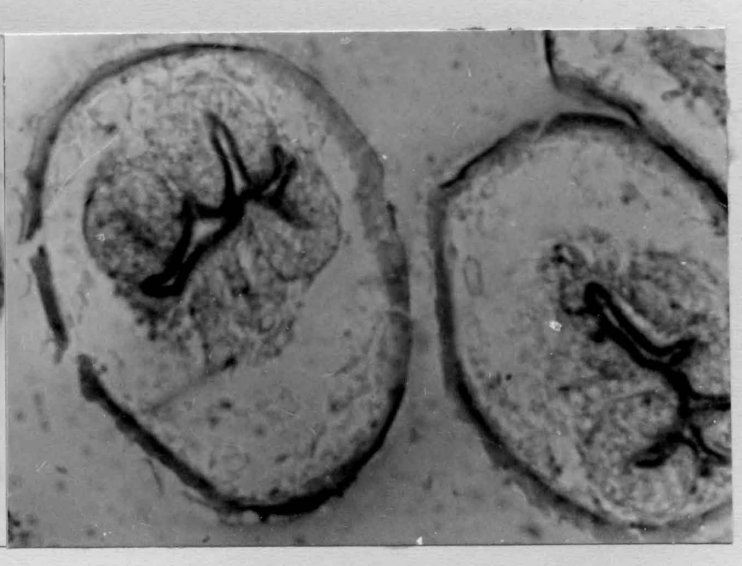

Fig. 16

Diastase digested sections
stained with PAS.
(Mag. 420x).

5-day cultured scolices in growth medium - rabbit serum

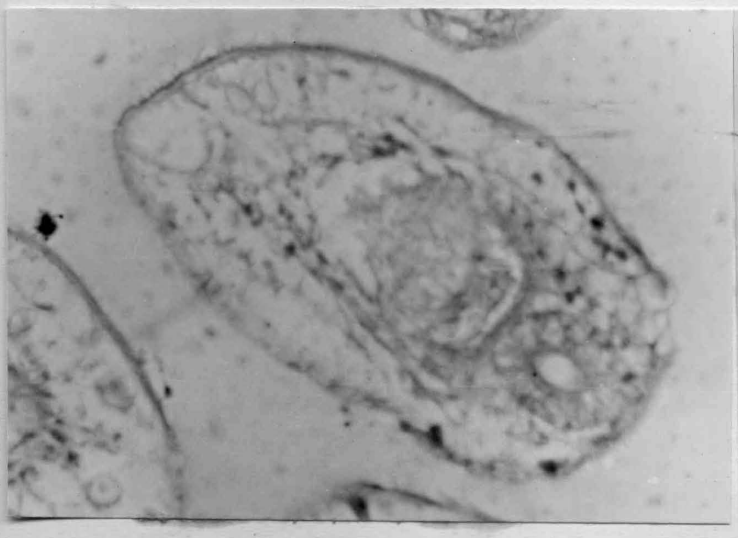

Fig. 17

Undigested sections
stained with PAS.
(Mag. 420x).

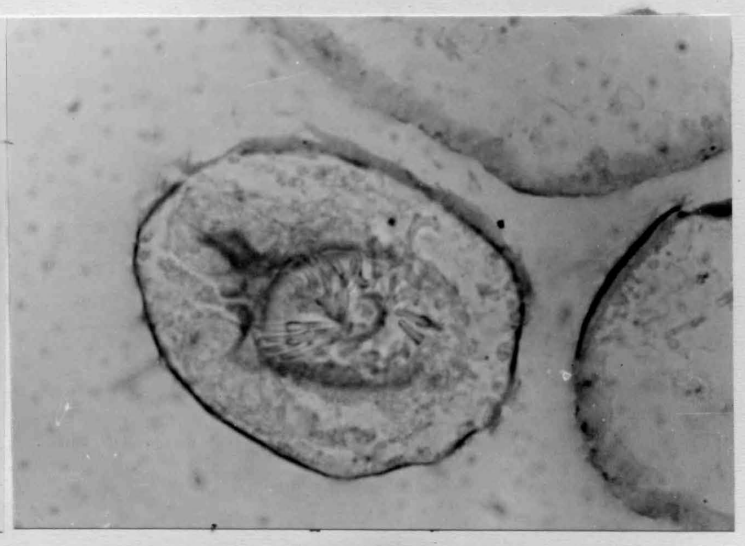

Fig. 18

Diastase digested sections
stained with PAS.
(Mag. 420x).

10-day cultured scolices in growth medium - rabbit serum

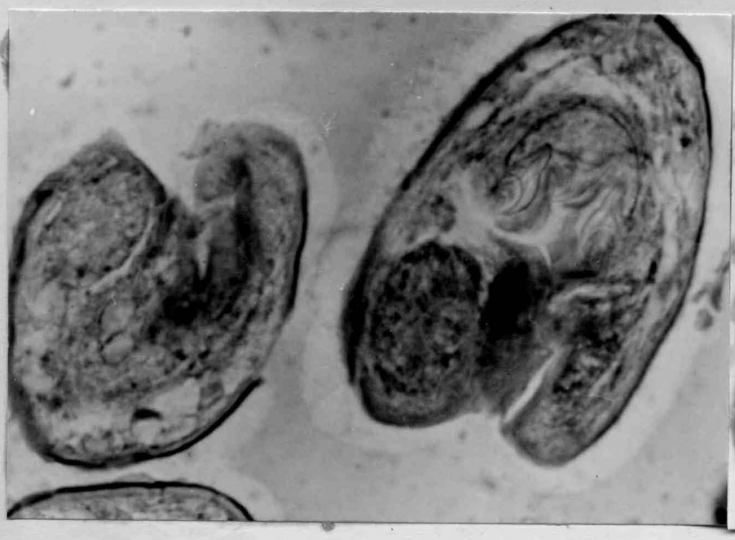

Fig. 19

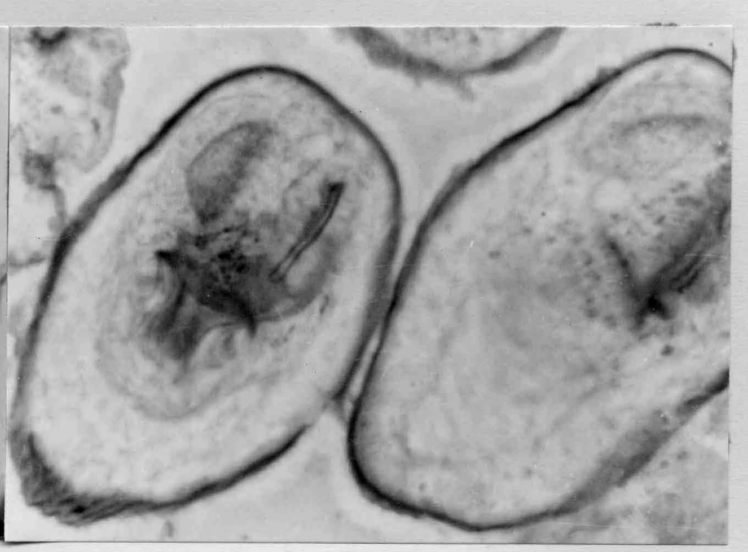

Fig. 20

Fresh scolices

Undigested sections stained with PAS. (Mag. 420x).

Diastase digested sections stained with PAS. (Mag. 420x).

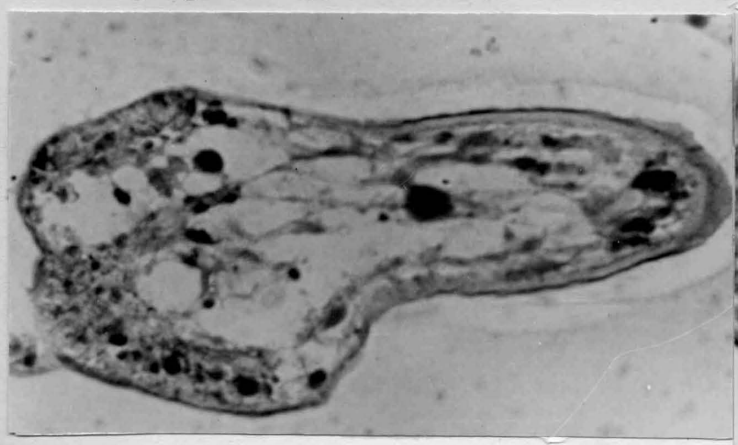

Fig. 21

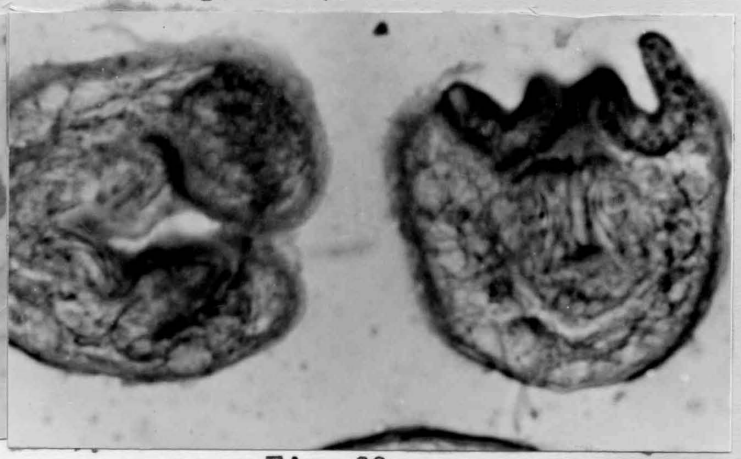

Fig. 22

5-day cultured scolices in hydatid cyst fluid. Undigested sections stained with PAS. (Mag. 420x).

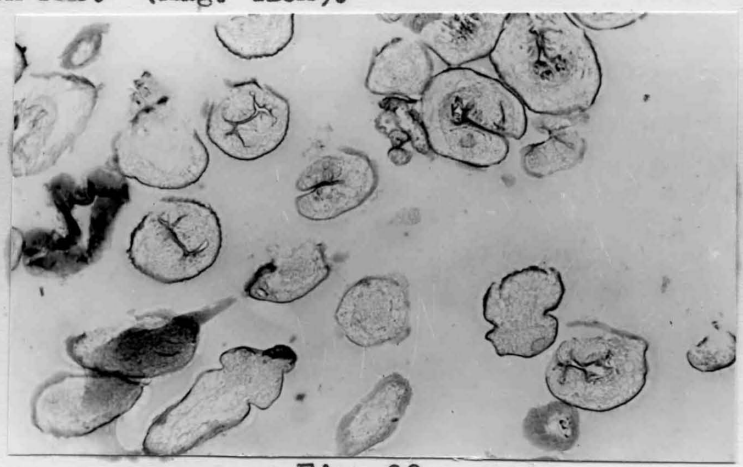

Fig. 23

5-day cultured scolices in hydatid cyst fluid. Diastase digested sections stained with PAS. (Mag. 100x).

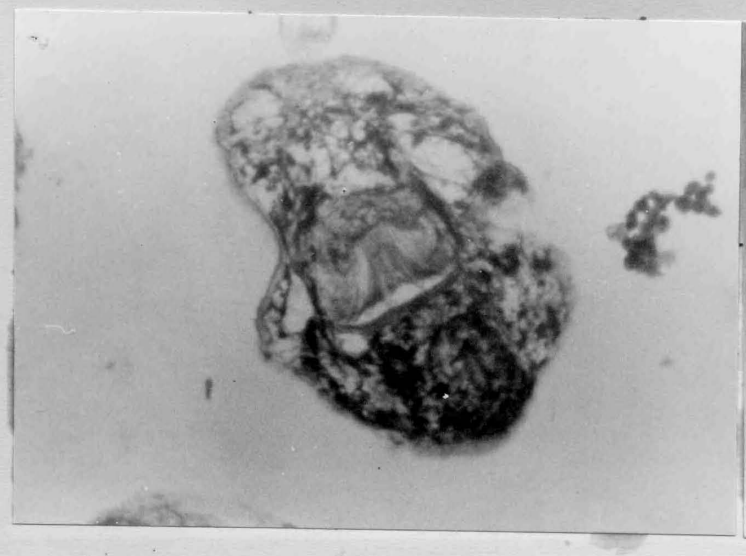

Fig. 24

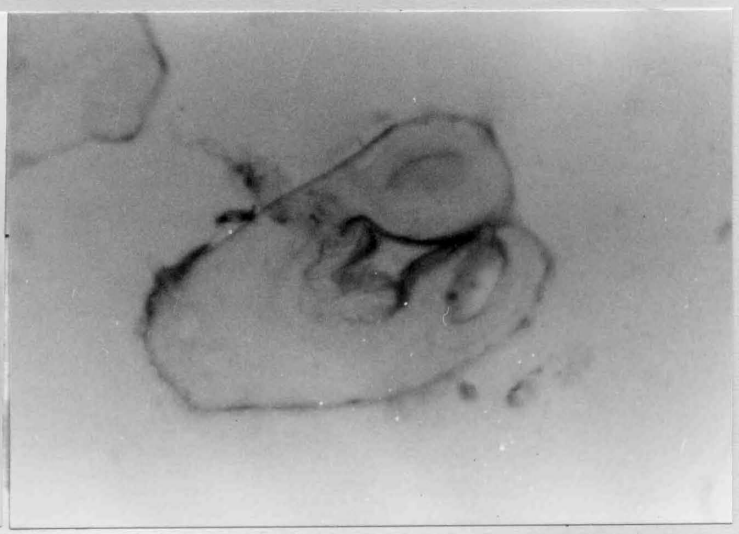

Fig. 25

5-day cultured scolices in medium 199

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

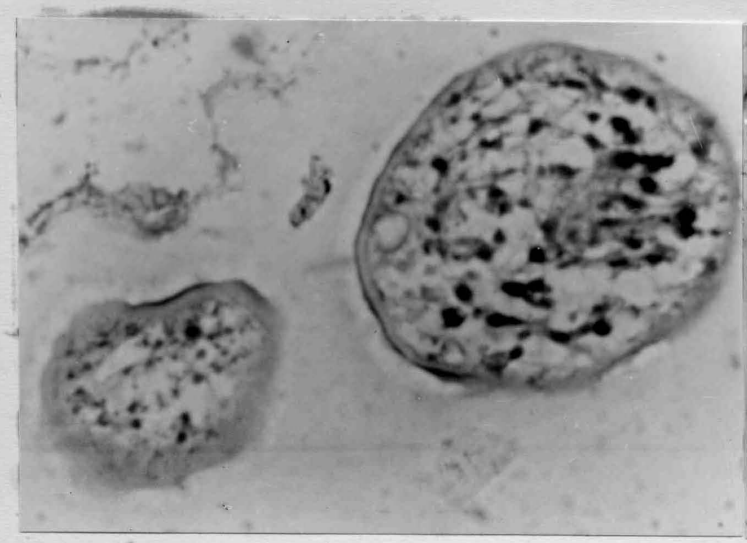

Fig. 26

Fig. 27

10day cultured scolices in medium 199

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

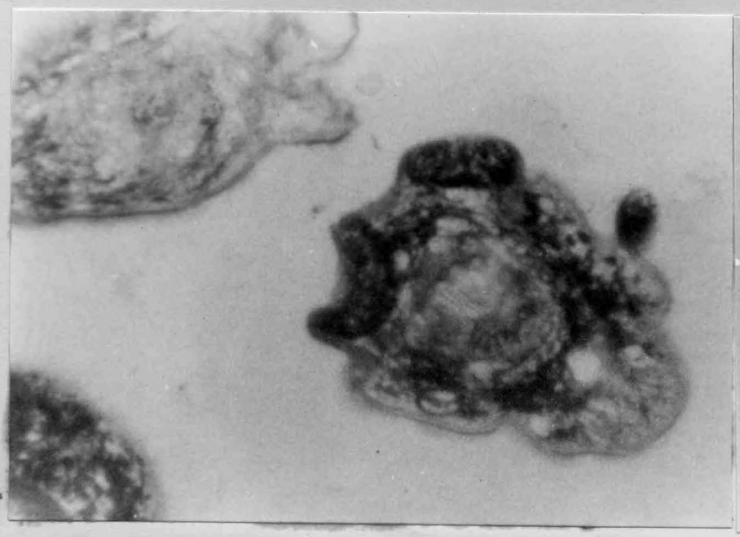

Fig. 28

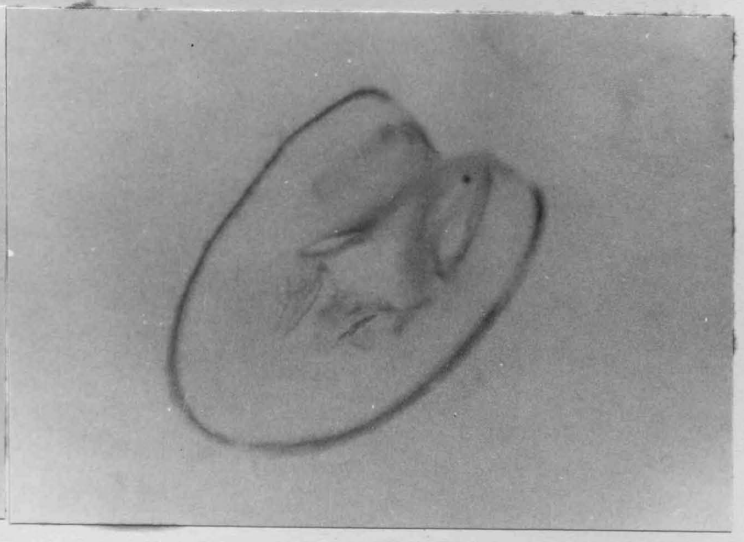

Fig. 29

5-day cultured scolices in Kreb-Ringer's solution

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

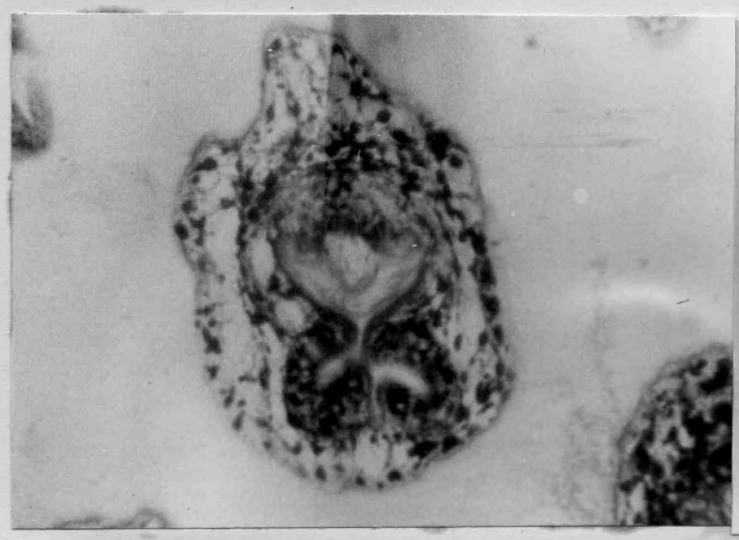

Fig. 30

Fig. 31

5-day cultured scolices in 0.154 M glucose in Kreb-Ringer's solution

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

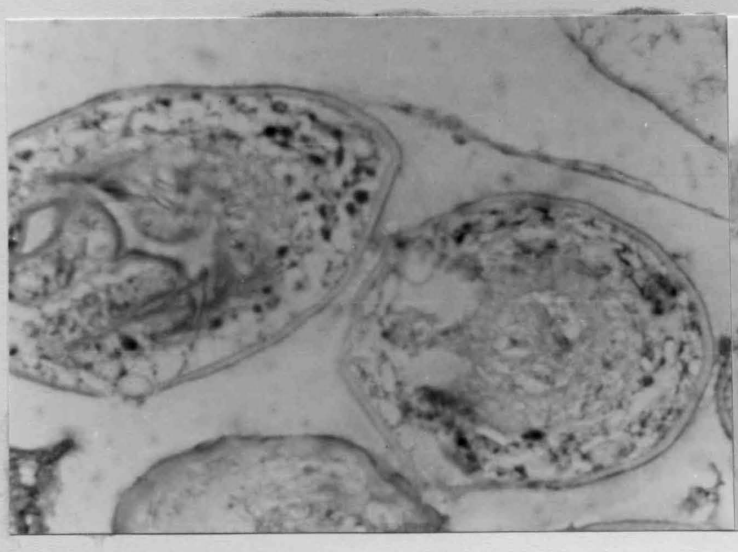

Fig. 32

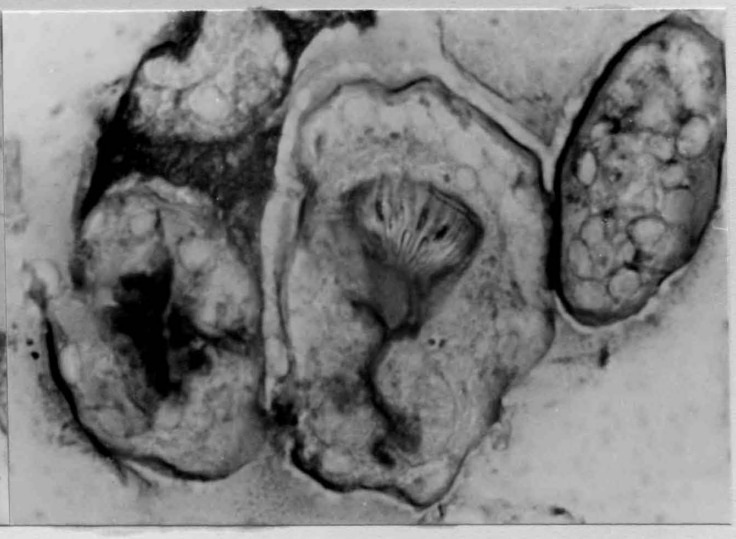

Fig. 33

10-day cultured scolices in 0.154 M glucose in Kreb-Ringer's solution

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

Fig. 34

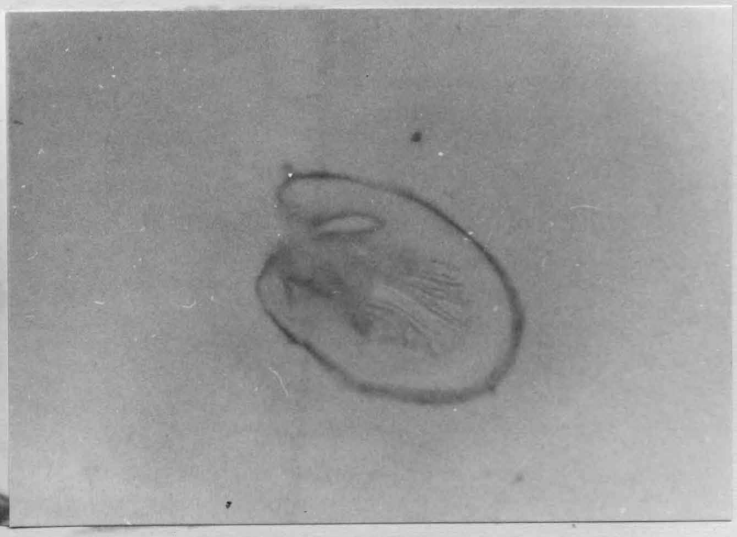

Fig. 35

5-day cultured scolices in 0.154 M galactose in Kreb-Ringer's solution

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

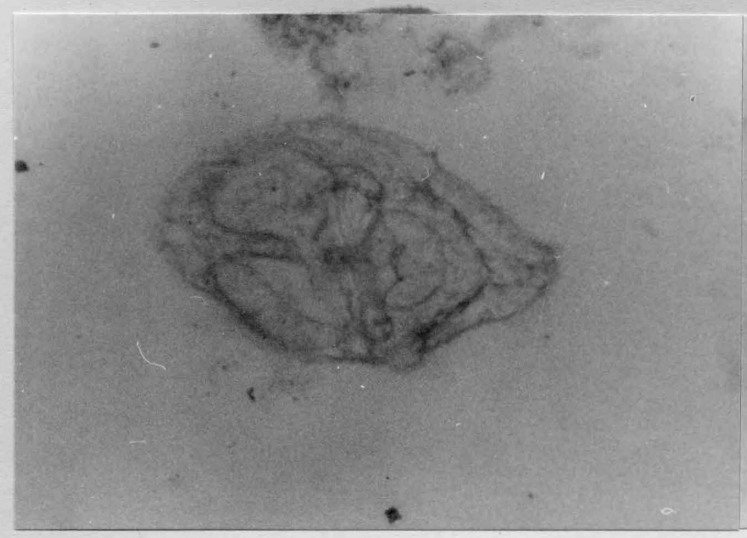

Fig. 36

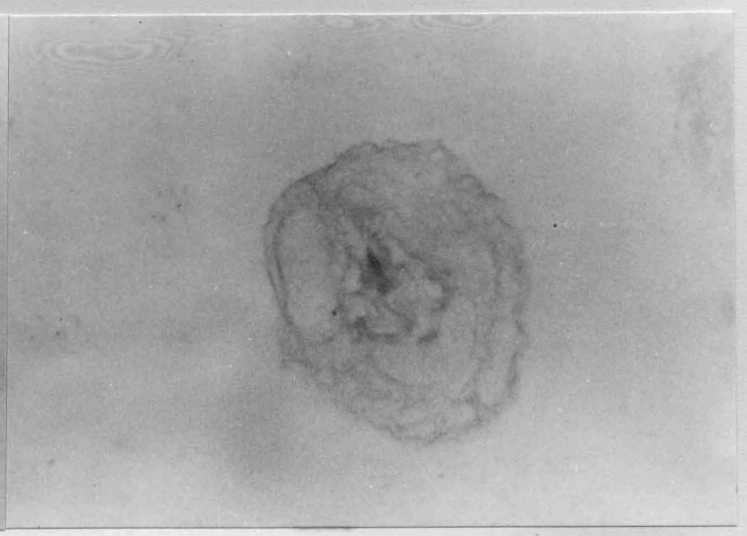

Fig. 37

5-day cultured scolices in 0.154 M glucosamine in Kreb-Ringer's solution

Undigested sections stained with PAS. (Mag. 420x).

Diastase digested sections stained with PAS. (Mag. 420x).

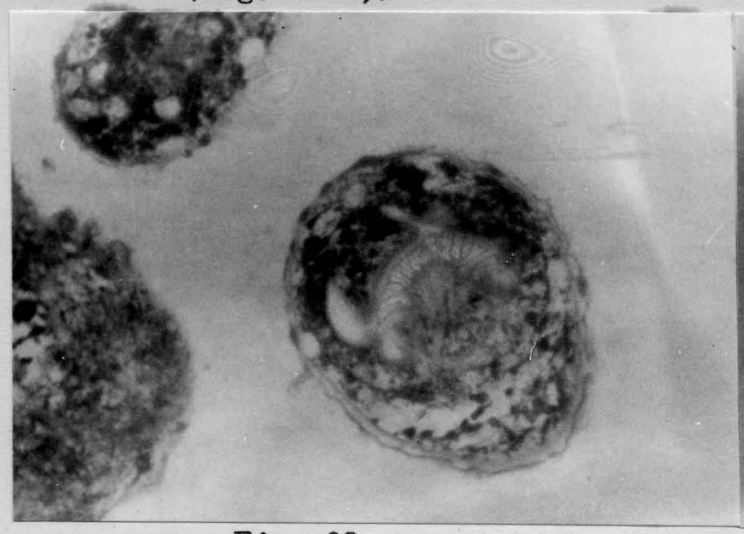

Fig. 38

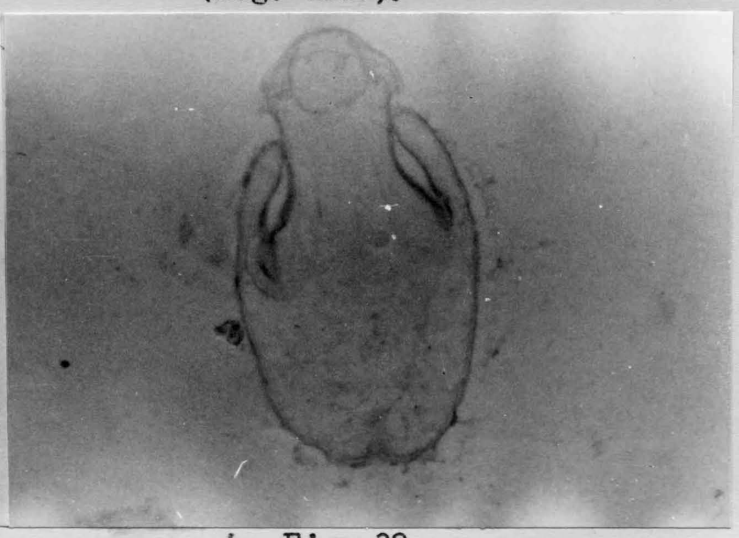

Fig. 39

5-day cultured scolices in 0.154 M glucose and 0.154 M galactose in Kreb-Ringer's solution

Undigested sections stained with PAS. (Mag. 420x).

Diastase digested sections stained with PAS. (Mag. 420x).

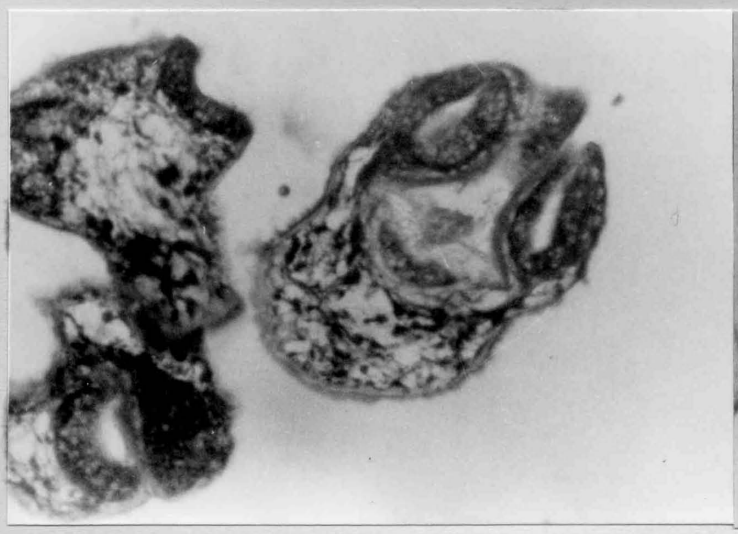

Fig. 40

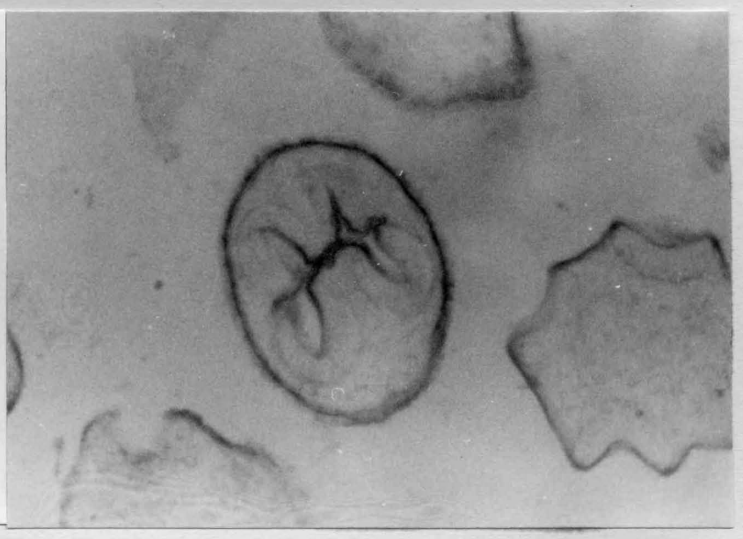

Fig. 41

5-day cultured scolices in GKN medium

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

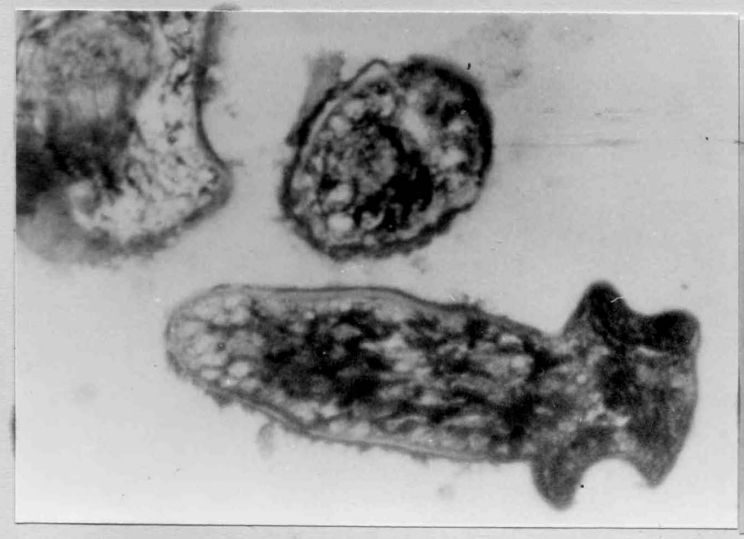

Fig. 42

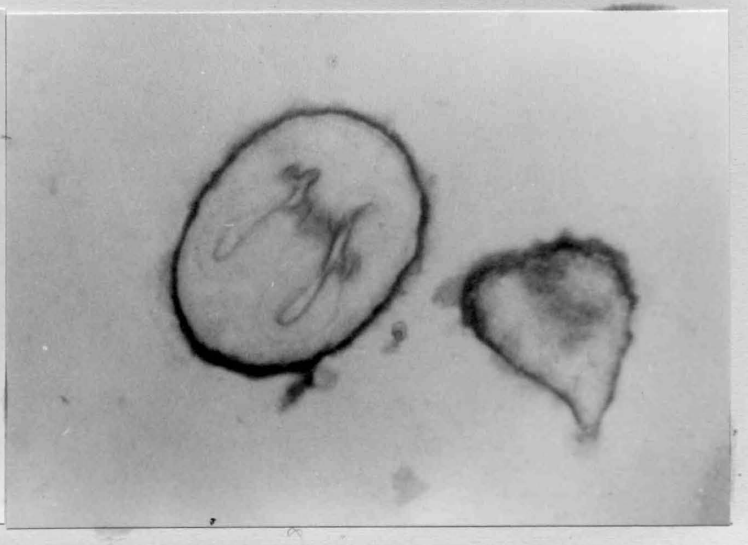

Fig. 43

5-day cultured scolices in lactalbumin hydrolysate

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

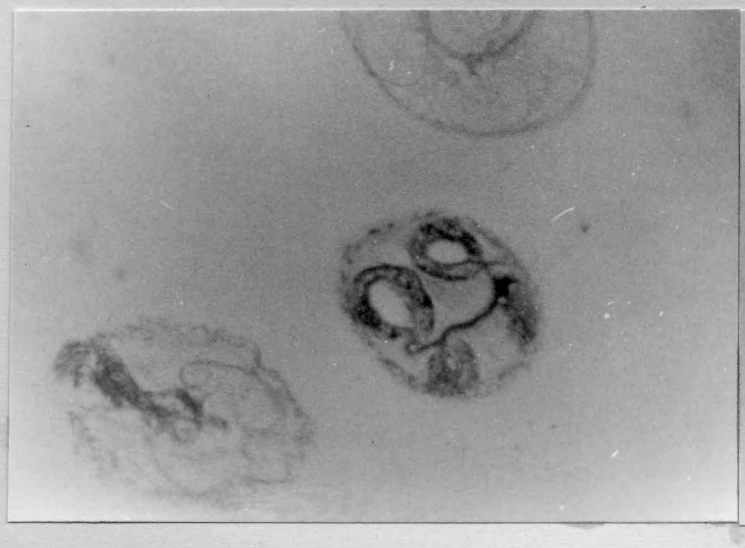

Fig. 44

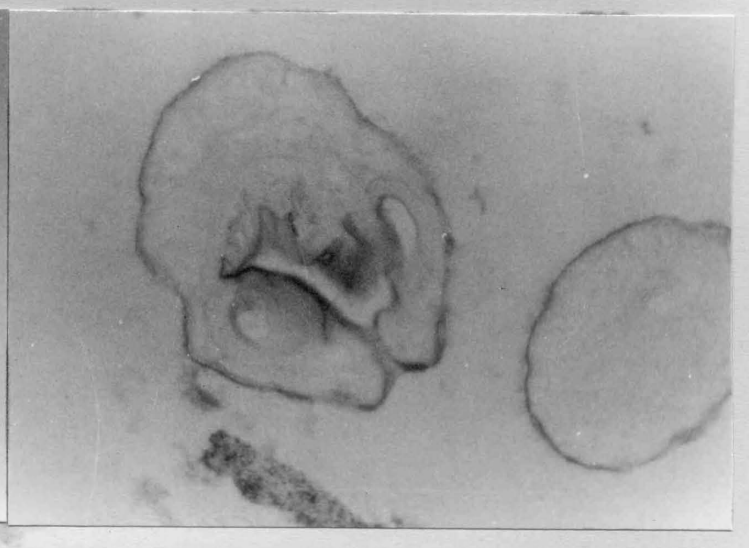

Fig. 45

5-day cultured scolices in isotonic saline

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

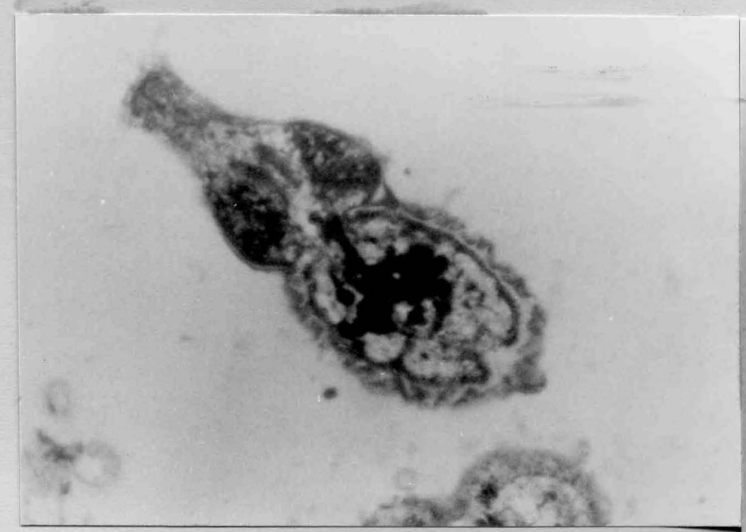

Fig. 46

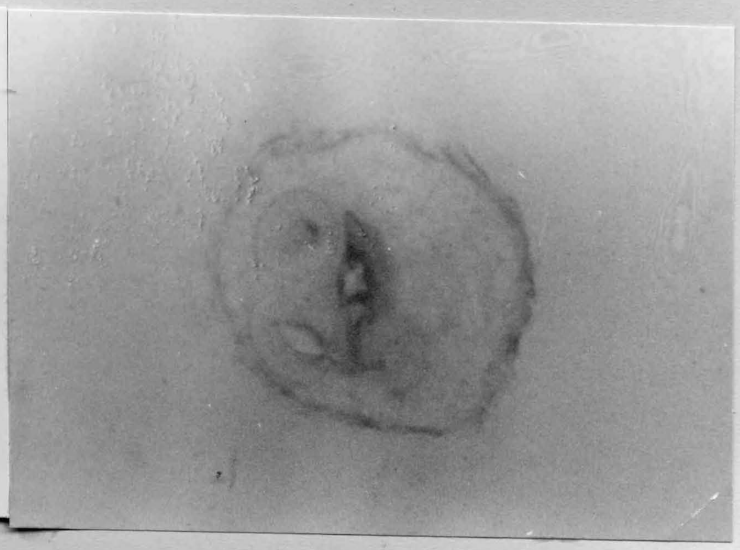

Fig. 47

5-day cultured scolices in 0.154 M glucose in isotonic

Undigested sections
stained with PAS.
(Mag. 420x).

Diastase digested sections
stained with PAS.
(Mag. 420x).

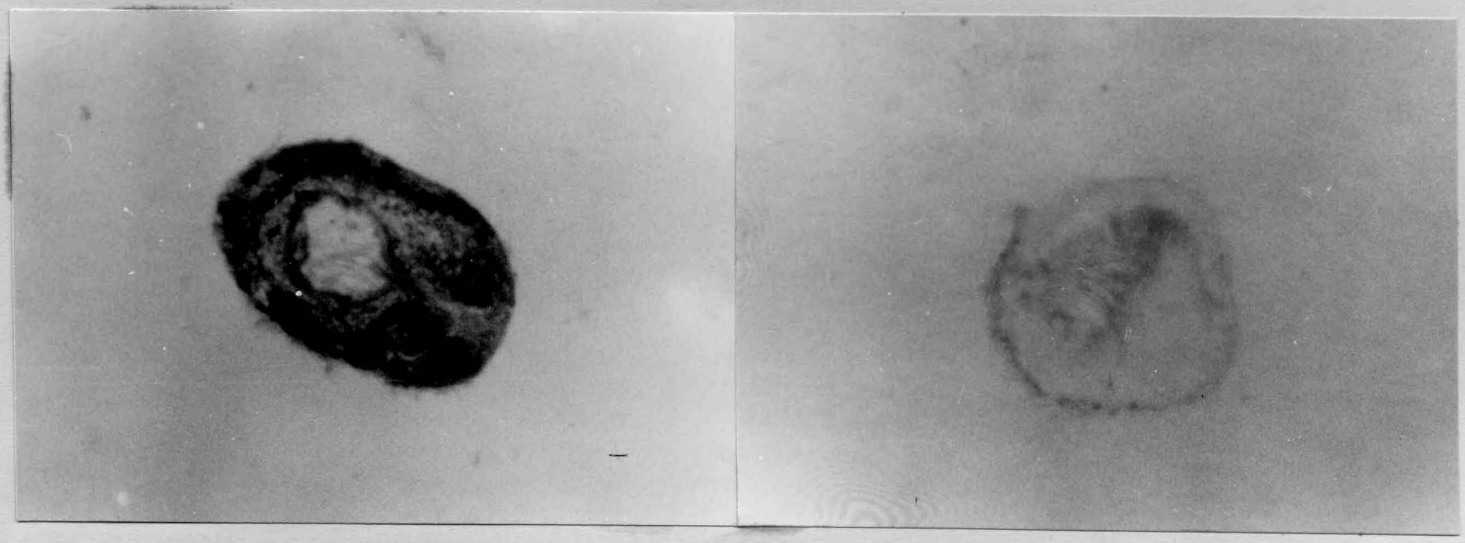

Fig. 48 Fig. 49.

5-day cultured scolices in 1% glucose

Undigested sections stained with PAS. (Mag. 420x).

Diastase digested sections stained with PAS. (Mag. 420x).

DISCUSSION

Echinococcus granulosus scolices were noted to develop in two different ways when cultured in vitro. Scolices cultivated in Parker's TC medium 199 plus bovine amniotic fluid were observed by Smyth(1961) to vesiculate and form cysts with laminated membranes and primordia of brood capsules. On the other hand, Webster and Cameron (1963) reported segmentation and the formation of excretory system primordia when scolices were reared in a complex medium simulating Parker's TC medium 199, but, supplemented with dog intestinal extract instead of bovine amniotic fluid. It seemed that the former had factors that inhibited vesiculation and initiated segmentation. Smyth (1962) tried to explain the difference in development, as well as, host specificity on the basis of the effect of certain bile salts on cuticular lysis. He demonstrated that bile from herbivores, which is rich in deoxycholic acid largely conjugated with glycine, caused lysis of the cuticle; while bile from the dog, which is relatively poor in deoxycholic acid and is largely conjugated with taurine, had no effect on the cuticle.

In this study, scolices were cultured in Parker's TC medium 199, but no lamination was observed inspite of the fact that vesiculation and long survival periods were obtained. The explanation for this was that rabbit serum did not contain the host factors which interacted with the parasite supplementary factors to produce lamination. Rabbit serum, however, was observed to retard vesiculation, in which case, segmentation was indirectly favoured.

The significance of the various forms of vesiculation was not yet known. Each of the forms might be a response to a certain specific stimulus. One

form, however, showed a tendency to segmentation (see results). It was noted in this investigation, as was noted by Smyth (1961), that all vesicle forms developed to a single final globular pattern.

The cuticular vesicles formation observed in detail in this study (figs. 9-12) and mentioned by Webster and Cameron (1963) might be responses to a variety of stimuli. The vesicles might indicate the beginning of lysing the cuticle or might be just a part of a normal physiological process.

The other part of this investigation dealt with the distribution of glycogen in fresh and cultured scolices in various media for different periods. It was observed that glucose and galactose were utilized by the scolices, while glycosamine was not. Glucose, however, was not observed to reciprocally inhibit galactose absorption as was reported by Read et al., (1963) for Hymenolepis diminuta. Agosin et al. (1957) reported that nonglycogen polysaccharides in the scolex contained glucosamine and galactose. The observation that galactose was utilized by the scolices while glucosamine was not still needed explanation. It might be that glucosamine caused the exhaustion of some of the enzymes needed in glycogenesis by using them in side reactions.

It seemed that the concentration factor, at least in the range 0.04M to 0.3M, did not affect glucose utilization by the scolices, as it was noted that glucose was equally utilized by the fifth day.

The reason for the partial depletion of parenchymal glycogen in 15-day cultured scolices in hydatid cyst fluid, and in 10-day cultured scolices in Krebs-Ringer's solution could be either that the enzymes necessary in glycogenesis were exhausted or that the necessary carbohydrates in the medium were used up.

It seemed that glycogenesis required the presence of ions other than sodium or chloride in the medium, since parenchymal and part of the sucker glycogen were depleted within 5 days in isotonic saline.

It was noted that parenchymal glycogen was used up first in all cultures and, then, sucker glycogen was depleted. The explanation speculated was that it seemed that the metabolic processess had direct access to parenchymal glycogen, but had no access to sucker glycogen. The latter seemed to be specialized for the acetabular metabolic processes.

The effect of the following drugs and enzymes on evagination, viability, vesiculation and glycogenesis in scolices was investigated because of the reasons mentioned below.

Ammonium molybdate was used because the molybdate ion was found by Phifer (1960) to inhibit phosphatase activity and hence interfere with the absorption of carbohydrates.

Physostigmine salicylate was used because it was noted by Schwabe et al. (1961) that it inhibited the activity of the enzyme acetylcholinesterase and hence interfered with permeability through the hydatid cyst wall.

Malonic acid was used because Read (1952) reported that it inhibited succinate oxidation and hence interfered with glycogenesis.

Adenosine triphosphate (ATP) was used because it contributed the high energy phosphate in the phosphorylation process and hence it affected glycogenesis.

Hyaluronidase, the spreading factor, was used because it promoted

absorption and diffusion through semi-permeable membranes. It was clear in this study that glycogenesis was accelrated in its presence.

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