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STUDIES ON THE METABOLISM OF THE
CESTODE, HYMENOLEPIIS DIMINUTA AND ITS
HOST, WITH SPECIAL REFERENCE TO THE SULFUR
AMINO ACIDS AND RELATED SUBSTANCES

by

SEYMOUR GARSON

A THESIS
SUBMITTED TO THE FACULTY
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II. INTRODUCTION TO THE PROBLEM

Recent reviews of the pertinent literature (Bueding, 1949; Geiman and McKee, 1949; von Brand, 1952) indicate that our knowledge of amino acid metabolism in the tapeworms is extremely limited. From the standpoint of host-parasite relations, there is virtually nothing known about the exact origin and mode of acquisition of the specific amino acids which participate in the synthesis of tissue and other proteins of the adult tapeworm. We have, in fact, only a few available analyses of the amino acids participating in the constitution of tapeworms (Kent, 1947; Aldrich, Chandler, and Daugherty, 1954). Moreover, the actual metabolic interconversions and degradations of amino acids in the tissues of the intact tapeworm, in situ, are essentially unknown.

The adult tapeworms, with few exceptions, inhabit the lumen of the vertebrate small intestine, and are completely devoid of a digestive tract. According to Wardle and McLeod (1952), it is generally assumed that the nitrogenous nutriment of tapeworms consists of cell-permeable products of the host digestion, such as amino acids which enter the parasite by diffusion through the body surface, and that cell-impermeable substances, such as proteins and peptones, are not utilizable. The demonstration, however, of the proteolytic enzymes, pepsin, cathepsin, and trypsin in the tissue fluids of Taenia saginata and Taenia solium (Smorodinzev and Bebeschin, 1936) leads
Wardle and McLeod to suggest the possibility that these tapeworms, particularly the larval forms, are able to utilize these proteolytic enzymes for the hydrolysis of protein derived from the host. Von Brand (1952), however, in referring to these enzymes described by Smorodinzev and Bebeschin, rejects this possibility and states, "It is much more likely that they are tissue enzymes used in movements of the parasites' own proteins."

Chandler (1943), in experiments with the rat tapeworm, *Hymenolepis diminuta*, has demonstrated that this parasite is essentially independent of the protein in the diet of its host, in the sense that elimination of protein from the host's diet had no evident effect on the establishment, growth, or reproductive capacity of the worms. Read (1950), in an extensive review of the literature on the physiology of the vertebrate small intestine, strongly suggested one probable means by which tapeworms acquire adequate quantities of nitrogenous nutrients independent of the host's diet. On the basis of much evidence gleaned from the literature, Read logically concluded that the physico-chemical nature of the intestinal lumen is in dynamic relation with the host tissues. In other words, diffusion through the mucosal cells, and secretions of the intestinal and associated glands provide the intestinal lumen with numerous substances many of which are capable of being resorbed by the cells lining the lumen. Thus, Read states, "Substances that take part in such a circulation
become available to a parasite in the intestinal lumen." One may, of course, interject that substances which are secreted and are not ordinarily resorbed by the intestinal mucosa may also, in one way or another, be available for utilization by the parasite.

The work of Mosenthal (1911) may be considered in support of the hypothesis that nitrogenous substances derived from the host may be available to intestinal parasites. That investigator, working with fistula dogs, observed that the intestinal secretion contained as much as 35% of the ingested nitrogen. In addition, more recent investigations have clearly demonstrated that, among many tissues analyzed, the proteins of the mucosa in the dog and in the rat show the highest rates of incorporation and release of various amino acids, regardless of whether the amino acid is administered orally or intravenously, in trace amounts or in considerable quantity (Tarver and Schmidt, 1942; Bloch, 1946; Friedberg, 1947; Tarver and Morse, 1948; Friedberg, Tarver, and Greenberg, 1948). These observations led to the conclusion (Friedberg, 1947; Friedberg, Tarver, and Greenberg, 1948) that a high rate of protein synthesis and turnover in the mucosa is the necessary result of the high rate of protein secretion into the intestine in the form of enzymes and mucous proteins which are lost in enormous quantities, unlike other body proteins. Although there is, apparently, no available information concerning the fate of these secreted proteins other than their
elimination in the feces, it is conceivable that some of these substances may possibly become available to intestinal parasites as amino acids through the normal digestive processes of the host or through the intervention of proteolytic microorganisms. In this regard, Porter and Rettger (1940) reported that, contrary to prevailing opinion, the stomach and small intestine of the albino rat under most dietary conditions contain appreciable numbers of viable bacteria and yeasts.

In spite of the evidences that nitrogenous substances may pass directly from the host to parasite, an active participation of the parasite in the acquisition of organic nitrogenous compounds cannot be ignored. According to Chandler, Read, and Nicholas (1950), the succus entericus of vertebrates is known to contain, in addition to proteins, such nitrogenous materials as amino acids, urea, creatine, creatinine, and ammonia. Daugherty (1954) has demonstrated the \textit{in vitro} synthesis of amino nitrogen from ammonia in \textit{Hymenolepis diminuta}, and more recent unpublished results (personal communication) indicate that this activity is exhibited by the worms \textit{in situ}.

The studies to be described here were undertaken to partially elucidate the nature of the mucosa-parasite relationship with respect to the acquisition of sulfur amino acids by \textit{Hymenolepis diminuta}, and to further determine the fate of these substances in the tissues of the parasite. This work was also initiated as an adjunct to a general program, now in progress in this laboratory, the aim of which is to explicate
various aspects of the metabolism of *Hymenolepis diminuta*. The choice of the sulfur-containing amino acids as the subject for the present metabolic study of *Hymenolepis diminuta* was occasioned by several considerations. Of initial significance was the fact that repeated paper-chromatographic analyses of aqueous worm extracts in this laboratory revealed the presence of several free sulfur-containing substances, such as glutathione, cystine, taurine, and a questionable amount of methionine. Among these substances, the large quantity of taurine (2-αminoethylsulfonic acid) that was found in the worm tissue, was of particular interest to the author. Although free taurine is known to occur in considerable quantities in the tissues of many invertebrates (Lewis, 1924; Sobotka, 1937; Baldwin, 1952), unpublished results from this laboratory have demonstrated, within the limits imposed by chromatographic analysis, the absence of free taurine in the tissues of various other helminths. The helminths which have been studied include the trematode, *Fasciola hepatica*, the acanthocephalan, *Macracanthorhyncous hirudinaceus*, and the cestodes, *Dipylidium caninum* and *Diphyllobothrium latum*.

In the vertebrates it is well known that taurine occurs in conjugation with cholic and other bile acids. The evidence for an enterohepatic circulation of the bile acids has been reviewed by Sobotka (1937) and it appears well established that a major portion of these bile constituents are resorbed during passage through the small intestine, while a small fraction is
lost with the feces. Bergström and Norman (1935) demonstrated the presence of labelled cholic and chenodesoxycholic acids as taurine conjugates in the bile of rats following the intra-peritoneal administration of cholesterol-4-C\textsuperscript{14}. These workers further demonstrated, however, that the feces contained a series of different labelled compounds none of which were bile acids or unchanged cholic acid. They conclude: "Thus the taurocholic acid that is the main labelled excretory product in the rat bile has not only been split, but the cholic acid has been further modified in various ways by the intestinal micro-organisms." Since the exact site in the intestine of the micro-organismic fractionation of taurocholic acid is apparently unknown, the possibility exists that the bile of the rat host may provide a source of the taurine that has been observed in H. diminuta.

In speaking of taurine, Sobotka (1937) stated, "It occurs in vertebrates as a specialized biliary product only." This statement, as we now know, is far from true. Awapara, Landua, and Fuerst (1950) have quantitatively demonstrated the presence of free taurine in the liver, kidney, muscle, heart, spleen, testis, brain, and ileum of the rat. "The data," state the authors, "suggests that taurine is a major component of the amino fraction of most organs." Subject to the demonstration of free taurine in the intestinal mucosa, it is conceivable, therefore, that diffusion of free taurine from the host mucosa into the intestinal lumen may serve as another source of
taurine for *H. diminuta*.

It is well established that taurine is a product of the metabolism of sulfur amino-acids in the vertebrates. In a series of papers, Virtue and Doster-Virtue (1937; 1938; 1939a; 1939b; 1941) demonstrated that when cystine, cysteine, cystine disulfide, cysteinesulfinic acid, cysteic acid, methionine, methionine sulfoxide, and homocysteine were fed to bile fistula dogs, given cholic acid in excess to deplete the taurine and to provide an ample supply for later conjugation, the production of taurocholic acid in the bile was significantly increased. This was taken as presumptive evidence that the sulfur of the aforementioned compounds was converted to taurine sulfur. Tarver and Schmidt later conclusively demonstrated the conversion of methionine sulfur to taurine sulfur by isolating significant quantities of radioactive taurine from the bile of fistula dogs fed \( \text{S}^{35} \)-methionine and cholic acid.

Although free taurine and its derivative, taurocarbamate, are found in the so-called neutral sulfur fraction of vertebrate urine, the greatest portion of the excreted sulfur consists of inorganic and ethereal sulfates. Fromageot (1947; 1951a; 1951b) has extensively summarized the results of the numerous investigations which have served to elucidate significantly the intermediary aspects of the vertebrate metabolic pathways leading from methionine sulfur to taurine and to sulfate. On the basis of present knowledge, the major steps in the animal degradation of methionine sulfur are as outlined
in Figure I. With respect to this scheme of intermediary events, it may be noted that the bulk of the sulfur needs of the free-living animal is supplied by the three amino acids, methionine, cystine, and cysteine, of which the latter two are subject to metabolic interconversion. Methionine is an indispensable amino acid for all animals that have been investigated while cystine and cysteine are known to be indispensable particularly since the sulfur of these two compounds is obtained from methionine (Tarver and Schmidt, 1939; du Vigneaud, Kilmer, Rachele, and Cohn, 1944). Among the microorganisms, however, nutritional sulfur requirements may range from the use of sulfate as the sole source of sulfur to an absolute requirement for cystine and methionine as well as for biotin, a sulfur-containing vitamin (Fruton and Simmonds, 1953).

In light of the previous discussion of sulfur amino acid metabolism, it was reasoned that the presence of the large amount of free taurine in *H. diminuta* might be the result of an active metabolism of some or perhaps all of the sulfur amino acids by this parasite. The additional presence in the worm tissue of cystine, glutathione (γ-glutamyl-cysteinyl-glycine), and possibly methionine appeared to support this view.

The studies to be described in the following pages were, therefore, initiated for the purpose of exploring possible sources of the sulfur-containing substances which were observed in *Hymenolepis diminuta*. The potential role of the host
Figure I. Metabolic Pathways Leading from Methionine Sulfur to Sulfate and Taurine.
bile was probed by means of experiments using bile duct-ligated rats. In vivo experiments, using $S^{35}$-labelled methionine, were designed to elucidate a possible relationship between the intestinal mucosa of the host and the parasite with respect to the acquisition of sulfur amino acids by the parasite. In vitro experiments, in which living worms were incubated in a medium containing $S^{35}$-methionine, were employed to study the uptake of this amino acid from the environment, and to follow the metabolic course of its sulfur in the worm. Methionine was chosen as the experimental amino acid because it is the only sulfur containing substance that would reasonably be expected, in the light of our present knowledge, to allow an investigation of the entire metabolic pathway ascribed to animals in their degradation of the sulfur amino acids. The use of the radioactive $S^{35}$-label has been shown by numerous investigators to be an extremely valuable method for the study of animal sulfur metabolism.
III. MATERIALS AND METHODS

Mature male albino rats of the Wistar and Sprague-Dawley strains were used throughout this investigation. The particular strain and the size of the animals used in each experiment are noted in the pertinent context. Except where it is otherwise stated, the rats were maintained on a complete pellet diet purchased from a local supply house.

The rats were infected with cysticercoids of *Hymenolepis diminuta* according to the procedure described by Addis and Chandler (1944). The cysticercoids were obtained from artificially infected grain beetles, *Tenebrio molitor*, and were fed to the rats by means of a pipette. The number of cysticercoids given to the rats ranged from 1 to 10 depending on the nature of the individual experiment. In all instances the worms were allowed to attain sexual maturity before the infected rats were used for experimental purposes. Most of the rats were used within three to eight weeks following infection. The strain of *H. diminuta* employed here is one which has been maintained in this laboratory for the past fifteen years.

In the recovery of the worms for use in the various experiments, the rats were killed by a blow on the head, and the small intestine was quickly removed. The worms were flushed from the intestine by injecting 0.89% saline solution into the intestinal lumen with a large syringe fitted with an extension of narrow diameter rubber tubing. The worms were
carefully cleansed of adhering intestinal contents, washed in five changes, at least, of the normal saline solution, and allowed to drain briefly on filter paper. Where the worm tissue was required solely for the purpose of amino acid and other constituent analyses, the worms were used either immediately or most often kept for brief periods of time in a freezer chest at \(-20^\circ\) C. The latter procedure did not yield any apparent changes in the results of the analyses. In the incubation studies, the worms were used immediately after being washed. Supplementary procedures used in the handling of the worm and host tissues are described in the sections devoted to the pertinent experiments.

Analyses of the amino acid constituents, both free and protein-bound, of the worm and host tissues were accomplished by means of one and two-dimensional paper chromatography. This procedure of analysis was adopted because of the ease with which the separation of complex mixtures of amino acids, particularly those resulting from the hydrolysis of proteins, could be achieved. The simplicity of the apparatus used in paper chromatography was also taken into consideration. In those experiments dealing with \(S^{35}\)-labelled amino acids, the semi-quantitative estimation of these substances on chromatograms could be readily obtained by means of a Geiger counter or by the preparation of radioautographs.

The procedure used in the preparation of host and worm tissues for the analysis of free amino acids is essentially
that of Awapara (1948). Homogenates were prepared in the ratio of 1 g. of tissue to 1 ml. of hot glass distilled water in an all-glass homogenizer of the type described by Potter and Elvehjem (1936). This device consisted of a heavy walled tube, conical at the closed end, into which was ground a tightly fitting pestle. With care, cell-free homogenates were readily obtained. In order to ensure complete precipitation of the protein-containing fraction, 9 ml. of absolute ethanol, for each gram of tissue used, was added to the homogenate, and this mixture was allowed to stand at room temperature for approximately two hours. The precipitate, or protein-containing fraction, was centrifuged down, and the clear supernatant was removed and thoroughly mixed with an amount of chloroform which was three times the volume of water plus alcohol originally added. Upon centrifugation the lipid-free aqueous layer, containing the free amino acids, separated from the alcohol-chloroform mixture. The aqueous layer obtained in this manner is henceforth referred to as the free amino acid fraction. In the later studies, particularly those concerned with the measurement of radioactivity in the free amino acid fraction, it was found that a more quantitative recovery of this fraction was obtained by allowing its separation from the alcohol-chloroform mixture to occur overnight at 4°C.

The precipitate, or the protein-containing fraction, obtained above was then prepared for the analysis of its constituent amino acids. Further purification of this fraction
was obtained by a series of extractions designed to give a residue free of lipid and certain other extraneous constituents. The precipitate was thoroughly stirred with 10 ml. of absolute ethanol, centrifuged, and the supernatant discarded. In similar manner, the precipitate was then successively extracted with 10 ml. aliquots of: cold 5% trichloroacetic acid, hot (75°C) 5% trichloroacetic acid, absolute ethanol-ether (1:1), and acetone. The acetone mixture was transferred to a filtering crucible (Selas No. 3013) from which the acetone was removed by suction. The recovered residue was washed with more acetone and stored in a desiccator over calcium chloride. This procedure yielded a fine powder.

Twenty-five mg. of this protein-containing powder were hydrolyzed in 2 ml. of a 1:1 mixture of 6N HCl and 80% formic acid. This type of hydrolysis mixture was recommended (Miller and du Vigneaud, 1937; Bakay and Toennies, 1951, Block and Bolling, 1951) to reduce humin formation and to minimize the possible destruction of cystine and methionine. Hydrolysis was carried out in hand-made tubes of heavy walled Pyrex which were sealed under vacuum and heated for 15 hours at 100°C. The tubes were then thoroughly cooled in an ice bath and broken open. The contents of the tubes were quantitatively transferred to small beakers and evaporated to near dryness on a steam bath. The residue was redissolved with glass distilled water and again evaporated. Subsequent
repetition of the later step was carried out only when required to ensure the removal of the excess acids. The last evaporation was carried to dryness and the residue was dissolved in 2.5 ml. of glass-distilled water. Aliquots of the latter solution were used for the analysis of the constituent amino acids.

Chromatographic analyses were carried out on 14 inch square sheets of Whatman #4 filter paper. For two-dimensional resolution, a suitable aliquot of the free amino acid extract or of the protein hydrolysate was applied by means of micropipette to a marked circular area, approximately 5 mm. in diameter, and 1½ inches in from both edges of the lower left hand corner of the paper. The amount of material that was spotted varied from 50 to 200 microliters and in any one case depended on the type of observation that was being made. The spot was held to the small circular area by repeated applications of 5 to 10 microliters which were dried with the aid of an infra-red lamp and a small air blower. In the preparation of one-dimensional chromatograms, the spots were applied 2 inches apart and in a line 1½ inches from the edge of the paper. The spots were routinely neutralized on the paper with ammonia vapors.

The chromatographic analyses were carried out at room temperature according to the ascending technique of Williams and Kirby (1948). With as little handling as possible, each spotted sheet was rolled into a single thickness cylinder,
stapled, and placed in a Pyrex jar of sufficient height and
diameter to accommodate the paper cylinder. The lower edge
of the paper cylinder was immersed in a buffered phenol
solvent, approximately \( \frac{1}{2} \) inch in depth, contained in a glass
orange juice squeezer at the bottom of the jar. The use of
these containers afforded an economical means of reducing
the amount of solvent needed. The jar was then immediately
covered with a square of plate glass. An airtight seal was
obtained by spreading petrolatum along the upper edge of the
jar before applying the glass cover. In order to maintain a
water saturated atmosphere in the jar during the phenol run,
a beaker of water was routinely included.

The method of preparing the phenol solvent was es-
sentially that of Berry and Cain (1949). Crystalline phenol
(Mallinkrodt, Analytical Reagent) was saturated and shaken
with an aqueous solution (pH 5.9) containing 6.3% sodium
citrate and 3.7% sodium phosphate. The emulsion formed was
then filtered and the upper layer used. The phenol solvent
was allowed to ascend for 21 hours at which time the paper
was removed, unstapled, and partially dried in a ventilated
oven at approximately 60\(^\circ\) C. for 2 to 3 minutes. In order to
avoid the destruction of amino acids by extended heat drying
(Brush, Bontwell, Barton, and Heidelberger, 1951), the re-
moval of the phenol solvent was completed under a hood at
room temperature. A one-dimensional resolution of the amino
acids was, thus, obtained.
For two-dimensional chromatography, the paper was turned 90°, and the above procedure was repeated with a 65% solution of 2, 4-lutidine (Matheson Co.) in glass distilled water as the solvent. The lutidine was allowed to ascend for 19 hours, following which the solvent was removed as described above. During the lutidine run a "brown front" is invariably formed which in some instances caused poor resolution of the amino acids. As also observed by Landua, Fuerst, and Awapara (1951), this front slows the solvent and causes an irregular solvent boundary. It was noted, quite accidentally, that less disturbance from the "brown front" was afforded if the papers were immersed in the lutidine solvent while still slightly moist from the water residue of the phenol solvent. This observation is possibly in line with Block's (1952) suggestion that the paper be hydrated by exposure to steam prior to the phenol and lutidine runs.

The papers were then sprayed lightly and evenly with a 0.2% solution of ninhydrin (triketohydridene hydrate) in water saturated n-butanol, and were dried as described above for the removal of the phenol solvent. Amino acids and certain related compounds were, thus, revealed as spots varying in color from purple to bright yellow. The use of special reagents for the detection of specific amino acids on the chromatograms is described in the appropriate context.

Chromatographic analyses were routinely prepared in duplicate. By strict adherence to the above chromatographic
procedures, it was found that the duplicate analyses of each sample gave quite comparable results.

All of the amino acids used in this investigation, with the exception of the $^{35}$S-labelled methionine, were obtained from the Nutritional Biochemical Corp., and were checked for chromatographic purity using the above ninhydrin solution as the color-developing reagent.
IV. EXPERIMENTAL

A. Qualitative chromatographic analyses of amino acids and related substances in experimental tissues.

As a means of providing an adequate background for the evaluation of later experimental results it was desirable to bring together in this initial section on experimentation the results of many chromatographic observations on amino acids and related substances which were made during the course of this investigation. Many of these observations resulted from planned studies, others were obtained incidentally. Together, however, they offered considerable guidance in the interpretation of various experimental aspects.

The behavior of various sulfur amino acids and related substances on one- and two-dimensional paper chromatograms was investigated in accordance with the previously described chromatographic procedure. This study was undertaken in preparation for the tissue analyses that followed. Of particular interest to the writer were the possible limitations which the chromatographic procedure might impose on the qualitative detection of these sulfur-containing substances.

Solutions of the following substances were prepared in glass distilled water; methionine, methionine sulfoxide, methionine sulfone, homocystine, cystine, cysteine hydrochloride, cysteic acid monohydrate, glutathione (reduced), and \(\alpha\)-alanine. Alanine was included among these substances since it occupies a position on two-dimensional chromatograms.
which serves as a convenient point of reference. Moreover, alanine is known to be a common constituent of animal tissues. Cystine and homocystine, which are poorly soluble in water, were completely dissolved with the aid of a small quantity of HCl. Aliquots of the solutions corresponding to 40 micrograms of each substance, or 60 micrograms in the case of taurine, were used in the preparation of the chromatograms. The two-dimensional positions assumed by some of the sulfur-containing substances, relative to that of alanine, are shown in Fig. II. It is seen that cystine disintegrates during two-dimensional chromatography, leaving a streak which extends from the point of origin in the lutidine direction, the heaviest concentration of material, however, remaining at the point of origin. Cystine and homocystine were also observed to undergo decomposition of a similar nature. These observations are in essential accord with those of Dent (1948) who further observed that cysteine, cystine, and homocystine are often totally undetectable on two-dimensional chromatograms. The destruction of cystine during chromatography may be readily circumvented by the procedure described by Dent (1947) in which small amounts (10-20 microliters) of 30% H$_2$O$_2$ and 0.02% ammonium molybdate are applied to the dried spot of known or unknown composition prior to development in the initial solvent. This procedure, according to Dent, quantitatively converts cystine to cysteic acid, and methionine to the sulfone (RSO$_2$CH$_3$). Extensive use was made of this technique in many
Figure II. Two-dimensional chromatograms of some sulfur-containing substances, showing positions of spots relative to alanine at which cross lines intersect. The circle in the lower corner of each chromatogram represents point at which solutions were applied to paper. Solvents, phenol and 2, 4-lutidine. Shown on chromatograms are spots representing:

A. cystine, alanine, methionine sulfone, methionine

B. glutathione (reduced), taurine, alanine, methionine

C. cysteic acid, alanine, methionine sulfoxide, methionine
aspects of this investigation, and with much success in the qualitative detection of cystine. The cysteic acid, once formed, is quite stable during the solvent runs, and assumes a two-dimensional position which is completely free of masking by other ninhydrin-reactive substances commonly found in biological materials. The results obtained with methionine by the use of this oxidation procedure were, on the other hand, subject to much variation. In most instances the oxidation of the methionine to methionine sulfone was not only incomplete, but the formation of the intermediate, methionine sulfoxide (RSO\textsubscript{2}CH\textsubscript{3}), was also detected on the chromatograms. Reduced glutathione was also found to react with H\textsubscript{2}O\textsubscript{2} and ammonium molybdate (Fig. III). This was of considerable diagnostic aid, particularly in those experiments involving radioautographs. This reaction has also been observed by Roberts (1949) who designated the reaction product as "oxidized" glutathione.

On one-dimensional chromatograms, using phenol as the solvent, the spot afforded by homocystine was quite discrete (Fig. IV). The results obtained with cystine and cysteine, however, were inconsistent. These substances were always observed to streak from the point of origin, but in some instances areas of concentration could be observed along the length of the streak as shown by the dash-lines in Fig. IV. Studies similar to the above were also carried out with many of the naturally occurring amino acids. Reference
Figure III. Two-dimensional chromatogram of glutathione oxidized with hydrogen peroxide and ammonium molybdate prior to development in phenol and 2, 4-lutidine solvents. Note that oxidation is incomplete. Streak in phenol direction represents non-oxidized (reduced) glutathione; intense spot represents reaction product.
Figure IV. One-dimensional chromatograms of some sulfur-containing substances and alanine. Developed in phenol. Spots shown from left to right are: cystine, cysteine, cysteic acid, homocystine, methionine, methionine sulfone, methionine sulf oxide, alanine.
chromatograms were, thus, obtained which were used for qualitative analyses of the amino acid and related contents of *H. diminuta* and the host mucosa. The amino acid "maps" of Dent (1948) and Awapara (1950) were also helpful in this respect. At this point, however, it should be emphasized that the chromatographic positions assumed by substances in pure solutions or in simple mixtures may vary to some extent when compared to the positions assumed by those same substances in complex biological mixtures. Further evidence for the identity of the various ninhydrin-reactive substances in the tissue preparations was, therefore, obtained by running known substances along with the unknown mixture, and noting the strengthening of various spots.

In the course of this investigation numerous two-dimensional chromatograms were prepared of the free amino acid fractions of *H. diminuta* and of the mucosa of the rat small intestine. Hydrolyzed protein fractions of *H. diminuta* were also chromatographed extensively. In all instances, the recovery and the preparation of the worms for chromatography were in accordance with the previously described procedure. After removal of the worms from the small intestine of the host, the intestine was quickly slit lengthwise, washed in several changes of isotonic saline, and the mucosa scraped off with a razor blade. Extraneous substances were thoroughly removed from these mucosal scrapings. The mucosal preparation was then thoroughly washed and the free
amino acid fraction prepared as previously described.

With the aid of the various reference materials and diagnostic procedures described above, it was possible to significantly elucidate the nature of the various ninhydrin-reactive substances found in the prepared fractions of the worms and the host mucosa. In Fig. V. are shown two-dimensional composite diagrams of the various spots observed in the free amino acid fractions of the worms and the mucosa, and in the protein hydrolysates of the worms. All of the spots shown in each of these composite diagrams were not readily detected as discrete entities on any one chromatogram because of the marked variation in the amounts of the individual substances present in the particular fraction. By chromatographing varying aliquots of any one kind of fraction, however, it was possible, in most instances, to ascertain the presence of almost all of these substances. This was generally the case, regardless of the animal strain (Wistar or Sprague-Dawley), animal size, or level of infection used. The composite diagrams may be compared with the representative chromatograms shown on Fig. VI.

Some comment may be made with respect to certain of the spots shown on the composite diagrams. The position occupied by spot 17, for example, may be taken up by citrulline and by glutamine. No attempt was made, however, to determine if one or both of these substances were responsible for this spot. The fact that spot 17 is not present in protein hydrolysates
Figure V. Two-dimensional Composite Chromatograms

A. Free amino acid fraction of *H. diminuta*
B. Protein hydrolysate of *H. diminuta*
C. Free amino acid fraction of rat intestinal mucosa

**Key**

1.∞ -Alanine

2. glutathione (reduced)

3. glutathione (oxidized)

4. aspartic acid

5. cysteic acid

6. glutamic acid

7. serine

8. glycine

9. taurine

10. tyrosine

11. methionine sulfoxide

12. valine

13. methionine and leucine

14. proline

15. unidentified

16. β-alanine

17. citrulline and/or glutamine

18. arginine

19. lysine

20. unidentified

21. threonine

22. unidentified

23. unidentified

24. unidentified

25. unidentified

26. methionine sulfoxide

Note: Spots indicated by dash lines appear following oxidation with hydrogen peroxide and ammonium molybdate.
Fig. VI. Representative Two-dimensional Chromatograms of Experimental Tissues.

A. Free amino acid fraction of *H. diminuta*
B. Free amino acid fraction of *H. diminuta* - oxidized
C. Protein hydrolysate of *H. diminuta*
D. Protein hydrolysate of *H. diminuta* - oxidized
E. Free amino acid fraction of rat intestinal mucosa
F. Free amino acid fraction of rat intestinal mucosa - oxidized
of the worm is of no diagnostic aid, since citrulline has not been definitely established as a constituent of proteins, and glutamine is probably converted to pyrrolidone-carboxylic acid during hydrolysis (Greenberg, 1951). Although spot 15 was not identified, its position approximates that of histidine and hydroxyproline as reported by Dent (1948). In addition, it may be noted that the position occupied by spot 15 nearly coincides with that of spot 26 (methionine sulfoxide) which is formed after the oxidation of the mucosa free amino acid fraction with $H_2O_2$ and ammonium molybdate according to the previously described procedure. In view of Dent's (1948) observation that some oxidation of methionine to methionine sulfoxide often occurs during the solvent runs, the possibility, therefore, exists that methionine sulfoxide is, to some extent, represented by spot 15. In addition to spot 26 (methionine sulfoxide), spots 3 ("oxidized" glutathione), 5 (cysteic acid), and 11 (methionine sulfone) also made their appearance following the use of the oxidation procedure on chromatograms of the intestinal mucosa. The appearance of methionine sulfone in oxidized free amino acid fractions of the worms was inconsistent, although the oxidation procedure invariably caused the appearance of oxidized glutathione and cysteic acid. Whether or not the absence of methionine sulfone and methionine sulfoxide from chromatograms of the worm protein hydrolysates, is truly indicative of the total absence of methionine from the proteins of the worm is questionable. Results to be described
would tend to indicate that methionine is present in the worm proteins in very small quantities. In view of the previously described difficulties which were encountered in the oxidation of known methionine to methionine sulfone, it is quite probable that small quantities of methionine would escape detection by this procedure. Spot 20, shown in later experiments to contain radioactivity, was observed in only a few instances and was possibly derived from glutathione. The writer has on several occasions noted that a spot of similar position is afforded by known glutathione on one and two-dimensional chromatograms. Landua, Fuerst, and Anapara (1951) observed that glutathione, under certain pH conditions, gave rise to multiple spots in phenol and in lutidine as the solvents. Spots 22, 23, 24, 25, and 27 were unidentified.

The presence of cystine on one-dimensional chromatograms of the free amino acid and protein fractions of the worms was confirmed by the use of the azide-iodine reagent of Chargaff, Levine, and Green (1948). The use of this reagent is based, according to the authors, on the ability of cystine, cysteine, and methionine to catalyze the oxidation of sodium azide by iodine in the following manner: \(2\text{NaN}_3 + \text{I}_2 = 2\text{NaI} + 3\text{N}_2\). After spraying chromatograms of the worm fractions with this reagent, cystine appeared as a distinct white area on the brown background afforded by the unaffected reagent. The presence of methionine in the worm fractions was not demonstrated with any certainty by the use of this reagent. It should be noted,
however, that the azide-iodine reagent is approximately five times more effective for the detection of cystine than it is for methionine.
B. Studies on sulfur metabolism in the worms and their hosts.

1. Investigation of the host bile as a possible source of taurine found in *H. diminuta*; related experiments.

In order to examine the possibility that the taurine content of *H. diminuta* is essentially derived from taurine conjugates of the host bile, an experiment was undertaken in which infected rats were subjected to bile-duct ligation. It was reasoned that a gross decrease in the taurine content of the worms, as a result of this procedure, would tend to indicate that the biliary secretion of the host is a major source of taurine found in the worms.

Thirteen rats of the Wistar strain, previously maintained on the regular pellet diet, were used approximately nine weeks after each rat was infected with 10 cysticercoids. All of the rats were found to be positive for infection by direct fecal smear. The animals were starved for 6 hours prior to surgery. Operations were performed under ether anesthesia without rigid aseptic technique. The bile ducts were doubly-ligated in 9 of the rats; 4 rats served as shams in which case the regular operative procedure was carried out with the exception that the bile ducts were not ligated. All of the rats showed a rapid return to normal activity after recovery from the anesthesia. The rats were then placed in separate cages and allowed to feed approximately 2 to 3 hours following surgery. Each rat was given a daily ration of finely ground and sifted pellets in the amount of 15 g. which was placed in a large stendor dish firmly mounted
on a petri dish base. Four of the rats with ligated bile ducts had 0.5 of desiccated Difco-Bacto Oxgall included and thoroughly dispersed in their daily food rations. The use of this commercial bile preparation was adopted in view of the fact that rat bile is not readily obtainable. Further, Ahrens and Craig (1952) have demonstrated the presence in ox bile of two taurine conjugates. The amount of food consumed by each rat was recorded daily. Two of the rats in each group were sacrificed on the third day following surgery and the remaining rats on the sixth day. The ligated bile ducts were in all instances observed to be intact and greatly distended.

The results obtained, in terms of the relative number of worms recovered in each of the experimental groups, were unexpected. These results along with other pertinent data are shown in Table I.

Those worms recovered from rats having ligated bile ducts, regardless of the type of food ration provided the rats, were, for the most part, dwarfed, and, in some instances partially mutilated. Where the worms had disappeared from the small intestines, masses of worm tissue in various states of disintegration were often found in the caecum and colon. The inclusion of the desiccated bile preparations in the diet of the one group of rats proved to be unsatisfactory as shown by the relative food consumption of the various groups. The data would, nevertheless, tend to indicate that a possible
<table>
<thead>
<tr>
<th>Group</th>
<th>Animal Day Sacrifised</th>
<th>Body Weight (g.)</th>
<th>Grams of food ration remaining per day</th>
<th>No. worms recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Diff.</td>
</tr>
<tr>
<td>(A)Shams</td>
<td>1</td>
<td>253</td>
<td>260</td>
<td>-7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>297</td>
<td>297</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>230</td>
<td>222</td>
<td>-8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>215</td>
<td>216</td>
<td>-1</td>
</tr>
<tr>
<td>(B)Ligated</td>
<td>1</td>
<td>242</td>
<td>232</td>
<td>-10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>229</td>
<td>217</td>
<td>-12</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>255</td>
<td>222</td>
<td>-33</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>205</td>
<td>185</td>
<td>-20</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>253</td>
<td>241</td>
<td>-12</td>
</tr>
<tr>
<td>(C)Ligated - bile</td>
<td>1</td>
<td>251</td>
<td>233</td>
<td>-18</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>232</td>
<td>218</td>
<td>-11</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>235</td>
<td>194</td>
<td>-41</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>201</td>
<td>178</td>
<td>-23</td>
</tr>
</tbody>
</table>

Table I: Recovery of Worms from Shams and Bile-duct Ligated Rats.
influence is exerted by the host bile on the maintenance of the worms. That some such situation does exist is, of course, quite conceivable. Conclusions of any sort are, however, precluded by the incompleteness of the results, and particularly by the many variables involved here. The question of direct or indirect cause is as significant here as it is in most experiments involving surgical intervention or extirpation.

The worms recovered from each rat were pooled and the free amino acid fractions prepared as previously described. Oxidized and non-oxidized two-dimensional chromatograms prepared from 100 microliter aliquots of each fraction showed no significant differences in the taurine or other sulfur-containing contents of worms from the sham animals and from those animals having ligated bile ducts.

On the basis of these results, it is not possible to conclusively eliminate the host bile as a possible source of the taurine found in the worms. One may, for example, argue that taurine obtained by the worms from the bile, prior to ligation of the bile duct, is firmly bound within the tissues of the worms by virtue of its highly ionizable-SO₂H grouping. However, the fact that taurine is readily obtained in the aqueous free amino acid fraction would appear to preclude this possibility. In this author's opinion it is more likely that the amount of taurine found in _H. diminuta_, at any one time, represents a balance between the taurine formed and/or obtained by the worms and the taurine lost from the worms.
In the course of this work several experiments were undertaken in order to repeat and to extend the above observations made with respect to the possible influence exerted by the host bile on *H. diminuta*. Repeated experiments, however, using available rats of the Sprague-Dawley strain could not be, for the most part, successfully completed. The majority of these animals, following bile-duct ligation, died from bile peritonitis resulting from ruptured bile ducts. This difficulty, on the other hand, was not encountered with rats of the Wistar strain.

An experiment, similar to that described above, was undertaken in which 7 rats of the Wistar strain were used 3 weeks following infection with 1 cysticercoid of *H. diminuta*. The bile ducts were doubly ligated in 4 of the rats, 3 rats serving as shams. The rats were allowed to feed ad libitum on ground and sifted pellets to which was added daily 1 drop of cod liver oil (Squibb) containing approximately 135 units Vitamin A (U.S.P.) and 13 units Vitamin D. The latter method of providing the vitamins was very effective since the rats immediately consumed the food on which the oil settled. All of the rats were sacrificed 17 days following surgery. The ligated bile ducts were all intact. The results are summarized in tabular form as follows:
<table>
<thead>
<tr>
<th>Group</th>
<th>Animal No.</th>
<th>Animal Initial Weight (g.)</th>
<th>Animal Final Weight (g.)</th>
<th>Animal Weight Diff.</th>
<th>Worm Recovery</th>
<th>Worm Wet Wt. (g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shams</td>
<td>1</td>
<td>360</td>
<td>397</td>
<td>+37</td>
<td>1</td>
<td>1.848</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>355</td>
<td>395</td>
<td>+40</td>
<td>1</td>
<td>1.737</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>325</td>
<td>352</td>
<td>+27</td>
<td>1</td>
<td>1.845</td>
</tr>
<tr>
<td>Ligated</td>
<td>1</td>
<td>353</td>
<td>348</td>
<td>-5</td>
<td>1</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>388</td>
<td>405</td>
<td>+17</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>320</td>
<td>300</td>
<td>-20</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>347</td>
<td>340</td>
<td>-7</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

Because of the small amount of tissue available, no attempt was made to assay the free amino acids and related substances of the single worm recovered from the bile-duct ligated rats. With regard to the overall changes in the animal weights shown above, it would appear that the conditions of this experiment were more conducive to the well-being of the animals than those of the previous experiment. Whether such factors as the method of feeding or the initial level of infection were involved is not known.

Results obtained from experiments using Sprague-Dawley rats were, as previously mentioned, mostly incomplete, and do not warrant detailed consideration here. It was of considerable interest, however, to note the results obtained from one experiment designed to study the possible influence of the host bile on the establishment of the worms. The rats in this case were subjected to bile-duct ligation 3 days prior to infection with 7 cysticercoids. In 18 days following infection
one surviving rat out of twelve bile-duct ligated animals was found to have its full complement of worms, although the total wet weight of these worms was approximately one-half that obtained from each of the shams. The ligated bile duct of this one animal was found to be intact and greatly distended. Two-dimensional chromatographic analyses revealed the presence of glutathione, taurine, and cystine as cysteic acid in the worms obtained from this one surviving bile-duct ligated rat.

Siperstein, Chaikoff, and Reinhardt (1952) showed that the absorption of cholesterol from the intestine of the rat stopped completely in the absence of bile. That the same results might be accomplished by the feeding of ferric chloride was suggested to Siperstein, Nichols, and Chaikoff (1953) since ferric chloride is known to precipitate bile salts in vitro. These workers then showed that the inclusion of 3% ferric chloride in the diet of birds served greatly to reduce the rise in plasma cholesterol resulting from cholesterol feeding. It was concluded by these authors that ferric chloride probably acted to precipitate bile salts in the intestinal tract.

It appeared of interest here to see if the addition of ferric chloride to the diet of rats infected with H. diminuta would affect the establishment, maintenance, and fat content of the worms. Experiments were, therefore, undertaken in which rats of the Wistar strain were placed on a ground pellet
diet containing 3% ferric chloride prior to and following infection with H. diminuta at the 1 and 3 cysticercoid levels. The rats were maintained on this diet for several weeks before being sacrificed. No significant differences were noted between the controls and the rats receiving ferric chloride with respect to the establishment, maintenance, and ether-soluble contents of the worms. It was found, however, that the rats receiving ferric chloride gained less weight than the controls, whereas the total weight of worms recovered from each of the ferric chloride rats was approximately the same as or greater than that obtained from the controls. It was also noted that the rats on the diet containing ferric chloride consumed considerably greater quantities of food than did the controls. It would, therefore, appear that the feeding of ferric chloride had a deleterious effect on the hosts, but not on the worms.
2. **In vivo** studies with S\(^{35}\)-labelled methionine.

These studies were undertaken in an attempt to provide some understanding of the extent to which the host, via the intestinal mucosa, might serve as a direct source of the sulfur-containing substances found in *H. diminuta*.

In these experiments fasted rats infected with *H. diminuta* were administered S\(^{35}\)-labelled methionine either orally or intravenously as described below. At varying intervals following administration of the labelled amino acid, the animals were sacrificed, the worms harvested, and the mucosal scrapings obtained as previously described. In one of the experiments to be described below liver samples were also collected. The worms and the host tissues thus obtained were freed of extraneous materials, thoroughly washed in 6 changes of isotonic saline solution, and carefully blotted with filter paper to remove excess wash fluid. Suitable quantities of the various tissues obtained from each rat were weighed out and transferred to small flasks containing 10 ml. of nitric acid. These preparations were allowed to stand overnight, following which they were carefully evaporated to within 1 to 2 ml. on a low temperature hot-plate. This procedure yielded perfectly clear solutions which were then quantitatively transferred to 10 ml. volumetric flasks and made to volume with distilled water. One ml. aliquots were removed to stainless-steel planchets and dried under an infra-red lamp. These samples were then counted in the Tracerlab Autoscaler employing a thin window Geiger-Müller tube.
All of the samples were prepared in duplicate, and counted for a sufficient period of time to give an error of less than 2 per cent. All of the radioactivity measurements were corrected for background and for self-absorption of the beta particles. The corrections for self-absorption were made from the empirically derived curve shown in Fig. VII. The data for this curve were obtained by adding varying quantities of a carrier methionine solution to a series of planchets containing equal aliquots of an $S^{35}$-methionine solution. The solutions were dried in the planchets and counted as described above. The planchets used here and throughout this work had a bottom surface area of 4.91 sq. cm. Dilutions of the administered solutions served as standards for the measurement of radioactivity. All of the results for whole tissues were calculated as follows:

\[
\frac{\text{counts/minute found per gram of tissue}}{\text{counts/minute administered per 10 grams animal weight}} \times 100
\]

The expression, per cent of dose per gram of tissue, as it is henceforth used, is defined in accordance with the above formula where the term, dose, is taken as the counts per minute administered per 10 grams of animal weight.
Figure VII. Self-Absorption Curve of $^{35}S$ in Methionine. Abscissa represents milligrams of methionine per 4.91 sq. cm.
(a) Preliminary observations—oral versus intravenous administration.

It was desirable, initially, to determine if the worms would incorporate sufficient radioactivity following the administration of $\text{S}^{35}$-methionine to the host to permit further experimentation on a feasible basis. It was further of interest to compare the relative uptake of $\text{S}^{35}$ in the intestinal mucosa and the worm following oral administration with that following intravenous administration.

Two rats of the Wistar strain were used approximately 6 weeks after infection with 7 cysticercoids of H. diminuta. The rats were fasted for five hours following which each received a dose of 11 mgm. of $\text{S}^{35}$-labelled methionine (approximately $4.2 \times 10^5$ counts/minute) in 0.5 ml. glass distilled water. One of the animals was fed the methionine solution by stomach tube, and the other received it by injection in the tail vein. Both of the animals were sacrificed 18 hours later. The choice of this time interval was based on the results obtained by Friedberg, Tarver, and Greenberg (1948) who showed that a maximum uptake of radioactivity by the protein fractions of the intestinal mucosa of the white rat occurred at approximately 15 to 18 hours following intravenous administration of $\text{S}^{35}$-methionine. Random samples of the pooled worms and the intestinal mucosa obtained from each rat were used for the assay of radioactivity as described above. The results are shown in tabular form as follows:
<table>
<thead>
<tr>
<th>Sample</th>
<th>Rat Wt. (g.)</th>
<th>Tissue Wet Wt. (g.)</th>
<th>% Dose* Per g. Tissue (g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 hr. oral-worm</td>
<td>258</td>
<td>2.661</td>
<td>6.78</td>
</tr>
<tr>
<td>mucosa</td>
<td>&quot;</td>
<td>1.384</td>
<td>49.83</td>
</tr>
<tr>
<td>18 hr. I.V.-worm</td>
<td>248</td>
<td>2.443</td>
<td>4.59</td>
</tr>
<tr>
<td>mucosa</td>
<td>&quot;</td>
<td>2.792</td>
<td>14.09</td>
</tr>
</tbody>
</table>

*Per 10 grams of animal weight.

It is seen from these results that sulfur administered either orally or intravenously to the host in the form of S\textsubscript{35}\textsuperscript{58} was incorporated into the worms and the intestinal mucosa. No proof is afforded by these results that the isotope was actually taken up by these tissues in the form of methionine, or that it was present in the form of methionine when the tissues were assayed for radioactivity. This situation was partially clarified by experiments to be described below. Perhaps the most significant aspect of these findings, however, is that the worms were able to obtain the isotopic sulfur directly from the host. The possibility is, therefore, suggested that one or more of the various sulfur-containing substances chromatographically detected in the worms is obtained by the worms in significant quantities from endogenous host sources. The further implications of these results will be discussed in another section in conjunction with the results of later experiments.
(b) The uptake of $S^{35}$ in host tissues and worms at various intervals following intravenous administration of $S^{35}$ -methionine to host.

These experiments were undertaken in order to study the pattern of incorporation of $S^{35}$ in the worms as compared to that in the intestinal mucosa following the intravenous administration of $S^{35}$ -methionine to the host. It was hoped that data obtained in this manner would clarify the possible role played by the mucosa with respect to the acquisition of endogenous sulfur by the worms.

Experiment (1)- Twelve rats of the Sprague-Dawley strain, weighing between 187 and 239 g. were used approximately 4 weeks after infection with 10 cysticercoids. The rats were fasted approximately 15 hours before each was injected in the tail vein with 3 mg. (approximately 26 microcuries) of L-methionine, labelled with $S^{35}$, in 0.5 ml. of glass distilled water. No food was given thereafter. The $S^{35}$-labelled methionine was obtained from Abbott Laboratories. At intervals of 1, 4, 8, 18, 24, and 48 hours following injection two animals were sacrificed. One gram samples, of the worms, mucosa, and liver obtained from each rat were accurately weighed out and used for the assay of radioactivity as previously described. Liver samples from the 1 hour rats were not obtained. It was decided to assay the liver samples as a result of the observation by Friedberg, Tarver and Greenberg (1948) that the proteins of the liver, over a period of 42 hours following the
intravenous injection of $^{35}$S-methionine, showed a lower rate of incorporation of the label than did the proteins of the intestinal mucosa, kidney, and plasma. Since the liver is generally recognized as an organ of high metabolic activity and as the primary site for the production of plasma proteins, it was of interest to see how the pattern of $^{35}$S incorporation in whole liver would compare with that of whole mucosa following the introduction of labelled methionine into the host's circulation. In view of the liver's possible contribution, via the bile, of sulfur-containing substances to the worms, it was, in addition, desirable to see if a relationship could be detected between the pattern of isotope incorporation in the liver and that in the worms. The results of the radioactivity assays are graphically shown in Fig. VIII.

It may be seen that the pattern of isotope incorporation shown by the worms closely resembles that of the intestinal mucosa during the first 18 to 20 hours following the intravenous administration of labelled methionine to the host. Perhaps the most surprising finding was the rapidity with which the isotope was initially made available to the worms by the host as shown by the significant amounts of radioactivity incorporated into the worms during the first hour. The fall in isotope concentration shown by the mucosa, at the end of 18 to 20 hours is in close agreement with the observations of Friedberg, Tarver, and Greenberg (1948) on the incorporation of $^{35}$S from methionine into the proteins of the rat intestinal
Figure VIII. Radioactivity in Experimental Tissues at Various Intervals Following the Intravenous Administration of $S^{35}$ to Rats Infected with H. diminuta. Note: Dose is defined as counts per minute administered per 10 grams of animal weight.
mucosa. In light of the introductory discussion to this work, it is quite conceivable that the bulk of isotope lost from the mucosa during this time occurred via the intestinal lumen, and thus served to sustain the rate of isotope uptake observed in the worms during the corresponding period. In this respect it would be of interest in future work to see if the absence of worms from the intestinal lumen would reflect any change in the rate of decrease of the isotope concentration in the mucosa. It was further seen that whole liver, in contrast to liver proteins as described above, showed an initial uptake of the label between 1 and 8 hours which exceeded that shown by the mucosa. This was followed by a rapid loss of activity from 8 to 18 hours and then a period of stabilization. The rapid turnover initially shown by the liver might be associated with the production of plasma protein, particularly since Friedberg, Tarver, and Greenberg (1948) have shown that the specific activity of liver protein is actually lower than the specific activity of plasma protein except for the first half hour following the intravenous administration of S\textsuperscript{35}-methionine to rats.

Experiment (2)—It appeared desirable to see if the results obtained in the above experiment, with respect to the worms and mucosa, were reproducible. Another experiment, similar in scope to the above, was, therefore, undertaken in which rats bearing single worm infections of H. diminuta were used.

Fifteen rats of the Sprague-Dawley strain, weighing between 210 and 277 g., were used approximately 6 weeks after
infection with 1 cysticercoid. The rats were fasted approximately 15 hours before each was injected in the tail vein with 2 mg. (approximately 36 microcuries) of L-methionine ($S^{35}$) in 0.5 ml. of glass distilled water. No food was given thereafter. At intervals of 1, 6, 12, 24, and 48 hours following injection three animals were sacrificed. The worm and the entire amount of mucosal scrapings obtained from each rat were accurately weighed out and used for the assay of radioactivity as previously described. Worms were not found in two of the 48 hour rats. The results of the radioactivity assays are shown in Fig. IX.

It may be seen that the results obtained here with respect to the patterns of isotope incorporation in the worms and mucosa were quite similar to those obtained in the previous experiment.
Figure IX (A) Radioactivity in Worms and Mucosa at Various Intervals Following the Intravenous Administration of $^{35}$-Methionine to Rats Infected with Single Worms.

Note: Dose is defined as counts per minute administered per 10 grams of animal weight.

(B) Worm/Mucosa Radioactivity Ratios. Each point represents the ratio value for tissues obtained from the same rat.
(c.) The distribution of $\text{S}^{35}$ among amino acids and related substances in worms and mucosa following intravenous administration of $\text{S}^{35}$-methionine to the host.

In conjunction with experiment (1) of the previous section several preliminary observations were made with respect to the distribution of $\text{S}^{35}$ among the free sulfur amino acids and related substances in the worms and mucosal tissues obtained at 8 and 18 hours after the hosts had been intravenously injected with the labelled methionine. The distribution of the isotope was also determined in the protein fractions of the 18 and 24 hour tissues. Portions of the tissues obtained at these intervals were used for the preparation of the free amino acid and protein fractions as previously described.

Protein hydrolysates were prepared in accordance with the procedure described in the section on materials and methods. Aliquots of 100 microliters were used to obtain one-dimensional chromatograms. The ninhydrin-reactive streaks produced on the paper were cut into serial sections of equal size, carefully inserted into planchets, and counted in a windowless gas flow counter. By this procedure it was determined that almost all of the radioactivity in the proteins of the 18 and 24 hour worm samples was located in cystine, with only a very small amount of the isotope detected in the form of methionine. In protein hydrolysates of the 18 and 24 hour samples of mucosa, however, there was a more equal distribution of the isotope between methionine and cystine, with the greater amount being present
in the form of methionine. The limitations imposed by this 
assay procedure do not permit true quantitative comparisons.

One dimensional chromatograms were prepared from 100 
microliter aliquots of the free amino acid fractions. Each 
chromatogram was then placed in contact with a sheet of Kodak 
No-Screen X-ray Film and kept in an X-ray exposure holder for 
approximately three months, at the end of which time the film 
was developed. The radioautographs thus obtained revealed the 
location of radioactive areas on the chromatograms. In the 8 
and 18 hour free amino acid fractions of the mucosa radioactive 
methionine and taurine were readily detected. In the 8 hour 
free amino acid fraction of the worms small amounts of meth-
ionine and taurine were detected, while in the 18 hour fraction 
no methionine was observed, and the taurine had greatly in-
creased in concentration. In each of these radioautographs an 
area of radioactivity, more intense in the worm fraction than 
in that of the mucosa, was observed in the position that is 
jointly occupied by glutathione, cystine, and sulfate on one-
dimensional chromatograms. It was not possible to determine 
at this time if more than one of these substances was present. 
Later analyses using two-dimensional chromatograms served to 
clarify this situation.

In Fig. X are shown radioautographs which were prepared 
from two-dimensional chromatograms of the 18 hour free amino 
acid fraction. One of the radioautographs was prepared from 
a chromatogram which had been oxidized with hydrogen peroxide
Figure X. Radioautographs prepared from (A) non-oxidized and (B) oxidized chromatograms of the free amino acid fraction of worms 18 hours following the intravenous administration of $^{35}$S-methionine to the host.

Key: (1) glutathione (reduced), (2) glutathione (oxidized), (3) taurine, (4) cysteic acid.

Note: Intense spots in the upper left and lower right corners are markers.
and ammonium molybdate as previously described. The quantity of cysteic acid formed by the oxidation of cystine, as shown here, was not as large as that observed in later analyses. The amount of oxidized glutathione shown on the radioautograph of the non-oxidized chromatogram was not sufficient to give a ninhydrin reaction on the chromatogram, and was probably formed during the chromatographic procedure. It may be seen that radioactive taurine was present in the worms in significant quantity at the end of 18 hours following the intravenous administration of S\textsuperscript{35}-labelled methionine to the host.

The above observations were supplemented by a more complete series of analyses in which 4 rats of the Sprague-Dawley strain, weighing between 220 and 233 g., were used approximately 6 weeks after infection with 3 cysticercoids of *H. diminuta*. The rats were fasted approximately 15 hours before each was injected in the tail vein with 2 mg. (approximately 36 microcuries) of L-methionine (S\textsuperscript{35}) in 0.5 ml. of glass distilled water. No food was given thereafter. The S\textsuperscript{35}-labelled methionine used in this experiment was obtained from Abbott Laboratories, and was found to contain a trace of impurity in the form of cystine as shown by a radioautograph prepared from a two-dimensional chromatogram of the injection solution. One animal was sacrificed at each of the following intervals after the injection of the labelled methionine: 1, 6, 24, and 48 hours. The worms and the intestinal mucosa were obtained from each rat and the free amino acid fractions
prepared, as previously described, from one gram samples of the tissues. Oxidized and non-oxidized two-dimensional chromatograms were prepared from 100 and 150 microliter aliquots of the free amino acid fractions obtained from the worms, and from 100 microliter aliquots of the fractions obtained from the mucosa in accordance with previously described procedures. Radioautographs of the chromatograms were obtained after an exposure time of approximately 1 month, and the positions of the radioactive spots were thereby determined. Cystine was, in all instances, detected as cysteic acid on the radioautographs prepared from the oxidized chromatograms. The appearance on these radioautographs of the oxidation products of glutathione and methionine served to confirm the presence of these substances. A composite of the observations made with respect to the presence on the various radioautographs of cystine, glutathione, taurine, and methionine is shown in the following table. The oxidation products of cystine, methionine, and glutathione are not listed separately.

<table>
<thead>
<tr>
<th>Substances Containing $S^{35}$</th>
<th>1 hr.</th>
<th>6 hr.</th>
<th>24 hr.</th>
<th>48 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>worm mucosa</td>
<td>worm mucosa</td>
<td>worm mucosa</td>
<td>worm mucosa</td>
</tr>
<tr>
<td>cystine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>glutathione</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>methionine</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>?</td>
</tr>
<tr>
<td>taurine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

Several comments may be made with respect to these
observations. By far the greatest concentration of radioactivity observed on the various radioautographs of the worm preparations was found in glutathione. At the end of one hour radioactive glutathione was not apparent in the mucosa. At later intervals the mucosa contained only trace quantities of glutathione. This may be seen in the radioautographs of the 48 hour preparations shown in Fig. XI. At the end of 24 and 48 hours the non-oxidized worm preparations revealed faint areas of radioactivity which closely corresponded in position to that of methionine. It was not possible, however, to identify these areas with any degree of certainty. Taurine was not apparent on the 1 and 6 hour radioautographs, but was detected at the end of 24 and 48 hour experimental periods in the worms and the mucosa. Radioautographs of the non-oxidized chromatograms prepared from the 24 and 48 hour worms also revealed a distinct area of radioactivity which, although not identified, was thought to be sulfate. The substance responsible for this spot was not ninhydrin-reactive, and appeared on the radioautographs in the region reported for sulfate by other workers. On radioautographs prepared from oxidized chromatograms, this unknown spot was masked by oxidized glutathione. Another spot, which was not identified, appeared on radioautographs prepared from oxidized and non-oxidized chromatograms of the 24 and 48 hour mucosa preparations. This spot shown on the radioautograph prepared from the 48 hour mucosa in Fig. XI is located in the approximate position reported for 2-
Figure XI. Radioautographs prepared from oxidized two-dimensional chromatograms of the free amino acid fractions of (A) H. diminuta, (B) rat intestinal mucosa 48 hours following the intravenous administration of $^{35}$S-methionine to the host.

Key:  
(1) glutathione (reduced)  
(2) glutathione (oxidized)  
(3) taurine  
(4) cysteic acid  
(5) methionine sulfoxide  
(6) methionine sulfone  
(7) unidentified
aminoethanesulfonic acid by Awapara (1953). The identification of this spot as 2-aminoethanesulfonic acid, however, would appear to be untenable since the latter is readily oxidized to taurine on treatment of the chromatogram with hydrogen peroxide. The implications of the results described above will be discussed in a later section.
3. The *in vitro* incorporation and the fate of $^{35}$S-methionine in *H. diminuta*.

The purpose of this study was to determine if the living worms, when removed from their hosts and introduced into a medium containing $^{35}$S-methionine, would actively metabolize the labelled amino acid.

In the preparation of this experiment sterile technique was maintained as far as possible to prevent utilization of the labelled amino acid by micro-organisms. All glassware was sterilized with dry heat; rubber stoppers and instruments were autoclaved. Sterile Tyrode's solution of pH 7.4 was used as the incubating medium to which the labelled methionine was added as described below. The incubation apparatus used in this experiment has been described in detail by Simmons (1952). This apparatus was equipped with a shaking device which kept the incubation tubes in motion at the rate of 83 excursions per minute. The labelled methionine used here was obtained from the Abbott Laboratories and was found to contain a trace of cystine on radioautographs prepared from chromatograms of the amino acid solution.

One rat of the Sprague-Dawley strain, weighing 327 grams, was used approximately 8 weeks after infection with 10 cysticercoids of *H. diminuta*. The animal was fasted for 15 hours at the end of which time it was sacrificed, and the worms recovered as previously described. The worms were thoroughly cleansed of extraneous materials, washed in 4 changes of
isotonic saline solution followed by 6 changes of the sterile Tyrode's solution. Two worms were placed in each of 4 Pyrex tubes, each tube containing 1 mg. (approximately 23 microcuries) of L-methionine ($S^{35}$) in 10 ml. of sterile Tyrode's solution. The tubes were then immediately sealed tightly with rubber stoppers and incubated with continuous shaking at 38°C. One tube was removed at the end of each of the following intervals: 1, 3, 5, 7 hours. The worms appeared to be in excellent condition on removal from the tubes. The two worms from each tube were thoroughly washed in 10 changes of isotonic saline solution, then individually blotted, pooled, weighed, homogenized and the free amino acid and protein-containing fractions prepared as previously described.

Ten mg. of each of the powdered protein-containing fractions thus obtained were accurately weighed out in duplicate in stainless steel planchets. Two ml. of 80% formic acid were introduced into each of the planchets which were then allowed to sit for approximately 15 minutes before they were dried down under an infra-red lamp. This procedure yielded more satisfactory plates of the protein-containing fractions than any other tried, and results obtained from duplicate preparations were always in excellent agreement. The planchets were then counted in the Tracerlab autoscaler and corrections made for background and self-absorption as previously described. In Table II are shown the results of the radioactivity assays expressed as the number of counts detected
per mg. of the protein-containing fractions prepared from the worms following the 1, 3, 5, and 7 hour incubation periods in the $S^{35}$-methionine medium.

Table II: Radioactivity Incorporated into Protein-containing Fractions of Worms After Incubation with $S^{35}$-Methionine

<table>
<thead>
<tr>
<th>Incubation period (hours)</th>
<th>Total Wet Wt. (g.)</th>
<th>Protein Fraction of Incubated Worms cts./min./mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.918</td>
<td>176</td>
</tr>
<tr>
<td>3</td>
<td>1.081</td>
<td>262</td>
</tr>
<tr>
<td>5</td>
<td>1.014</td>
<td>349</td>
</tr>
<tr>
<td>7</td>
<td>1.222</td>
<td>439</td>
</tr>
</tbody>
</table>

In view of the fact that these results were obtained with intact animals it would appear reasonable to suppose that the uptake of the labelling agent, as shown for each of the incubation periods, essentially represented true incorporation into the proteins by peptide bond formation and hence protein synthesis. That such might be the case is strongly suggested by the linear increase in the uptake with time which would, likewise, tend to eliminate adsorption of the labelled amino acids on the proteins as the possible cause of the above observations (Greenberg, 1951). It is not possible, however, to conclude from these results that the uptake of the labelling agent into the protein-containing fractions was solely in the form of methionine.
In order to assay the distribution of $\text{S}^{35}$ among the amino acid constituents of the various protein-containing fractions, hydrolysates were prepared as previously described from 25 mg. of each of the fractions. One-dimensional chromatograms were prepared from 50 microliter aliquots of the protein hydrolysates. The ninhydrin-reactive streaks were cut into serial circular sections corresponding to the levels shown in Fig. XII; these sections were then inserted into planchets, as previously described, and counted in a windowless gas flow counter. The results are shown in Table III. True quantitative estimations cannot be obtained by this procedure, particularly since the radioactive substances assayed are not confined to compact areas along the length of the streak, but this procedure does afford a convenient means of determining the approximate relative intensities of these radioactive areas. It may be seen from Table III that the major portion of the radioactivity in the protein-containing fractions of the 1, 3, 5, and 7 hour incubated worms was in the form of methionine (levels 10, 11, and 12 of Table III and Fig. XII), whereas considerably less of the label was contained in cystine (levels 3 and 4). These results were confirmed on radioautographs prepared from two-dimensional chromatograms of the various hydrolysates.

Aliquots of the free-amino acid fractions in amounts of 100 microliters were used for the preparation of two-dimensional chromatograms. Radioautographs of the chromatograms were obtained, as previously described, after an exposure time of
Fig. XII: One Dimensional Chromatograms Prepared from Protein Hydrolysates of Worms Incubated in $\text{S}^{35}$-Methionine, Showing Levels at which Sections of Chromatograms Were Cut.

<table>
<thead>
<tr>
<th>Incubation Periods</th>
<th>Counts Per Minute Minus Background Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 4 14 7 3 6 5 3 7 18 57 29</td>
</tr>
<tr>
<td>3</td>
<td>2 3 28 18 6 9 10 9 9 35 52 37</td>
</tr>
<tr>
<td>5</td>
<td>8 7 30 16 3 6 5 6 12 40 81 49</td>
</tr>
<tr>
<td>7</td>
<td>5 5 36 17 3 8 8 7 7 48 123 53</td>
</tr>
</tbody>
</table>

*Table III: Distribution of $\text{S}^{35}$ on 1-dimensional Chromatograms Prepared from Protein Hydrolysates of Worms Incubated in $\text{S}^{35}$-methionine.*
approximately 3 weeks. In this manner the $^{35}\text{S}$ label was readily detected in methionine, methionine sulfoxide, glutathione, and cystine (as cysteic acid) on chromatograms prepared from the free-amino acid fractions of the 1, 3, 5, and 7 hour incubated worms. The largest and most intense spots shown on the radioautographs were those of methionine and methionine sulfoxide. At the end of the 1 and 3 hour incubation periods the concentration of methionine was greater than methionine sulfoxide, as shown by the size and intensity of the spots on the radioautographs; at 5 and 7 hours the concentration of methionine sulfoxide exceeded that of methionine. The significance of these findings with respect to the metabolism of the worms is questionable in view of Dent's (1948) observation that some oxidation of methionine to methionine sulfoxide may occur during the development of the chromatogram. Radioautographs of the 5 hour incubation preparations revealed a discrete spot which increased in intensity at the end of 7 hours and appeared in the close vicinity of the position assumed by taurine on the corresponding chromatograms. It was not possible, however, to identify this spot with any degree of certainty.
V. DISCUSSION AND CONCLUSIONS

It is evident from this investigation that *H. diminuta* is capable of acquiring organic sulfur directly from its host. This has been, in part, shown by the fact that $S^{35}$ intravenously administered as methionine to the fasted host was readily acquired by the worms. It has been further shown that the labelling agent, in one hour following the injection, was contained in the glutathione and cystine constituents of the worm tissues. Although the possible intervention by intestinal microorganisms cannot be overlooked, the rapidity with which the isotopic label was initially taken up by the tapeworms, would appear, at least, to indicate that the transfer of the labelled substances from host to parasite did not require the mediation of the intestinal flora. These findings appear in partial support of Chandler's (1943) observation that *H. diminuta* is essentially independent of the protein in the diet of its host, and tend to confirm Read's (1950) hypothesis, at least with regard to *H. diminuta*, that nitrogenous substances which pass from the host tissues into the lumen of the small intestine, either by diffusion or active transfer, become available as nutriment to the parasite. Although Chandler, Read, and Nicholas (1950) have stated that a variety of nitrogenous compounds, including amino acids, are normal constituents of the gastric juice which passes into the small intestine, the observation by Friedberg, Tarver, and Greenberg (1948) that the incorporation of $S^{35}$-methionine into the gastric mucosa is negligible would tend to minimize the
potential significance of the gastric secretion as a source of the radioactivity which was observed in the tissues of *H. diminuta*. Likewise, the extent to which the pancreatic juice might contribute to the parasite significant amounts of the isotope in the fasted animal would appear to be questionable when it is considered that the pancreas normally secretes its juice in large quantities only during the passage of the acid chyme from the stomach into the duodenum. With regard to the biliary secretion, the evidences obtained in the course of this investigation strongly indicate that the worms are totally independent of the host bile in their acquisition of sulfur amino acids and related substances. It would, therefore, appear, as was indicated by the results obtained from the *in vivo* experiments, that the worms acquired the isotope-labelled substances in the most significant amounts via the cells of the intestinal mucosa. Further evidences in support of the latter argument will be discussed below.

In those experiments in which it was attempted to evaluate the potential significance of the host bile as a source of the taurine and other sulfur-containing substances chromatographically detected in the worm tissues, it was found that ligation of the host bile duct resulted, in many instances, in the loss of worms from the intestinal lumen. Although it is quite conceivable that the host bile does exert a direct influence on the metabolism of the worms, particularly in view of the complexity of functions which have been attributed to the hepatic secretion,
the nature of the experimentation involved in the present work precludes the possibility of a definite conclusion. It is, in other words, clearly recognized that any attempt to eliminate the flow of bile into the intestinal lumen, either by ligation of the bile duct or by other surgical procedure, introduces many variables which are impossible to evaluate. For example, the deleterious effects of biliary obstruction on the host, in the case of bile-duct ligation, make it entirely possible that alterations in the overall metabolism of the host are reflected on that of the parasite.

It has been suggested by the work of Edgar (1941) that bile salts play a possible role in the establishment of tapeworm infections. Edgar demonstrated that bile salts obtained from cattle were quite effective in causing the rapid evagination of the scolex from the cysticercus of *Taenia pisiformis*. In the present work, however, it was found that ligation of the bile duct in a single rat prior to infection with *H. diminuta* had no effect on the establishment of the worms in the lumen of the intestine. It is, therefore, not possible at the present time to assert a relationship between the biliary secretion of the host and the physiological activities of the worms; further work is, obviously, required to obtain adequate explanations of the pertinent observations which have been made in the course of this investigation.

Perhaps the most striking aspect in the pattern of isotope uptake shown by the worms, following the intravenous administration
of $^{35}$S-methionine to the host, was the rapid initial rise in the incorporation of the labelling agent into the worm tissues. This observation strongly suggests that the labelled substances acquired by the worms were in the form of relatively simple molecules, in which case the intestinal mucosa is indicated as the logical source of these substances. As previously mentioned, Read (1950) has described the large mass of evidence showing the surprising extent to which the cells lining the intestinal lumen permit two-way passage of a great many substances. Moreover, the work of Friedberg, Tarver, and Greenberg (1948), as previously discussed, indicates the presence in the intestinal mucosa of mobilized amino acid molecules in amounts far exceeding that of most other tissues. Further, the close similarity observed in the uptake patterns of the worms and the mucosa may constitute additional support for the intestinal mucosa as the primary source of the radioactivity found in the worm tissues. In this regard, it may be noted that the worms did not reflect the pattern of isotope uptake shown by the liver at any time during the 48 hour experimental period. On the other hand, the possibility does exist that the \textit{in vivo} pattern of isotope incorporation demonstrated by the worms was essentially a manifestation of a maximum rate at which the worms were capable of incorporating the labelled substances. That such may be the case was, perhaps, shown in the experiment in which radioactivity assays of the worm tissues were compared following the oral and intravenous administration of $^{35}$S-methionine to the host; it was seen that the
quantity of radioactivity detected in the worms at 18 hours following oral administration was not as great as would be expected when compared to that found in the worms at 18 hours following intravenous administration. On the other hand, the incorporation of the labelled substances into the intestinal mucosa proceeded at a much higher rate following oral administration than it did following intravenous administration.

At the end of 1 and 6 hours following the intravenous administration of $S^{35}$-methionine to rats infected with H. diminuta, the isotopic label was, by a combination of chromatographic and radioautographic procedures, readily detected in the worm tissues in the tripeptide, glutathione, and to a lesser extent in cystine. At the end of 24 and 48 hours, the label was additionally present in taurine and in barely detectable quantities of methionine. As a result of these observations, it is not possible to arrive at a definite conclusion with regard to the actual organic form or forms in which the sulfur label was acquired by the worms. In view of the inability to demonstrate a consistent appearance of methionine in the worm tissues, either when the worms were removed from well-fed hosts or when $S^{35}$-methionine was intravenously administered to fasted hosts, it was initially thought that the worms were not capable of significantly incorporating or utilizing methionine. On the basis of the incubation study, however, in which large quantities of methionine were readily incorporated into the free amino acid and protein fractions of the worm tissues, it appeared that other explanations must exist for
the small and often questionable quantities of methionine
found in the worms under *in vivo* conditions. It is, for ex-
ample, conceivable that the normal diet of the host contains
methionine in such quantities as to be entirely absorbed by
the cells of the intestinal mucosa. In view of the fact that
the sulfur of methionine is readily converted to cystine sul-
fur in vertebrate tissues (Tarver and Schmidt, 1939), it is
therefore, possible that the organic form in which sulfur is
most significantly made available to the worms is that of
cystine. On the other hand, the possibility also exists that
the failure to readily demonstrate the presence of methionine
in the tissues of freshly harvested worms may be a manifesta-
tion of the worm's own metabolic capacities. That such may
well be the case, would appear to be demonstrated by the re-
sults obtained from the *in vitro* study in which it was found
that the worms were capable of actively metabolizing methionine
labelled with $^{35}$S, as indicated by the appearance in the worm
tissues of radioactive cystine, glutathione, and possibly taurine.

The chromatographic detection of radioactive methionine
sulfoxide in the worms incubated in a medium containing $^{35}$S-meth-
ionine would appear to be of doubtful significance in view of
Dent's (1948) observation that methionine is, to some extent,
ocasionally oxidized to the sulfoxide during the development
of the chromatogram. Moreover, Dent has stated that methionine
sulfoxide is only rarely found in biological materials. In this
regard, it may be noted that radioactive methionine sulfoxide
was not detected in the worm tissues in the course of the in vivo studies. Nevertheless, the possibility can not be overlooked that the sulfoxide was a true product of the worms metabolism under the conditions of the in vitro study, particularly when it is considered that results obtained from such studies may be at variance with what usually occurs in an in vivo situation. When it is, further, considered that the incubation medium, in the present in vitro study, contained a relatively large quantity of methionine, and that considerably more oxygen was made available to the worms than exists in the host intestine, the opportunities for irregular metabolic behavior on the part of the worms become more apparent. In spite of the hazards entailed in the comparison of observations made from in vivo and in vitro experiments, however, any attempt to merge the results obtained from these two types of studies in the present investigation leads to the conclusion that the in situ worms are capable of actively metabolizing, at least, some of the sulfur amino acids.

The results obtained from the in vitro study, in which whole worms were incubated in a medium fortified with S\textsuperscript{35}-methionine, are noteworthy in another regard. Between 1 and 7 hours of incubation, it was seen that the worm proteins exhibited a striking linear increase in the accumulation of radioactivity. In accordance with the criteria set forth by Greenberg (1951) for the true incorporation of amino acids into proteins, it is believed that the above observation essentially represented peptide
bond formation and hence protein synthesis, rather than ad-
sorption of the labelled substances on the proteins. That
such is probably the case is indicated by the fact that signif-
icant quantities of radioactive glutathione, a tripeptide, ac-
cumulated and were apparently synthesized in the worm tissues
in the course of both the in vivo and in vitro studies.

It is hoped that, at least, some of the observations made
in the course of this work will serve as a basis for future ex-
perimentation. Perhaps one of the more significant aspects of
this investigation was the possible demonstration of in vitro
peptide bond formation, and hence protein synthesis in the in-
tact worms. In this regard it would be of much interest to see
if amino acids, other than methionine, are likewise incorporated
into the worm proteins under similar in vitro conditions. Since
peptide-bond formation is known to be inhibited by anaerobic con-
ditions and by respiratory poisons (Greenberg, 1951), convenient
means are, thereby, afforded for the further study of the pre-
sumed in vitro synthesis of proteins in H. diminuta. With re-
gard to an active metabolism of various sulfur amino acids and
related substances in H. diminuta, it is hoped that future work
will include detailed studies of the specific enzyme systems
which are known to be associated with these substances in other
tissues.
SUMMARY

Observations on the behaviour of various sulfur amino acids and related substances on one and two-dimensional paper chromatograms have been presented.

Qualitative two-dimensional chromatographic analyses of the free amino acid and protein fractions of *Hymenolepis diminuta*, and of the free amino acid fraction of the rat intestinal mucosa were carried out with special reference to the sulfur-containing constituents. Cystine, glutathione, taurine, and small quantities of methionine were detected in the free amino acid fraction of *H. diminuta*. Cystine and trace amounts of methionine were detected in the protein fraction of *H. diminuta*. Cystine, methionine, taurine, and small quantities of glutathione were detected in the free amino acid fraction of the intestinal mucosa of the rat. Other amino acids and ninhydrin-reactive substances detected in the various fractions are shown in composite and representative chromatograms.

Rats having their bile ducts ligated following infection with *H. diminuta* were, in many instances, found to lose their worms. Recovered worms were often dwarfed and partially mutilated. Chromatographic analyses of the free amino acid fractions of the recovered worms showed no significant differences in the content of taurine and other sulfur-containing constituents as compared to controls. A single rat having its bile duct ligated prior to infection with *H. diminuta* was found to have its full complement of worms; no significant differences
were detected in the free sulfur-containing constituents of the recovered worms as compared to controls.

The inclusion of 3% ferric chloride in the diet of rats infected with *H. diminuta* had no apparent effect on the establishment, maintenance, and ether-soluble contents of the worms. It was observed, however, that rats on the ferric chloride diet gained significantly less weight than the controls, but yielded worms of the same or greater weight.

Preliminary observations on the uptake of S\(^{35}\) administered as methionine to rats infected with *H. diminuta* revealed that a slightly larger percentage of the administered dose was incorporated into the worms 18 hours after oral administration than was incorporated into the worms at the same period following intravenous administration. A similar comparison with regard to the rat intestinal mucosa showed a much greater incorporation of the labelling agent following oral administration.

The patterns of incorporation of radioactive sulfur into tapeworms, intestinal mucosa and liver were determined up to 48 hours following the intravenous administration of S\(^{35}\)-methionine to rats infected with *H. diminuta*. A period of rapid incorporation of the labelling agent occurred in all three tissues during the first hour. Concentration of the isotope in the liver reached a maximum in 8 hours, following which it sharply declined before stabilization was obtained at 18 hours. Subsequent to the initial rapid rise in activity, the mucosa showed a slower accumulation of the isotope to 18 hours, followed by
a gradual decline to the end of the 48 hour experimental period. Radioactivity in the worms rose gradually between 1 and 48 hours.

At the end of 1 and 6 hours following the intravenous administration of S$^{35}$-methionine to rats infected with *E. diminuta*, the isotopic label was, by a combination of chromatographic and radioautographic procedures, detected in the worm tissues as glutathione and cystine; in the intestinal mucosa as cystine and methionine. At the end of 24 and 48 hours the label was additionally detected in the worm tissues as taurine and trace amounts of methionine; in the mucosa as taurine and glutathione.

Whole worms incubated for periods of 1, 3, 5, and 7 hours in Tyrode's solution containing S$^{35}$-labelled methionine showed, after the first hour of incubation, a linear increase in the incorporation of the label into their proteins. It is suggested that this observation represents peptide bond formation, and hence protein synthesis. At the end of each of the incubation periods the isotope was detected in the worm proteins principally in the form of methionine, and to a lesser extent in cystine. Among the free amino acids and related substances of the worm tissues, the isotope was detected in cystine, glutathione, methionine, and methionine sulfoxide. An unknown radioactive substance was also detected which, on two dimensional chromatograms, assumed a position closely approximating that occupied by taurine.
VII. BIBLIOGRAPHY


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