OBSERVATIONS ON THE IN VITRO SURVIVAL OF ECHINOCOCCUS GRANULOSUS SCOLICES

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ECHINOCOCCUS GRANULOSUS

Hadidian

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ABSTRACT

The object of this investigation was to maintain viable scolices of Echinococcus granulosus outside of their host for extended periods, and to determine physical and chemical conditions favorable to their in vitro survival and development.

The presence of bacteria was found to be markedly deleterious to the existence of hydatid scolices in vitro.

Scolices maintained in vitro, in non-nutrient balanced salt solutions, were found to tolerate temperature ranges of 5°-37°C. Maximum survival, 58 days, was obtained at refrigerator temperature (5°-9°C.).

Solutions of double strength, 1/4 strength, and 1/2 strength Krebs-Ringer's reduced the survival time of scolices considerably.

Omission of NaCl from Kreb-Ringer's solution had a profound influence upon the in vitro survival of scolices. The effect was less drastic when KH_2PO_4 or $MgSO_4$ was omitted. On the other hand omission of $CaCl_2$ or KCl did not appear to have any marked effect.

Survival of scolices of E. granulosus in a non-nutrient medium at 30°C. did not seem to be favored by conditions of reduced oxygen tension.

In buffer mixtures of different salt compositions,

scolices were found to tolerate a pH range of 6-8 with the optimum lying between 7.0 and 7.4. In culture media they were found to tolerate a pH as low as 3.6.

Addition of glucose, maltose, galactose, valine, and nicotinic acid to Krebs-Ringer's solution reduced the survival time of scolices *in vitro*. Phenylalanine, lysine, ascorbic acid, thiamine-HCl, CEE, and liver extract had no effect, and hydatid fluid and sheep serum definitely prolonged their survival.

Maximum period of survival, 70 days, was obtained with sheep serum incubated at 30°C.

Evagination and vesiculation, though extremely variable, were found to take place in almost all media tried.

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INTRODUCTION

Interest in Echinococcus granulosus (Batsch, 1786), the smallest intestinal cestode parasite of dogs has been stimulated by the knowledge of its relation to human disease. Since its discovery and identification, the larval form of E. granulosus has been observed in the tissues of a wide range of mammalian hosts, including man, in which it is known to be the cause of hydatid disease.

The ease with which experimental investigations concerning growth, nutrition, respiration, and other aspects of physiology can be conducted with free living animals is not possible in the case of parasites such as E. granulosus. This parasite cannot be observed continuously within its host nor can it be kept alive outside of its host's body for more than a few hours. Even in the most favorable artificial media its life is, as a rule, brief and abnormal, particularly so because it is susceptible to attack by certain bacteria and fungi. Relatively little attention has been directed to overcoming these experimental difficulties, and as a consequence the physiology and metabolism of this parasite, like that of other cestodes, is poorly understood.

The greatest single obstacle confronting the investigator of the physiology and metabolism of E. granulosus, therefore,

is this inability to keep the parasite alive and healthy for prolonged periods apart from its host.

Parasites which normally reside in and/or derive sustenance from different organs of different hosts during their course of development undoubtedly encounter many changing nutritional and physical conditions which may effect growth and development. Duplicating these factors in their correct sequence and in time is, perhaps, necessary for successful *in vitro* cultivation. The problem then of keeping parasitic helminths alive *in vitro* appears as essentially one of imitating the natural conditions as closely as possible, although it is likely that some substances present in the normal environment are not necessarily favorable.

The present experiments were designed to study the effects of certain physical and chemical environmental factors upon the larval scolices of *E. granulosus* with the object of ultimately substituting a more or less defined medium for the normal parasitic habitat. With even limited success, *in vitro* conditions that would permit reasonable survival or partial development would facilitate many types of investigations on the nutrition, metabolism, and embryology of this important parasite of man and domestic animals. Finally, the *in vitro* cultivation of larval scolices of *E. granulosus* might further research aimed at the medical treatment of echinococcosis by permitting *in vitro* screening of potentially useful drugs.

The purpose of this investigation then was to maintain viable scolices of *E. granulosus* outside of their host for

extended periods and to determine physical and chemical conditions which might favor their *in vitro* survival and development.

Life Cycle

There is no doubt that hydatid disease has existed and has been noted in human beings since the earliest times. For a long time, however, hydatid cysts were regarded as manifestations of other morbid processes. They were thought to be degenerated glands, collections of pus or mucus or even distended lymph spaces.

The fact that the hydatid cyst was of animal origin was first recognized by Francesco Redi (1626-94) and Edward Tyson was the first to give a satisfactory account of its parasitic nature. The next important advance occurred in 1782 when Goerze accurately described the hydatid cyst, recognized its larval scolices as tapeworm heads and regarded the parasite as a form distinct from the well known cysticercus. In 1808, Rudolphi gave the name "echinococcus" to the common vesicular hydatid.

Von Siebold (1853) was the first to study the life cycle of parasite experimentally. He found that adult mature taeniid cestodes were produced in 27 days by infecting dogs with scolices obtained from typical hydatid cysts. In this way he proved the relationship between the cystic larval form and the adult tapeworm.

It remained for Leuckart (1867), however, to first completely and accurately describe the life history and morphology of the parasite. He experimented with artificially infected pigs and by means of direct observations and by

analogy with the development of the other cestodes cleared up many of the undecided points.

Echinococcus granulosus belongs to the Order Cyclophyllidea of cestodes. As an adult, it inhabits the small intestine of the dog, wolf or jackal. The fox, however, is an unsuitable definitive host as has been shown by the work of Matoff and Jantscheff (1957) and of Bronzini and Bertoline (1904). Dévé (1904) with some difficulty infected the domestic cat experimentally although subsequent workers have shown that sexual maturity is not reached in that host.

Morphologically the adult parasite consists of a head or scolex, a neck and three segments. The head is small, is armed with a double row of chitinous hooklets, posterior to which are four suckers. The overall length is 3-4 mm. (Fig. 1). The adult tapeworm attaches itself to the intestinal mucosa of the definitive host.

The ova are small, measuring 36 uby 39 u. They have a radially striated shell which surrounds the oncosphere or hexacanth embryo. (Fig. 2). As the terminal egg-filled proglottid matures, it is shed and passes out with the feces. If the ova are then ingested by a suitable intermediate host the larval stage of development takes place.

The natural intermediate hosts of greatest epidemiologic consequence are the sheep, the ox and the pig. Many other animals, however, may serve as natural hosts, and, experimentally, a large number of species have been infected. This wide range

of intermediate hosts' plus the ubiquity of the dog accounts for the cosmopolitan distribution of the disease. In so far as the epidemiology of hydatid disease is concerned, human infections represent a dead end. Upon being ingested by a suitable host, the striated shell of the ovum is dissolved. The hexacanth embryo, thus liberated, passes through the wall of the small intestine and, from the radicles of the portal vein, is carried to the liver. The liver is the most common site of infection. Some larval parasites by pass or pass through the hepatic capillary bed, however, and make their way into the inferior vena cava whence they are carried via the right heart to the lungs, which is the second most frequent location of cysts. Occassionally larvae pass through the pulomonary capillary bed and are filtered out in other organs.

The hydatid cyst, which then develops, consists of an outer laminated membrane, or ectocyst, which is non-living, and a thin inner living germinal membrane or endocyst. In the matur ecyst delicate sacs or brood capsules arise from the nucleated germinal layer. Within these brood capsules the scolices or future tapeworm heads develop. As many as forty scolices may be present in a single brood capsule. (Fig. 3).

In addition to healthy, normal scolices, it is common to find within brood capsules degenerated scolices, which are characterized by their dark brown color and shrunken appearance (Fig. 4). If ruptured, the thin brood capsule

membrane tends to turn inside out, with the scolices on the outside (Fig. 5).

Due to the invagination of the hooklets and suckers, the larval scolex, itself, is ovoid in shape. The protoplasm is clear and the "calcareous corpuscles" and nuclei are distinctly visible. Some differentiation of structure is apparent. A single row of hooklets, averaging 36 in number, and the four suckers may be distinguished readily (Fig. 6). Flame cells in the invaginated scolex, are seen only with difficulty. The average scolex measures 160 u by 120 u and is bearly visible to the naked eye. The brood capsule, with its contained scolices, may measure up to 1.5 mm. in diameter and is readily visible. If liberated within the body of the intermediate host, each scolex has the capacity to develop into another complete hydatid cyst.

In Vitro Cultivation of Cestodes

Although the usefulness of techniques for the *in vitro* cultivation of tapeworms in the elucidation of their complex parasite-host relationships is obvious, relatively little success has been achieved by previous investigators. A number of attempts have been made to maintain tapeworms alive apart from their hosts.

As early as 1734 Frisch had kept plerocercoids of *Schistocephalus solidus* alive for more than 2 days in river water while Fabricius (1780) reported the survival of *Proteocephalus percae* in sea water for 14 days. Abildgaard (1793) maintained plerocercoids of *Schistocephalus solidus* for 8 days in fresh tap water. Knock (1862) found that holdfasts of bothriocephalid tapeworms from fishes live 8 days in aqueous solution of egg albumin. Pintner (1880) similarly found that many tapeworms from marine fishes lived 5 to 6 days in sea water plus a trace of egg white. Zschokke (1888) reported survival of tapeworms from selachian fishes for 24 hours in sea water plus host gut mucus. Loennberg (1892) found that *Triaenophorus tricuspidatus* lived 28 days in a slightly acid pepsin-peptone solution of NaCl at 10°C. in total darkness, 14 days when glucose was added, and 4 days in peptone alone. Tower (1900) obtained survival periods of 5 days with *Moniezia expansa* in 10% albumin, plus 5% beef extract and 2% glucose in tap water. Ortner-Schonbach (1913) kept *Calliobothrium coronatum* alive for 7 days in saline plus

monosaccharides. Le Bas (1924) found that plerocercoids of Diphyllobothrium latum lived 7 days in Ringer-Locke solution. Coutelen (1929) succeeded in keeping individual coenurids of Multiceps serialis alive for 20 days with increase in volume in sterile NaCl solution, plus fresh horse serum renewed daily, at 37°C. Cook and Sharman (1930) reported the survival time of Moniezia trisonophora at 37°C. as 2 days in distilled water, 12 days in M/10,000 NaOH in carbonate-free Ringer's solution, with their immediate death in tap water.

Wardle (1932) observed that the plerocercoids of Diphyllobothrium latum and Triaenophorus and adults of Bothriocephalus scorpini tolerate balanced salines better than low molecular concentrations of the component salts. Wardle (1934) in a range of saline and nutrient media, pH 7.5 at 18-24°C., obtained maximum longevity values for plerocercoids of Nybelinia in sterilized double Locke's solution (456 hours); the longevity in serum saline gel was 192 hours; in sterilized Locke-bouillon, 200 hours; in Locke-glucose solution 408 hours. The most promising media were nutrient gels used under aseptic conditions. There was little or no indication of larval growth, however, in any of these early experiments. Prolonged survival times were, for the most part, limited to larvae of pseudophyllidean tapeworms.

Mendelsohn (1935) kept larvae of Taenia crassicollis, alive for 35 days in a sterile nutrient fluid of 7 drops of balanced saline, 2 drops of chicken embryo extract, and 3 drops of filtered horse serum at 37.5°C. and reported the

invagination of the head with no evidence of sucker formation. Markov (1938) obtained maximum survival of plerocercoids of Diphyllobothrium latum in fish broth 63 days and in glucose with added vitamins 56 days. In glucose without vitamins they lived only 30 days. Young plerocercoids 1-2 mm. lived only half as long as more mature ones. von Brand (1933) reported the survival of Moniezia expansa for half a day in Ringer's solution alone, while Munoz Fernandez and Saucedo Aranda (1945) maintained the same parasite for 6-24 hours in Tyrode's solution at 37-38°C. Hoeppli, Feng and Chu (1938) found that cysticerci of Taenia taeniaeformis and plerocercoids of Diphyllobothrium erinacei lived in various nutrient serum solutions at 37°C for a maximum period of 18 days without undergoing any development. Wilmoth (1938-1945) obtained survival of larvae of Taenia taeniaeformis in Tyrode's modification of Ringer-Locke solution for a period of 14 days at 37°C., under oligoseptic conditions, the media being changed at 20 hour intervals, and for periods of 16, 18, and 24 days in modified Ringer's and Tyrode's solutions. In more complicated media, with or without amino nitrogen sources survival time was considerably less. Survival time, longest in simple media (576 hours), was shortened by anaerobiasis, which led to small decreases in the pH of the medium. The larvae were able to withstand gradual pH changes from 9.3 to 4.6 and abrupt changes from 6.5-6.8 as well as a temperature range of 14 to 37°C. Reid and Boles (1949) kept Hymenolepis nana and H. diminuta alive for 10

days in Locke's solution with 1/3 strength of a nutrient medium consisting of 19 amino acids and 23 other constituents. Smith (1954) reported survival of Raillietina cesticillus for 60 hours in liquid solution of Ringer's glucose corn starch at 40°C.

In the experiments noted above, the longevity of the worms in artificial media was a mere fraction of their longevity in the host, and in no case did normal growth and activity take place. Stunkard (1932), however, obtained some evidence of growth with specimens of Crepidobothrium loennbergi in sterile isotonic salt-glucose solution, with Hottinger broth added, at pH 7.3 and room temperature, media being changed every 12 hours. In one experiment, young specimens lived more than 32 days and increased 3 to 4 times in length. The terminal portion of the body became segmented, but the proglottids were abnormal and sterile. Addition to the medium of salt extracts of intestinal mucosa, pancreas and liver of Necturus, sterilized by filtration, did not appreciably alter the rate of growth or the time of survival. Exclusion of free oxygen by anaerobic culture method did not affect the results. Fresh serum from Necturus was definitely toxic to the worms.

Green and Wardle (1941) were able to keep Hymenolepis fraterna from rats alive and active for 20 days, (considerably exceeding the normal longevity of 11 days in the rat host) in a dilution of Baker's tissue culture medium (10 drops to 5 cc. of Tyrode's solution).

Joyeux and Baer (1938, 1942) obtained partial development of plerocercoids of Ligula intestinalis in a variety of media including saline with ascitic fluid, with horse serum, and with ascitic fluid and horse serum; but development was abnormal in that, although some larvae underwent oviposition, the testes failed to undergo spermatogenesis. The addition of various extracts from testes or pituitary, or from the tissues of a normal Ligula itself, also failed to promote spermatogenesis.

The only reasonably successful efforts have been those of Smyth (1946 - 1956) with certain Pseudophyllidean worms. Smyth (1946) cultured plerocercoids of Schistocephalus solidus under completely aseptic conditions in a variety of balanced salines, glucose-salines, and in nutrient peptone broth. He obtained maximum survival and normal behavior of plerocercoids in peptone broth at room temperature (16°-19° C), and induced their development to sexually mature adults, with the normal occurrence of spermatogenesis, oogenesis, vitellogensis, and shell formation by raising the temperature of cultivation in peptone broth to 40°C. Later Smyth (1953, 1954) succeeded in inducing fertilization in vitro, in this species, with the production of 77% fertile eggs, capable of hatching into normal coracidia. These latter experiments were performed using cellulose tubing suspended in tubes of horse serum at 40°C.

Smyth (1947b) also cultivated unidentified plerocercoids of the Family Diphyllobothridae aseptically at

40°C in various liquid nutrient and saline media under sterile conditions, and obtained the most successful results with peptone broth plus 10% horse serum in which larvae remained active and behaved normally for 10 days.

In experiments with the larvae of Ligula intestinalis, Smyth (1947a) obtained maximum survival and activity, as well as spermatogenesis oogensis, vitellogensis, and shell formation in peptone broth at 40°C. Using one ligulid larva per tube of 50 cc. of undiluted horse serum, renewed every 24 hours, Smyth (1948) succeeded in rearing the larvae to adult stage with a production of some fertile eggs. Fragments of larvae similarly cultivated, developed and produced eggs as efficiently as complete larvae, but there was no regeneration of tissue in either anterior or posterior regions.

In later experiments with Ligula, Smyth (1949) found that certain bacterial contaminations of the media, accidentally produced, had no apparent ill effect upon the plerocercoid development in vitro in nutrient solutions. Also partial development of Ligula proceeded in non-nutrient media such as Locke's solution, but complete development as far as oviposition apparently required infected media, as if some bacterial product were necessary for normal worm development. Fragments of larvae with or without holdfasts underwent development to the stage of oviposition in nutrient media as readily as complete plerocercoids.

Information about the in vitro survival and cultivation

of Echinococcus granulosus is limited. In 1906, Dévé (1926) tried unsuccessfully to maintain larval vesicles of E. granulosus in vitro. He, later (1926) was able to keep hydatid scolices viable for a period of fourteen days, at 37°C., in bacteriologically sterile hydatid cyst fluid plus fresh unheated equine serum. In this medium he observed vesiculation of scolices with considerable increase in size.

Coutelen (1927) found further that hydatid scolices lived for thirty-one days in hydatid cyst fluid plus ascitic fluid, became vesicular, and reached sizes 24-35 times their original volumes.

Dévé (1928), particularly concerned with his failure to obtain actual lamination of the cuticle in the small vesicles derived from scolices, did finally succeed in maintaining scolices for forty-three days in a mixture of equal parts hydatid cyst fluid and human ascitic fluid at 37°C. In this medium, he observed the formation of relatively thick, laminated cuticles about larval vesicles. This work has not been confirmed by subsequent investigation.

In preliminary experiments, Schwabe (unpublished data) maintained viable hydatid scolices in vitro for as long as 47 days in physiological salt solutions alone. Vesicularization with imbibition of water and size increase occurred but there was no evidence of cuticular lamination.

MATERIALS AND METHODS

I. Materials

A. Scolices: The scolices were obtained from hydatid cysts found in livers and lungs of cattle and sheep slaughtered at the Beirut Municipal slaughter house. Some scolices were also obtained from human lung and liver hydatid cysts removed by surgical operation at the American University Hospital.

B. Media:

1. Isotonic solution:

The basic salt solution used was Krebs-Ringer's (100 parts of 0.9% NaCl (0.154M), 4 parts of 1.15% KCl (0.154M), 3 parts of 1.22% $CaCl_2$ (0.11M), 1 part of 2.11% KH_2PO_4 (0.154M), 1 part of 3.82% $MgSO_4.7H_2O$ (0.154M)) prepared according to P.P. Cohen (Umbreit *et al.*, 1949).

2. Hypertonic and Hypotonic solutions:

The hypotonic and hypertonic solutions were obtained by preparing Krebs-Ringer's solutions in one half, one fourth and double strengths.

3. Solutions with different hydrogen ion concentrations: Solutions with different pH values were obtained by preparing buffers according to the procedure of Umbreit *et al.*, (1949): Hydrogen ion concentrations were checked with a Beckman pH meter.

The pH 4 and pH 5 buffers contained 0.2M $KHC_8H_4O_4$ and 0.2M NaOH, those of pH values 6.0, 7.0, 7.4, 0.067M KH_2PO_4

and 0.067M Na_2HPO_4, and those of pH values of 8.0, 9.0, 10.0, borate-KCl and 0.2M NaOH.

4. Krebs-Ringer's solutions with altered salt composition:

Krebs-Ringer's solutions were also prepared with the omission of single ingredients.

All of the above media were sterilized by autoclaving at 121°C. and 15 lbs. pressure for 15 minutes.

5. Krebs-Ringer's solutions containing crude tissue preparations, biological mixtures and individual metabolites:

a. 50% Chick Embryo Extract (CEE):* 8 to 9 day old embryos were removed aseptically from the egg, washed with Hank's solution cut into thin slices with scissors and homogenized in a sterile-sieved syringe for 60 seconds in 1 cc. of chilled Hank's solution (adjusted to pH 7.2) per gram of embryo tissue. After standing at 5°C for approximately 1 hour, the homogenate was centrifuged at 2000 r.p.m. for 15 minutes at room temperature. The supernatant extract was removed with a volumetric pipette, without disturbing the sediment, was placed in rubber-stoppered test tubes and stored at -20°C. All steps were performed with aseptic precautions and the extract was tested for sterility.

A final concentration of 20% CEE was used in these experiments. This was obtained by mixing 3 ml. of sterile Krebs-Ringer's solution with 2 ml. of CEE.

b. Liver Extract: Powder liver extract, Lilly (Eli Lilly and Company, Indianapolis, U.S.A.) was used

*CEE was kindly supplied by the Department of Bacteriology, American University of Beirut.

in a final concentration of 20%. The solvent was sterile Krebs-Ringer's solution.

c. Serum: Seitz-filtered serum, derived from a single sheep, was obtained and stored in the frozen state. Serum media were composed of 1 cc. of serum to 4 cc. of sterile Krebs-Ringer's solution.

d. Hydatid fluid: The hydatid fluid was obtained aseptically from bovine cysts, diluted to a final concentration of 20% with sterile Krebs-Ringer's solution and stored in the freezer.

e. Monosaccharides and disaccharides: Solutions of 0.09% d(+) maltose, d(+) galactose, and glucose (Nutritional Biochemical Corporation, Cleveland Ohio), were prepared sterilized by autoclaving at 121°C. and 15 lbs. pressure for 15 minutes and were stored in the refrigerator until used.

f. Vitamins: Vitamins used were ascorbic acid, nicotinic acid, and thiamine HCl (Nutritional Biochemical Corporation, Cleveland Ohio), in concentrations of 0.62 mg., 0.6 mg., and 8.0 micrograms per 100 ml. of sterile Krebs-Ringer's solution, respectively.

g. Amino acids: Solutions of 0.01% phenylalanine, 0.022% lysine, and 0.024% valine (Nutritional Biochemical Corporation, Cleveland Ohio) were prepared in Krebs-Ringer's solution and stored in the freezer until used.

C. Antibiotics: The sodium salt of crystalline penicillin G and streptomycin sulfate B.P. (Glaxo Laboratories Ltd., Greenford, England) were dissolved in sterile Krebs-

Ringer's solution and other media in concentrations of 500 units and 0.1 mg. per milliliter, respectively.

II. Methods

A. Recovery of scolices: The surface of the hydatid cyst was seared with a hot spatula, the hydatid cyst fluid, with suspended brood capsules and scolices, was aspirated with a sterile veterinary syringe and collected in a sterile flask. The supernatent fluid was removed with a sterile Pasteur pipette, and the scolices were washed three times by suspension in sterile Krebs-Ringer's solution. The scolices were then transferred by sterile Pasteur pipettes to culture tubes.

B. Inoculation of culture media: Except for the experiments on anaerobiasis where low O_2 tension was required, 12.5 cm. x 1.2 cm. screw cap tubes were used for cultures routinely.

C. Culture methods: Two different culture methods were utilized routinely:

1. Aerobic cultures: A suspension of aseptically collected scolices were placed in a series of culture tubes each containing 5 ml. of the medium under investigation. Antibiotics were routinely added (see above).

2. Anaerobic cultures: Suspensions of aseptically collected scolices were inoculated into a series of Thunberg tubes each of which contained 5 ml. of Krebs-Ringer's solution. Antibiotics were routinely added (see above). Thunberg tubes are provided with sidearm stoppers arranged to hold materials

to be added to the main tube after evacuation. Two lines of sterile grease were put on the ground glass joint. The cap was then pressed firmly into place and the joint rotated a few degrees. In this manner no air was trapped in the grease. The side outlet was attached to a vacuum pump. The sidearm cap was turned so that the hole drilled in its ground glass joint coincided with the evacuation outlet. The tubes were evacuated for 3 to 5 minutes, the tube being constantly tapped to release dissolved gas, and inclined at a low angle to minimize "bumping". After evacuation, the tube was closed off by turning the sidearm.

An alternate method for anaerobic culture consisted of carefully layering sterile paraffin oil on top of scolex suspensions in screw cap tubes. The suspending fluid was Krebs-Ringer's solution.

Suspensions were routinely incubated at 30°C. Experiments to determine possible effects of temperature upon scolex survival were run using bacteriological incubators at 37°C. and 30°C., room temperature (14-21°C.) and refregerator temperature (5-9°C.).

The pH of all media was measured at the beginning and end of each experiment with a Beckman pH meter.

D. Sterility Test: The components of all media were initially tested for sterility by inoculation into thioglycollate broth at 37°C. At the termination of an experiment cultures were similarly inoculated into thioglycollate broth, and occasionally were checked by Gram stain. As scolex cultures were examined microscopically, they were routinely

checked for presence of gross bacterial or mold contamination.

E. Examination of Cultures: All cultures were examined at regular intervals, usually once every four days. An aliquot was taken with a sterile Pasteur pipette and put on a slide under a coverslip and examined with low power microscope for activity and morphological appearance. Routine counts were made of the living, dead, invaginated, evaginated, normal, and swollen scolices in each preparation. Occasionally high power microscopy was used to detect the beating of the flame cells.

F. Evaluation of Cultures: The criteria used for the evaluation of cultures were the maximum period of survival of any scolices, and the percentage survival, percentage evagination and percentage vesiculation at any given time.

RESULTS

The Effects of Physical Environmental Factors on the In Vitro Survival of Echinococcus Granulosus Scolices.

a. Temperature.

The results of 7 experiments presented (Table I) demonstrated that survival of Echinococcus granulosus scolices maintained in vitro in non-nutrient balanced salt solutions was favored by low temperatures. The maximum survival time, 58 days, was obtained at refrigerator temperature (5-9°C.), whereas at 37°C. scolices failed to survive beyond the 20th day of incubation, except in one preliminary experiment, not included with this group, in which one of 4 tubes contained a few viable scolices on the 36th day.

Scolices maintained at room temperature (14-21°C.) and at 30°C. did not differ from one another appreciably in their survival times. In both, the scolices exhibited normal contractile movements as well as regular beating of flame cells for a maximum period of 44 days.

With respect to percentage survival of scolices, the results obtained with all four incubation temperatures were quite satisfactory up to the 4th day of incubation. By the 8th day a large number of scolices incubated at 37°C. had died. All cultures at the other temperatures examined still showed a high percent survival, but on the following days, the percentage of living scolices decreased gradually in each instance.

Microscopic examinations of scolices revealed the presence of 6 major morphologic forms in culture media. These were 1) invaginated and normal in size, 2) evaginated and normal in size, 3) invaginated and swollen, 4) evaginated and swollen, 5) invaginated and vesiculated, and 6) evaginated and vesiculated (Figs. 7, 8, 9, 10, 11, 12). Minor differences were observed among the evaginated swollen and evaginated vesiculated scolices. In some only the anterior end was swollen, in some the posterior and in some both parts were swollen or vesiculated. In addition to these main types, living scolices with cuticular bubble-like swellings were observed, as were dead scolices in various stages of degeneration (Figs. 13, 14).

Higher incubation temperatures would appear to favor both evagination and vesiculation. As seen in Table II and III, 98% of the scolices were evaginated and 90% swollen or vesiculated by the 8th day of incubation at 37°C. At refrigerator temperature, however, rare evaginated scolices were seen beginning on the 8th day, but swelling or vesiculation rarely, if ever, occured at that temperature (Tables II, and III).

The initial pH of all tubes which contained Krebs-Ringer's solution was approximately 5, and, at the termination of most experiments, approximately 6. Qualitative tests with Nessler's reagent at the conclusion of experiments gave evidence of the presence of ammonia in all of the tubes examined.

No bacterial or mold contamination was observed in these experiments.

b. Hydrogen ion concentration.

Four experiments were run with scolex suspensions suspended at 30°C. in buffer solutions at pH's of 4, 5, 6, 7, 7.4, 8, 9, and 10.

At the lower pH values (4 and 5), all scolices were found to be degenerated, shedding hooklets and dead on the 2nd day of incubation (Fig. 15). Higher pH levels (9 and 10) were found to be equally unfavorable to survival. It should be noted, however, that the suspending buffers were often markedly different in chemical composition. It is, therefore, likely that these effects did not result for pH alone.

At pH 6, 30% of scolices were living on the 4th day of incubation. On the 8th day, 0.9% were only living, and on the 10th day of incubation all scolices were dead.

Up to the 8th day of incubation, the percentage survival of scolices at pH 7.0, 7.4, and 8.0 was high. On the 12th day of incubation the percentage of living scolices at pH values 7.0 and 7.4 were still high (92.5% and 93.4%, respectively) but that at pH 8.0 had fallen to 58.5%.

By the 28th day all scolices maintained at pH 8.0 were dead, while some of those maintained at pH 7.0 and 7.4 survived for another 4 days.

Evagination of scolices appeared to be somewhat favored at higher hydrogen ion concentrations.

The pH's did not change throughout these experiments,

nor was there evidence of bacterial contamination.

c. Anaerobiasis.

Survival of scolices of Echinococcus granulosus in a non-nutrient medium at 30°C. did not seem to be favored by conditions of reduced oxygen tension. The maximum period of survival observed in these cultures was 16 days, which was much shorter than the maximum survival time of 44 days in cultures maintained under aerobic conditions.

Values given in Table VII indicated a very high survival rate of scolices up to the 8th day of incubation (100-93.7%). The survival percentages, however, had decreased markedly by the 12th and 16th days, and no scolices were found to have survived beyond the 18th day of incubation.

Upon microscopic examination invaginated normal and invaginated swollen forms predominated.

The pH of the suspending medium increased from 5 to 5.5. There was no evidence of bacterial contamination in any of the tubes.

d. Ionic Environment.

The omission of NaCl from Krebs-Ringer's isotonic solution seemed to have a profound influence on the in vitro survival of E. granulosus scolices. The results of 4 experiments indicated that 73% of the scolices were dead by the 4th day of incubation, with only 2.5% living on the 8th day and all dead by the 9th day (Table X). Omission of NaCl from Krebs-Ringer's solution, without the substitution of an equal volume of water resulted in large relative increases in the

concentrations of the other salts, while at the same time the toxicity of the solution was unaltered.

Results obtained when either KH_2PO_4 or $MgSO_4$ was omitted were less drastic. Maximum survival time of scolices in the former medium was 16 days, while the percentage survival of scolices was high during the entire survival period.

The omission of $CaCl_2$ or KCl from Krebs-Ringer's solution did not appear to have any marked effect upon the survival of scolices. Scolices remained viable for a maximum period of 38 days, although the percentages for the last 8 days were quite low (Table X).

No bacterial contamination was observed. During the experiments the pH in most tubes increased from about 5.0 to about 6.0.

e. Osmotic concentration.

When the concentration of the components of the Krebs-Ringer's solution were doubled, a marked reduction in survival time of scolices incubated at 30°C. occurred. As Table XIII indicates, survival time of 44 days in normal Krebs-Ringer's solution was shortened to 12 days in the double concentration solution.

Hypotonic solutions of one-half and one-fourth times Krebs-Ringer's solution were likewise found to be unfavorable for the survival of scolices, although the maximum periods of survival (21 and 19 days respectively) exceeded that obtained in double strength Krebs-Ringer's solution.

Evaginated swollen and evaginated vesiculated forms

predominated with scolices swelling almost as readily in the hypertonic solution as in isotonic and hypotonic media.

The final pH varied from 5 to 6. There was no evidence of bacterial contamination.

TABLE I

Average Percentage Survival of Scolices of *Echinococcus granulosus* in Krebs-Ringer's Solution at Different Temperatures

Temperature	Average Percentage Survival of Scolices at days														
	1	4	8	12	16	20	24	28	32	36	40	44	48	52	56
Refrigerator 5 - 9°C	100.0+ (6)x	100.0	100.0	99.0	98.7	95.6	88.2	78.0	55.0	46.3	42.0	38.9	30.0	25.2	15.1
Room 14 - 21°C	100.0 (6)	100.0	99.0	98.0	94.5	82.8	74.5	53.4	31.5	20.0	18.5	14.2			
30°C	99.8 (16)	99.0	96.7	88.0	75.8	61.5	51.5	46.1	29.4	19.5	5.8	3.2			
37°C	95.6 (7)	84.0	27.1	23.0	18.8	9.2									

\+ Mean of experiments

x Number of experiments

TABLE II

Average Percentage Evagination of Scolices of *Echinococcus granulosus* in Krebs-Ringer's Solution at Different Temperatures

Temperature	Average Percentage Evagination of Scolices at days														
	1	4	8	12	16	20	24	28	32	36	40	44	48	52	56
Refregerator 5°- 9°C	0.0+ (6)x	0.0	2.0	3.6	22.3	35.0	42.6	48.3	54.7	55.0	57.2	59.6	60.0	60.5	62.5
Room 14°-21°C	23.1 (6)	83.0	86.7	88.4	92.0	98.5	98.7	100.0	100.0	100.0	100.0	100.0			
30°C	0.0 (16)	56.0	68.8	70.2	73.0	82.3	87.5	93.9	98.7	100.0	100.0	100.0			
37°C	4.5 (7)	74.7	98.0	99.8	100.0	100.0									

\+ Mean of experiments.

x Number of experiments.

TABLE III

Average Percentage Vesiculation of Scolices of Echinococcus granulosus in Krebs-Ringer's Solution at Different Temperatues

Temperature	Average Percentage Vesiculation of Scolices at days														
	1	4	8	12	16	20	24	28	32	36	40	44	48	52	56
Refrigerator 5 - 9°C	0.0+ (6)*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Room 14 - 21°C	0.0 (6)	58.0	67.3	78.4	85.8	88.1	90.8	96.9	100.0	100.0	100.0	100.0			
30°C	0.0 (6)	41.0	59.3	63.4	67.8	82.5	88.0	92.7	99.3	100.0	100.0	100.0			
37°C	0.0 (7)	42.0	90.0	98.0	100.0	100.0									

+ Mean of experiments

* Number of experiments

TABLE IV

Average Percentage Survival of Scolices of *Echinococcus granulosus* in Different Buffer Mixtures at 30°C.

Buffer Mixtures	Average Percentage Survival of Scolices at days								
	1	4	8	12	16	20	24	28	32
pH 6.0	98.6+ (4)*	30.0	0.9						
pH 7.0	99.0 (4)	98.6	94.3	92.5	54.7	26.2	6.5	4.6	2.0
pH 7.4	100.0 (4)	99.0	96.2	93.4	57.0	28.2	8.5	5.7	3.4
pH 8.0	99.2 (4)	98.4	84.5	58.5	31.5	15.8	1.6		

TABLE V

Average Percentage Evagination of Scolices of *Echinococcus granulosus* in Different Buffer Mixtures at 30°C.

Buffer Mixtures	Average Percentage Evagination of Scolices at days								
	1	4	8	12	16	20	24	28	32
pH 6.0	0.0+ (4)*	88.0	100.0						
pH 7.0	0.0 (4)	10.8	33.5	61.0	63.2	67.0	83.5	85.4	88.0
pH 7.4	0.0 (4)	12.5	38.6	50.7	58.5	80.0	80.0	81.0	82.3
pH 8.0	0.0 (4)	27.8	46.2	48.0	67.5	83.4	86.7		

\+ Mean of experiments

* Number of experiments

TABLE VI

Average Percentage Vesiculation of Scolices of *Echinococcus granulosus* in Different Buffer Mixtures at 30°C.

Buffer Mixtures	Average Percentage Vesiculation of Scolices at days								
	1	4	8	12	16	20	24	28	32
pH 6.0	0.0+ (4)x	46.0	55.5						
pH 7.0	0.0 (4)	27.0	36.0	40.2	43.4	48.2	49.6	5L.3	53.4
pH 7.4	0.0 (4)	28.2	31.4	40.0	41.5	45.1	47.3	50.4	52.8
pH 8.0	0.0 (4)	20.0	23.8	48.0	56.5	58.3	60.2		

TABLE VII

Average Percentage Survival of Scolices of *Echinococcus granulosus* in Krebs-Ringer's Solution Under Conditions of Reduced Oxygen Tension at 30°C.

Reduced Oxygen Tension	Average Percentage Survival of Scolices at days											
	1	4	8	12	16	20	24	28	32	36	40	44
Paraffin Oil Method	100.0+ (4)x	98.5	93.7	29.1	15.5							
Thunberg Method	98.0 (4)	94.2	90.5	36.3	16.4							
Control	99.8 (16)	99.0	96.7	88.0	75.8	61.5	51.5	46.1	29.4	19.5	8.8	3

\+ Mean of experiments

x Number of experiments

TABLE VIII

Average Percentage Evagination of Scolices of *Echinococcus granulosus* in Krebs-Ringers Solution Under Condtions of Reduced Oxygen Tension at 30°C.

Reduced Oxygen Tension	Average Percentage Evagination of Scolices at days											
	1	4	8	12	16	20	24	28	32	36	40	44
Paraffin Oil Method	8.0+ (4)x	31.9	36.4	43.0	50.0							
Thunberg Method	12.0 (4)	44.5	54.5	60.0	71.4							
Control	0.0 (16)	56.0	68.8	70.2	73.0	82.3	87.5	93.9	98.7	100.0	100.0	100.0

TABLE IX

Average Percentage Vesiculation of Scolices of *Echinococcus granulosus* in Krebs-Ringer's Solution Under Conditions of Reduced Oxygen Tension at 30°C.

Reduced Oxygen Tension	Average Percentage Vesiculation of Scolices at days											
	1	4	8	12	16	20	24	28	32	36	40	44
Paraffin Oil Method	0.0+ (4)x	15.0	22.1	44.7	100.0							
Thunberg Method	0.0 (4)	15.3	34.8	52.3	100.0							
Control	0.0 (16)	41.0	59.3	63.4	67.8	82.5	88.0	92.7	99.3	100.0	100.0	100.0

\+ Mean of experiments

x Number of experiments

TABLE X

Average Percentage Survival of Scolices of *Echinococcus granulosus* in Different Salt Solutions at 30°C.

Krebs-Ringer's Solution	Average Percentage Survival of Scolices at days											
	1	4	8	12	16	20	24	28	32	36	40	44
- KCl	100.0$^{+}_{x}$ (4)	98.0	86.0	76.0	67.0	47.7	30.2	5.8	3.0	1.0		
- $CaCl_2$	100.0 (4)	98.5	89.0	86.0	84.0	60.5	20.5	5.9	2.1	0.5		
- NaCl	100.0 (4)	27.0	2.5									
- KH_2PO_4	100.0 (4)	98.0	96.2	94.0	93.5							
- $MgSO_4$	100.0 (4)	97.0	89.0	54.0	46.0							
Normal Krebs-Ringer's (Control) Solution	99.8 (16)	99.0	96.7	88.0	75.8	61.5	51.4	46.1	29.4	19.5	5.8	3.2

\+ Mean of experiments

x Number of experiments

TABLE XI

Average Percentage Evagination of Scolices of Echinococcus granulosus in Different Salt Solutions at 30°C.

Krebs-Ringer's Solution	Average Percentage Evagination of Scolices at days											
	1	4	8	12	16	20	24	28	32	36	40	44
- KCl	1.5+ (4)x	55.0	79.0	84.5	91.5	96.4	100.0					
- $CaCl_2$	2.5 (4)	60.5	76.0	81.3	87.5	94.5	98.0					
- NaCl	2.0 (4)	80.0	100.0									
- KH_2PO_4	2.2 (4)	50.0	75.2	87.6	95.0							
- $MgSO_4$	1.0 (4)	29.6	62.0	78.3	90.5							
Normal Krebs-Ringer's (Control) Solution	0.0 (16)	56.0	68.8	79.2	73.0	82.3	87.5	93.9	98.7	100.0	100.0	100.0

+ Mean of experiments

x Number of experiments

TABLE XII

Average Percentage Vesiculation of Scolices of *Echinococcus granulosus* in Different Salt Solutions at 30°C.

Krebs-Ringer's Solution	Average Percentage Vesiculation of Scolices at days											
	1	4	8	12	16	20	24	28	32	36	40	44
- KCl	0.0+ (4)x	16.7	58.5	66.8	75.2	72.3	88.6	100.0				
- $CaCl_2$	0.0 (4)	48.5	68.0	75.0	79.8	89.0	100.0					
- NaCl	0.0 (4)	32.6	100.0									
- KH_2PO_4	0.0 (4)	53.2	69.0	72.6	77.3							
- $MgSO_4$	0.0 (4)	11.2	33.6	52.4	76.0							
Normal Krebs-Ringer's (Control) Solution	0.0 (16)	41.0	59.3	63.4	67.8	82.5	88.0	92.7	99.3	100.0	100.0	100.0

+ Mean of experiments

x Number of experiments

TABLE XIII

Average Percentage Survival of Scolices of *Echonococcus granulosus* in Different Concentrations of Krebs-Ringer's Solution at 30°C.

Krebs-Ringer's Solution	Average Percentage Survival of Scolices at days											
	1	4	8	12	16	20	24	28	32	36	40	44
Double Strength	94.3[+] (6)[x]	84.7	51.2	9.0								
1/2 Strength	95.3 (6)	94.1	81.0	75.6	21.6	3.0						
1/4 Strength	90.7 (6)	90.0	60.7	48.3	11.3							
Normal Krebs-Ringer's (Control) Solution	98.8 (16)	99.0	96.7	88.0	75.8	61.5	51.5	46.1	29.4	19.5	5.8	3.2

+ Mean of experiments

x Number of experiments

TABLE XIV

Average Percentage Evagination of Scolices of Echinococcus granulosus in Different Concentration of Krebs-Ringer's Solution at 30°C.

Krebs-Ringer's Solution	Average Percentage Evagination of Scolices at days											
	1	4	8	12	16	20	24	28	32	36	40	44
Double Strength	31.8+ (6)x	58.4	72.5	79.8								
1/2 Strength	14.1 (6)	64.7	76.9	81.5	95.5	100.0						
1/4 Strength	20.7 (6)	72.7	83.9	99.0	100.0							
Normal Krebs-Ringer's (Control) Solution	0.0 (16)	56.0	68.8	70.2	73.0	82.3	87.5	93.9	98.7	100.0	100.0	100.0

+ Mean of experiments

x Number of experiments

TABLE XV

Average Percentage Vesiculation of Scolices of *Echinococcus granulosus* in Different Concentrations of Krebs-Ringer's Solution at 30°C.

Krebs-Ringer's Solution	Average Percentage Vesiculation of Scolices at days											
	1	4	8	12	16	20	24	28	32	36	40	44
Double Strength	0.0+ (6)x	44.9	54.0	66.0								
1/2 Strength	2.5 (6)	40.1	54.4	55.2	61.7	100.0						
1/4 Strength	4.0 (6)	46.7	57.9	79.9	98.0							
Normal Control	0.0 (16)	41.0	59.3	63.4	67.8	82.5	88.0	92.7	99.3	100.0	100.0	100.0

\+ Mean of experiments

x Number of experiments

TABLE XVI

Average Percentage Survival of Scolices of *Echinococcus granulosus* in Different Nutrient Media at 30°C.

Media	Average Percentage Survival of Scolices at days																
	1	4	8	12	16	20	24	28	32	36	40	48	52	56	60	64	68
Glucose	98.3+ (6)x	95.5	92.2	66.7	55.0												
Maltose	99.5 (6)	98.5	88.7	83.0	69.5	39.8	27.5										
Galactose	99.2 (4)	98.5	96.0	91.5	31.8	27.4	12.5										
Lysine	98.4 (4)	96.0	84.8	68.5	64.2	61.0	57.0	54.0	27.4	14.7							
Valine	99.0 (4)	96.0	70.8	70.0	12.5	3.0	0.5										
Phenylalanine	99.1 (4)	97.0	91.6	84.8	66.8	52.0	42.0	30.0	10.0	3.5	1.0						
Ascorbic	96.8 (4)	92.0	90.4	82.9	49.8	41.1	36.5	32.7	13.1	8.8	4.3						
Nicotinic Acid	98.6 (4)	95.5	81.4	58.0	7.0												
Thiamine HCl	99.3 (4)	97.0	95.2	81.0	17.0	5.5	5.0	4.5	4.0	3.5	3.						
CEE	100.0 (4)	98.0	96.0	96.5	96.0	95.5	91.0	88.5	86.5	63.0	6.						
Liver extract	99.0 (4)	98.0	95.0	81.5	70.2	55.8	28.7	22.7	6.5	4.5	2.						
Serum	99.8 (4)	99.3	98.5	94.2	86.7	83.6	72.9	71.2	69.1	63.8	57.	1	31.5	.6	6.2	4.5	2.0	1.2
Hydatid Fluid	99.7 (4)	98.3	95.9	93.5	86.7	78.1	61.3	58.2	48.9	29.4	14.	5	8.3	.9	2.5	1.3	0.6	
K - R 30°C	99.8 (16)	99.0	96.7	88.0	75.8	61.5	51.5	46.1	29.4	19.5	5.	2						

+ Mean of experiments

x Number of experiments

TABLE XVII

Average Percentage Evagination of Scolices of Echinococcus granulosus in Different Nutrient Media at 30°C.

Media	Average Percent Evagination of Scolices at day																	
	1	4	8	12	16	20	24	28	32	36	40	44	48	52	56	60	64	68
Glucose	2.0+ (6)x	71.9	86.0	94.0	96.5													
Maltose	3.0 (6)	71.0	79.9	85.2	89.0	95.8	100.0											
Galactose	2.0 (6)	78.0	90.0	94.4	95.0	100.0	100.0											
Lysine	5.0 (4)	61.2	86.3	94.1	98.3	98.5	98.9	99.0	99.0	100.0								
Valine	0.0 (4)	38.0	87.8	95.0	96.0	100.0	100.0											
Phenylalanine	4.0 (4)	36.6	42.5	53.1	83.0	83.0	91.0	92.0	100.0	100.0								
Ascorbic Acid	8.0 (4)	70.6	80.3	85.3	88.6	89.1	90.9	91.1	94.1	94.1								
Nicotinic Acid	1.0 (4)	54.5	71.4	88.5	100.0													
Thiamine HCl	2.0 (4)	48.1	89.0	97.0	99.0	100.0	100.0	100.0	100.0	100.0								
CEE	0.0 (4)	13.7	28.9	49.0	77.3	91.0	94.6	97.6	98.0	99.0								
Liver Extract	4.0 (4)	54.3	67.2	77.9	90.2	94.5	100.0	100.0	100.0	100.0								
Serum	0.0 (4)	23.1	54.4	66.5	79.8	83.7	91.6	92.8	96.8	100.0			100.0	100.0	100.0	100.0	100.0	100.0
Hydatid Fluid	0.0 (4)	36.4	61.9	86.9	89.2	90.7	97.2	100.0	100.0	100.0			100.0	100.0	100.0	100.0		
K - R 30°C	0.0 (16)	56.0	68.8	70.2	73.0	82.3	87.5	93.9	98.7	100.0								

+ Mean of experiments

x Number of experiments

TABLE XVIII

Average Percentage Vesiculation of Scolices of Echinococcus granulosus in Different Nutrient Media at 30°C.

Media	Average Percentage Vesiculation of Scolices at days																	
	1	4	8	12	16	20	24	28	32	36	40	44	48	52	56	60	64	68
Glucose	0.0	63.7	77.0	91.5	95.5													
Maltose	0.0+ (8)x	55.0	64.0	66.0	88.7	77.1												
Galactose	0.0 (6)	76.1	86.0	93.3	97.0	100.0												
Lysine	0.0 (6)	58.5	72.3	84.0	85.0	86.0	90.0	95.2	97.8	100.0								
Valine	0.0 (4)	39.8	79.8	89.0	100.0	100.0	100.0											
Phenylalanine	0.0 (4)	32.0	59.0	78.3	85.5	87.5	93.0	97.5	100.0	100.0								
Ascorbic Acid	0.0 (4)	42.6	61.6	64.8	68.8	76.9	78.0	82.0	87.8	89.3								
Nicotinic Acid	0.0 (4)	62.0	68.8	71.5	84.9													
Thiamine HCl	0.0 (4)	41.1	77.0	85.0	87.0	100.0	100.0	100.0	100.0	100.0								
CEE	0.0 (4)	39.0	60.8	63.4	65.0	73.4	84.0	90.0	90.0	93.0	95.0							
Liver Extract	0.0 (4)	57.8	73.3	77.9	80.0	89.3	94.0	100.0	100.0	100.0	100.0							
Serum	0.0 (4)	3.2	48.6	67.6	74.4	73.5	91.6	88.6	89.1	93.3	96.8		100.0	100.0	100.0	100.0	100.0	100.0
Hydatid Fluid	0.0 (4)	38.6	73.2	94.5	97.3	97.9	98.0	98.3	98.8	100.0	100.0	100.0	100.0	100.0	100.0	100.0		
$K_{30°C}^{R}$	0.0 (16)	41.0	59.3	63.4	67.8	82.5	88.0	92.7	99.3	100.0	100.0							

+ Mean of experiments

x Number of experiments

The Effects of Individual Metabolites on the Survival of Echinococcus Granulosus Scolices In Vitro.

a. Monosaccharides and disaccharides:

Up to the 8th day of cultivation, the results obtained in 6 experiments indicated a favorable effect of glucose maltose, and galactose upon the survival of scolices *in vitro*. The percentage survival in these media was 92.2, 88.7 and 96.0, respectively. These rates did not differ appreciably from the controls. On the 12th day values indicated a reduced viability in glucose (66.7%), whereas in maltose and galactose the percentage survival was still high (83% and 91.5%).

By the 16th day, however, there was a sharp drop in the percentage of living scolices in galactose (31.8%), and on the 25th day all scolices maintained in galactose and maltose media were dead, while those in glucose had died between the 16th and the 20th days.

In all cases, the results obtained with Krebs-Ringer's solution alone were superior to those with Krebs-Ringer's solution with added monosaccharides.

A high percentage of scolices were found to be evaginated and swollen or vesiculated with considerable increase in size as early as the 4th day of incubation (Fig.16).

The pH rose from an average of 4.5 to 5.6 during these experiments.

Culture media at the termination of experiments, was positive to Nessler's reagent indicating the production of NH_3 by the scolices.

There was no sign of bacterial contamination, although in some tubes, microscopic examination revealed the presence of molds and yeasts.

Quantitative determinations on glucose, maltose, and galactose, (Somogyi-Nelson Method) showed the disappearance of considerable amounts of these sugars from the culture media during these experiments.

b. Amino acids.

Of the essential amino acids, phenylalanine, lysine, and valine in concentrations of 0.01%, 0.022%, and 0.024%, respectively, (corresponding to normal values in human blood) were chosen because they were found to be constituents of the hydatid cyst wall (Cmelik and Briski, 1952).

Phenylalanine and lysine, in the concentrations used, produced a slight temporary stimulating effect on the survival of scolices in vitro. As Table XVI indicates, a high percentage of scolices were viable up to the 20th day with the percentage falling off gradually until the day when the experiments were terminated.

In no case, however, did added amino acids maintain life in vitro longer than Krebs-Ringer's solution alone, nor was there any additional evidence of development.

Ammonia production by scolices in the presence of these amino acids was confirmed by their positive reaction to Nessler's.

The pH rose from 4.6 to 6.6 in lysine medium and to 5.8 in phenylalanine and valine.

Bacteria were absent from the culture media. There were, however, signs of mold growth in some tubes.

c. Vitamins.

Three vitamins, thiamine HCl, ascorbic acid, and nicotinic acid, were chosen for addition to Krebs-Ringer's solution because they were reported from hydatid cyst fluid by Latif and el Kordy, 1946.

In both ascorbic acid and thiamine HCl, some scolices survived for a maximum period of 42 days and, therefore, resembled the control. Up to the 12th day of incubation the percentage of living scolices in both was very high (Table XVI).

Nicotinic acid, however, had an apparently unfavorable effect upon scolex survival. Only 7% of scolices were living on the 16th day, and on the 20th day of incubation all scolices were dead.

The rise in pH obtained with thiamine HCl and ascorbic acid was from an initial of 4.75 to 5.8 at the termination of the experiments. With nicotinic acid the pH rise was from 3.6 to 4.8.

All cultures were negative for bacteria, but some were heavily contaminated with molds and yeasts.

The Effects of Crude Tissue Preparations and Biological Mixtures on the Survival of E. Granulosus Scolices In Vitro.

Experiments were set up to ascertain whether chick embryo extract (CEE) would prolong the survival of E. granulosus scolices in vitro and promote their development.

The scolices in Krebs-Ringer's solution plus CEE were viable for a maximum period of 44 days with no sign of development beyond vesiculation. However, cultures examined up to the 32nd day of incubation showed unusually high survival rates when compared to the other media and to the controls. Figures given in Table XVI indicated a survival of 86.5% in CEE medium on the 32nd day of incubation whereas in all other media the percentages were very low except in sheep serum which also showed a high percentage of 69.1. In the control, the corresponding value was only 29.4%. By the 40th day, however, the percentage survival had fallen to 6. Upon examination under the microscope and with Gram's Stain cultures showed evidence of heavy contamination with molds.

The pH remained constant at a value of about 6 throughout these experiments.

Evagination and vesiculation did not seem to be particularly favored in the presence of CEE in view of the low percentage obtained up to the 12th day of cultivation.

Survival of scolices in the presence of liver extract did not differ appreciably from the controls. Results presented in Table XVI indicated a survival period of 44 days in liver extract medium with the percentage survival falling off

gradually from the 16th day.

Microscopic examination of scolices in this medium revealed the presence of many bizzare morphologic forms in addition to the usual types observed in other media. Most of the evaginated scolices were vesiculated, with a considerable increase in size at their anterior end, leaving a very narrow stalk in the posterior end containing the "calcareous corpuscles" (Fig. 17); There were also some evaginated, vesiculated scolices considerably increased in size and with a knob at the posterior end (Fig. 18). Furthermore, upon careful observation, some scolices in these media, were found with 2 or 3 constrictions simulating proglottisation (Figs. 19; 20).

The pH at the termination of the experiments had increased from 4.8 to 5.7.

There was no sign of bacterial or fungal growth in these cultures.

The utilization of hydatid fluid and sheep serum in culture media prolonged the survival time of scolices beyond that of the controls. These were the most successful media for scolex survival. In hydatid fluid, scolices were maintained for a period of 64 days and in sheep serum for a period of 70 days. Vesiculation with imbibition of water occured and although there was size increase, there was no evidence of cuticular lamination.

DISCUSSION

The conditions which had to be met in attempting to maintain the scolices of Echinococcus granulosus in vitro primarily concerned bacterial sterility, nutritional requirements and physical factors such as temperature, pH, oxygen tension, ionic composition of the media and osmotic concentration.

Obtaining bacteria-free conditions was an important concern initially and one that had hampered most earlier investigations. Gross bacterial contamination appeared to be deleterious to the existence of hydatid scolices in vitro possibly because of the altered environmental conditions produced by their consumption of nutrients and excretion of metabolic end products. Although most scolices seemed to succumb more quickly in the presence of bacteria, Schwabe (unpublished observations) was successful in maintaining scolices under anaerobic conditions in grossly contaminated hydatid cyst fluid at room temperature for as long as 9 days.

The addition of antibiotics to sterile media aided considerably in maintaining bacteria-free conditions, although yeast and mold contamination of cultures of nutrient media frequently occurred.

The establishment of E. granulosus scolices in cultures free from microorganisms represents the important

first step toward the development of a physically and chemically defined medium.

If cestodes are to be reared throughout their life history or maintained apart from their hosts for long periods it is obvious that they must be supplied with suitable nourishment, for they cannot live indefinitely upon their own reserves.

Very little is known concerning the nutritional requirements of cestodes, as advances in this field are necessarily linked with the technique of *in vitro* cultivation. However, there is considerable evidence available indicating that several species of cestodes utilize glucose (Hopkins, 1952; Markov, 1939; Read, 1956; Wardle, 1937). The early experiments of von Brand (1933) and Wardle (1937) suggested that glucose played an important part in cestode metabolism. Arabinose, galactose, glucosamine, fructose, and maltose did not appear to be converted into polysaccharide by *Moniezia expansa*, whereas glucose was (Wardle 1937). Markov (1939), in his experiments on the glycogen content of the strobilae of *Eubothruim rugosum* and *Triaenophorus nodulesus* and of plerocercoids of *Ligula intestinalis* and *Diphyllobothrium latum*, found under starvation conditions (i.e. after cultivation in non-nutrient salines) a marked and significant loss in glycogen and, in nutritive media, a rise in glycogen with a corresponding loss of glucose from the medium. This constituted evidence that glucose was a source of energy for some cestodes. Under starvation conditions glycogen reserves were almost

completely consumed within a few days.

The scolices of Echinococcus granulosus likewise appeared to utilize glucose as evidenced by the disappearance of measureable amounts of glucose from the medium at the end of each experiment, although Agosin et al. (1957), in a few experiments, presented evidence to the contrary. Similar disappearance of measurable amounts of galactose and maltose from the culture media gave further evidence that these sugars were also being utilized by the scolices. The possibility, however, that a certain amount of these sugars could be lost in the process of autoclaving must not be overlooked.

The utilization of these monosaccharides, and with it increased metabolism of scolices leading to exhaustion of their energy reserves, might perhaps explain the reduced survival time of scolices in vitro upon addition of 0.09% glucose, maltose, and galactose to saline media. Agosin et al. (1957), however, failed to observe any stimulation of oxygen uptake by scolices upon addition of glucose to the Ringer's solution. Ammonia production by scolices upon addition of 0.09% glucose, maltose or galactose in the present studies was not significantly increased. Smyth (1947), in his work with Schistocephalus (unpublished observations) also suggested a detrimental effect of 0.1% glucose, upon in vitro survival of this worms.

Quantitative determinations on total amino nitrogen in cultures containing phenylalanine and lysine, at the

termination of experiments , showed a higher concentration of amino acids than was originally present. Possibly scolices were liberating amino acids into the culture medium or the increase noted resulted from the disintegration of dead scolices.

There is some evidence to suggest that vitamins are essential for normal development in cestodes. Hager (1941) noted that lack of vitamin G (B_2) in the host's diet caused practically complete cessation of egg production in Hymenolepis diminuta. Chandler (1943) and Chandler and Addis (1944) likewise found that lack of vitamin G produced marked stunting in the same species. Smyth (1948) believed that vitamins present in the peptone broth were the essential factors responsible for spermatogenesis in Ligula in vitro and that their absence in the media used by Joyeux and Baer accounted for his failure.

Addition of single vitamins to Krebs-Ringer's solution did not seem to alter the survival time of scolices of E. granulosus.

High early mortality and short survival time of scolices in the nicotinic acid medium, may have resulted from the highly acidic conditions (pH 3.8) rather than to the presence of the vitamin.

The addition of hydatid cyst fluid and sheep serum to Krebs-Ringer's solution provided an environment which was reasonably similar to that to which they were accustomed. In these media they survived for longer periods than in any

of the other media, although no further development was observed.

Wilmoth (1945) found that larvae of Taenia taeniaeformis could tolerate temperatures over a considerable range (14-37°C.), while Smyth (1946) reported that the development of plerocercoids of Schistocephalus solidus into sexually mature adults occurred when the temperature of cultivation was raised to 40°C. Voge and Heynemann (1957) reported the normal development of cysticercoids of Hymenolepis nana at environmental temperatures as high as 40°C. Scolices of Echinococcus granulosus, in isotonic Krebs-Ringer's solution were able to tolerate a considerable range of temperatures 5°-37°C. The prolonged period of survival at low temperatures (58 days) and shortened survival at higher temperatures (20 days) could probably be explained on the basis of their different metabolic rates.

At temperatures ranging from 5°-9°C. activity of scolices seemed to slow down as evidenced by cessation of normal contractile movements and pulsations of the flame cells. Upon stimulation by warmth normal movements were resumed. At high temperatures scolices exhibited normal contractile movements and rapid beating of their flame cells suggesting that they were using up energy reserves at a greater rate.

According to Hobson (1948), there is little evidence that parasitic worms are benefited in vitro by anaerobic conditions. In fact, Wilmoth (1945) observed a marked reduction

in the survival period of larvae of Taenia taeniaeformis in Tyrode's solution under anaerobic conditions.

Results similar to those reported by Wilmoth (1945) were found with the scolices of Echinococcus granulosus. The fact, however, that scolices were able to live for as long as 20 days in the absence of oxygen suggested that these organisms are facultative anaerobes. Farhan et al (1959) have shown that the scolices of E. granulosus are efficient, however, in the utilization of oxygen even under low environmental oxygen tensions.

Scolices of Echinococcus granulosus suspended in various buffers tolerated pH extremes of 3.6 to 9 although survival was markedly in the neighborhood of pH 7. This tolerance is not suprising in view of the fact that the scolices are exposed to quite variable hydrogen ion concentrations during transfer to their normal habitat in the definitive host. The possible effects of buffers of varying chemical composition has not been studied.

It has been assumed by most workers that the osmotic pressure of an internal parasite resembles to that of its environment (Hobson, 1948). Little work has been done, however, on the effect of variations of external osmotic pressure on the length of time for which a cestode can survive under artificial conditions. Wardle (1932) found that plerocercoids of Diphyllobothrium latum and Triaenophorus and adults of Bothriocephalus scorpi tolerated balanced salines better than low molecular concentrations of the component

salts. On the other hand maximum longevity values for plerocercoids of Nybelina were obtained in double strength Locke's solution (Wardle, 1934), and for some Pseudophyllidean tapeworms by 3/4 strength Locke's solution (Smyth, 1946).

The scolices of Echinococcus granulosus did not seem to be very sensitive to changes in osmotic pressure. They withstood for prolonged periods concentrations of balanced salt solutions of between 0.308 M and 0.0385 M. However, it should be pointed out that in both hypotonic and hypertonic solutions, particularly in the latter where the concentration of the components of Krebs-Ringer's solution was doubled, the survival time of scolices was considerably reduced. Of considerable interest was the observation that scolices in vitro were able to imbibe water against a substantial osmotic differential. The mechanism involved is certainly a subject for further study.

The scolices of Echinococcus granulosus seemed to be indifferent to the absence of $CaCl_2$ and KCl from Krebs-Ringer's solution. Neither did they seem to be very sensitive to the absence of KH_2PO_4 and $MgSO_4$ although survival time was reduced considerably in these latter cases.

The marked reduction in percentage survival as well as of survival time in the absence of NaCl seemed to point to the importance of Na^+ in the environment of the scolices.

A number of chemical and physical substances were found to stimulate evagination in cestodes.

Clarenberg (1932) obtained active evagination of the hold fasts of Cysticercus bovis with bile, pancreatin extract, trypsinogen, sodium taurocholate, or sodium glycocholate solutions at 40°C. Smith (1954) observed evagination of cysticercoids of Raillietina cesticillus under mechanical pressure in chicken bile or a detergent in water, Ringer's solution, sodium taurocholate or iron bile salts. Butning (1927), de Waele (1934) and Edgar (1940, 1941) reported very effective evagination of Cysticercus pisiformis in a bile salts solution. Sawada (1959) obtained best results for the cysticercoids of Raillietina kashiwarensis with 0.1% pancreatin, at pH 7 and at temperatures of 40-42°C. De Waele (1935) considered evagination of hydatid scolices to be a function of temperature and not of pH and bile salts.

In the present studies, evagination of Echinococcus granulosus scolices cultured in different media, though extremely variable, suggested that scolices cultured in certain media, or under certain physical conditions were perhaps potentially capable of a high degree of evagination. In the great majority of cases, the marked variation observed in percentage evaginated from scolices cultured under apparently identical conditions further suggested a natural variation in the scolices used in each experiment.

It has been found that tapeworms become flaccid and expanded in hypotonic media, and flaccid and contracted in hypertonic ones (Wardle and McLeod, 1952).

The swelling or vesiculation of the scolices of

Echinococcus granulosus did not seem to be governed by the laws of osmosis. This phenomenon was observed almost in all media under investigation. Moreover swollen or vesiculated scolices were seen as readily in hypertonic solutions as in isotonic and hypotonic media.

Other Phenomena Observed

Many scolices appeared to emit bubble like objects from either the anterior end or posterior, or both. These according to Ross (1927) were the "spherules bulleuses" described by Coutelen, the exact nature of which was not clear. Coutelen (1927c) considered them to be either thrown out as a result of the change from the high intracystic pressure of the scolices, or to be an indication of the commencing degeneration of the scolex.

The appearance of bubbles seemed to indicate a response by the scolices to an unfavorable stimulus, in agreement to the findings of Ross (1927) who similarly reported seeing their occurrence most numerously shortly before their death.

In some cases these bubbles were very much emphasized. They were clear and structureless as Ross (1927) had found. In some nutrient media, however, it was possible to see the fine granulations which Coutelen (1927c) had at times found, of the same nature as that of the scolex.

In one set of experiments with phenylalanine, ascorbic acid, thiamine-HCl and valine, it was interesting to find the

appearance of "twin-scolices" on the 21st day of incubation (Fig. 21). It was thought that these media had the potentiality to induce the development of new scolices, but as long as this phenomenon did not appear in subsequent experiments it suggested an abnormality of that particular batch of scolices. Although the fact that it appeared only in those media may have some significance which needs further elucidation.

CONCLUSION

The object of this investigation was to maintain viable scolices of Echinococcus granulosus outside of their host for extended periods, and to determine physical and chemical conditions which might favor their in vitro survival and development.

The elimination of bacteria was an important concern primarily for their deleterious effect upon the scolices, as well as for rendering the medium undefined.

Scolices maintained in vitro, in non-nutrient, balanced salt solutions, at different temperatures seemed to be favored at low temperatures.

Oxygen tension was found to be an important controlling factor. Survival of scolices in vitro were favored by aerobic conditions, although survival for a considerable period of time under conditions of low oxygen tensions suggested their being facultative anaerobes.

Behavior and maximum survival of scolices cultured in normal, double strength, 1/2 strength, and 1/4 strength Krebs-Ringer's appeared to be quite different, which suggested that within these limits osmotic pressure must be controlled.

A pH of about 7.4 was found to be optimum. Hydrogen ion consentrations below pH 6 and above pH 8 could not be tolerated by the scolices. The results, however, were not

conclusive in view of the fact that the suspending buffers were often markedly different in chemical composition and molarity, and therefore the effects produced were not necessarily due to pH.

The scolices were indifferent to the absence of $CaCl_2$ or KCl, they were somehow sensitive to the absence of KH_2PO_4 or $MgSO_4$. However, they could not tolerate the omission of NaCl from the medium.

The stimuli causing evagination and vesiculation seemed to be many and varied, not the least of which was a natural variation of the scolices.

Hydatid fluid and sheep serum were the most successful media for scolex survival.

The failure to obtain growth and prolonged survival in presence of CEE, liver extract, amino acids, vitamins, the shortened survival in sugars, as well as liberation of amino acids by the scolices into the medium are questions which possibly can be answered in the light of further investigations.

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PLATE I

Fig. 1. Adult Echinococcus granulosus (Mag. 18x).

PLATE II

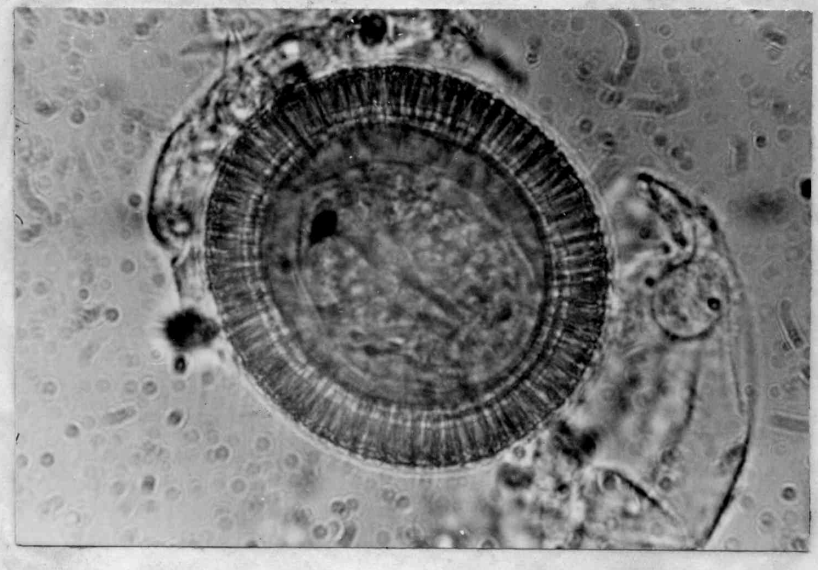

Fig. 2. Ovum of E. granulosus showing the radially striated shell surrounding the oncosphere or hexacanth embryo. (Mag. 970x).

PLATE III

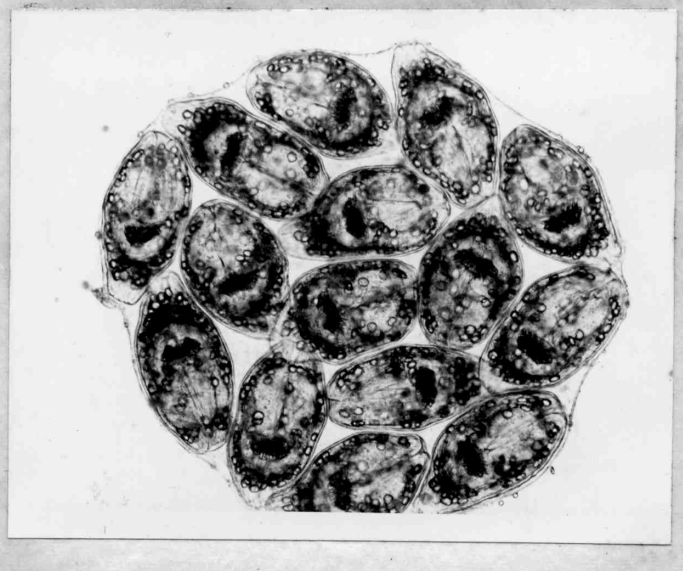

Fig. 3. Larval Scolices of E. granulosus within the brood capsule. (Mag. 100x).

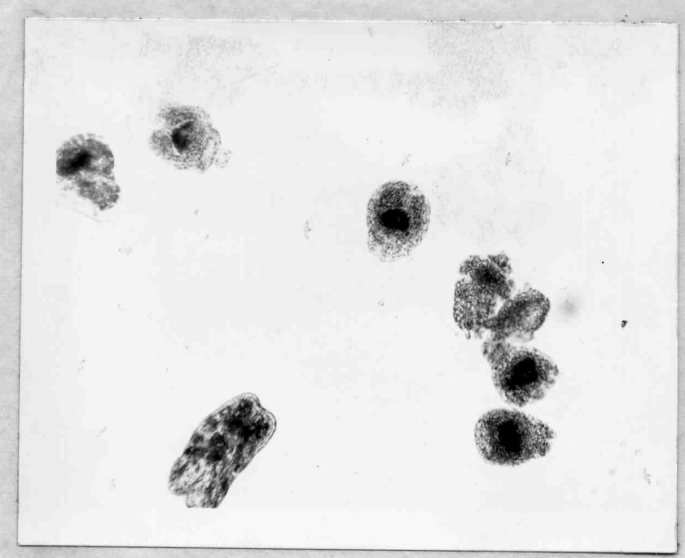

Fig. 4. Degenerated scolices characterized by their brown color and shrunken appearance. (Mag. 100x).

PLATE IV

Fig. 5. Brood capsule membrane turned inside out; with the invaginated scolices on the outside. (Mag. 100x).

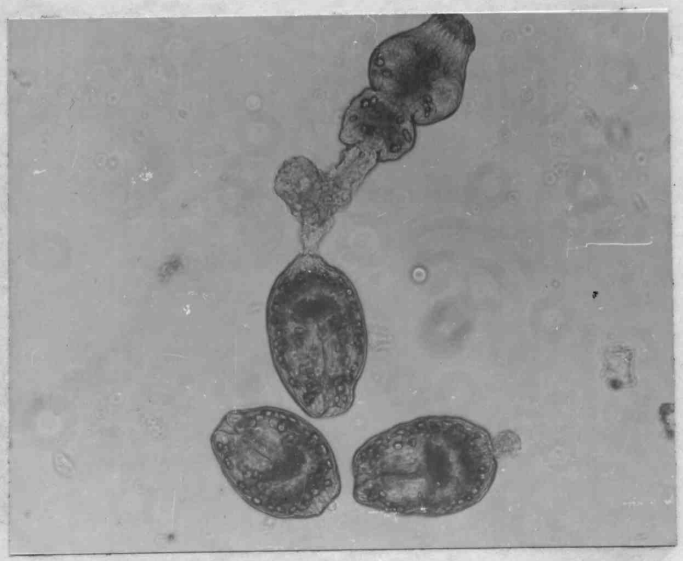

Fig. 6. Invaginated scolices of E. ganulosus showing "calcareous corpuscles"; hooklets and suckers. (Mag. 100x).

PLATE V

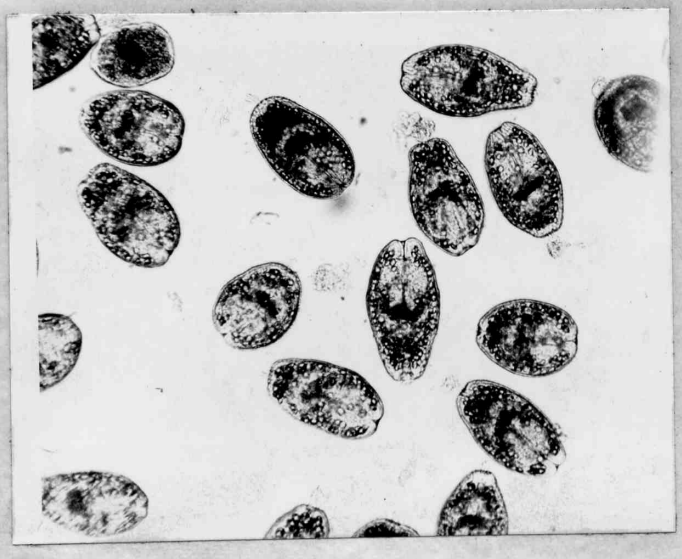

Fig. 7. Invaginated scolices of E. granulosus normal in size. (Mag. 100x).

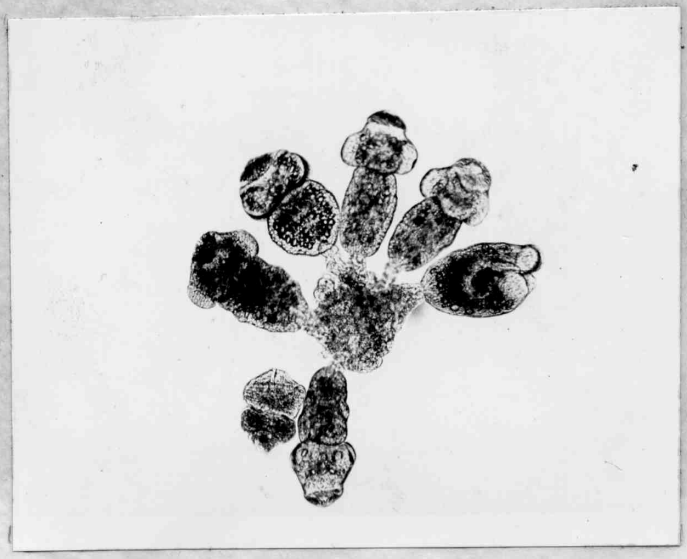

Fig. 8. Evaginated scolices of E. granulosus normal in size. (Mag. 100x).

PLATE VI

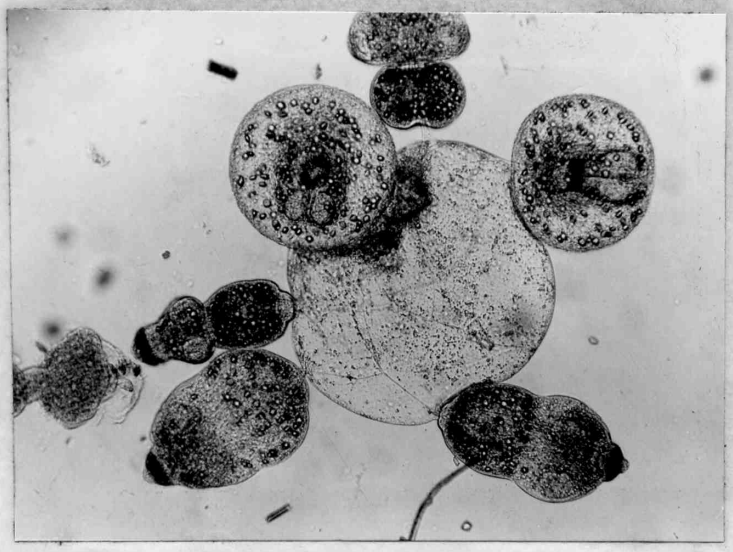

Fig. 9. Invaginated and swollen scolices of E. granulosus. (Mag. 100x).

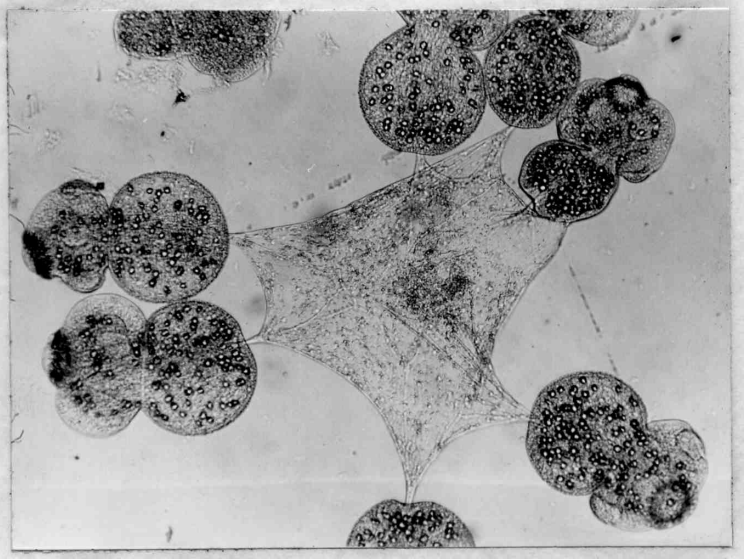

Fig. 10. Evaginated and swollen scolices of E. granulosus. (Mag. 100x).

PLATE VII

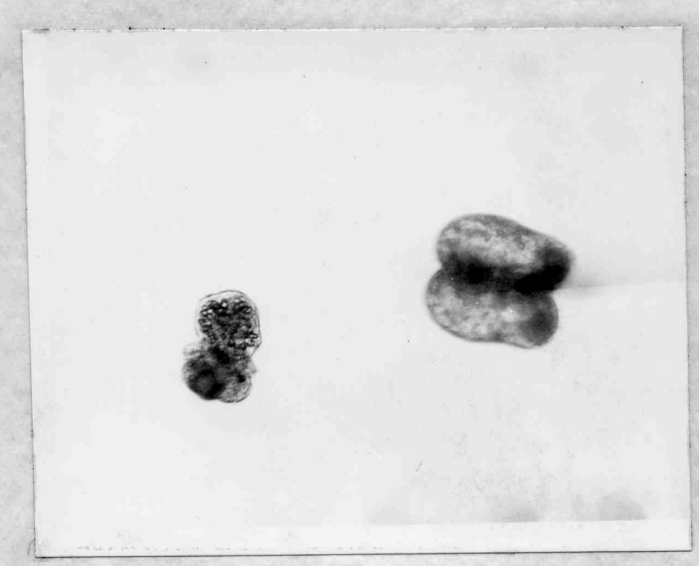

Fig. 11. Invaginated and vesiculated scolices of E. granulosus. (Mag. 100x).

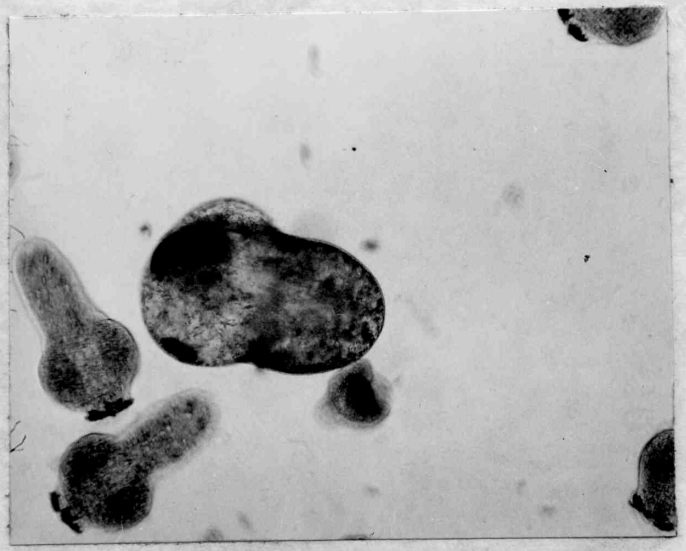

Fig. 12. Evaginated and vesiculated scolices of E. granulosus. (Mag. 100x).

PLATE VIII

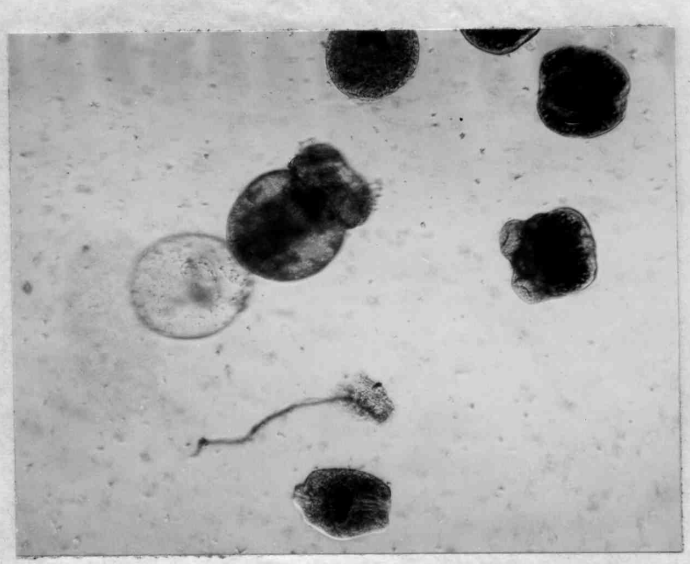

Fig. 13. Evaginated scolex of E. granulosus showing cuticular bubble-like swelling. (Mag. 100x).

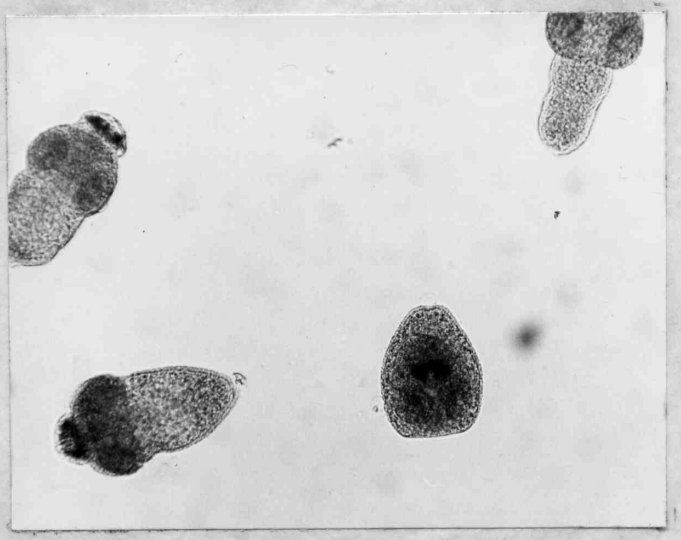

Fig. 14. Dead scolices of E. granulosus in various stages of degeneration. (Mag. 100x).

PLATE IX

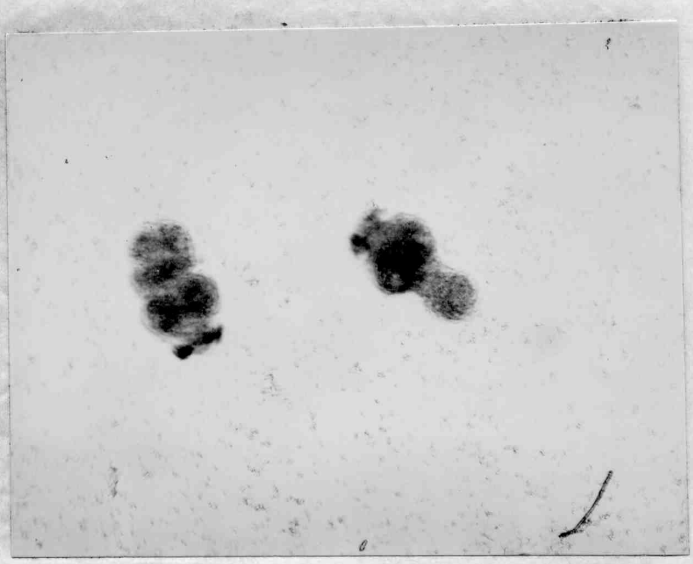

Fig. 15. Degenerated scolices of E. granulosus with shedded hooklets. (Mag. 100x).

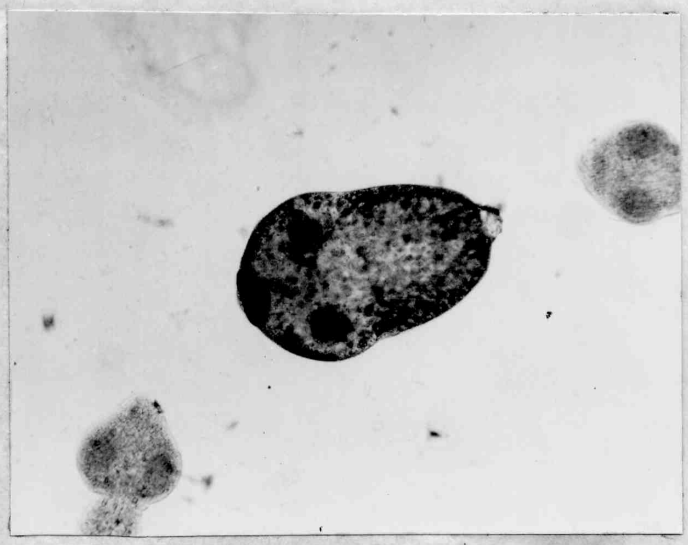

Fig. 16. Evaginated and swollen scolices of E. granulosus with considerable increase in size. (Mag. 100x).

PLATE X

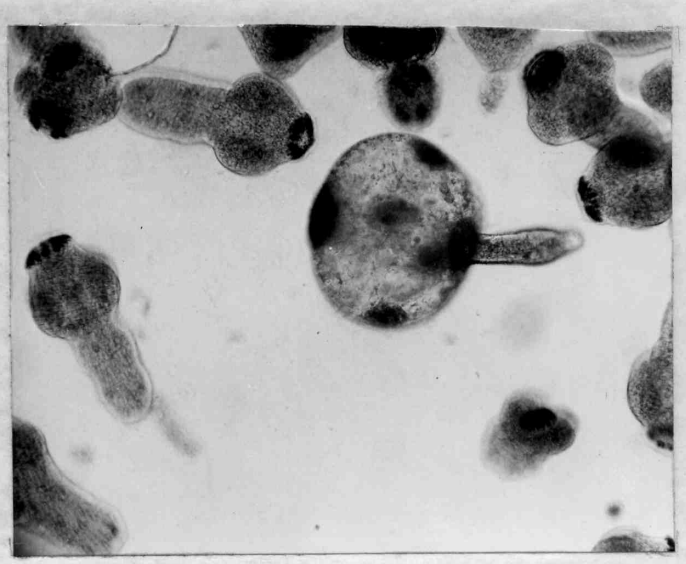

Fig. 17. Evaginated and vesiculated scolex of E. granulosus with considerable increase in size at the anterior end and with a narrow stalk at the posterior. (Mag. 100x).

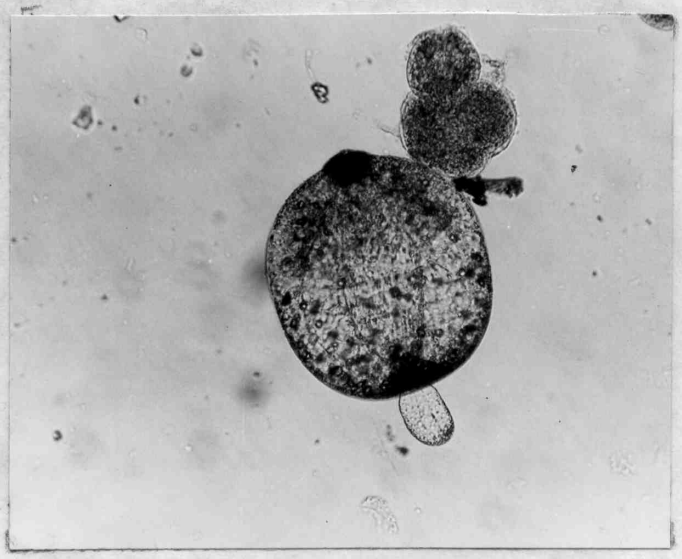

Fig. 18. Evaginated and vesiculated scolex of E. granulosus; considerably increased in size and with a knob at the posterior end. (Mag. 100x).

PLATE XI

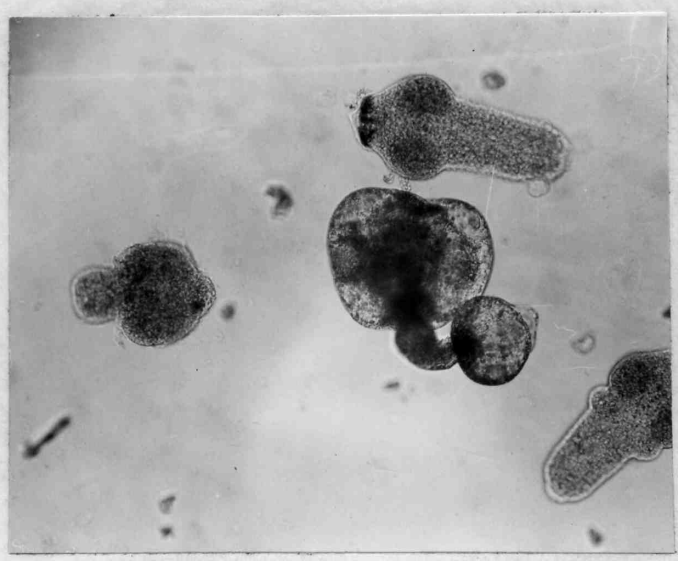

Fig. 19. Evaginated scolex of E. granulosus with constrictions simulating proglottisation. (Mag. 100x).

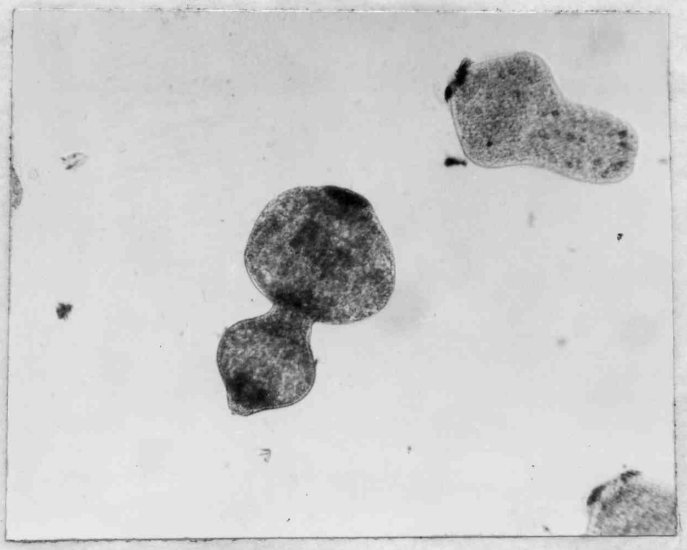

Fig. 20. As Fig. 19.

PLATE XII

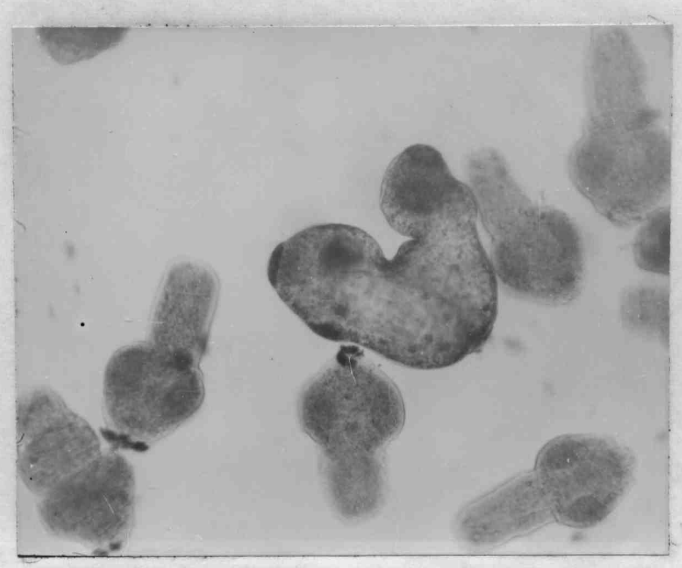

Fig. 21. "Twin-Scolices" of E. granulosus. (Mag. 100x).