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The Physiology of Activation of Larval Moniliformis dubius (Acanthocephala)

by

Kalman Horvath

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
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DOCTOR OF PHILOSOPHY

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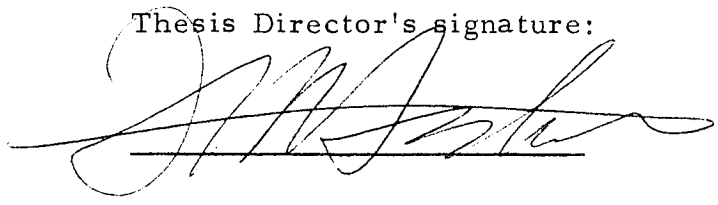

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INTRODUCTION

"A major feature of parasitism is the provision by the host of the environment for the parasite. What it is that is unique in the physics and chemistry of the environment of parasites is not known." (Rogers, 1962)

In the environment of parasites there are a number of conditions which make it unique and different from those encountered by free-living organisms. Rogers, quoted in the above paragraph, analyzed and compared the differences and concluded that "the presence of special substances, special combination of substances which are not unique in themselves, physical properties and stability" are the factors which are unique to the parasites' environment. These conditions, such as osmolarity, viscosity, oxidation reduction potential, amino acid composition and buffering capacity and the like, would differ from one host species to another and could account for the suitability of certain hosts, while excluding others.

One of the most remarkable features in the parasite life cycle is the role of the environment provided by the host which presumably controls and certainly stimulates initiation of the adult development of parasites. This is especially striking in those cases where the parasite remains essentially in a state of diapause and resumes growth and develops to sexual maturity only in certain definitive hosts. While we recognize some of the compounds which signal the initiation of renewed development, we know very little about the mode

of action of such substances.

A. Literature Review

The acanthocephalan, Moniliformis dubius, was chosen as the subject of study of the effects of host environmental factors on its ability to infect and establish in the definitive host.

1. Life history and development

Moniliformis dubius, Meyer 1933 -- an intestinal parasite of rats -- completes its larval development in the haemocoel of cockroaches of the genus Periplaneta. The life cycle is initiated by the consumption of viable eggs by the intermediate host, followed by development to an infective stage within the cockroach. In nature, rats acquire the infection by eating the intermediate host. The developmental stages of this parasite in the cockroach were first described by Burlingame and Chandler (1941). Moore (1946) described the life cycle in detail and further examined the changes in the morphology of the larval forms during development. He adapted the terminology of Van Cleave (1935, 1946, 1947) for the various stages, calling the egg or shelled embryo, the acanthor, and the developing stages within the cockroach the acanthellae. Later the name cystacanth was proposed by Chandler (1949) for the infective stage, which is the most advanced stage found in the cockroach. In the haemocoel of the cockroach the

parasite is surrounded by a capsule (cyst) derived from the haemocytic reaction of the host. The formation and role of this "cyst" has been reveiwed by Nicholas (1967). More recently, King and Robinson (1965) re-examined the larval development of M. dubius in the cockroach and described the effects of parasite burden and host maintenance conditions on the length of development to the infective stage. They found that the rate of development of the parasite was not significantly influenced by the number of larvae in the haemocoel of infected hosts. In cockroaches kept at lower temperatures however, the rate of development was markedly reduced. They also reported that development was faster in cockroaches infected during the summer, even though they were kept under the same conditions as the winter group. This suggested that seasonal variation in the host's physiology, independent of the temperature, may effect changes in the rate of the parasite's development. Under the conditions of the present study, development from egg to egg-producing adults takes approximately 120 days.

2. Biochemistry and physiology

The acanthor stages of some acanthocephalans are quite resistant to environmental conditions. Survival under extreme conditions of dryness and cold has been reported for Macracanthorhynchus hirudinaceus (Spindler and Kates, 1940; Kates, 1942), a parasite of terrestrial intermediate and definitive hosts.

This would be of definite survival value, since great fluctuations of temperature are common in that environment. In the case of Polymorphus, an acanthocephalan utilizing aquatic invertebrates as intermediate hosts, the tolerance for cold and dryness is considerably less (Hynes and Nicholas, 1963) reflecting perhaps the relative stability of the aquatic environment.

The requirements for hatching of acanthors in the gut of the intermediate host have been studied by a few authors. The gastric mill, by its grinding action, may effect the release of the acanthor of Polymorphus minutus from the egg (Crompton, 1964a); however, the pH, osmolarity and CO_2 tension in the gut may play a role in this process. Edmonds (1966) was able to effect the hatching of acanthors of Moniliformis dubius by exposing them to 0.25 M sodium bicarbonate. He stated that this treatment, while effective, may not have any relation to the actual hatching factors in the gut of the intermediate host, since the composition of the contents of the cockroach gut was not known.

Polysaccharides serve as reserves for energy production in most animals, when an exogenous supply of nutrients is not available. Glycogen is present in the acanthor of Macr. hirudinaceus in increasing amounts during development in the insect (von Brand, 1939, 1940). Miller (1943) reported an initial drop in glycogen content of acanthors of the same parasite upon hatching,

but the polysaccharide was resynthesized as development reached the late acanthella (cystacanth) stage. The development of the acanthocephalans in the haemocoel of the intermediate host has not been investigated from a physiological or biochemical point of view beyond the above references. There is some information available on the development and role of the cysts which apparently arise by lamellar overlaying of host haemocytes on the acanthors and which seem to provide protection to the developing larvae against other host responses (Crompton, 1964a; Mercer and Nicholas, 1967).

The cystacanth in the haemocoel of the cockroach appears dormant. Its proboscis, which lies inverted within its body, is motionless. Were it to remain in this state, it would certainly be passed through the intestine of the rat host without being able to establish itself in its required habitat. The cystacanth is activated in the small intestine of the rat. In the course of natural infections, the cyst is digested, or at least partly removed, in the stomach of the host and only the inactive larva reaches the small intestine. The removal of the cyst does not activate the cystacanth, its proboscis remains inverted and immobile. Exposure to bile *in vivo* or to salts of bile acids *in vitro* causes the organism to evert its proboscis, leading to eventual penetration of the intestinal tissues by the proboscis and the subsequent attachment by the hooks

of the proboscis. After these events rapid growth occurs culminating in sexual maturity. Graff and Kitzman (1965) showed that bile or bile salt causes activation of the cystacanth, and, in addition to this, they demonstrate the synergistic effect of increased levels of CO_2 and anaerobiosis on the activation phenomenon.

The chemical composition of acanthocephalans has been studied by many authors. Fisher (in press) observed and estimated the changes in body composition of M. dubius during the twenty week period following the infection of the rat. Earlier, glycogen was shown to be the main carbohydrate reserve in adult acanthocephalans from definitive hosts (von Brand, 1939; von Brand and Saurwein, 1942; Laurie, 1959; Graff and Allen, 1963). Read and Rothman (1958) found a diurnal fluctuation in polysaccharide content of M. dubius which seems to reflect on the availability of carbohydrate in the intestinal environment. They demonstrated that the growth of this parasite stopped following the omission of carbohydrate from the diet of the host. The return of the rat hosts to a carbohydrate-containing diet was followed by the resumption of parasite growth. Thus they established that M. dubius has a growth requirement for carbohydrates obtained from the diet of the rat host.

Protein, lipid and inorganic ion composition of acanthocephalans has received some attention in the past years, along with the ability of these parasites to accumulate various organic

compounds. The significance of the studies was the demonstration of uptake of host dietary lipids (Van Cleave and Rausch, 1950; Crompton, 1964b) and the active transport of amino acids (Rothman and Fisher, 1964; Edmonds, 1965).

Intermediary metabolism of acanthocephalans has been studied in considerable detail. The synthesis of glycogen by M. dubius from starved rat hosts was demonstrated by Laurie (loc. cit.). He found that glucose, fructose, mannose and maltose stimulated the synthesis of this polysaccharide. While Laurie also showed the presence of trehalose in M. dubius, he could not demonstrate the synthesis of this disaccharide. It was only later (Fisher, 1964) that the synthesis of trehalose was accomplished with homogenates of M. dubius and Macr. hirudinaceus, in incubation mixtures fortified by adenosine triphosphate (ATP) and uridine diphosphoglucose (UDPG).

Graff (1964) studied the uptake and incorporation of glucose into glycogen by adults of M. dubius and noted that worms from hosts which were starved 24 hours prior to his experiments incorporated more glucose into glycogen than those from unstarved controls. Carbohydrates, required by M. dubius for growth, occur only during a relatively short period of time in the intestine of the rat following feeding during any twenty-four hour period. The carbohydrate stores of the worm are utilized during the time

the intestine contains essentially no carbohydrates. The rapid re-synthesis of glycogen, while substrates are available, is pre-requisite for the maintenance of the parasites's metabolism between the meals of the host.

To test the effects of the physical conditions of the intestine on the synthesis of glycogen by M. dubius, Kilejian (1963) studied the role of CO_2 under anaerobic conditions on glycogenesis in vitro in these parasites. She found that, in contrast to the tapeworm Hymenolepis diminuta, CO_2 had no effects on the anaerobic synthesis of glycogen by M. dubius.

The enzymes of glycolysis have been studied in a few acanthocephalans. The presence of aldolase, triose phosphate dehydrogenase and lactic dehydrogenase in M. dubius was reported by Read (1961). In addition to these, Dunagan and Scheifinger (1966) reported the demonstration of six glycolytic enzymes from Macr. hirudinaceus, these being hexokinase, glucose phosphate dehydrogenase, glutamic dehydrogenase, phosphoglucomutase, phosphoglucose isomerase and phosphofructokinase. The present author questions the classification of some of these enzymes, especially glutamic dehydrogenase, as glycolytic enzymes. The demonstration of enzyme activity in homogenates may not reflect the role of the enzyme in the metabolism of the whole animal. Evidence for the functional glycolytic pathway was presented by Graff (1964) and

Bryant and Nicholas (1965) who demonstrated the labeling of glycolytic intermediates from radioactive glucose by intact and homogenized adult M. dubius.

The operation of the citric acid pathway was shown to be limited to only the dicarboxylic acid portion by Graff (1964, 1965) and Bryant and Nicholas (loc. cit.) in M. dubius. The latter authors showed that the metabolism of succinate and acetate involves intermediate steps in the dicarboxylic acid intermediates of the Krebs cycle. They found that some of the label from glycolytic intermediates will occur in glutamate; however, they suggested that this would not occur under normal operation of the pathway in intact animals, but can be ascribed to the use of high concentrations of intermediates and the types of worm preparations such as minces homogenates and sonicates, which make the conditions of their assays unphysiological.

Graff (1965) reported the fixation of CO_2 by whole adult M. dubius. He demonstrated the occurrence of label from $H^{14}CO_3^-$ in malate, fumarate, succinate, lactate, pyruvate, oxaloacetate, aspartate and alanine, and in one experiment radioactivity was observed in serine. Label from propionate-1-^{14}C did not appear in any of the above mentioned compounds. Based on this, he speculated that pyruvate or phosphoenolpyruvate may serve as the acceptor in CO_2 fixation.

The endproducts of aerobic and anaerobic metabolism of carbohydrates by M. dubius were reported to be lactate, acetate and formate by Laurie (1957, 1959). In contrast, Crompton and Ward (1967a, b) reported that the major anaerobic endproduct of glucose metabolism of M. dubius was ethanol, while lactate and acetate were of minor importance. They also reported that the formation of succinate was very limited and of little significance in the overall carbon balance of glucose metabolism in M. dubius. However, succinate formed the major endproduct of carbohydrate metabolism in Polymorphus minutus, another acanthocephalan. Examination of their data reveals that they did not account for the major portion of the glucose metabolized by M. dubius and thus, there exists the possibility of an error in the low values for succinate reported.

Bryant and Nicholas (1966) studied the terminal oxidations of succinate and NADH (nicotinamide adenine dinucleotide, reduced) by M. dubius. Based on studies employing redox dyes and inhibitors, they postulated a scheme for electron transport in this organism. They found that both succinate and NADH reduced cytochrome b in particulate preparations. Nicholas (1967) proposed that the terminal oxidation of this cytochrome could be similar to that shown by Kmetec et al. (1962) in the roundworm Ascaris, although the results of his work published with Bryant (loc. cit.) could not warrant such comparison, mainly because of the differences in technique and the type

of preparation of particulate fractions. The present author cannot reconcile the findings of Crompton and Ward (loc. cit.) and the proposal of Nicholas for the terminal oxidative pathway in M. dubius. Reexamination of the relationship of succinate formation to the operation of the terminal oxidative pathway may clairfy this aspect of the biochemistry of M. dubius.

B. The Problem

The acanthocephalan, Moniliformis dubius, probably requires the presence of host bile for initial establishment in the intestine of the definitive rat host. Graff and Kitzman (1965) found that these worms did not infect surgically altered hosts whose bile duct had been diverted to the caecum, thus having no bile present in the small intestine where M. dubius attaches. These authors found, however, that activation in vitro with bile salts resulted in the eversion of the proboscis of the cystacanths and the ingestion of these "activated" parasites by surgically-altered hosts resulted in the establishment of an infection in the small intestine. The same authors showed that activation in vitro was enhanced by anaerobiosis and increased levels of CO_2 in the gas phase. These findings prompted the present author to ask two questions. (1) How do bile salts effect activation of the cystacanth and what are the physiological parameters of the mechanism of their action? (2) What is

the mode of action and role of anaerobiosis and increased CO_2 level in enhancing synergistically the bile salt activation of these larval parasites?

MATERIALS AND METHODS

A. Maintenance of the Hosts

The definitive hosts, Holtzman white rats (Holtzman Rat Co.), were maintained in groups of ten to twenty in 12" X 15" X 18" size wire mesh cages in air-conditioned quarters. They were provided "Purina Laboratory Chow" (Ralston Purina Co.) and tap water *ad libitum*. Only male rats were used.

Periplaneta were maintained in painted 5-15 gallon aquaria on a diet of fresh apples, pulverized rat chow and water.

B. Maintenance of the Life Cycle

Eggs of *Moniliformis dubius* were obtained from gravid female worms and concentrated by sedimentation in tap water. After blotting with filter paper to remove excess moisture, the eggs were mixed in a ratio of approximately 1:1 with fresh minced apple and this mixture was presented to cockroaches which were denied food and water for the previous week. Complete development to the cystacanth stage in the haemocoel of the cockroaches required approximately 90 days at 24-28°. Male rats weighing 60-90 grams were infected with twenty five cystacanths. Adult parasites were recovered from the intestine of rats 40-45 days later.

C. Handling of Biological Materials

Infective cystacanths were removed from the haemocoel of cockroaches by flushing the haemocoel with Krebs-Ringer saline buffered at pH 7.4 with 0.025 molar tris-(hydroxymethylamino) methane-maleate, hereafter referred to as KRT. In experiments involving the use of cystacanths, the cyst wall was removed by expressing the intact cystacanths through a narrow tipped pipette which allowed passage of the larvae but impeded the passage of the cysts. The larvae were examined under a dissecting microscope for possible anatomical damage and/or abnormalities and only anatomically perfect larvae were selected for experiments. In incubations requiring rapid transfer of parasites into different incubation media, the larvae were placed in a short glass cylinder having a stainless steel mesh bottom. The use of this container facilitated the rapid rinsing of the cystacanths following radioactive label uptake experiments. It also permitted the extraction of larvae by immersion of the cylinders and the parasites in a given volume of 70% ethanol. Wet and ethanol-extracted dry weight ratios were determined using groups of fifty cystacanths, which were extracted for 18 hours in 70% ethanol and dried at 95° for 24 hours.

All incubations were performed in KRT or KRT with Na-bicarbonate except where otherwise noted. The gas phases during incubations were established by equilibrating the media for five

minutes with either air, air-CO_2 (95:5), N_2 (100), N_2-O_2-CO_2 (90:5:5) or N_2-CO_2 (95:5). Bicarbonate was added to all CO_2-equilibrated preparations in concentrations to maintain pH 7.4 at 37° following gas phase equilibration.

D. Preparation of Cell-Free Homogenates

Weighed cystacanths were homogenized in glass distilled water at 0° in all glass homogenizers. The homogenate was centrifuged at 12,000 X g in a Sorvall RC2B centrifuge for thirty minutes at 2° and the supernatant was used as a crude enzyme preparation.

E. Chemical Assay Techniques

Polysaccharide was isolated as the 30% alkali-stable 52% ethanol precipitatable fraction and was estimated colorimetrically by the phenol-sulfuric acid method of Dubois et al. (1956).

Glucose was measured by the glucose oxidase technique using "glucostat" (Worthington Biochemical Corporation) according to the method of Fisher (1965).

Protein was estimated by the colorimetric method of Lowry et al. (1951).

F. Measurement of Surface Tensions

The capillary rise method of surface tension measurement was utilized, as described by Bull (1951). It consists of measuring the

the rise (h) of the fluid above the surface in a capillary of radius (r). At equilibrium the surface tension (T) is calculated by the formula:

$$T = \frac{h\ r\ g\ p}{2 \cos \alpha}$$

where (g) is the acceleration of gravity, (p) is the density of the liquid and (α) is the angle of contact of the fluid with the capillary wall. In these measurements the angle of contact was assumed as 0^{o} (a valid assumption for completely wetting liquids), thus the cos α equals unity. Furthermore, the densities of liquids examined were all considered to be equal since the measurements were to compare the fluids at equal solute concentrations. The units of surface tension were expressed as dynes/centimeter.

G. Enzyme Assay Techniques

1. Hexokinase was measured according to the method of Di Pietro and Weinhouse (1960). The reaction was followed spectrophotometrically, measuring the reduction of NADP (nicotinamide adenine dinucleotide phosphate) by the coupled reaction of glucose-6-phosphate dehydrogenase at 340 mμ wavelength in a Zeiss PMQ II spectrophotometer. The assay medium contained (in 3.0 ml volume) 100.0 μmoles of tris-(hydroxymethylamino)methane·HCl pH 7.4, 50.0 μmoles of glucose, 100.0 μmoles of $MgCl_2$, 10.0 μmoles of

ATP (adenosine triphosphate) and 50 µgrams of glucose-6-phosphate dehydrogenase (specific activity 135 µmoles/min/mg protein).

2. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were measured by the method of Kornberg and Horecker (1955) and Horecker and Smyrniotis (1955). The reaction mixture (3.0 ml) contained 200.0 µmoles of triethanolamine-HCl buffer pH 7.5, 50.0 µmoles of $MgCl_2$ NADP (nicotinamide adenine dinucleotide phosphate) 1.0 µmole and substrate, either glucose-6-phosphate or 6-phosphogluconate 5.0 µmoles. The reaction was followed spectrophotometrically at 340 mµ wavelength measuring the increase in optical density due to the formation of NADPH.

3. Glucose-6-phosphate isomerase was estimated by the method of Slein (1955), estimating the amount of fructose-6-phosphate formed by the method of Roe (1934). The assay media contained, in a total volume of 1.0 ml, 100.0 µmoles of tris-(hydroxymethylamino)methane·HCl buffer pH 8.0, 200.0 µmoles of $MgCl_2$ and 0.5 µmoles of glucose-6-phosphate.

4. Aldolase was measured by the method of Warburg and Christian (1943) in a reaction coupled with glyceraldehyde-3-phosphate dehydrogenase. The assay mixture of 3.0 ml contained 200.0 µmoles of tris(hydroxymethylamino)methane·HCl buffer pH 8.0, 50.0 µmoles of Na-arsenate, 50.0 µmoles of glycine, 600.0 µmoles of cysteine, 0.5 µmoles of NAD, 25.0 µmoles of fructose-1,

6-diphosphate and 1.0 mg of glyceraldehyde-3-phosphate dehydrogenase (specific activity 40 μmoles/minute/mg protein). The reduction of NAD was followed at 340 mμ wavelength in a spectrophotometer.

5. α-glycerophosphate dehydrogenase was measured by the method of Beisenherz et al. (1955). The reaction mixture (3.0 ml) contained 150.0 μmoles of triethanolamine·HCl buffer pH 7.5, 30.0 μmoles of dihydroxyacetone-phosphate and 0.5 μmoles of NADH. The oxidation of NADH was followed spectrophotometrically at 340 mμ wavelength.

6. Enolase was measured by the method of Grisolia (1955) by following the change in optical density at 240 mμ due to double bond formation. The assay medium (3.0 ml) contained 300.0 μmoles of tris(hydroxymethylamino)methane·HCl buffer pH 7.3, 100.0 μmoles of $MgCl_2$ and 15 μmoles of 2-phosphoglyceric acid.

7. Pyruvate kinase was measured by the method of Bucher and Pfleiderer (1955), following the oxidation of NADH by the coupled reaction of lactic dehydrogenase at 340 mμ wavelength spectrophotometrically. The assay medium consisted of 3.0 ml total volume and included 160.0 μmoles of tris(hydroxymethylamino) methane·HCl buffer at pH 7.5, 0.15 μmole of NADH, 0.23 μmole of ADP (adenosine diphosphate), 0.65 μmole of PEP (phosphoenol pyruvate, sodium salt), 7.0 μmoles of $MgSO_4$, 75.0 μmoles of KCl

and 35.0 µgrams of LDH (lactic dehydrogenase, specific activity of 615.0 µmoles/minute/milligram protein). As a control, rabbit muscle pyruvate kinase was used.

8. Lactic dehydrogenase was measured according to the method of Kornberg (1955). The assay medium contained 100.0 µmoles of phosphate buffer pH 7.4, 0.2 µmole of NADH, 1.0 mole of Na-pyruvate, 1.0 µmole of NaCl in a total volume of 3.0 ml.

9. PEP-carboxykinase was measured according to the technique of Utter and Kurahashi (1954), as modified by Prescott and Campbell (1965). The assay mixture (2.0 ml) contained 250.0 µmoles of tris(hydroxymethylamino)methane·HCl pH 7.16, 50.0 µmoles of oxaloacetate, 50.0 µmoles of Na-$H^{14}CO_3$ (specific activity of 0.1 µcuries/µmole), 5.0 µmoles of $MnCl_2$ and 2.0 µmoles of ATP (adenosine triphosphate) or ITP (inosine triphosphate). This enzyme was also assayed in combination with malic enzyme to separate and estimate the incorporation of CO_2 due to each system.

10. Malic enzyme was initially measured by the method of Ochoa (1955a), following the formation of NADPH spectrophotometrically at 340 mµ. In addition to this method, this enzyme was also assayed in combination with the PEP-carboxykinase method of Utter and Kurahashi as modified by Prescott and Campbell (loc. cit.). This assay included in addition to the components listed previously (part 9) 50.0 µmoles of malate and 1.0 µmole of either

NAD (nicotineamide adenine dinucleotide) or NADP (nicotinamide adenine dinucleotide phosphate). The reaction was followed by measuring the radioactivity incorporated into malate from $H^{14}CO_3^-$ by the malic enzyme, and the same incorporated into oxaloacetate by the PEP-carboxykinase enzyme, by the exchange reactions. Separation of the incorporation due to each enzyme was achieved by specific decarboxylation of oxaloacetic acid by Al^{+3}. The difference in activities, resulting from this treatment which liberates the label from oxaloacetate as $^{14}CO_2$ in an acidic medium, was assigned to PEP-carboxykinase activity.

11. Malic dehydrogenase was measured by following the oxidation of NADH according to the method of Ochoa (1955b). The assay medium of 3.0 ml contained 80.0 μmoles of glycylglycine buffer at pH 7.4, 0.15 μmole of NADH and 0.75 μmole of oxaloacetate.

H. Isotope Assay Techniques

Only ^{14}C labeled substrates were used in these experiments. Soluble samples and slurries of insoluble preparations were dried on planchets and counted in a Nuclear Chicago gas flow counter with proper controls to correct for self absorption. Chromatograms were scanned for radioactivity in a Vanguard Model 880 Autoscanner and the radioactive spots which were localized were estimated by liquid scintillation spectrometry. Samples in aqueous solutions were

solubilized by the use of Beckman Bio-Solve in the toluene base scintillator before counting in the scintillation spectrometer.

I. Chromatographic Techniques

Amino acid, organic acid and neutral fractions of worm extracts or media were separated by the sequential use of Dowex 50 (H^+) and Dowex 2 (OH^-) resins. Amino acids were not chromatographed. Organic acids were chromatographed on Whatman #1 paper ascending in the solvent of Cheftel (1952, 1953) consisting of ethanol:ammonium hydroxide:water (80:5:15). Neutral compounds or total extracts were chromatographed on Whatman #1 or #3 papers ascending in Friedman's solvent (ethyl acetate:pyridine:2-butanone:water, 50:36:36:30) as reported by Fisher (1964). Reducing compounds were visualized by a modified silver nitrate-sodium hydroxide technique of Trevelyan et al. (1950).

J. Source and Grade of Chemicals

All chemicals were reagent or analytical grade chemicals from Fisher Scientific Corporation or Mallinckrodt Corporation. Radioisotopes were purchased from New England Nuclear Corporation. Enzymes and cofactors were obtained from Sigma Chemical Corporation or California Corporation for Biochemical Research; the latter was also the source of the conjugated bile salts used. Cholic and deoxycholic acids were enzyme grade reagents from Mann Research

Laboratories. The gas mixtures were prepared and analyzed by Big Three Industrial Gas Corporation.

RESULTS AND DISCUSSION

A. Wet and Dry Weights of Cystacanths

Initial studies on the cystacanth of Moniliformis dubius established that using fifty parasites as the experimental group would satisfy the requirements for reproducibility of assays, since the organisms were too small to use individually. That number also allowed conservation of the limited supply of experimental material. To express internal concentration of metabolites and storage products, the determination of wet and ethanol-extracted dry weights of the cystacanths was necessary. Samples of fifty parasites were selected at random from several cockroach hosts to avoid bias in securing the experimental groups. The larvae were placed in KRT and transferred to single preweighed wet millipore filters. After removal of excess fluid by suction, the worms and the wet filter were weighed, and the weight of the worms was calculated by difference. Dry weights of the cystacanths were obtained by direct weighings. The same procedure was used to obtain the wet and dry weights of both active and inactive cystacanths. The magnitude of the standard deviation from the mean weight of the cystacanths was caused by the evaporative loss of the fluid during the weighing procedure. This deviation is small for the weights of cystacanths obtained following extraction with 70% ethanol and drying at 95^{o} from

18-24 hours. The wet weights of intact cystacanths and the wet and dry weights of active and inactive cystless cystacanths are shown in Table I. The data in later experiments were calculated using water content and dry weights based on these values. This was necessary in order to avoid the extensive handling of cystacanths before experiments and also to conserve the time that is required if all groups of cystacanths are to be weighed immediately before each experiment. Examination of the water content of cystacanths before and after activation resulted in the finding that active cystacanths have a higher water content (78%) than inactive controls (45%). These differences were caused by uptake of water by the active cystacanths following exposure to bile salt solutions. Inactive cystacanths maintain a constant relative water content (45%) through experiments lasting up to 90 minutes. The possible role of the uptake of water in the mechanism of bile salt activation of cystacanths will be discussed later.

B. Bile Salts Causing Activation and Their Effects on the Motility of Cystacanths in vitro

Graff and Kitzman (1965) demonstrated that activation of the cystacanths, as defined by the eversion of the proboscis and its active movements, occurs in the small intestine of the rat host and it can also be achieved by exposure of the cystacanths to solutions of

TABLE I

Wet and Extracted Dry Weights of Intact, Active and Inactive Cystless Cystacanths

Condition	Samples	Wet weight mg / 50 cystacanths	Dry weight
Inactive, intact	5	23.96±0.84	----
Inactive, cystless	9	3.80±0.15	----
Active, cystless	9	5.13±0.42	----
Active, cystless	9	5.13±0.51	1.163±0.081
Active, cystless	9	----	1.138±0.031

bile salts *in vitro*. They demonstrated that Na-taurocholate, Na-glycocholate and Na-cholate would activate the parasites during a four hour exposure to 0.25% (weight/volume) concentrations of these bile salts at 37°, but activation did not take place at 24-26° during the four hour incubation period. In initial studies, it was noted that cystacanths can be activated at room temperature (22°) by a 0.25% bile salt solution if the length of exposure to the bile salts is extended to eight or ten hours. Thus, elevated temperature appears only to enhance the rate at which activation takes place. Other factors, such as amino acid concentrations in the gut of the rat, oxygen tensions and the level of carbon dioxide may play a role in determining the rate of activation, but they are not required for activation to occur. These conditions would not restrict the activation of *M. dubius* to the rat intestine, since injection of bile salts into the haemocoel of infected cockroaches will cause activation of the parasites contained therein. From these observations it appears that the activation of the cystacanth is triggered by only bile salts.

The composition of mammalian bile varies in terms of the predominant bile salt. Herbivores, with the exception of bovids, have glycine conjugated cholate as the primary bile salt, whereas carnivores and the bovids possess taurine conjugated cholate. Unconjugated bile salts occur in the bile only under certain pathological conditions of the liver. Other bile salts, largely cholate derivatives

resulting from bacterial metabolism of the bile salts in the large intestine occur in the bile of some species. It was essential to examine the effects of different bile salts on the cystacanth in order to determine which properties of bile salts play the important role in cystacanth activation. Other bile salts which occur in the bile of some animals are deoxycholate, chenodeoxycholate and to a small extent lithocholate. Initially, unconjugated cholate and deoxycholate were used in experiments to study the effects of these salts on the activation of the cystacanths. The parasites were incubated in solutions of 0.01, 0.05, 0.10 and 0.25% (weight/volume) concentrations of bile salts in KRT at 37°. They were observed for seven hours and the activation and the maintenance of motility of the cystacanths in the two solutions were compared. Both cholate and deoxycholate activated the cystacanths, however, those exposed to the latter were bloated and nonmotile at the end of the experiment, while those in cholate appeared unchanged in size and were actively moving. Microscopic examination of the cystacanths from the deoxycholate solution revealed generalized swelling and blister-like separation of the tegument over the entire body of the animal. This swelling was reminiscent of the condition of the cystacanths exposed to hyposmotic stress such as distilled water. While exposure to distilled water will cause the eversion of the proboscis of the cystacanth, motility of the larvae was never observed by this author.

Deoxycholate is unique among the bile salts in its property of forming coordination compounds with fatty acids. This property is quite distinct from the general detergent effects displayed by bile salts. It appears probable, that the deleterious effect of deoxycholate is caused by its action on membrane components, possibly the lipids or lipoproteins, of the cystacanths, resulting in excess permeability to water and causing the observed swelling. A similar hypothesis was proposed by Smyth and Haslewood (1963) for the deleterious effects of deoxycholate on the larval forms of the tapeworm Echinococcus granulosus. Cholate (3α, 7α, 12α-trihydroxycholanate) and deoxycholate (3α, 12α-dihydroxycholanate) differ only in the presence or absence of the hydroxyl radical at position seven on the cyclopentanophenanthrene ring. It was hypothesized that other bile salts showing similar differences may provide some answers to the question of structural requirements in the bile salt molecule which are prerequisite for the activation of the cystacanth. In addition to the aforementioned bile salts, chenodeoxycholate (3α, 7α-dihydroxy-cholanate) and lithocholate (3α-hydroxycholanate) were obtained as the glycine and taurine conjugates. Cystacanths, ten for each experimental solution, were exposed to 0.25% solutions of these bile salts in KRT at 37°. They were examined periodically to note the rate of activation of the cystacanths. The experiment was continued after two hours of observations to a final observation at

nineteen hours of exposure. The results are presented in Table II. The data are expressed as the number of motile cystacanths for the first two hours and as the total cystancanths still moving at the end of the nineteen hour exposure. Maximal activation and longest maintenance of motility were found in the solution of tauro- or glycocholate. Conjugates of chenodeoxycholate caused the activation of the cystacanths. However, these lost their motility by the end of the experimental period, only three showing some feeble muscle movements. Conjugates of deoxycholate activated some of the cystacanths; however, these resembled the larvae exposed to unconjugated deoxycholate, namely that they were swollen and nonmotile by the end of the observation period. Lithocholate, which is essentially insoluble in KRT, will activate the cystacanths following a prolonged exposure to a suspension of this salt in KRT, suggesting that perhaps only a very small amount of this bile salt is needed for activation. Two other compounds were tested for potential activating capacity, based on the similarity of their structure to bile salts. Dehydrocholate (3, 7, 12-trioxocholanate), a derivative of hydroxycholanate and oubain (3-0-rhamnosyl-1β, 5, 11α, 14, 19-pentahydroxy 20:22-cardenolide), a member of the digitalis-like glycoside group, were employed in 0.25% concentrations to test their effects on the cystacanths. Both compounds, following prolonged exposures up to 24 hours, will activate the cystacanths, while controls exposed to

TABLE II

Activation and Maintenance of Motility of Cystacanths

Bile salt (0.25%)	activated						motile
	10'	15'	20'	30'	60'	120'	19 hrs
Na-taurocholate	1*	6	7	10	10	10	10
Na-taurochenodeoxy cholate	0	8	8	10	10	10	3
Na-taurodeoxycholate	0	0	0	0	0	6	0
Na-glycocholate	0	0	1	7	10	10	5
Na-glycochenodeoxy cholate	0	4	8	8	10	10	0
Na-glycodeoxycholate	0	0	0	0	3	3	0

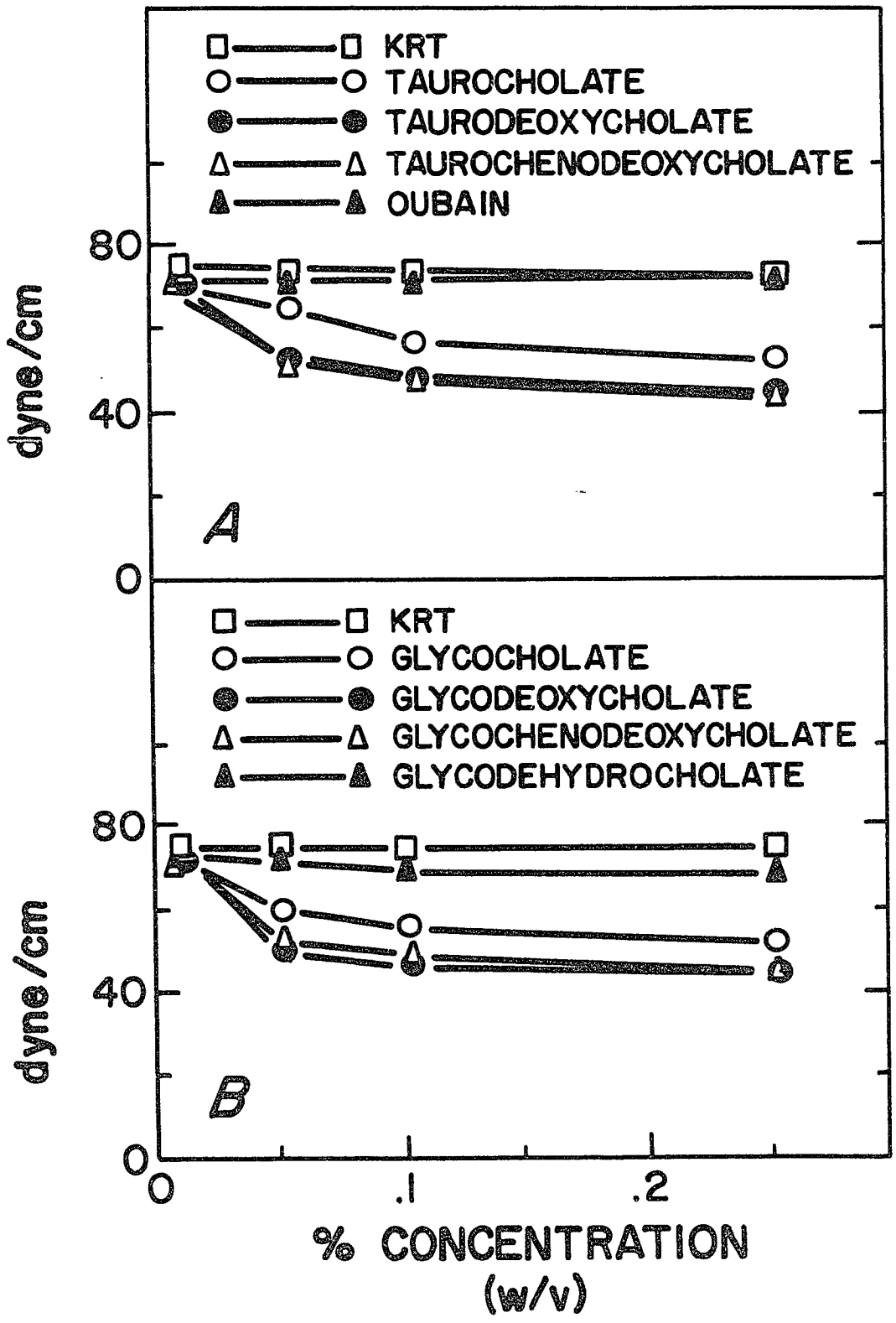
* Numbers represent the active and motile cystacanths at the time intervals indicated.

only KRT are inactive and motionless following this experimental period.

It was suggested by many authors (see Voge, 1967) that it is the surface active property of bile salts which causes the activation of larval parasites. To examine the surface tension lowering action of the bile salts and allied compounds used in these studies, measurements of the surface tensions of various concentrations of these solutions were performed by the capillary rise technique. The values obtained were graphed and are shown in Figure 1. Examination of these values shows that dehydrocholate and ouabain did not lower the surface tensions of the solutions appreciably, yet they still exhibited activating capacity. This would suggest that surface active properties may not be absolute requirements for the action of bile salts, but that perhaps some other property--perphaps chemical--may play the actual trigger role. To test this possibility, the effects of a series of detergents over a wide concentration range were examined in order to determine if they were able to cause the activation of the cystacanths. Cationic, anionic and neutral detergents used in these studies did not activate the cystacanths. Furthermore, it was evident as the experiments progressed that certain morphological changes were taking place in the cystacanths. These changes, quite different from activation, were opacity seen after application of cetylpyridinium chloride, blistering of the

Figure 1.

Surface tensions of bile salt solutions in KRT at 25°.
A. Taurine conjugated bile salts.
B. Glycine conjugated bile salts.


KRT
TAUROCHOLATE
TAURODEOXYCHOLATE
TAUROCHENODEOXYCHOLATE
OUBAIN
80
40
0
dyne/cm
A
KRT
GLYCOCHOLATE
GLYCODEOXYCHOLATE
GLYCOCHENODEOXYCHOLATE
GLYCODEHYDROCHOLATE
80
40
0
dyne/cm
B
0
.1
.2
% CONCENTRATION
(w/v)

tegument caused by alkyl aryl sulphonate (Haemosol) and disruption of the cystacanth caused by Na-lauryl sulphate. Some detergents did not cause any visible changes in the morphology of the larvae. Following the exposure of the parasites to the various detergent solutions, those which appeared least changed were exposed to bile salt solutions to test capacity for activation. The results of these experiments are shown in Table III, where morphological changes noted at time intervals are marked as "x". The number of cystacanths responding to bile salt activation is shown in the last column. It was very evident that detergents caused changes in the ability of the cystacanths to respond to bile salt action. These changes do not necessarily appear to be related to the structural confirguration or surface acting capacity of these detergents, but appear to be related to the actual concentration ranges of each compound. The surface tension lowering activity of these compounds was measured by the same technique is used to estimate that of the bile salts. These data, shown in Figure 2, indicate that the range of surface tensions overlaps that of the bile salt solutions. These results imply the possibility of a lesser role for surface action in the actual activation mechanism.

C. Metabolism of Glycogen Before and After Activation

The cystacanth in the haemocoel of the cockroach is the most

TABLE III

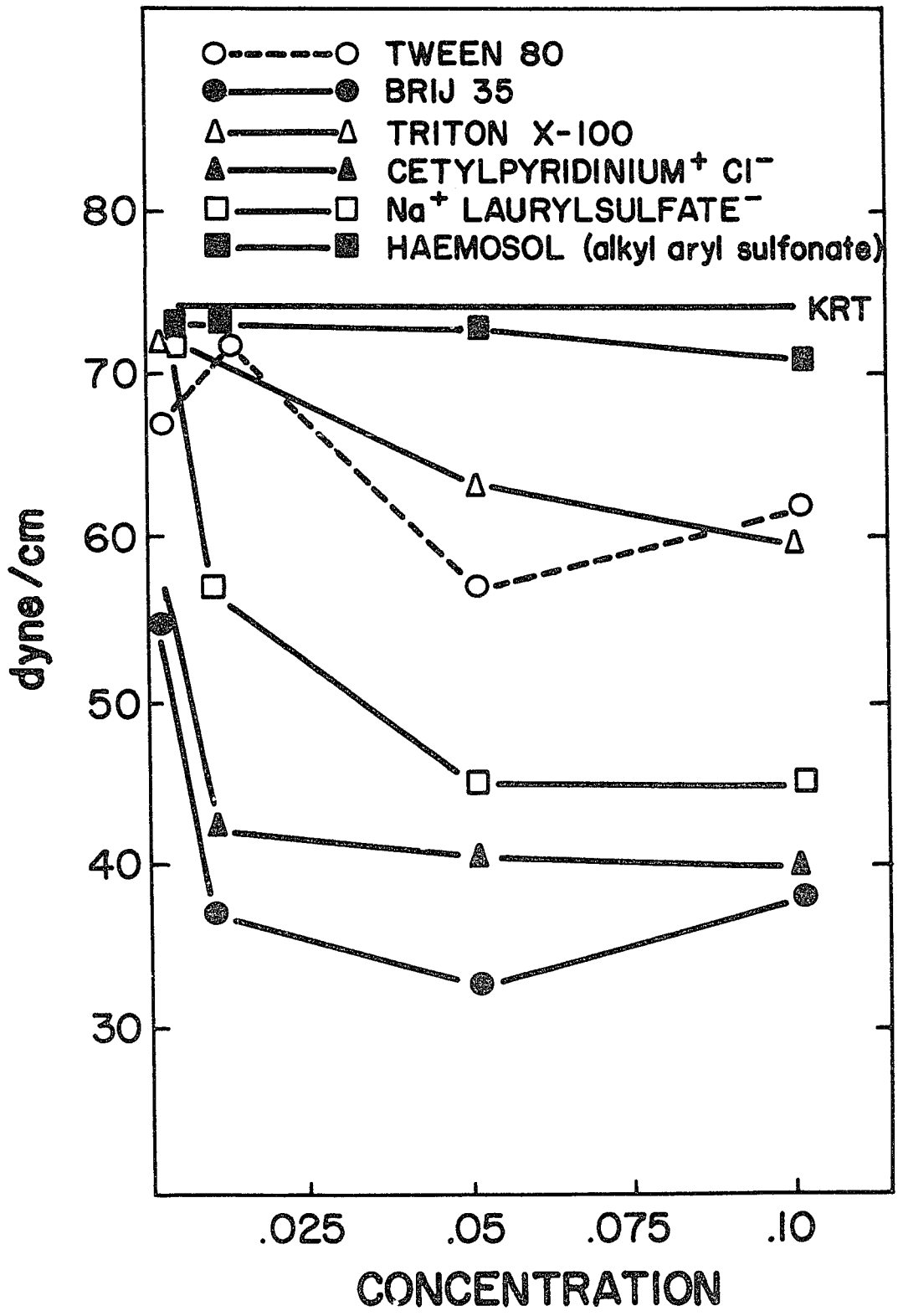
Effects of Surface Active Agents on the Cystacanths

Agents	% conc.	Time				Responds to bile salts
		60'	90'	120'	420'	
Tween 80	.1	-	-	-	-	10*
	.05	-	-	-	-	10
	.01	-	-	-	-	10
	.001	-	-	-	-	10
Brij 35	.1	-	-	-	-	0
	.05	-	-	-	-	1
	.01	-	-	-	-	6
	.001	-	-	-	-	9
Haemosol	.1	-	-	x**	x	0
	.05	-	-	x	x	0
	.01	-	-	-	x	9
	.001	-	-	-	x	10
Triton X-100	.1	-	x	x	x	0
	.05	-	x	x	x	0
	.01	-	-	x	x	0
	.001	-	-	-	x	6
Na-lauryl-SO_4	.1	x	x	x	x	0
	.05	-	x	x	x	0
	.01	-	-	x	x	0
	.001	-	-	-	x	0
Cetylpyridinium chloride	.1	x	x	x	x	0
	.05	x	x	x	x	0
	.01	x	x	x	x	0
	.001	-	x	x	x	0
Na-taurocholate (control)	.1	1	6	7	8	10
	.05	-	2	2	2	10
	.01	-	-	-	-	10
	.001	-	-	-	-	10

* Numbers represent the cystacanths activated and motile.
** Represents morphological changes not related to activation.

Figure 2.

Surface tensions of detergent solutions in KRT at 25°.

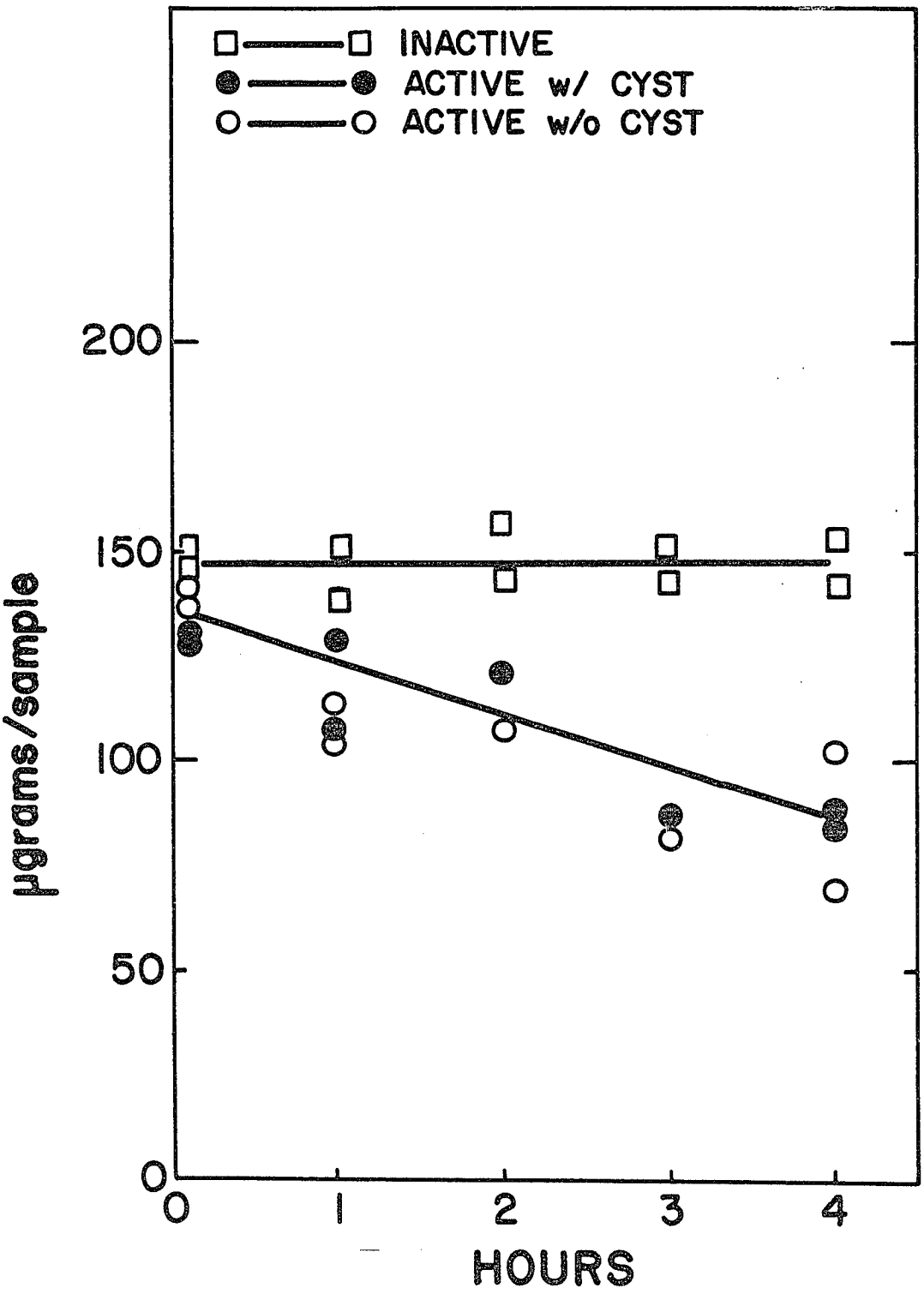

TWEEN 80
BRIJ 35
TRITON X-100
CETYLPYRIDINIUM+ Cl−
Na+ LAURYLSULFATE−
HAEMOSOL (alkyl aryl sulfonate)
KRT
80
70
60
50
40
30
dyne/cm
.025
.05
.075
.10
CONCENTRATION

advanced stage to be found in that host. During the approximately 90 days of development its size has increased from about 0.2 millimeters to 2.0 millimeters. Following activation, rapid growth of the parasite results in a 20-30 centimeter adult worm, representing an appoximate one hundred fold increase in size. The initiation of growth and development to adult stage appeared to be triggered by bile salts. As growth requires energy, the metabolism of the cystacanth before and after activation was examined to see if there were any differences in the rate and character of utilization of exogenous and endogenous metabolites. Since carbohydrates were shown to be required for growth of M. dubius (Read and Rothman, 1958), the examination of the metabolism of glucose and glycogen was undertaken.

Studies on the initial glycogen content and utilization by the active and inactive cystacanth were carried out at 37° in KRT. The parasites were incubated for various periods of time up to four hours and the level of glycogen was determined after the incubations. The values for the active and inactive cystacanths are shown in Figure 3. It was apparent that inactive cystacanths showed no appreciable metabolism of their glycogen during the four hour incubation period, while the active cystacanths depleted approximately 35% of their carbohydrate stores in the same time. The experiment was repeated with active cystacanths and the glycogen content was determined and

Figure 3.

Glycogen levels in inactive and active cystacanths incubated in KRT at 37°.


INACTIVE
ACTIVE w/ CYST
ACTIVE w/o CYST
200
150
100
50
0
µgrams/sample
0
1
2
3
4
HOURS

expressed as the percent of the dry weight. The results are shown in Table IV. In the same experiment at activation, one and two hours following activation the worms were exposed to a 10^{-3} M glucose-^{14}C U. L. (specific activity of 0. 5 μcuries/μ mole). The incorporation of glucose into glycogen during five minutes was estimated by measuring the label appearing in glycogen. The results, shown in Table V, indicate that the rate of incorporation of glucose into glycogen increases as the total level of glycogen decreases.

D. Effects of the Gas Phase on Glycogen Metabolism

It has been demonstrated that HCO_3^- or CO_2 affects the activation of various stages of parasites (Edmonds, 1966; Graff and Kitzman, 1965). Since activation increases the rate of metabolism of glycogen, it was of interest to find out if any conditions which enhance activation may be correlated with their ability of enhancing the metabolism of the parasites.

Cystacanths were activated in 0. 25% (weight/volume) Na-taurocholate in KRT, following a preincubation of one hour at 37°. They were transferred to KRT without exogenous glucose and equilibrated with the gas phase to be tested. All media gassed with CO_2 (5%) contained 0. 015 M Na-bicarbonate in addition to the tris (hydroxymethyl)aminomethane-maleate buffer to maintain pH 7. 4. The activated parasites were allowed to remain at 37° under these

TABLE IV

Glycogen Content of the Cystacanths Following Activation

Condition	µgrams of glycogen*	% dry weight
At activation	143.1	12.9
	121.2	10.7
	143.1	12.2
1 hour following activation	121.5	11.0
	106.6	9.1
	108.0	9.5
2 hours following activation	93.1	8.5
	98.5	8.4
	95.8	8.3

* Based on samples of fifty cystacanths.

TABLE V

Incorporation of Glucose-C^{14} into Glycogen by Active Cystancanths (during a five minute pulse label following starvation in KRT).

Condition	mμmoles of glucose incorporated*	% glycogen
at activation	0.20 0.17 0.16	less than 0.1
1 hour after activation	4.75 4.35 5.42	0.7 0.7 0.9
2 hours after activation	15.20 17.30 12.78	2.9 3.2 2.4

* Based on samples of fifty cystacanths.

conditions for three hours. Following this incubation period they were extracted with 70% ethanol, dried and the glycogen was estimated. The results, expressed as micrograms of glycogen/ sample of 50 cystacanths are shown in Table VI. The examination of the data for differences based on the standard deviation from the mean showed that utilization of glycogen under the gas phase of air, air-CO_2 (95:5) and N_2 was essentially the same; however, the use of N_2-CO_2 (95:5) as the gas phase resulted in a significantly greater glycogen depletion ($P=0.01$). Thus, if activation requires the increase of metabolism, these results would provide at least a part of the answer to the observation on the effects of CO_2 in enhancing activation. The effects of the gas phase on the turnover of glycogen were examined in an experiment of relatively shorter duration, since the levels of glycogen had to be maintained identical in all samples studied to be able to use direct comparison of values obtained, without biasing the results by widely differing levels of glycogen in the groups metabolizing under the different gas phases. During thirty minutes of incubation the level of glycogen does not change significantly in any of the samples of the cystacanths with respect to the gas phases employed. Activated cystacanths were incubated under the various gas phases as in the previous experiment, but in the presence of 10^{-3} M glucose-^{14}C U. L. (specific activity of 0.5 μcuries/μmole). Following the termination of the

TABLE VI

Effects of the Gas Phase on Glycogen Utilization of the Cystacanths of M. dubius

Sample	Gas phase	µgm Glycogen / Sample	Mean	% Utilized
Group I*				
1	N_2:CO_2 (95:5)	73.80		
2		95.40	85.80±8.98	58
3		88.20		
4	Air:CO_2 (95:5)	118.80		
5		111.60	121.20±8.98	40
6		133.20		
7	N_2 (100)	149.40		
8		117.80	134.40±10.63	32
9		126.00		
10	Air	117.00		
11		127.80	132.00±14.27	34
12		151.20		
13	(t_o control, Group I)	212.00		
14		223.00	201.33±23.30	0
15		169.00		
Group II*				
1	N_2:CO_2 (95:5)	40.30		
2		40.30	40.80±0.68	71
3		41.70		
4	Air:CO_2 (95:5)	----	---------	--
5		77.80		
6		----		
7	N_2 (100)	71.30		
8		71.30	72.48±1.69	50
9		74.90		
10	Air	86.40		
11		84.90	76.38±13.15	47
12		57.80		
13	(t_o control, Group II)	134.80		
14		142.80	144.93±9.27	0
15		157.20		

* Groups I and II differ only in the length of infection of the intermediate host; group I being higher in glycogen level is also the oldest. This aspect was not followed in these studies.

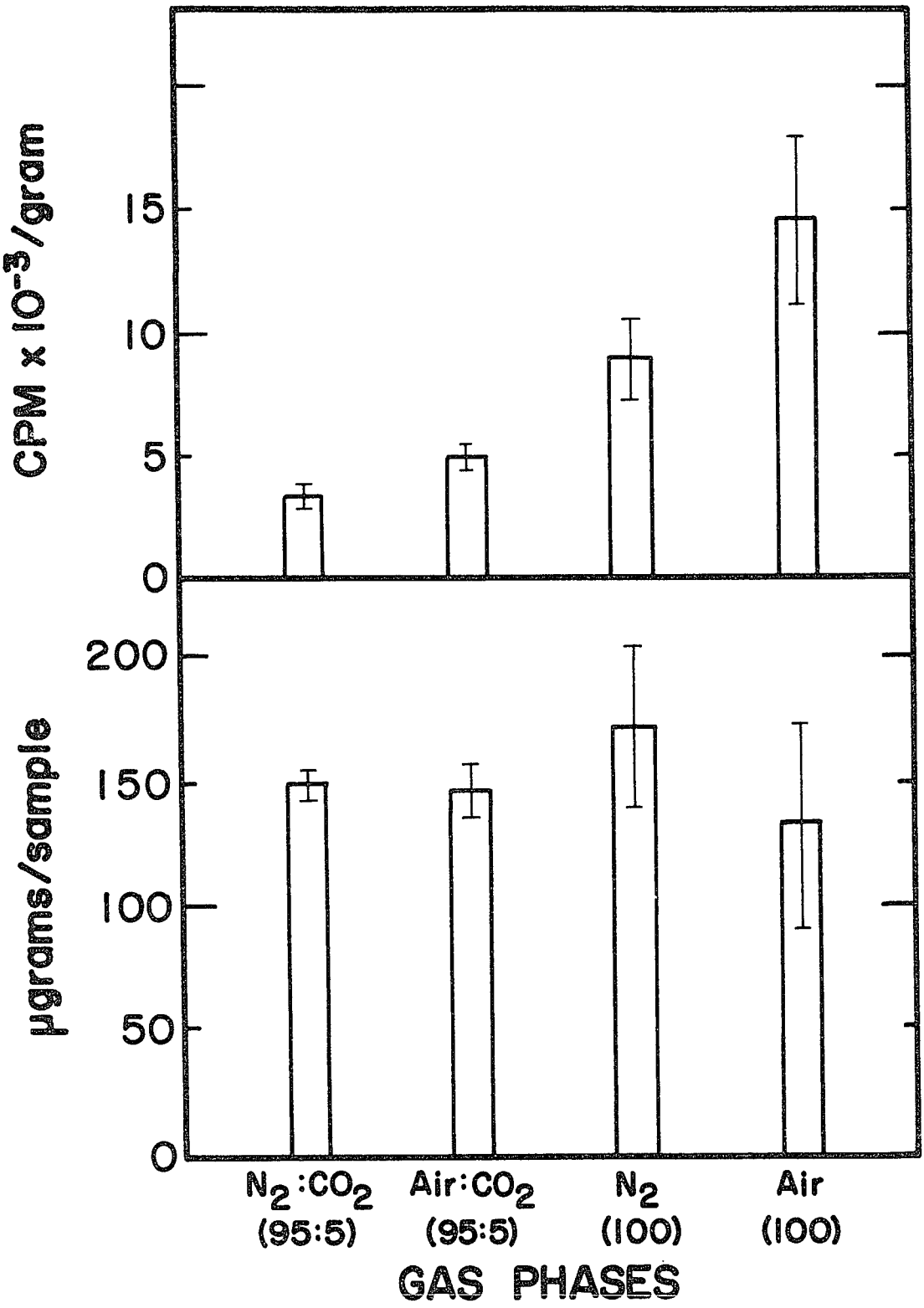
experiment at thirty minutes, the specific activity of the glycogen of the cystacanths was determined. The results, shown in Figure 4, indicate that the lowest amount of incorporation occurred under N_2-CO_2 (95:5), followed by air-CO_2 (95:5), N_2 (100) and air which gave the highest rate of incorporation. The gas phases thus not only affect the breakdown of glycogen, but also the incorporation of glucose into glycogen by the cystacanths. How this may affect the rate of activation cannot be answered from these experiments.

E. The Effects of Activation on Glucose Metabolism

In the intermediate host the cystacanth is surrounded by the cyst. To determine the possible role of the cyst on the amount of free glucose available to the organism, intact and mechanically excysted cystacanths were incubated in KRT at 37^{o} in 1.0 mM glucose-C^{14} U. L. (specific activity of 0.5 μcuries/μmole) for five minutes. Following three rinses in KRT the cystacanths were extracted with 70% ethanol and the radioactivity of the extracts was determined by counting aliquots of the ethanol. The medium was also counted following dilution in ethanol. This procedure minimized self-absorption by the salts in KRT. Based on these data the total uptake of glucose by the cystacanths was calculated. Chromatography of aliquots of the ethanol extracts showed that essentially all radioactivity recovered from the cystacanths resides in glucose; thus, metabolism

Figure 4.

Effects of the gas phase on the incorporation of glucose-^{14}C into glycogen by active cystacanths.
A. Specific activities of the glycogen.
B. Glycogen content of the cystacanths.

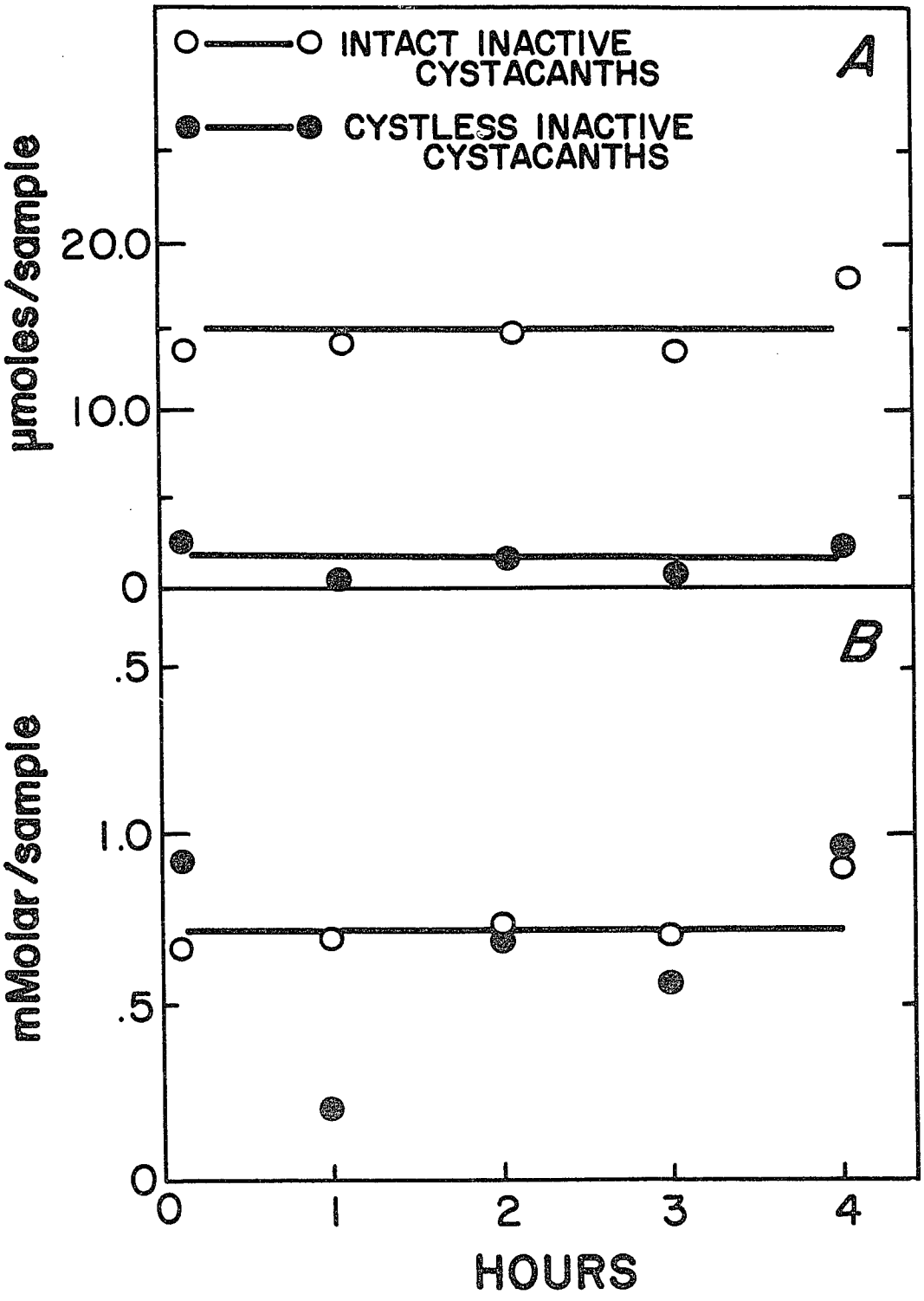

CPM x 10^{-3}/gram
15
10
5
0
µgrams/sample
200
150
100
50
0
N_2:CO_2 (95:5)
Air:CO_2 (95:5)
N_2 (100)
Air (100)
GAS PHASES

of the inactive cystacanth during a five minute incubation in negligible. The total uptake of glucose by inactive cystacanths is shown in Figure 5. It is evident that even after four hours of incubation under starvation conditions in KRT the accumulation of glucose is unchanged in both intact and cystless cystacanths.

To test the possibility that the mammalian saline used in these experiments may have had adverse effects on the cystacanths, intact cystacanths were exposed to 1.0 mMolar glucose in Pringle's insect saline at 25°. Following extraction of the worms in ethanol, the uptake of glucose was calculated based on the radioactivity of the extracts. During the five minute incubation period in glucose-^{14}C, the intact inactive cystacanths accumulated 13.85±2.85 µmoles of glucose. Increasing the incubation to ten minutes resulted in the uptake of 13.92±1.01 µmoles of glucose. Chromatography of the extracts showed that essentially all radioactivity resided in glucose; thus, metabolism could be discounted. These values indicated that the cystacanths were able to establish an equilibrium with respect to glucose concentration, since the values for five and ten minutes were identical. Furthermore, the comparison of these values to those given in Figure 5 shows that the internal concentration is of the same order of magnitude, in spite of the change in the saline-buffer system. Based on this, the possible deleterious effects of KRT can be discounted.

Figure 5.

Glucose uptake by intact and cystless inactive cystacanths.
A. Total uptake by fifty cystacanths incubated in hourly five minute pulses of 1.0 mMolar glucose-^{14}C.
B. Internal molar concentrations of glucose in cystacanths.


INTACT INACTIVE CYSTACANTHS
CYSTLESS INACTIVE CYSTACANTHS
A
B
μmoles/sample
mMolar/sample
20.0
10.0
0
.5
1.0
.5
0
0
1
2
3
4
HOURS

The effects of activation on the metabolism of glucose were examined in a series of experiments, using mechanically decysted cystacanths. The parasites were activated in a 0.25% Na-taurocholate solution in KRT, following a preincubation of one hour at 37°. They were incubated for five minutes in a 1.0 mMolar glucose-^{14}C U. L. solution in KRT. The larvae were extracted with ethanol, and the extracts were chromatographed. The scanning of the chromatograms revealed that the active cystacanths metabolize glucose at a very high rate; the radioactivity residing in metabolic intermediates (approximately 70-90% of the total activity of the extracts), while the activity remaining as glucose was greatly diminished. Thus, in addition to the effects of activation on the glycogen metabolism of the cystacanths, which showed that active cystacanths metabolized their glycogen at a rate significantly higher than inactive cystacanths, the same conclusion can be made concerning the metabolism of exogenous glucose.

F. Enzymes of Glucose Metabolism of the Cystacanths

The demonstration of enzyme activity or its lack in a homogenate or cell-free supernatant may not reflect at all the possible role of the enzymes thus detected in the metabolism of the whole animal. Evidence for the glycolytic pathway in adult M. dubius was presented by Graff (1963), and by Bryant and Nicholas (1965) who showed

labeling of intermediates of this pathway in both adult male and female homogenate and intact organisms by the use of radioactive glucose-C^{14}. In addition to these studies, the presence of aldolase, triose phosphate dehydrogenase and lactic dehydrogenase was shown by Read (1961), in adult worms only. There were no published findings concerning the enzymes of glucose metabolism in the cystacanth stage of this parasite. In the present study some enzymes of the glycolytic, pentose phosphate shunt and Krebs cycle enzymes of the cystacanths were assayed, using the techniques outlined earlier. Since the assay conditions were designed for enzymes from other sources, the results are only indicative in a qualitative not quantitative manner. The enzyme activities noted, expressed in Table VII as mμmoles of substrate converted/minute/mg protein corrected for control values, indicated the presence of all enzymes sought, with the exception of pyruvate kinase. The assay for pyruvate kinase was repeated, using adult worm homogenates, and again showed no activity of this enzyme. The same assay medium was satisfactory to demonstrate the activity of rabbit muscle pyruvate kinase, even in the presence of M. dubius homogenates; thus, the assay medium and the parasite preparations did not inhibit the action of the known enzyme. The apparent lack of this enzyme in M. dubius, suggested to the present author the need to investigate the possible means available for the parasite to circumvent this

TABLE VII

Glycolytic, Pentose Phosphate Shunt and Krebs Cycle Enzyme Activities in M. dubius Cystacanth Homogenates

Enzyme	specific activity mμmoles/minute/mg protein
Hexokinase	50
Hexose phosphate isomerase	480
Glucose-6-phosphate dehydrogenase	1370
6-phosphogluconate dehydrogenase	1040
Aldolase	1250
α-glycerophosphate dehydrogenase	470
Enolase	320
Pyruvate kinase	0
Lactic dehydrogenase	5900
Malic dehydrogenase	4500

step in the glycolytic pathway.

It appeared that glycolysis proceeds to the level of phosphoenol pyruvate in M. dubius. This exclusion of the direct formation of pyruvate from this precursor leaves open another pathway which includes the fixation of CO_2, the formation of oxaloacetate and then its conversion by some route to pyruvate. While CO_2 fixation in adult M. dubius was reported by Graff (1965), that by the cystacanths or its mechanism in either of these stages of this organism were not reported. To continue the investigation, the study of the mode of CO_2 fixation was undertaken; a step also warranted by the effects of CO_2 on the activation of the cystacanths, a phenomenon noted but not explained by Graff and Kitzman (1965).

G. Carbon Dioxide Fixation by Adult and Larval M. dubius

The ability of M. dubius to fix CO_2 into glycolytic and Krebs cycle intermediates was shown by Graff (1965). He postulated that CO_2 is fixed at either pyruvate or phosphoenol pyruvate level. He based his interpretation on the distribution of label from $H^{14}CO_3^-$ into the various metabolic intermediates. He excluded the fixation of CO_2 into propionate and its subsequent incorporation into succinate through methylmalonyl CoA by showing that propionate is not metabolized by adult M. dubius. The results of Bryant and Nicholas (1965) showed that acetate-1-^{14}C did not appear in

oxaloacetate nor in its transamination product--aspartate; thus, the presence of an active oxaloacetic carboxylase can be excluded. These findings limit the pathways of CO_2 fixation by M. dubius to essentially two enzymes, the malic enzyme of Ochoa, fixing CO_2 into pyruvate to form malate with the oxidation of NADPH, and/or the enzyme PEP carboxykinase, fixing CO_2 into phosphoenol-pyruvate to give oxaloacetate and producing stoichiometric quantities of high energy phosphate as ITP, ATP or GTP. The examination of these two pathways was considered important for two reasons: one being the demonstrated importance of CO_2 in the gas phase on the activation of the cystacanths, the other being the apparent absence or very low activity of pyruvate kinase in both adult and larval M. dubius. This latter finding alone makes the CO_2 fixation by PEP carboxykinase very important, since it would serve as the connecting link to the dicarboxylic acid portion of the Krebs cycle, especially if the formation of pyruvate and its metabolism to other intermediates or endproducts is hindered. This role of PEP-carboxykinase would assure the formation of oxaloacetate and malate, the latter of which could then be metabolized to the various intermediates and endproducts of glucose metabolism identified in M. dubius.

To examine the activity of PEP-carboxykinase in homogenates of M. dubius, the technique of Utter and Kurahashi (1954) was

employed. This assay, based on the exchange incorporation of label from $H^{14}CO_3^-$ into oxaloacetate or malate, gives the total incorporation due to both PEP-carboxykinase and malic enzyme. The activities of each enzyme can be isolated by the selective omission of cofactors required by one or the other enzyme and by the selective decarboxylation of oxaloacetate by Al^{+3} ions under acidic conditions. The activity remaining in the assay medium following decarboxylation is assigned to CO_2 fixation by the malic enzyme. The assay components with the exception of the cofactors are referred to as control; the appropriate cofactors added and the resultant changes in CO_2 incorporation are shown in Table VIII for both adult and cystacanths preparations. From these data one can conclude that incorporation of CO_2 by the PEP-carboxykinase pathway diminishes if the nucleotide triphosphate cofactor is omitted from the medium. The addition of NAD or NADP dinucleotide cofactors enhances the incorporation of CO_2 by the malic enzyme pathway. Both enzymes are present in adult and cystacanths preparations; however, the malic enzyme activity of the cystacanths is very low, in comparison to that of the adult worms. From these data it would appear that PEP-carboxykinase is the major CO_2 fixing enzyme in M. dubius. This finding is consistent with the assumption that there is no direct formation of pyruvate from phosphoenolpyruvate by pyruvate kinase, since the activity of this enzyme could not be demonstrated

TABLE VIII

Malic Enzyme and PEP-carboxykinase Activities in M. dubius

Assay conditions	Time	µmoles of CO_2 incorp. /mg protein		
		total	malic enz.	PEP-CO_2kinase
Adult				
control	10 min	0.51	0.00	0.51
+ATP	10 min	6.58	0.21	6.27
	20 min	7.95	0.27	7.48
+NADP	10 min	0.92	0.12	0.80
	20 min	1.50	0.75	0.75
+NAD	10 min	0.61	0.57	0.04
	20 min	1.50	1.40	0.10
Cystacanth				
control	10 min	0.08	0.00	0.08
+ATP	10 min	11.20	0.20	11.00
	20 min	15.50	0.00	15.50
+NADP	10 min	0.48	0.02	0.46
+NAD	10 min	0.11	0.02	0.09

in homogenates of either adults or cystacanths. Furthermore, it is consistent with the pattern of label distribution from $^{14}CO_2$ fixation by whole adult M. dubius reported by Graff, where the highest amount of label was found to occur in aspartate, which is derived from oxaloacetate by transamination. Utter and Kurahashi (loc. cit.) reported that PEP-carboxykinase requires Mn^{+2} ions for optimal activity and that the enzyme operates at the highest rate when ITP or GTP are used in lieu of ATP. These characteristics of PEP-carboxykinase in M. dubius were examined, using the omission of Mn^{+2} and the substitution of ITP for ATP in the assay system. The results of CO_2 incorporation by this system, shown in Table IX, confirm the requirement of the enzyme for Mn^{+2} ions and the selectivity for nucleotide triphosphate cofactor in M. dubius. Using ATP in the assay system reduces the incorporation to approximately forty percent of the level obtained with ITP, while the omission of Mn^{+2} reduces it to less than twenty percent in the adult and less than ten percent in the cystacanth; thus, it appears that these worms possess a PEP-carboxykinase.

H. Endproducts of Glucose and Glycogen Metabolism

Crompton and Ward (1967) identified succinate and lactate in addition to ethanol in the endproducts of carbohydrate metabolism of adult M. dubius. In the present study both succinate and lactate

TABLE IX

Specificity of PEP-carboxykinase for Cofactors

Assay condition	Time	μmoles of CO_2 incorp. /mg protein	
		adult	cystacanth
Control	10 min	0.01	0.00
+ATP $+Mn^{+2}$	"	4.50	12.80
+ITP $+Mn^{+2}$	"	9.20	33.90
+ITP $-Mn^{+2}$	"	1.92	2.88

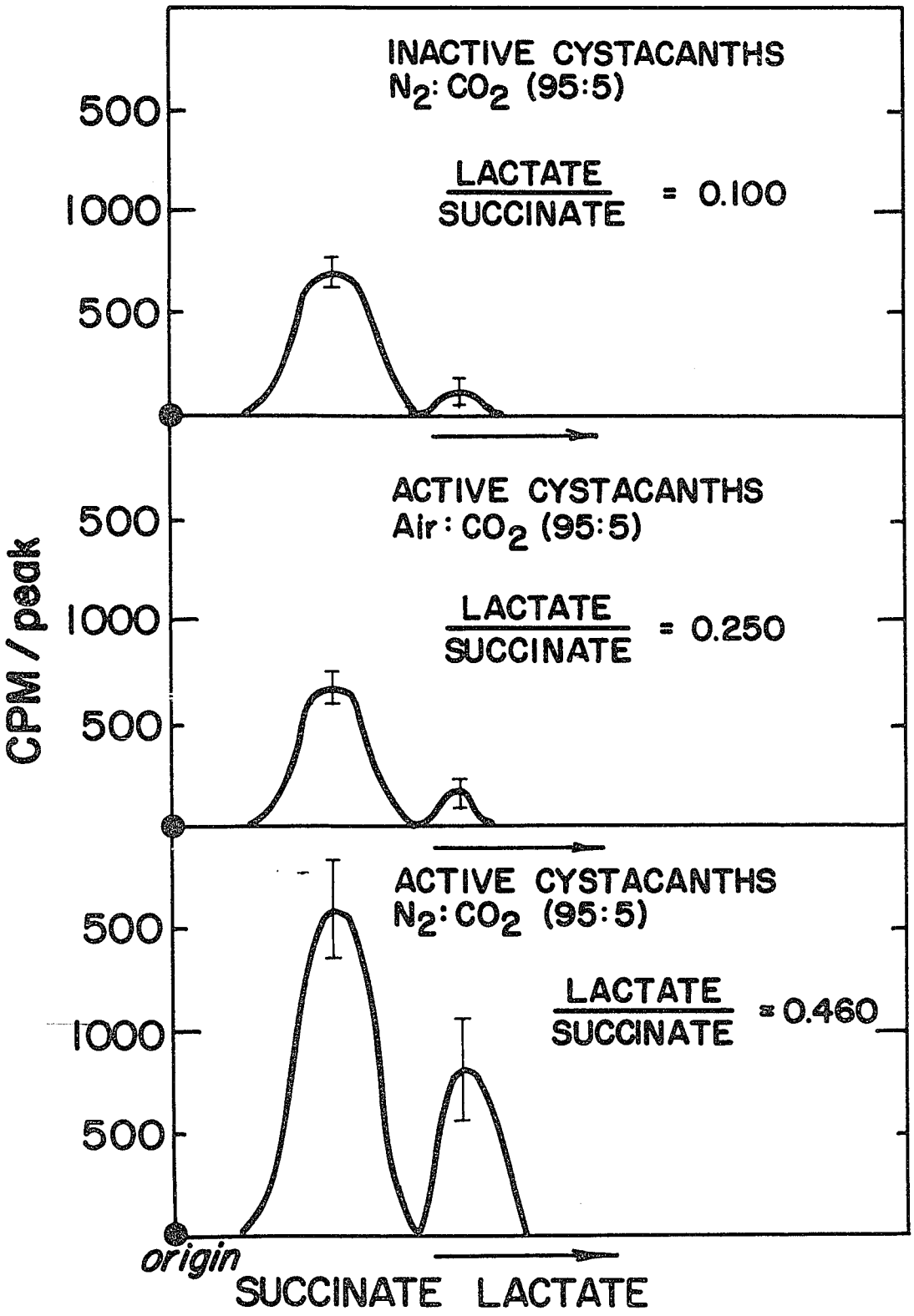
were identified as endproducts of carbohydrate metabolism in the cystacanths of the same parasite. It was of interest to determine the role of succinate formation in the metabolism of the cystacanth of this acanthocephalan in order to attempt to correlate the noted effects of CO_2 and anaerobiosis on activation and carbohydrate metabolism of these worms. Based on the enzyme pattern of PEP metabolism discussed earlier, CO_2 could have two different roles in the pathway of carbohydrate metabolism. The fixation of CO_2 by PEP-carboxykinase and the decarboxylation of malate formed from oxaloacetate requires only catalytic amounts of CO_2, since the pathway could operate as a cyclic sequence of events; CO_2 being first fixed, then removed and able to reenter the fixation reaction again. Two of the reported endproducts, lactate and ethanol, may be formed by this operation of the pathway; however, to form succinate the pathway from PEP would require the fixation of stoichiometric quantities of CO_2 for each molecule of succinate produced. An interesting aspect of these two ways of CO_2 fixation is that the label from $^{14}CO_2$ fixation in oxaloacetate or malate would be lost by decarboxylation of malate by the malic enzyme, unless randomization of the label into carbons 1 and 4 of fumarate and therefore malate could not take place. Thus in the proposed pathway any hindrance or interference in the randomization process would result in the lowering of the amount of label reaching pyruvate and

consequently alanine, lactate or ethanol. It was concluded that the amount of $^{14}CO_2$ incorporated into lactate and succinate by active and inactive cystacanths might give some insight into the role of succinate formation and the effects of the gas phases on the activation and metabolism of larval M. dubius. Recalling that only N_2:CO_2 (95:5) gave increased rate of glycogen breakdown, but not N_2 or air:CO_2 (95:5), it was hypothesized that CO_2 is required in stoichiometric quantities--a condition which must be accompanied by anaerobiosis, in order for the possible formation of succinate by the cystacanths. Active and inactive cystacanths were incubated in KRT with 15.0 mMolar $NaH^{14}CO_3$ at 37° under a N_2:CO_2 (95:5) gas phase. The total amount of $^{14}CO_2$ incorporated into the endproducts of metabolism was determined following chromatography of the media. The same experiment using active cystacanths under an air:CO_2 (95:5) gas phase was also performed. The results of the experiments are shown in Figure 6, where the total radioactivity of lactate and succinate is represented by the relative heights of the peaks in the schematic of the chromatograms. The specific activities of these compounds were not determined.

Comparison of the activities of the endproducts showed that the activated parasites incorporated twice the amount of $^{14}CO_2$ into succinate (1650 CPM) as did the inactive larvae (750 CPM). Thus, this suggests that an increase in succinate formation is correlated

Figure 6.

Chromatogram profiles corrected for total peak activity of lactate and succinate endproducts of the carbohydrate metabolism of the cystacanths.
(Solvent – ethanol: NH_4OH : water, 80 ÷ 5 ÷ 15)


INACTIVE CYSTACANTHS
N_2: CO_2 (95:5)
LACTATE / SUCCINATE = 0.100
ACTIVE CYSTACANTHS
Air : CO_2 (95:5)
LACTATE / SUCCINATE = 0.250
ACTIVE CYSTACANTHS
N_2: CO_2 (95:5)
LACTATE / SUCCINATE = 0.460
500
1000
500
CPM / peak
origin
SUCCINATE LACTATE

with the phenomenon of activation by bile salts. Incorporation of CO_2 into lactate was ten times higher (800 CPM vs. 70 CPM) by the activated parasites indicating the possibility of increased turnover in fumarate, where randomization occurs. Under aerobic conditions (air:CO_2, 95:5) the level of activity in succinate produced by the active worms was reduced to the level formed by the inactive parasites (700 CPM vs. 750 CPM), suggesting that oxygen may block at least partially the formation of succinate; or perhaps it enhances its reoxidation to fumarate. Lactate under these conditions contained radioactivity intermediate (170 CPM) between active (800 CPM) and inactive (70 CPM) parasites under N_2:CO_2. The ratio of activity in lactate to that in succinate is the lowest in the inactive cystacanths (.100) and highest in the active cystacanths (.460), both measured under a N_2:CO_2 (95:5) atmosphere. The ratio of lactate to succinate based on radioactivity in active cystacanths is intermediate (.250) between the active and inactive preparations if the gas phase is aerobic (air:CO_2, 95:5).

A further suggestion of pathway differences was seen in the metabolism of malate-^{14}C by a "particulate" preparation of active and inactive cystacanths, which was isolated in 0.27 Molar mannitol by sedimentation of the 3000 X g supernatant at 12'000 X g at 2^{o}. The incubation media contained 20.0 µmoles of phosphate buffer pH 7.4 and 1.0 µmole of ADP in addition to the particulate fraction

prepared from 100.0 milligrams of cysta[illegible]
volume. Malate-4-^{14}C was added (5.0 [illegible]
was stopped after fifteen minutes incubation [illegible]
of the reaction mixtures following deprotein[illegible]
indicated that the active cystacanth prepara[illegible]
activity into pyruvate (1350 CPM) than did the [illegible]
preparations (900 CPM). This would tend to s[illegible]
that the randomization of the label is hinde[illegible]
cystacanths; thus there in a greater loss of rad[illegible]
resulting from the decarboxylation of malate b[illegible]
To test in detail the possible reasons for th[illegible]
preparation and characterization of mitochond[illegible]
required. However, due to the small size and [illegible]
cystacanths this was not feasible.

Further evidence in favor of the possible [illegible]
creased succinate formation as activation occurs [illegible]
ments with amytal. The addition of this barbit[illegible]
medium of KRT containing 0.25% Na-tauroch[illegible]
increase of activation time of the cystacanths [illegible]
to more than two hours, i.e. an approximately [illegible]
crease. The concentration of amytal (10^{-4}M [illegible]
could inhibit the transfer of H^+ from NADH thro[illegible]
chondrial oxidase system to fumarate in the pr[illegible]

with the phenomenon of activation by bile salts. Incorporation of CO_2 into lactate was ten times higher (800 CPM vs. 70 CPM) by the activated parasites indicating the possibility of increased turnover in fumarate, where randomization occurs. Under aerobic conditions (air:CO_2, 95:5) the level of activity in succinate produced by the active worms was reduced to the level formed by the inactive parasites (700 CPM vs. 750 CPM), suggesting that oxygen may block at least partially the formation of succinate; or perhaps it enhances its reoxidation to fumarate. Lactate under these conditions contained radioactivity intermediate (170 CPM) between active (800 CPM) and inactive (70 CPM) parasites under N_2:CO_2. The ratio of activity in lactate to that in succinate is the lowest in the inactive cystacanths (.100) and highest in the active cystacanths (.460), both measured under a N_2:CO_2 (95:5) atmosphere. The ratio of lactate to succinate based on radioactivity in active cystacanths is intermediate (.250) between the active and inactive preparations if the gas phase is aerobic (air:CO_2, 95:5).

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prepared from 100.0 milligrams of cystacanths in a total of 0.5 ml volume. Malate-4-^{14}C was added (5.0 μcuries) and the reaction was stopped after fifteen minutes incubation at 37°. Chromatography of the reaction mixtures following deproteinization and desalting indicated that the active cystacanth preparations incorporated more activity into pyruvate (1350 CPM) than did the inactive cystacanth preparations (900 CPM). This would tend to support the hypothesis that the randomization of the label is hindered in the inactive cystacanths; thus there in a greater loss of radioactivity as $^{14}CO_2$ resulting from the decarboxylation of malate by the malic enzyme. To test in detail the possible reasons for this difference, the preparation and characterization of mitochondrial preparations is required. However, due to the small size and limited number of cystacanths this was not feasible.

Further evidence in favor of the possible requirement for increased succinate formation as activation occurs was seen in experiments with amytal. The addition of this barbiturate to the activation medium of KRT containing 0.25% Na-taurocholate resulted in the increase of activation time of the cystacanths from 15-20 minutes to more than two hours, i.e. an approximately five or six-fold increase. The concentration of amytal (10^{-4}M) used in these studies could inhibit the transfer of H^+ from NADH through some mitochondrial oxidase system to fumarate in the process of succinate

formation. Thus, this potential hindrance of succinate formation by amytal may be considered as circumstantial evidence for a mechanism of activation which may include modifications in mitochondrial metabolism, namely the production of succinate. The system of NADH oxidation by mitochondrial preparations of the cystacanths has not been worked out in detail, and study of such mechanisms would require the availability of larval materials in quantities not readily available.

CONCLUSION

The activation of the cystacanth of Moniliformis dubius was defined by Graff and Kitzman (1965) as the eversion of the proboscis of the organism following exposure to bile salt solutions in vitro or to bile in vivo. Based on the results of the studies reported in the previous section, the present author feels that the definition of activation should be amended to include the increase in the rate of carbohydrate metabolism, the change in the nature of the endproducts formed and the increased permeability to water in addition to the eversion of the proboscis. These physiological changes are caused directly or indirectly by bile salts. The review of the chemistry of these compounds suggested two possible ways by which the activation of the cystacanths occurs; surface action and some type of structural requirement for the bile salt molecule.

The series of bile salts examined included some with essentially no surface active properties, yet even these compounds activated the cystacanths. The rate of activation, on the other hand, seemed to be related to the detergent power of bile salts, although taurocholate which was of intermediate detergent activity gave the fastest activation. The concentration of bile salts required for activation was not studied directly, however, the activation of cystacanths by lithocholate, which was essentially insoluble in KRT, suggested that

only trace amounts are required.

The detergents examined in these studies did not activate the cystacanths; furthermore, they caused morphological changes not related to activation and reduced the number of cystacanths which were able to respond to bile salt activation. These effects appeared to be correlated to their concentrations, but not to their structure or mode of action. Microscopic examination of the cystacanths which were treated with detergents revealed anomalous changes of the morphology which appared to be similar to changes caused by chenodeoxycholate or deoxycholate. These two bile salts, while activating the cystacanths, reduced the length of motility in vitro and caused swelling and blistering of the organisms. Bile salts are detergents, but because of their structure they are quite different in physical properties from the typical ionic detergents (Hofmann and Small, 1967). The hydrophilic (OH^-) and hydrophobic (CH_3) portions of these molecules are juxtaposed on the cyclopentanophenanthrene ring, while typical detergents have a hydrophobic flexible body which ends in one or more ionic groups. Thus, the use of detergents in attempts to activate the cystacanths allowed examination of the effects of lowering the surface tension of the medium but did not test the effect of the structural requirement found in bile salts. The use of the cardenolide, oubain, on the other hand allowed testing the effects of structure, this compound being structurally similar to bile salts,

while it had essentially no surface tension lowering capacity. The fact that cystacanths are activated by oubain suggests that the structural requirement for the bile salt molecule is essential to elicit the activation, while detergent capacity only enhances the rate of the activation process. The uptake of water by the cystacanth during and following activation, which results in increasing its volume, may play a role in the evagination of the proboscis, which is operated by a hydraulic muscular mechanism. However, no attempt was made to localize the fluid taken up by the cystacanths and was assumed to be distributed throughout the whole organism. Since the inactive cystacanths did not change their internal water content significantly during similar incubations in KRT only, the bile salts elicited a change in permeability of the organism. One must note, however, that acanthocephalans will take up water from the medium when removed from their hosts and that there are no satisfactory salines for in vitro incubations of these parasites (van Cleave and Ross, 1944). This pehnomenon may be a reflection of a requirement by these worms for some compounds or components of the natural environment, the gut, to maintain their water balance unaltered. The gut contains amino acids, proteins and lipids in addition to the electrolytes replaced by the salines. The effects of these compounds on the cystacanths was not studied.

In the natural definitive rat host, taurocholate is the major bile

salt found in the small intestine (Bergstrom et al., 1963). Moore (1946) reported that M. dubius will not establish in rabbits or guinea pigs under laboratory conditions. The failure of these infections may have been caused by the bile salts found in these animals, i.e. chenodeoxycholate in guinea pigs and deoxycholate in rabbits, since the present findings indicate a deleterious effect of these bile salts on the cystacanths in vitro. However, other physiological factors of the small intestine may have also played a role. A similar suggestion of host specificity and unsuitability was advanced by Smyth and Haslewood (1963) when they demonstrated the deleterious effects of deoxycholate-containing bile on the larval tapeworm Echinococcus granulosus.

The mode of action of bile salts in eliciting the increase in carbohydrate metabolism may involve modification of mitochondrial function. This view is supported by the results showing differences in the metabolism of malate-4-^{14}C by particulate fractions of active and inactive cystacanths and by the change in the ratios of labeled CO_2 corporated into lactate and succinate by intact active and inactive cystacanths. The mechanism of bile salt action could involve either permeability change in mitochondrial membranes or a change in the succinate forming system, or possibly a combination of both. The suggestion that the succinate-forming system is modified appears to be supported by the observation that amytal, a barbiturate

interfering with electron transfer from NADH, increased the time required for activation of the cystacanths. Furthermore, the admission of air (O_2) to the incubation system also increases the time required to activate the parasites. Both of these conditions interfere with the formation of succinate; thus, their effects on activation may be the result of such action. To test this hypothesis in detail, mitochondrial preparations should be studied to define in detail the electron transport system and terminal oxidations of M. dubius cystacanths, and as mentioned earlier such preparations are not feasible at the present due to the limitations of the size and availability of parasites and also by the paucity of mitochondria therein (Byram, unpublished).

Bryant and Nicholas (1966) suggested a terminal oxidative pathway in adult M. dubius based on results of studies using inhibitors and redox dyes; however, their study was not sufficiently precise to represent adequate description of such pathways. The mitochondrial isolations in their studies were not characterized sufficiently to ascertain purity and structural integrity; furthermore, the techniques of isolation they used were not adequately proven to be satisfactory for such preparations from acanthocephalans. It has been shown (Harlow, 1968) that isolation techniques developed for mammalian liver mitochondria are unsuitable for satisfactory preparation of mitochondria from the tapeworm Hymenolepis diminuta. Studies of

this nature have not been performed on any acanthocephalans.

It is the opinion of the present author that the actual mode of action of bile salts on activation of larval parasites and the corresponding enhancement and modification of carbohydrate metabolism will have to be done on an organism other than M. dubius. Smyth and Haslewood (1963) reviewed the role of bile and bile salts as activators or hatching requirements in other parasitic systems. They found that certain coccidians, trematodes, cestodes and nematodes require bile salts for the hatching of eggs or for larval activation. While there has been no published information that bile salts cause physiological changes in these parasitic organisms which would correspond to the changes noted in the cystacanths of M. dubius, it is possible that a closer examination of these organisms will yield a system better suited for study than M. dubius. Furthermore, bile salts cause changes in the metabolism of adult parasites resulting in a decrease of production of acidic endproducts of carbohydrate metabolism (Rothman, 1958). Bile salts also act similarly to cardiac glycosides on isolated heart preparations (reviewed by Horrall, 1938) and numerous other cases. These possibilities will allow one to find the optimal system in which the actual mode of bile salt activation on the metabolism may be determined.

It is of interest to note that M. dubius appears to possess an enzyme pathway bypassing the pyruvate kinase step, a pathway

similar to that reported for other organisms producing large amounts of succinate or its volatile derivatives (Bueding and Saz, 1968). By the participation of stoichiometric quantities of CO_2 in the anaerobic formation of succinate in this type of pathway, the mode of action of CO_2 in enhancing the activation is apparently connected to the ability and requirement of the organism for succinate production. This view is further supported by the results of the studies on glycogen utilization, where the gas phase of N_2:CO_2 (95:5) produced the fastest rate of glycolysis, while anaerobiosis without increased CO_2 level or an aerobic system with high level of CO_2 did not differ significantly from an aerobically incubated system. Any other role for CO_2 or anaerobiosis will also have to be reexamined when the isolation and characterization of mitochondria from M. dubius has been successfully completed.

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