IN VITRO SURVIVAL OF ISOPARORCHIS HYPSELOBAGRI
UNDER AEROBIC AND ANAEROBIC CONDITIONS

DISSERTATION
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ALIGARH
1974
This is to certify that the work presented in this dissertation has been carried out by Wajih Ahmad Nizami and is suitable for the award of the Master of Philosophy degree in Zoology of the Aligarh Muslim University, Aligarh.

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AMU ALIGARH
ACKNOWLEDGEMENT

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INTRODUCTION
The idea of culturing trematode parasites outside their hosts has long held a strong fascination for many parasitologists. According to Read (1950) "the cultivation of parasitic helminths outside the host presents one of the most difficult and most challenging problems facing parasitologists today".

There are several techniques of in vitro culture of digenetic trematodes and considerable progress has been made in this field in the past one or two decades. These culture methods may be expected to help our understanding of the host-parasite relationship. The most successful techniques, however, are too complicated to be regarded as standard laboratory procedure.

Due to this difficulty in studying the living parasites, the physiological and biochemical knowledge is based on short term experiments. The parasites, freshly removed from the host, and kept in simulated in vitro physiological conditions, do not remain alive for a very long time. As a result, our knowledge of the biochemistry, physiology and morphogenesis of trematodes is very limited.

As the digenetic trematodes are exclusively endoparasitic and the complexity of their life cycle creates hazards in culture methods, in vitro culture of trematodes is a hazardous and challenging field of
research, and one faces several major difficulties in vitro culture of trematodes. Trematodes live in some biological habitat, which has complex physico-chemical properties, such as pO₂, pCO₂, pH, amino acid and sugar contents, temperature and osmotic pressure. The nature of many of the parasitic habitats and their physico-chemical properties are imperfectly known, and attempts should be made to obtain information about the same with some degree of accuracy for successful in vitro culture of parasites.

Secondly, all species feed on complex biological materials, such as intestinal contents, bile, blood etc., whose nutritional properties are difficult to replace by artificial media.

Thirdly, these parasites live in habitats which contain some microflora, and treatment with antibiotic procedures may be necessary before axenic culture can be attempted. However, it has been found that different trematodes react differently in their response to antibiotics and if these are utilized for maintaining sterility in in vitro culture there is possibility of either inhibition or retardation of metabolic activities of the parasites.

Due to continuous anabolism and catabolism the metabolic waste products are excreted by these worms,
which should be continuously removed from the culture medium, since their accumulation generally have adverse effects on parasite during in vitro culture. Special precautions are necessary to be taken to ensure dispersion and dilution of toxic by-products. A Stoll (1959) pointed out that "Slight agitation of culture vessel markedly improves culture results".

Lastly the selection of suitable media to simulate as closely as possible the conditions which normally exists in situ in the host. The exposure to appropriate physico-chemical conditions in balanced salt solutions is sufficient to induce considerable differentiation and maturation in parasites.

In addition to this, trigger stimuli, temperature and pH do play an important role in successful culture techniques.

In spite of these difficulties, in vitro culture techniques have made considerable progress in the past few years and there is every hope that within the next decade a large number of parasite species can be cultured in the laboratory. The knowledge of in vitro culture yields a clear insight into the nature of the host-parasite relationship, which helps in the elucidation of the nutritional requirements, effect of host secretions,
host immunological reactions, trigger stimuli, effect of anthelminthic and other biochemical events.
HISTORICAL REVIEW
AND
STATEMENT OF PROBLEM
The cultivation and survival of helminths in vitro has received much attention in recent years. Literature on the subject has been reviewed by Bertzen (1966), von Brand (1966), Clegg and Smyth (1967), Read and Simmons (1963), Silverman (1965), Smyth (1962), Weinstein (1958, 1960 & 1966), Woodruff (1963) and more recently by Taylor and Baker (1968).

It has been observed that in vitro survival of trematode parasites under aerobic and anaerobic conditions varies markedly from species to species. Ross & Bueding (1950) have shown that Schistosoma mansoni, when incubated in serum ultrafiltrate at 37°C, survives only five days under anaerobic condition while 12 days under aerobic condition. Rohrbacher (1957) observed that Fasciola hepatica, cultured in Tyrode at 37°C, survived 13 days under aerobic condition and 20 days under anaerobic condition, but on adding liver extract to the Tyrode, the survival time increased under aerobic conditions from 13 to 17 days. Robinson (1956 & 1960) has shown that in horse or guinea pig serum supplemented with 0.1% glucose with Tyrode saline, S. japonicum and S. mansoni survive 28 to 56 days and continue to lay eggs for at least 14 days. When Schistosoma mansoni was cultured in NCTC 109 (Senft & Senft, 1962), the worms laid eggs for 20 days, after which the rate of egg production deteriorated.
These observations clearly indicate that the contents of the culture medium do play an important role in the survival of a parasite in vitro, whether they are cultured under aerobic or anaerobic conditions.

Different workers have used different gaseous phases for the establishment of anaerobic conditions in vitro. For cestodes Bertzen and Mieller (1904) used 90% nitrogen and 10% carbon-di-oxide; Bertzen (1962), used 95% nitrogen and 5% carbon-di-oxide. Rohrbacher (1957) established anaerobic conditions for Fasciola hepatica with complete replacement of air by nitrogen gas. Different in vitro survival times have been reported by various workers for the adult trematodes, Churchill & Crowther (1961), Burton (1962); Dawas & Mueller (1957); Ito & Komiya (1955); Lee & Chu (1935); Mao & Lyu (1957); Robinson, (1950, 1957, 1958, 1960) Senft & Senft, (1962), Senft & Weller, (1956). The variations in the survival time are due to the culture media, gas phase, different types of culture vessels used or types of apparatus and other physico-chemical conditions.

Various workers have choosen different parameters to indicate survival and growth of parasites. In earlier experiments the movement of the parasite was used as a useful survival parameter. Rohrbacher (1957) used color of the parasite. Bell & Smyth (1958) have correlated mitotic activity in the parasite with their
phase of development, thus providing a useful technique for estimating growth of worms in vitro. Mykytowyeyz and Dudzinski (1965) used weights as growth and survival parameters.

According to Newsome & Robinson (1954), and Robinson (1956, 1960), frequent change of culture media will increase the survival time in order to avoid the accumulation of metabolites which drop the pH rapidly. Clegg (1961) designed a continual flow system for the culture of schistosomes. Smyth (1959) developed a dislysis sac technique for the removal of the metabolites.

The metabolites excreted during in vitro culture have been studied and investigated by a number of workers; Weinland and von Brand (1926); Flury and Leeb (1926); von Brand (1928, 1950, 1966); Van Grembergen & Pennoit DeCooman (1944); Stephenson (1957); Bueing et al., (1947); Goil (1958, 1961); Mansour (1959); Senat (1963); Ehrlich, Rijavec & Kurelec (1963); Kurelec (1964), Prichard & Schofield, (1968), Moss (1970) and more recently by Lutz and Siddiqi (1971).

It has been observed that various trematodes excrete different quantities of metabolites under aerobic and anaerobic conditions. The main constituents of the excretory products of the trematodes are volatile fatty acids, higher fatty acids, amino acids, organic acids,
ammonia and urea. Lutz and Siddiqi (1971) have shown that proline, alanine, histidine, phenylalanine, serine, lysine, urea and ammonia are excreted in the protonephridial fluid of *Fasciola gigantica*.

According to Moss (1970) different excretory metabolites reflect the differences in the diet of trematodes.

Knowledge of the *in vitro* metabolism of trematode parasites under aerobic and anaerobic conditions and their habitat is necessary for a clear understanding of the physiology of these animals.

*Isoparorchis hypselobagri* is a common parasite of the swim bladder of *Wallago attu*. The swim bladder is a peculiar biotope whose physico-chemical properties are very little known. von Brand (1966) divides parasitic habitats into environments possessing very little or no molecular oxygen and habitats in which excessively high oxygen tensions are encountered.

Most of the parasites live in environments with high oxygen tension; for example, parasites of vertebrate lungs have almost the same oxygen pressure as free living animals. Another habitat rich in oxygen is blood; especially the arterial blood of dog, rabbit, man and fish, which have $pO_2$ equal to 70-100 mmHg, as reported
by Frederieq (1893); Firket (1910), Dill, Edward & Florkin (1932), and Barcroft et al. (1923). While venous blood of horse, man, duck, have 37-40 mmHg, (von Buddenbrook, 1939) and of fish 2-14 mmHg (Root, 1931), (Dill, Edward and Florkin 1932).

The $O_2$ tension of various parasitic habitats have been tabulated by von Brand (1952). Rogers (1949) reported that in the small intestine of rat, oxygen tension ranges from 8.9 to 30.2 mmHg which is biologically significant, and depends upon the distance from the pylorus; while Chaigneau and Charlet-Lery (1957) observed that intestine of large vertebrates is poor in oxygen or is sometimes completely oxygen free. Schloesing and Richard (1898) have shown that fishes living near the surface of water have, in their swim bladders, gas which is much like air while at greater depth the $O_2$ becomes an increasing dominant component; while Hufner (1892) observed that bottom dwelling fishes of lakes have 99% nitrogen. Later Sundnes (1963) added a significant piece of information, that is the bottom dwelling fishes have high pO$_2$. Wittenberg (1958) determined higher pO$_2$ and lower inert gases in the swim bladder of the physostomes. Hoar and Randle (1970) reviewed available literature on the content of the gases in swim bladders. These observations indicate that oxygen is a conspicuous gas in the swim bladders of physostomes.
All stages in the life of the digenetic trematodes, which have been studied *in vitro* have been found to utilize oxygen when it is available. Vernberg (1963) and von Brand (1966) reviewed the available information and concluded that adult trematodes are facultative aerobes. According to Bueding (1949) von Brand (1952) and Goil (1958) some of them are quite tolerant to lack of oxygen. The oxygen consumption in trematodes have been studied by a number of workers which shows that $O_2$ consumption varies from species to species, Van Grembergen (1949), Bueding (1950), Lazarus (1950), Read & Yogore (1958), Goil (1958c), Schimomura (1959), Freeman (1962), Taft and Fried (1968) and Bruce *et al.* (1971).

There have been relatively few studies on the respiration of adult digenetic trematodes; von Brand (1966), Vernberg (1963), Smyth (1966), Florkin and Sheer (1968) and Erasmus (1972) reviewed the available data on oxygen consumption in trematodes. The small amount of evidence available indicates that the respiratory rate is dependent (i.e., directly proportional) on the external oxygen tension, glucose and temperature, as pointed out by Harnisch, (1932), Van Grembergen, (1949), Vernberg (1963), Bueding (1949), Mansour (1959), Bruce *et al.* (1971), Bueding (1950), Vernberg and Hunter (1961), and Richards *et al.* (1970).
Although much research has been done on trematode physiology during the last twenty years, still there are many gaps and lacunae in our knowledge of the subject. In India most of the work so far done in parasitology has been on the morphology and taxonomy of various groups of parasites. Only Goll (1953-73) has published some preliminary observations in this field which has otherwise remained completely neglected. Research on trematode physiology is a difficult task, because trematodes of veterinary or clinical importance have to be kept alive in vitro for long periods. Further, their life cycles are very complex. One of the most exciting developments during the past decade in parasitology has been the realisation that the nutritional and environmental requirements of trematodes can be sufficiently met by artificial systems. In vitro techniques allow the nutrition, physiology and biochemistry of a parasite to be studied in isolation from the interacting physiology of the host. Tools are now available for more rigorous attempts to study physiological phenomena of the natural environment. The study of this aspect will be rewarding in increasing our knowledge and understanding of the actual mechanism of pathogenicity and metabolic processes in parasites.
Isoparorchis hypselobagri, (Billet, 1898) a trematode parasite in the swim bladder of Wallago attu, provides a unique opportunity to study some aspects of physiology, primarily the respiratory metabolism during in vitro culture. In this respect several questions arise and must be answered. Does the swim bladder of the fish provide aerobic environment to the parasite? Does the parasite survive better under aerobic or anaerobic conditions during in vitro culture? Does the parasite take up oxygen? Is their any effect of temperature and glucose on the oxygen consumption? What are the end products of the metabolism which are released by the parasite into the culture medium during in vitro culture? The present investigation is an attempt to answer some of these questions.
MATERIALS AND METHODS
ANALYSIS OF THE SWIM BLADDER GAS:

The gas of the swim bladder was analysed by the method of Krogh (1908), which has advantages of simplicity, flexibility, and ease of construction; and is accurate to within 1 - 2%. The apparatus was fabricated in the laboratory.

Fabrication of Krogh Gas Analyzer

The apparatus was made from a 1 ml pipette, graduated to 0.01 ml. The mouth end of the pipette was blown into a cylindrical bulb, 6 cm long and 7 mm in outer diameter. The opening of the pipette into the bulb was constricted in order to prevent dripping. This pipette was encased in a water filled glass tube of 1 inch diameter, to prevent the rapid changes in temperature during handling, and the whole apparatus was clamped to a stand in such a manner that it could be manoeuvred easily. Ten cm long, soft polyethylene capillary tubing was attached to the delivery end of the pipette. It was plugged with a glass stopper at the other end. A small screw clamp served to regulate the volume contained in the polyethylene tube, in order to control the position of the gas sample in the pipette.
Collection of Gas Sample: The samples were collected from the living fishes from Ganges river at Narora and Fanethi Canal, and the samples were analysed on the spot.

The samples of the swim bladder gas were collected in 3 ml syringes, over 1 ml of acidified sodium chloride solution (saturated NaCl in water + methyl red indicator (0.5% in 95% alcohol) and a few drops of HCl). This solution is low in oxygen dissolving capacity. To avoid the contamination of the sample with air, the needle of the syringe was held upside down and was injected at the mid-lateral line, just beneath the gill region about half an inch deep into a freshly caught fish. By gently moving the piston upwards, about 3 ml gas of the swim bladder was taken into the syringe. Syringes containing gas samples were placed in a downward position on stands especially designed for the purpose.

The pipette of the gas analyzer was filled with acidified NaCl solution by sucking on the polyethylene tubing and the clamp was screwed. The tubing was closed with glass stopper without trapping air bubbles. The whole apparatus was kept at 60° angle (Fig. 1), and a sample gas bubble was introduced into the pipette bulb containing NaCl solution. The clamp screw was loosened to draw the bubble slowly into the graduated part of the pipette. Once inside, the
a vertical position anticlockwise. With a medicine dropper the NaCl solution was withdrawn from the bulb, which was filled with oxygen absorbent "OxSorbent" (Burrell Corp.) as Hoar (1956) recommended. The apparatus was then rotated to its original position (60° angle) in a clockwise direction, and the bubble was pushed back into the bulb by tightening the screw clamp. The whole apparatus was gently moved about the horizontal position and the bubble was brought more fully into contact with "OxSorbent" by moving it to and fro in the bulb. After two minutes the bubble was taken into the capillary tubing of the pipette, and the volume of the bubble was read again. The reduction in the volume of the gas bubble represents the volume of the oxygen in the sample.

After each determination, the pipette was rinsed and refilled with fresh NaCl solution. Oxygen content was determined both as percentage of the swim bladder gas and as partial pressure of $O_2$ in mmHg.
(B) **IN VITRO SURVIVAL OF ISOPARORCHIS HYPSELOBAGRI**

Erlenmeyer flasks (50 ml) with cotton plugs were used for aerobic cultivation. For anaerobic studies, 100 ml Erlenmeyer flasks with a rubber stopper with two stopcocks were used; one for gas inlet and the other for gas outlet. The glassware was acid cleaned, and finally washed with double distilled water. All glassware and instruments were sterilized under 15 lb pressure per square inch for 20 minutes.

The medium in which the parasites were maintained for **in vitro** culture, contained the following electrolytes and the pH was adjusted to 7.0

<table>
<thead>
<tr>
<th>Electrolyte</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>100 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>NaH(_2)PO(_4)</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>5 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>19.9 mM</td>
</tr>
</tbody>
</table>

For the maintenance of axenic conditions, penicilline G (Na Salt), streptomycine sulphate and mycostatin were provided in the medium in the following concentrations:

- **Penicilline G (Na Salt)** 100 units/ml
- **Streptomycine Sulphate** 100 µg/ml
- **Mycostatin** 20 p.p.m.
Sintered or fritted glass and asbestos filters (Seitz type) were used for the sterilization of the medium in order to avoid bacterial and fungal contamination.

In some experiments mammalian blood was also added to the above medium to see if the parasites fared better in its presence. For this study, ACD solution (Acid citrate dextrose) was used as an anticoagulant for buffalo blood. The blood was collected from the local slaughter house and 5 ml blood per litre of saline was used in this study in both, aerobic and anaerobic in vitro cultures.

The worms were collected from the swim bladder of *Wallago attu* from the local fish market. The worms were washed three times with sterile culture medium and original wet weight of parasite was recorded on a single pan balance. Only active flukes were used for experiments. One fluke was placed in culture flask containing 20 ml sterile medium, and the culture medium was changed every alternate day to avoid bacterial or fungal growth, and every 10th day, the weight of the parasites was recorded.

Anaerobic conditions were established by using nitrogen as the gas phase. Both the stopcocks of the culture flask were kept open in order to replace
air by the nitrogen gas. The nitrogen gas was allowed to bubble in the medium for about 3 minutes, and the outlet stopcock was closed first, so as to establish the nitrogen gas pressure, followed by the closure of the gas inlet stopcock.

In changing the culture medium during anaerobic experiments, the fluid was gently poured in, to prevent excessive contamination with air, and again air was replaced with nitrogen in the manner mentioned above.

Prior to daily examination of flukes, the flasks were swirled to disperse the waste products more evenly throughout the solution.

(C)  OXYGEN CONSUMPTION

The oxygen consumption was determined by the Scholander (1950) respirometer. The respirometer was fabricated in the laboratory. The basic element of the apparatus (Fig. 2), consists of a respiration chamber (A), thermodenrometer or compensation chamber (B), plexiglass manometer block (C), measuring device for oxygen (D), and temperature controlling device (E). For the measurement of gas
volumes an Agla micrometer (Welcome Lab. London) was used, in which one small division of the micrometer was equal to 0.20 ul. A single Warburg flask (15 ml) was used as the respiratory chamber, containing 0.2 ml 20% KOH in the centre well and 2 ml saline medium in the flask with parasite. The thermobarometer was used containing only 2 ml of medium. The temperature of the Scholander tank assembly was controlled by an aquarium automatic thermostatic control which is accurate to the extent of ± 2°C.

(D) ANALYSIS OF EXCRETORY PRODUCTS BY SPOT TESTS

The flukes were incubated aerobically at 23°C in 20 ml of sterile medium for 6 hrs., in 100 ml Erlenmeyr flasks plugged with sterile cotton. At the end of the incubation period, the flukes were removed and the medium was concentrated in a dialyzing tube No. 4465-A2 (Arthur H. Thomas, U.S.A.) to 5 ml. This 5 ml incubation solution was tested micro-chemically to determine qualitative nature of either compounds or classes of compounds excreted by the parasite during in vitro incubation.
A few drops (0.1 ml) of the incubation medium were used in each test for aminoacids, protein, hemoglobin, formaldehyde, formic acid, methanol, sulphur compounds o-dioxymethylene, oxomethylene compounds, etc. and tested by the methods of Feigl (1954).
RESULTS
RESULTS

(A) ANALYSIS OF THE SWIM BLADDER GAS

The gas of the swim bladder of *Wallago attu* was analyzed by the Krogh's gas analyzer, and was found to contain a sufficient quantity of oxygen which is biologically significant. The oxygen content ranges from 22.0 mmHg to 73.9 mmHg or 2.9 per cent to 7.9 per cent of the swim bladder gas. It was also found that the presence or absence of the parasites in the swim bladder does not influence the partial pressures of oxygen. The results of these experiments are shown in Table I. It can be seen from the results that small amounts of oxygen were always present in the swim bladder. However, the fishes collected from different localities have different oxygen content in their swim bladders. This clearly indicates that *Isoparorchis hypselobagri* always lives in an oxygen rich environment.

(B) IN VITRO SURVIVAL OF ISOPARORCHIS HYPSELOBAGRI

The criteria of normal survival used in these experiments were quadrifold.

1. Movement of the parasite.
2. pH changes of the medium.
3. Color of the parasite, and
4. Weight of the parasite.
These experiments were carried out using simple electrolyte solution containing glucose (19.9 mM). These preliminary experiments in vitro culture studies revealed that *Isoparorchis hypselobagri* survive longer under aerobic rather than under anaerobic conditions. On the 10th, 20th, 30th and 40th day, the survival of *Isoparorchis hypselobagri*, under aerobic condition is 100%, 100%, 84% and 44% respectively; while under anaerobic conditions it is 80%, 83%, 1% and 0% respectively. Under aerobic conditions the maximum survival was for 49 days whereas in anaerobic conditions it was only for 30 days (Fig. 3, Table II).

In the present study, the weight of the parasite was used as a parameter. On the 10th, 20th, 30th and 40th day, the parasite lost 8.9%, 17.3%, 24.5% and 37.3% of its wet weight respectively in the presence of glucose in saline, but on adding blood to the medium the weight loss was reduced to half of that of glucose saline.

However, in those experiments in which blood was not added to the medium, the dark appearance of the fluke ceca disappeared after two or three days. Usually the dark pigment was regurgitated, but its quantity was less in worms kept in the blood free medium while greater in the blood containing medium.
Freshly prepared medium was adjusted to pH 7.0 but actively metabolizing worms lower the pH of the medium to 4.5 to 5.5.

To determine the day to day condition of the worms, during in vitro culture, certain subjective evaluation of the flukes were made. The movements of the head and neck were noticed with special attention on the mode of attachment of the worm to the walls of the culture flask. Healthy and freshly collected flukes showed rapidity in their wriggling movements. After a few days of living in vitro conditions, the flukes showed a gradual decrease in their size and slowed down their movement. If no movement was noticed in any part of the worm and color of the parasite changed from red to white, it was considered to be dead.

Besides this, mature worms continued to lay eggs during the first ten days, after which the egg production deteriorated and had an amorphous cell organization, and embryonic development and cleavage was completed inside the egg shell.

(c) OXYGEN CONSUMPTION

Isoparorochis hypselobagri lives in an oxygen rich environment as can be seen from the results mentioned above, as well as in vitro studies revealed that it
survives well in aerobic environment. As a consequence, oxygen consumption of the parasite in relation to in vitro culture time was also determined. These results indicate that with the passage of time the oxygen consumption of worms decreases gradually. On the 10th, 20th, 30th, and 40th day, the rate of oxygen consumption decreases 15.53%, 25.26%, 50.80% and 68.54% respectively. It has been observed that parasites in vitro culture show a declining rate of oxygen consumption with increasing age of the culture. As can be seen from Fig. 4 and Table III this decline is quite pronounced.

The metabolic-temperature response of *Isoparorchis hypselobagri* has been studied over a temperature range of 20–45°C. It has been observed that oxygen consumption increases from 20 to 40°C but at 45°C the oxygen consumption decreases, Table IV and Fig. 5). The effect of the presence or absence of glucose on consumption was also noticed. This study revealed that exogenous glucose has marked effect on the oxygen consumption, which increases when glucose is provided in the medium. This indicates that the parasites utilize the glucose in the carbohydrate metabolism, Table No. V and Fig. 6.
(D) ANALYSIS OF THE EXCRETORY PRODUCTS

The excretory metabolites were analysed micro-chemically to determine their qualitative nature. Results of these tests on culture medium of *Isoparorchis hypselobagri* are shown in Table VI. Positive reactions were obtained for aminoacids, amines, amides, ammonia, aldehyde, glycine (aminoacetic acid) L-alanine, ascarbic acid, L-aminocarboxylic acids, hemoglobin, nitrogen, tyrosine and reducing materials. Acetaldehyde, alcohols, formaldehyde, O-dioxo methylene and oxomethylene and formic acid were not detected.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Percentage $O_2$</th>
<th>$pO_2$ in mm Hg</th>
<th>No. of parasites in swim bladder</th>
<th>No. of samples analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>7.4</td>
<td>56.3</td>
<td>None</td>
<td>8</td>
</tr>
<tr>
<td>2.</td>
<td>7.9</td>
<td>73.9</td>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td>3.</td>
<td>6.4</td>
<td>48.7</td>
<td>None</td>
<td>13</td>
</tr>
<tr>
<td>4.</td>
<td>5.4</td>
<td>41.1</td>
<td>Three</td>
<td>9</td>
</tr>
<tr>
<td>5.</td>
<td>6.9</td>
<td>52.5</td>
<td>Two</td>
<td>7</td>
</tr>
<tr>
<td>6.</td>
<td>5.4</td>
<td>41.1</td>
<td>None</td>
<td>6</td>
</tr>
<tr>
<td>7.</td>
<td>5.6</td>
<td>42.6</td>
<td>None</td>
<td>7</td>
</tr>
<tr>
<td>8.</td>
<td>2.9</td>
<td>22.0</td>
<td>None</td>
<td>9</td>
</tr>
</tbody>
</table>
### TABLE II. Percentage survival of *Isoparorchis hypselobagri* under aerobic and anaerobic conditions

<table>
<thead>
<tr>
<th>Days</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>100%</td>
<td>80%</td>
</tr>
<tr>
<td>20</td>
<td>100%</td>
<td>53%</td>
</tr>
<tr>
<td>30</td>
<td>84%</td>
<td>1%</td>
</tr>
<tr>
<td>40</td>
<td>44%</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>0 day</td>
<td>10th day</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>----------</td>
</tr>
<tr>
<td>Rate of $O_2$ Consumption in $ul$ $O_2/ 100 mg$ wet weight / hr. in relation to in vitro culture time.</td>
<td>37.2</td>
<td>31.3</td>
</tr>
</tbody>
</table>
**Table AI. Oxygen Consumption in Ulo/100 g wet weight at different temperatures**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
<th>75 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>43.9</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>43.6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>43.0</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>42.4</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>41.8</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>41.2</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>40.6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

*"*
TABLE V. Oxygen Consumption in ul O$_2$ / 100 mg. of wet weight in the presence and absence of glucose.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Without Glucose</th>
<th>With Glucose</th>
<th>Percentage increase in O$_2$ consumption.</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>6.4</td>
<td>9.6</td>
<td>50.0</td>
</tr>
<tr>
<td>30</td>
<td>11.0</td>
<td>18.2</td>
<td>65.4</td>
</tr>
<tr>
<td>45</td>
<td>16.8</td>
<td>27.0</td>
<td>60.7</td>
</tr>
<tr>
<td>60</td>
<td>20.2</td>
<td>37.2</td>
<td>84.1</td>
</tr>
<tr>
<td>75</td>
<td>34.6</td>
<td>46.0</td>
<td>32.9</td>
</tr>
<tr>
<td>90</td>
<td>39.8</td>
<td>52.6</td>
<td>32.1</td>
</tr>
<tr>
<td>Reaction</td>
<td>Sensitivity</td>
<td>Method Reported</td>
<td>Reagent 1</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td>----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Negative</td>
<td>1 ug</td>
<td>10 ug</td>
<td>Chromatogram gold</td>
</tr>
<tr>
<td>Positive</td>
<td>5 ug</td>
<td>1 ug</td>
<td>Neutrophilic anlge</td>
</tr>
<tr>
<td>Positive</td>
<td>0 - 10 ug</td>
<td>5 ug</td>
<td>Neutrophilic medium</td>
</tr>
</tbody>
</table>

*Table VI. Microbial tests on medium in which the organisms were incubated at 33°C. (+2°C) for 6 hrs.*
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Chemicals or Group Test</th>
<th>Reaction</th>
<th>Sensitivity Reported</th>
<th>Reason/Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Formic acid</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Oxomethylene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Hydroxide salts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Sodium hydroxide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Nitrilotriacetic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>L-amino-carboxylic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>L-anthranilic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Acetic acid (trimethyl C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Sodium nitroprusside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>0 - Dihydroxy-and</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table VI continued.*
<table>
<thead>
<tr>
<th>Reaction</th>
<th>Sensitivity</th>
<th>Method</th>
<th>Reagents or Group Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia Positive</td>
<td>Phosphomolybdate acid</td>
<td>11.</td>
<td>Reduction materials</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>10.</td>
<td>Nitro HNO3</td>
</tr>
<tr>
<td>Positive</td>
<td>1-MITRO-4-NAPHTHAL</td>
<td>16.</td>
<td>TRYOSINE</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>15.</td>
<td>Succinic acid</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>14.</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>13.</td>
<td>Homoeptablin</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>12.</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Negative</td>
<td>Chromomotorphic acid</td>
<td>0.14 ug</td>
<td></td>
</tr>
<tr>
<td>Reaction</td>
<td>Sensitivity</td>
<td>Method</td>
<td>Reagents or Group Test</td>
</tr>
</tbody>
</table>

Table VI (continued)
DISCUSSION
An analysis of the metabolic activities of parasites is necessarily based on experiments conducted in vitro because today our experimental techniques for in vivo experiments are confined to particular aspects of the problem. In order to draw conclusions about the type of activity endoparasites show in vivo, it is necessary to take into consideration the results of both, in vitro experiments and the true nature of their habitats.

According to von Brand (1966) parasitic habitats can be divided into environments offering only very little or no molecular oxygen; habitats in which more or less plentiful supply of oxygen is available; and finally habitats in which excessively high quantity of oxygen is found.

The swim bladder of *Wallago attu* is also a peculiar and not much explored biological niche for parasites. The results obtained in the gas analysis of the swim bladder revealed that it is an oxygen rich environment. These results are very close to the oxygen content of the arterial blood than to that of the intestine. It becomes evident that *Isoparorchis hypselobagri* lives in an oxygen rich environment, which is biologically significant. In addition to this, in vitro experiments prove that this parasite survives longer in aerobic conditions than under anaerobic conditions.
During aerobic experiments the parasites remain more active and do not loose the colour of their tissue hemoglobin as rapidly as they do in anaerobic conditions.

These preliminary in vitro experiments on I. hypselobagri which were carried out in simple glucose containing saline, show that if this parasite would be kept in a chemically defined medium the survival time will be increased and the weight loss counteracted. Even otherwise I. hypselobagri survives 49 days under simple conditions while Fasciola hepatica (Rohrbacher 1957), Hematolechus medioplexus (Burton 1962); and Schistosoma mansoni (Robinson 1956, 57, 58, 60) survive 30 days, 50 hours and 56 days respectively in a chemically defined medium. This clearly indicates that I. hypselobagri can be easily used as an experimental animal for biochemical, immunological and chemotherapeutic studies, and will prove an extremely useful tool in the hands of parasite physiologists and will help in elucidating many physiological aspects of tremadoces in general.

The fact that the fluke can be kept alive under in vitro conditions in a defined medium is an encouraging beginning. But the constituents are still not optimal which resulted in gradual deterioration and death of the worms. It appears that a medium ought to be devised in order to prolong in vitro survival and determine the exact nutritional requirements of the parasite.
Adult helminths are generally considered to be facultative aerobes and use oxygen if available. Bueding (1949) observed that adult *Schistosoma mansoni* could survive anaerobically but they did better in the presence of oxygen. Rohracher (1957) noticed that the adult *Fasciola hepatica* lost the colour of hemoglobin rapidly under anaerobic conditions. All parasites studied so far under aerobic conditions consume oxygen and produce carbon dioxide regardless of whether they lead a primarily aerobic or anaerobic life in their normal habitat.

The data available on oxygen consumption in trematode parasites indicate that the oxygen content of the habitat of the parasite plays an important role. *Schistosoma mansoni* consume more oxygen than *Fasciola hepatica* and *Gigantocotyla adunca*. It is suggested that the experimental conditions under which the present measurement of the oxygen uptake of *Isoparorchis hypselobagri* was made, were approximately very close to the conditions inside the swim bladder of *Wallago attu*. Since the oxygen tension in the swim bladder ranges from 22 - 70 mmHg and oxygen is consumed by *Isoparorchis* as supported from the results of the present study, there is a every likelihood that this trematode might have aerobic type of metabolism.
In the case of *Fasciola gigantica*, Lutz and Siddqi (1967) have presented a convincing evidence that it possesses hemoglobin quite different from the host. Similarly *Isoparorchis hypselobagri* also possesses hemoglobin which is quite different from the fish (Unpublished results), and may be playing an important role in oxygen transport.

It has been reported that the culture forms of protozoa and cestodes show a declining rate of oxygen consumption with increasing age of culture but the reason for this decline is still unknown. In this study, (Fig. 4 and Table III), decline in oxygen consumption is quite pronounced in the present case also, but it is difficult to advance definite reasons for it.

Various workers have studied various factors such as size of the worms, temperature substrates, atmospheric pressure, and different inhibitors which effect the rate of respiration. Temperature is one of the most important factor. The metabolic temperature curve of the adult trematode is closely related to the body temperature of the host (Vernberg and Hunter, 1961). From the present study it is evident that oxygen consumption in *I. hypselobagri* is greatly influenced by the temperature. The rate of respiration increases gradually from 20 to 40°C, but at 45°C the rate of respiration
decreases. It appears that high temperatures are deleterious to the well being of the trematode.

A comparison of the oxygen uptake by *I. hypselobagri* in the absence and presence of glucose, suggests that the parasite oxidizes glucose. The increase in oxygen uptake produced by the addition of glucose to the medium is from 32 to 50%. The same fact was observed by Bueding (1950) in the case of *Schistosoma mansoni*. Pasooe (1968) found that glucose increased the oxygen uptake of sporocyst of *Microphallus pygmaeus*. On the contrary, Vernberg and Hunter (1963) found that glucose did not increase the oxygen uptake in the case of *G. adunca*. *Isoparorchis hypselobagri* provides a peculiar opportunity to study whether it has aerobic type of metabolism. It may be possible that oxygen is either utilized in the terminal respiratory metabolism or oxidation of the protein metabolism.

Most of the metazoan parasites which live in predominantly anaerobic environment and can not metabolize carbohydrate more efficiently through oxidative process, produce volatile fatty acids as the end products. Lutz and Siddiqi (1971) reported free amino acids, Mansour (1959) found lactic acid and pyruvic acid, Kurieo and Rijaveo (1966) found certain intermediates of the ornithine cycle and other
nitrogenous substances. Lahoud et al. (1971) found volatile fatty acids in the end products of various trematodes.

Isoparorohis hypselobagri which lives in an oxygen rich environment also excreted certain organic substances which dropped the pH of the culture medium. Keeping this fact in mind, it was decided to analyze the culture medium in order to examine the end products of its metabolism. A large number of amino acids and other complex molecules were detected in the incubated medium (Table VI). The results of the microanalysis of medium clearly indicate that nitrogen metabolism is operative in the parasite, but the particular processes which lead to the excretion of amino acids, amines, ammonia, and other nitrogenous substances have not been studied. Presence of hemoglobin in the medium is probably due to the fact that it is present as tissue hemoglobin and is lost by simple diffusion. Excessive accumulation of ammonia in the worm would undoubtedly be harmful and thus it is excreted into the medium. Amino acid synthesis and discharge are particularly interesting in I. hypselobagri. The present results provide a good opportunity for further research on this problem which should prove to be fruitful and revealing.
ABSTRACT

(a) The gas of the swim bladder of *Wallago attu* contains a biologically significant quantity of oxygen and *Isoparorchis hypselobagri* lives in an oxygen rich environment.

(b) *In vitro* culture *Isoparorchis hypselobagri* survives longer under aerobic rather than under anaerobic conditions in a balanced salt solution containing glucose, although it continues to lose weight.

(c) When blood is added to the culture medium, the rate of weight loss of the parasite is reduced to half of that in glucose saline, with greater production of hematin.

(d) Oxygen consumption of the parasite in relation to *in vitro* culture time decreases gradually. The metabolic temperature response of the parasite is quite pronounced. Oxygen consumption increases with the increase in temperature from 20° to 40°C, but at 45°C the oxygen consumption decreases. Glucose has a marked effect on oxygen consumption, which increases when glucose is provided in the medium. This indicates that the parasite utilizes glucose in the carbohydrate metabolism.

(e) The excretory metabolites of the parasite consist of amino acids, amines, ammonia, and other nitrogenous compounds.


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Stephenson, W. (1947) Parasitology 38: 116-


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FIGURES
Fig. 1: Krogh's Gas Analyzer
Fig. 2: Scholander Respirometer
Fig. 3: In vitro survival of Isoparorchis hypselobagri
Fig. 4: Rate of Oxygen Consumption in $\mu$L/100 mg. wet weight/hour in vitro culture time.
Fig. 5: Rate of Oxygen Consumption in μl/100 mg. wet weight at different temperatures.
Fig. 6: Oxygen Consumption in $\mu$ $O_2/100$ mg. wet weight in the presence or absence of Glucose.