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THE EFFECT OF *IN VIVO* ALTERATION OF BILE ACID COMPOSITION ON *HYMENOLEPIS MICROSTOMA* (DUJARDIN 1845)

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THE EFFECT OF *IN VIVO* ALTERATION OF BILE ACID COMPOSITION ON *HYMENOLEPIS MICROSTOMA* (DUJARDIN 1845)

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CHAPTER I

INTRODUCTION

Physiological and biochemical differences between hosts and their cestodes have been shown to have adverse effects on the latter (Larsh, 1946; Read and Voge, 1954; Schiller, 1959; Litchford, 1963), as have intraspecies host alterations (Beck, 1952; Landt, 1955; Landt and Goodchild, 1962; Read and Simmons, 1963). Smyth (1962, 1963) and Smyth and Haslewood (1963) have stated that differences in bile constituents, viz., the kind, type of conjugation, and concentration of bile acids, may play a major role in the determination of host specificity for *Echinococcus granulosus*. Further, Smyth (1962, 1963), on the basis of his studies with *E. granulosus* protoscolices, has postulated that biochemical differences in bile may be a primary controlling factor in the life cycle of other intestinal parasites as well. In addition, rat bile has been shown to be a necessary factor for the normal growth and maturation of *Hymenolepis diminuta* (Goodchild, 1958, 1960, 1961a, 1961b; Vilar-Alvarez and Goodchild, 1961; Goodchild and Vilar-Alvarez, 1962), as well as the *in vivo* survival of *Giardia duodenalis* (Bemrick, 1963).

Hymenolepis microstoma, established in the laboratory by Dvorak,

Jones, and Kuhlman (1961), promises to be a useful tool for additional research on cestode physiology, biochemistry, and host-parasite relationships. This cestode, primarily an inhabitant of the mouse bile duct (Dvorak et al., 1961; Litchford, 1963), offers a unique opportunity to investigate the physiological and biochemical interactions between a parasite and its host. Litchford (1963) noted short-term differences between individuals of this worm when grown in the laboratory mouse, rat, and hamster, and postulated that these growth characteristics may be due to biochemical differences in the bile of the hosts.

Factors, such as hormones, known to influence cholesterol levels have been investigated by a number of workers interested in a better understanding of bile acid dynamics, since bile acids are the principal metabolic endproducts of cholesterol metabolism (Thompson and Vars, 1953; Eriksson, 1957a, 1957b; Strand, 1962). The administration of propylthiouracil, an antithyroidal drug, is accompanied by changes in the excretory pattern of bile acids in bile fistula rats (Strand, 1962). Therefore this research was undertaken to investigate the response of H. microstoma in the laboratory mouse to an in vivo environment of altered bile acid composition brought about by thiourea administration.

CHAPTER II

MATERIALS AND METHODS

Hymenolepis microstoma was obtained from the University of Tennessee colony and maintained in Tribolium confusum and laboratory mice. Maintenance and infection of beetles followed the procedures of Voge (1963, 1964) and Litchford (1963). All cysticercoids used throughout the investigation were of a similar age since Evans (1963) demonstrated that dry weight and infectivity of this worm varies with cysticercoid age. Adult, male and female laboratory mice were given a single cysticercoid by stomach tube and sacrificed 15 days after infection. The experimental mice were given 0.5% (w/v) thiourea in their drinking water for 30 days prior to larval administration and also during the 15 day infection period. To obtain sufficient quantities of bile for analysis, all mice were starved 24 hours before necropsy, allowing an accumulation of from five to ten µl of bile in the gall bladder.

At necropsy individual gall bladders were removed, washed quickly in several rinses of distilled water, blotted, cut, and the bile collected in small pyrex cylinders. Aliquots of this bile were used for the assay of cholic acid (3α, 7α, 12α-trihydroxycholanic acid) and chenodeoxycholic (3α,7α-dihydroxycholanic acid) and deoxycholic (3α, 12α-dihydroxycholanic acid) acids, following the technique of Mosbach et al. (1954) as amended by Haust and Beveridge (1958). After extraction of the acids, this procedure allows for the simultaneous quantitative

determination of these bile acids by utilizing UV absorption spectrophotometry, but this test does not differentiate between the isomeric dihydroxycholanic acids occurring in mouse bile, viz., chenodeoxycholic and deoxycholic (Danielsson and Kazuno, 1959). Accordingly, the values listed are the sums of these dihydroxycholanic acids. A Beckman DU spectrophotometer was used to measure the optical density of the extracted bile acids. The amounts of the individual acids were calculated by the methods of Umbreit, Burris, and Stauffer (1964), as the absorption spectra of these bile acids overlap at the wavelengths used (320 mμ for cholic, with a slit width of 0.3 mm; 385 mμ for chenodeoxycholic and deoxycholic, with a slit width of 0.14 mm). Cholic and deoxycholic acids (Nutritional Biochemical Corporation) were used as standards.

Worms were removed from the bile duct and their wet weight recorded after washing in Ringers solution and blotting on filter paper. Dry weight of individual worms was determined after drying at 105 C for 18 hours, as suggested by Hopkins and Hutchison (1960). The dried worms were stored at -20 C until used for the sequential determination of lipid, carbohydrate (glycogen and anthrone sensitive sugars), protein, and non-protein nitrogen. In some cases, especially within the experimental group, individual dried worms did not give adequate concentration and volume of homogenate necessary for the tests; therefore some of the dried worms were pooled and analysed jointly.

The air-dried, defatted tissue was rehomogenized in distilled water to give a dry weight concentration of approximately 4 mg/ml. One ml of homogenate was added to four ml of 10% (w/v) trichloroacetic acid and to five ml of 80% (v/v) methanol in centrifuge tubes. After a 30 minute interval the tubes were centrifuged at 2200 x g for 20 minutes.

The supernatant and precipitate of both procedures were separated and analysed in the following manner. The trichloroacetic acid precipitate was dissolved in 0.1 N NaOH by heating for 30 minutes at 80 C; and, after suitable dilution, protein concentrations were determined using the method of Lowry et al. (1951). Crystalline human serum albumen (Nutritional Biochemical Corporation), first precipitated with trichloroacetic acid and then dissolved in 0.1 NaOH, was used as a standard. The optical density was measured with a B&L Spectronic 20 at 750 mμ. The supernatant of this fraction was analysed for non-protein nitrogen by nesslerization after acid digestion (Klett Clinical Manual; Taras, 1958). The amount of glycogen was determined by precipitation with 80% methanol followed by deproteinization as outlined by Kemp and Van Heijningen (1954). The concentration of soluble sugars in the supernatant was determined by the anthrone technique outlined by Mokrasch (1954).

Where applicable, validity of the data was determined by Student's t-test and P values of 0.05 or less were considered significant using a single-tailed table.

CHAPTER III

RESULTS

The bile acid concentrations from normal and thiourea-treated mice are outlined in Table 1. As selective absorption processes may alter the concentration of individual bile components during gall bladder storage (Read, 1950; Smyth and Haslewood, 1963), it should be emphasized that these data are from gall bladder bile collected after a 24 hour starvation period. The mean concentration of cholic acid in the thiourea-treated mice is significantly lower when compared to the normal value. Conversely the concentration of the dihydroxycholanic acid fraction (chenodeoxycholic and deoxycholic) increases significantly with thiourea treatment. The approximate molarity of the individual acids indicates this change on a per mole basis. The changes in individual bile acids within the normal and thiourea-treated mice are not reflected in either the overall total concentration or in total molarity. Both of these parameters indicate that there is no change in bile acid concentration between the groups of mice, but these are misleading when the individual values of concentration and molarity are considered. Another indication of a difference in bile acid composition between the host groups is the bile acid ratio. In normal animals the ratio of cholic to chenodeoxycholic and deoxycholic is approximately 3/1, while in the thiourea-treated animals it decreases to approximately 2/1. Therefore H. microstoma, grown in mice treated with thiourea, is exposed to an increased

TABLE 1

QUANTITATIVE DATA ON BILE
FROM NORMAL AND THIOUREA-TREATED MICE[a]

Host and Bile Acid	Concentration (mg/ 100 ml)[b]	Approx. Moles/Liter	Approx. Total (mg/ 100 ml)	Approx. Total Moles/Liter	Approx. Bile Acid Ratio
Normal					
Cholic	1287.6 ± 268.6 (28) P < .05[c]	0.0315	1678	0.0414	3.3/1
Chenodeoxycholic Deoxycholic	390.4 ± 116.8 (28) P < .005[c]	0.0099			
Thiourea-treated					
Cholic	1174.4 ± 138.2 (26)	0.0287	1698	0.0420	2.2/1
Chenodeoxycholic Deoxycholic	552.4 ± 49.3 (26)	0.0133			

a = gall bladder bile; 24 hour starvation period

b = $\bar{X} \pm SD$ (N)

c = statistical significance between means of normal and thiourea-treated animals

level of dihydroxycholanic acids and a decreased level of trihydroxycholanic acid.

The effects of thiourea treatment on infection rates and certain physical measurements are presented in Table 2. In all infected mice of both groups the scolex was attached in either the common bile duct or cystic duct, although in most cases the posterior portion of the strobila extended into the lumen of the duodenum. The infection rate is higher in the normal group, and within both groups there appears to be a relationship between infection rate and sex. In each case the infection rate for female mice is lower than the corresponding value for the males. In normal mice 18 of 21 males (85.7%) and 19 of 23 females (82.6%) were infected. In the thiourea-treated group 22 of 31 males (70.9%) and 15 of 28 females (53.6%) contained worms at necropsy (Table 2). Also, the wet and dry weights of worms from normal hosts were significantly greater than those from thiourea-treated animals. The thiourea-treated mice exhibited a higher dry weight to wet weight ratio than did worms from normal mice.

The data of the chemical analyses of worms from both groups of mice are presented in Table 3. Absolute amounts of lipid and protein of worms from thiourea-treated mice are significantly lower than those from worms grown in normal animals. Also the absolute values of non-protein nitrogen, glycogen, and anthrone sensitive carbohydrate are lower in worms from thiourea-treated mice than from the normals, but the small differences noted are not significant. The lipid and protein content, as per cent of dry weight, for worms from experimental animals are also lower than normal values; while non-protein nitrogen and anthrone sensitive sugar values are slightly larger. The glycogen content is also larger than the corresponding value for the controls.

TABLE 2

THE INFECTION RATE AND PHYSICAL MEASUREMENTS OF HYMENOLEPIS MICROSTOMA FROM NORMAL AND THIOUREA-TREATED MICE

Measurement	Host		
	Normal	Thiourea-treated	P values
Infection rate (per cent)	37/44 = 84.1 18/21 males 19/23 females	37/59 = 62.7 22/31 males 15/28 females	
Wet weight [a] (mg)	53.2 ± 29.2 (37)	32.9 ± 17.8 (37)	<.005
Dry weight [a] (mg)	13.3 ± 6.9 (37)	9.7 ± 4.4 (37)	<.005
Dry weight/ wet weight (per cent) [a]	25.4 ± 2.4 (37)	33.3 ± 11.1 (37)	<.005

a = $\bar{X} \pm SD$ (N)

TABLE 3

COMPARISON OF THE CHEMICAL COMPOSITION OF
HYMENOLEPIS MICROSTOMA FROM NORMAL AND THIOUREA-TREATED MICE

	Host		
Fraction	Normal	Thiourea-treated	P values
Lipid [a] as % dry wt	4.591 ± 1.590 (37) (34.5)	2.943 ± 1.370 (37) (30.3)	$<.005$
Protein [a] as % dry wt	5.400 ± 1.500 (35) (40.6)	3.639 ± 1.150 (37) (37.5)	$<.005$
Non-protein nitrogen [a] as % dry wt	0.528 ± 0.287 (23) (4.0)	0.443 ± 0.246 (36) (4.6)	$.2 > P > .3$
Glycogen [a] as % dry wt	1.599 ± 1.640 (35) (12.0)	1.434 ± 0.918 (37) (14.8)	$.4 > P > .3$
Anthrone CHO [a] as % dry wt	0.204 ± 0.183 (31) (1.5)	0.157 ± 0.083 (37) (1.6)	$.1 > P > .05$

a - data are given in milligrams as $\bar{X} \pm SD$ (N)

CHAPTER IV

DISCUSSION

Normally the final concentration and composition of bile acids in the enterohepatic circulation are the result of an intimate relationship between enzyme systems of the liver and those of bacteria in the intestine (Bergstrom, Danielsson, and Samuelsson, 1960). In the laboratory rat and mouse, cholesterol is catabolized in the liver to cholic acid, which makes up approximately 75% of the total bile acids, and chenodeoxycholic acid. Under the influence of intestinal microorganisms, cholic acid loses its 7α hydroxyl, yielding deoxycholic, which is reabsorbed and enters the enterohepatic circulation. This acid is then rehydroxylated to cholic by a 7α hydroxylase system in the liver. By injecting labeled deoxycholic acid, Danielsson and Kazuno (1959) showed that the rehydroxylation of deoxycholic acid requires 72 hours to be 90-100% complete in the laboratory mouse. In addition trace amounts of two other trihydroxycholanic acids have been reported from mouse bile, and have been demonstrated to arise from bacterial action on chenodeoxycholic acid. The bile acids in mouse bile are present exclusively as taurine conjugates (Danielsson and Kazuno, 1959). Therefore, mouse bile contains at any one time in the enterohepatic circulation, cholic and chenodeoxycholic as primary bile acids and deoxycholic as a microbial metabolite.

Bile salts are known to be necessary for the activation and excystment of certain cestode larvae, but various cestode species differ

in their response to bile salts as evidenced by their pattern of excystment. Maximum excystment is related to stomach emptying time (in reality, how long the larvae are exposed to the action of the constituents of gastric juice) and subsequent exposure to the proper concentration of trypsin and bile salts. Variation in time of exposure and concentration of host secretions, particularly to bile salts, may result in the impaired establishment of certain cestode species (Rothman, 1959a). Smyth and Haslewood (1963) pointed out that the salts and conjugated forms (glycine and taurine) of certain dihydroxycholanic acids have a marked lytic effect on the cuticle and induce the accumulation of large amounts of cytoplasmic fat in the protoscoleces of E. granulosus. Although they carried out only a limited number of experiments related to changes in concentration of bile acids, the effects noted in the protoscoleces were dependent on the concentration of bile acids used.

In view of the effects of bile acids on larval cestodes mentioned above, the bile acid changes noted in this study (Table 1), correlated with a lowered infection rate in mice treated with thiourea (Table 2), indicate that a certain concentration and ratio of bile acids are necessary for the maximum establishment of H. microstoma. Accordingly, the difference in infection rate is due to the alteration of individual bile acid concentrations, probably the increase in the dihydroxycholanic acid fraction; but changes in host physiological mechanisms associated with hypothyroidism, such as a decrease in secretion of digestive juice and rate of gastrointestinal motility, may also be contributing factors.

The alteration of bile acid concentrations also results in physical and chemical changes in H. microstoma (Tables 2 and 3). The

difference in wet weight is due primarily to a decreased amount of water, as cestodes, in general, have little or no capacity to osmoregulate (Read and Simmons, 1963). A higher dry weight to wet weight ratio for worms from experimental hosts confirms this. The lower dry weight values (Table 2), are the result of depressed levels of lipid and protein (Table 3). No other significant differences in chemical constituents are noted between the groups of worms. It is pertinent that Ashmore and Nesbitt (1955) have demonstrated that liver microsome activity may be either increased or decreased by changing the concentration of deoxycholic acid used. Thus H. microstoma requires a particular level of certain bile acids for its normal metabolism, primarily in regard to its protein and lipid components.

In general, the results of the chemical analyses of 15-day-old H. microstoma from normal mice are consistent with those of Jones et al. (1963). They found that H. microstoma resembles H. diminuta in dry weight to wet weight ratio; but differs from it in glycogen and protein content, which are lower, and in total lipids, which are higher. The ages of worms used for their comparisons were not stated, and this may assume some importance as Roberts (1961) has reported that the major chemical components of H. diminuta change with age and intensity of infection. Nevertheless some comparisons can be made, even in the absence of their data. The normal value of 0.25, observed in this experiment for dry weight to wet weight ratio of 15-day-old worms (Table 2), is very similar to that of the same age H. diminuta (Fairbairn et al., 1961). Using the data of Roberts (1961), for single worm infections of 15-day-old H. diminuta, total lipids account for 23.3% and total carbohydrate 40.4% of the dry weight. Thus the data of this experiment are in accord with Jones et al. (1963) for these two chemical parameters; total lipids of H. microstoma

is higher and total carbohydrates much lower in comparison to H. diminuta. Conversely, total protein in H. microstoma, from this study, is higher than the value given by Roberts (1961) for 15-day-old H. diminuta, and is therefore inconsistent with Jones et al. (1963) observation. This difference may be related to the analytical procedure employed for measuring protein or to the fact that the comparison is of relative values; H. microstoma used here were taken from hosts starved 24 hours prior to necropsy. In intestinal cestodes host starvation leads, in a short period of time, to a tremendous decrease in the amount of stored carbohydrate. Thus in terms of relative values, amounts of other tissue components should increase in proportion.

Rothman (1958, 1959b) demonstrated that bile salts, added in vitro with and without glucose, inhibit the anaerobic metabolism of H. diminuta and Oochoristrica symetrica, have no effect on it in H. citelli, and stimulate the metabolism of Taenia taeniaeformis and larval T. crassiceps. The degree of inhibition, when it occurred, was partially dependent on the concentration of bile salts and on pH. The higher concentrations of bile salts, alone, or in conjunction with a lowered pH tended to be the most powerful inhibitors. Interestingly, he observed no effect on the metabolism of H. diminuta when galactose was used as a substrate. This may indicate a selective effect of bile salts on the absorptive site for glucose. Assuming that H. microstoma is sensitive to changes in exogenous carbohydrate, as are other cestodes (Read and Simmons, 1963), then, if bile acids or changes in their concentration are involved with glucose transport, some discernible difference should have been noted in glycogen content between the two groups of worms. Actually there was no significant difference in absolute amounts, although glycogen as per cent of dry

weight, was higher in worms from experimental hosts.

How changes in bile acid concentration affect lipid and protein content of H. microstoma at the molecular level is not shown or even suggested by this study. Data concerning the interrelationship and degree of dependency of the major metabolic pathways on each other are scarce for cestodes, but soon may be forthcoming (Graff et al., 1965; Lumsden, 1965). As there was no significant difference in the non-protein nitrogen fraction between the two groups of worms, this can indicate no apparent change in the gross "amino acid pool" of experimental H. microstoma. This suggests that bile acids are not involved in amino acid transport but more likely in pathways of protein synthesis. Very little information is available concerning the molecular action of bile salts on cestodes, except in reference to their surface-active properties (Rothman, 1958). Rothman (1963) has demonstrated the presence of mitochondria in the cuticle of cestodes, and these structures are known to be easily fragmented by bile salts. Smyth and Haslewood (1963), in discussing the observed difference in effect of chenodeoxycholic in comparison to deoxycholic on the cuticle of E. granulosus, suggested that "...certain parts or areas of the cuticle may have a molecular structure which renders it susceptible to the lytic effect of a particular surface-active agent."

This data, which represents the first of its type in terms of actual values for H. microstoma, is generally in accord with that available for other cestodes (Read and Simmons, 1963). Also the results of this study lend weight to the concept that very subtle changes in the host fluids surrounding a parasite and in the host regulatory mechanisms governing its composition often result in rather dramatic and sometimes drastic changes in the parasite.

In view of the above discussion alteration of individual bile acid concentration in the laboratory mouse, i.e., an increase in the dihydroxycholanic acid fraction and a decrease in the trihydroxycholanic acid fraction, results in the impaired establishment and subsequently the normal metabolism and growth of H. microstoma in vivo.

CHAPTER V

SUMMARY

The concentration of the major bile acids in the laboratory mouse was significantly changed by the administration of an antithyroidal drug, thiourea. The dihydroxycholanic acid fraction (chenodeoxycholic and deoxycholic acids) was increased and the trihydroxycholanic acid fraction (cholic acid) decreased.

Hymenolepis microstoma, grown for 15 days in mice treated with thiourea, exhibited physical and chemical differences when compared to worms of the same age from normal mice. The infection rate and wet and dry weight were lowered, and the dry weight to wet weight ratio was increased in worms from experimental animals. In chemical constituents, the lipid and protein components in absolute amounts for worms from thiourea-treated mice were significantly depressed. No other significant changes were noted in the various fractions analysed.

The changes noted in bile acid concentrations in the experimental mice were implicated as being limiting factors for the initial establishment and maintenance of normal metabolism of H. microstoma in vivo.

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