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CYTOLOGICAL STUDIES ON THE
ABSORPTIVE SURFACES OF CESTODES

by

Richard D. Lumsden

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
Doctor of Philosophy

Thesis Director's signature . . .

A handwritten signature in dark ink, appearing to read "Clark P. Read". The signature is fluid and cursive, with a horizontal line drawn underneath the name.

Houston, Texas

May, 1965

"Tapeworms are unfavorable objects for histological study, since ... the true relations of various cellular elements are difficult to distinguish, and ... easily distorted by fixatives, producing puzzling artifacts." (Wardle and McLeod, 1952, The Zoology of Tapeworms)

ABSTRACT

The host-parasite interface, in the case of cestodes, is defined morphologically as a cell membrane circumscribing a complex coenocytic integument composed of a peripheral cuticle, underlying muscular and secretory cells. Studies with the electron microscope reveal the cuticle of certain cyclophyllidean, tetraphyllidean and trypanorhynch cestodes (Hymenolepis diminuta, Calliobothrium verticillatum and Lacistorhynchus tenuis) as an anucleate syncytium, whose apical and basal surfaces are defined by triple-layered plasma membranes. The former is projected as a series of closely spaced microvilli. The cuticular matrix contains mitochondria and other characteristic inclusions of typical cytoplasmic ground substance. Subcuticular cells continuous with the cuticular layer possess morphological specializations consistent with the synthesis and elaboration of a protein secretory product.

Tissue section autoradiographs prepared after in vitro exposure of worms to tritiated proline and leucine indicate high levels of amino acid incorporation by the subcuticular cells. A significant portion of the incorporated label is subsequently secreted into the cuticular matrix. Studies with tritiated glucose and galactose suggest that the subcuticular cells, and perhaps the cuticle, are also engaged in the synthesis of non-glycogen polysaccharide.

Electron microscopy of thin sectioned tapeworm tissues reveals polymorphism of glycogen deposits consistent with previous observations of molecular weight heterogeneity of the polysaccharide

fraction isolated from H. diminuta and other helminths. Two types of glycogen particles are distinguishable morphologically: (1) single granules (beta particles) 200-400 A in diameter and (2) rosettes (alpha particles) comprised of varying numbers of 200A particles, with an aggregate diameter of 600 to 2000 A. Mixtures of these particles occur in certain parenchymal cells, though the alpha particles tend to predominate in cells storing large quantities of polysaccharide while the beta particles are characteristic of cells thought to possess elevated levels of carbohydrate metabolism. Tissue section autoradiography following in vitro administration of H3-glucose indicates a high rate of glycogen turnover in tissues containing this polysaccharide primarily in the beta form compared with those containing a preponderance of alpha glycogen. It is suggested that the alpha configuration constitutes a reserve depot carbohydrate in cestode tissues, while the beta particle represents a source of more readily mobilizable hexose.

Evidence is presented for the derivation of some subcuticular cells from medullary centers of mitotic proliferation; the stem cells appear to migrate peripherally, undergoing differentiation and subsequent attachment to the basal cuticular surface. Expansion of the integument in a rapidly growing proglottid is visualized as the product of additive cytological multiplication and biosynthesis of macromolecular constituents of the cuticular matrix by the subcuticular cells.

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I

INTRODUCTION

The universal, obligate and highly specific commitment of cestodes to a parasitic mode of existence is reflected to a large degree in the extent of physiological integration achieved between these helminths and their hosts. Though often defined primarily in nutritional terms, the basis of this symbiotic relationship may well include dependence on a wider spectrum of environmental factors impinging directly on specific physiological mechanisms. Several endoparasites studied thus far, notably tapeworms, appear unable to carry out certain homeostatic functions normally associated with the physiology of free living forms, particularly those operations involved with maintaining steady state levels of free pool metabolites (Read, Rothman, and Simmons, 1963). It has been suggested by these and other investigators that specific physiochemical parameters in the environment of endoparasites may (1) effect feedback control of various essential processes in the development of the symbiont (Smyth, 1952; Rogers and Sommerville, 1957; Rothman, 1959), (2) compensate for a lack of regulatory ability on the part of the parasite (McGue and Thorson, 1964; Thorson et al., 1964; Read et al., 1963), or (3) close critical lesions in its biosynthetic mechanisms (Trager, 1952, 1954, 1959; Siddiqui and Trager, 1964). The successful establishment and maintenance of the host-parasite relationship would depend, therefore, not only on the presence of specific host-provided chemical entities but further on the degree of possible interchange which could occur between the environment of the parasite, specified

in turn by the physiology of the host organism, and the parasite's metabolic pathways. It is immediately obvious from a large number of studies on free living organisms that the superimposition of exogenous control on a biological process is not a feature unique to parasitism. Rather it is a corollary of informational deletion and the means by which the organisms so affected compensate for such deficiencies which ultimately may be expected to distinguish the parasitic condition.

Read, Rothman and Simmons (1963) have defined the host-parasite interface as "(that) region of chemical juxtaposition of regulatory mechanisms of both host and parasite". Implicit in this concept of the host-parasite interface is a structural entity, which, in the case of the cestodes, might be interpreted as including the parasite's body surface. Lacking an alimentary organ system, the interchange of materials between the tapeworm and its environment involves their passage directly through the worm's integument. Pursuant to an understanding of the microecological aspects of the host-cestode relationship, knowledge of the tapeworm outer surface, the cuticle and cellular elements of the subcuticle assumes paramount significance. However, that the cestode surface is yet but poorly defined is best evidenced by the following statement of Read, Rothman and Simmons (1963):

"It cannot be decided as yet whether the interface, in the case of a cestode, should be considered to include a specific bounding membrane or the entire integument, which (previous) studies have shown to be acellular."

According to the classical view, adult tapeworms are devoid of a true epithelial epidermis, the integument consisting of a trilaminar cuticle overlying the cellular components of the subcuticle. A surface epithelium resembling turbellarian ectoderm is present in the postembryonic coracidium, but this, according to Schauinsland (1886), Leukart (1886) and many subsequent investigators, degenerates or is cast off during subsequent development and replaced by the cuticle. Though there has been considerable controversy over homology and ontogeny, it is generally accepted by most helminthologists that the definitive cestode cuticle is an acellular, non-living investment secreted by elements of the cortical tissues.

While Salensky (1874) and others (eg: Wagener, 1855; Monticelli, 1892, 1893; Cerfontaine, 1899) believed the cuticle represented a degenerate epidermis, containing bodies interpreted as nuclei or remnants thereof, Blochmann (1896, 1897) regarded the tapeworm surface as a non-cellular exudate of the underlying subcuticular cells, which in turn constituted a secondarily displaced ("eingesenkt") layer of surface ectodermal tissue. A similar idea regarding the origin of the adult cuticle was advanced by Johnstone (1911) and supported by Fuhman (1931). Other workers have suggested that the subcuticular cells, rather than representing a modified epidermal epithelium, are differentiated parenchymal cells variously specialized for secretory, absorptive, neuromuscular and sensory functions (Ziegler, 1905; Balss, 1908; Young, 1908; Pratt, 1909; Vogel, 1930; Vernard, 1938; Hyman, 1951). Poche (1924) termed the

cuticle of Archigetes a "pseudodermis", in accord with his belief that the surface layer was deposited by mesodermal parenchymal cells, a view subsequently supported and extended by Wisniewski (1930).

Early descriptions of tapeworm histology especially pertinent to current concepts of the structure and cytological relationships of the cestode integument include those of Schiefferdecker (1874), Kahane (1880), Zograf (1892, cit. in Pratt, 1909), Pintner (1880), von Linstow (1891, 1893), Zerneck (1895), Steudener (1877) and Zschokke (1888). Schiefferdecker (1874) described a system of cuticular pores, which he postulated was of importance in cestode alimentation. Pore canals in the tapeworm cuticle were described by Zschokke (1888) as "nombreux..... présente une fine striation, perpendiculaire à la surface du corps..." This author stated further "J' ai vu sortis par ces canaux de très fines fibrilles protoplasmiques, prolongement directs des cellules sous-cuticulaires". The free surface of the cuticle has been variously described as bearing cirri, "pseudo-cilia", hair-like processes ("Comidien") or "microtriches" (Zograf, 1892; Zschokke, 1885; Steudener, 1877; Moniez, 1884; Minckert, 1906; Rees, 1958; Rothman, 1963; Siddiqui, 1963). Schiefferdecker (1874) and Zschokke (1888) believed these peripheral filamentous structures represented projections of subcuticular cells which reached the surface via intracuticular canaliculi.

Recent histochemical studies on a number of tapeworm species indicate that the cuticular matrix is largely proteinaceous, containing as well some non-glycogen polysaccharide, perhaps

mucopolysaccharide or oligosaccharide units conjugated as glycoprotein. Monne' (1959) suggested that the cuticle of Diphyllbothrium latum, Mesocetoides lineatus, Moniezia expansa, Cittotaenia pectinata and Taenia taeniaeformis was composed of protein and mucopolysaccharide, based primarily on staining properties with acidic aniline blue before and after deamination, thiazin dyes and the periodate acid-Schiff method for the demonstration of vic-glycols. Waitz and Schardein (1964) in a series of similar studies with Hymenolepis diminuta, H. nana, Hydatigera taeniformis and Dipylidium caninum, additionally demonstrated the presence of high concentrations of SH groups (presumably as protein-bound cysteine) in the cuticle and subcuticular cells using the DDD-azo method of Barrnett and Seligman (1952). Further evidence for the presence of protein in the tapeworm cuticle can be inferred from the work of Bogitsh (1963) who reported that the cuticle of Hymenolepis microstoma stained intensely following histochemical application of the Millon reaction (for the detection of tyrosine) and mercuric-bromphenol blue (Mazia et al., 1953)(for general proteins) to paraffin embedded sections. Cheng and Jacknick (1964) reported that the cuticle of H. diminuta stained uniformly with pyronin Y, a reaction which the authors claim was diminished by preincubating the sections in ribonuclease.

Enzymic activity has been associated histochemically with the cestode cuticle. Such includes demonstration of alkaline and acid phosphatase (Erasmus, 1957 a and b; Kilejian, et al., 1961; Rogers, 1947; Yamao, 1952; Bogitsh, 1963), cytochrome oxidase

(Waitz and Schardein, 1964), succinic dehydrogenase (Hedrick, 1956; Waitz and Schardein, 1964; Rothman and Lee, 1964), and other enzymes associated with the tricarboxylic acid cycle (Rothman and Lee, 1964).

The physiological activity of the cuticle has not been satisfactorily elucidated. Pratt (1909) and Monne* (1959) suggested that the primary function of this structure is to resist the action of the host's digestive enzymes, thus preventing enteric destruction of the worm's tissues. Additionally, various investigators (eg: Schiefferdecker, 1874; Schopfer, 1932) have implied that the cuticle may impart some factor of selectivity to the interchange of materials with the host, though any determinant role of the cuticle in the absorption of exogenous substances by these parasites has not been clearly demonstrated. Threadgold (1962, 1963) stated that the cuticle of Dipylidium might possess a "digestive function".

Alimentation in cestodes was formerly assumed to involve simple diffusion of relatively low molecular weight compounds from the lumen of the host intestine across the cuticle into the parenchymal tissue. A passive role of the cuticle in this process has been postulated by Wardle and McLeod (1952):

"It (the cuticle) is without pore canals, and the passage of water, glucose, etc. through it can be only by osmosis. Presumably its laminated nature favors such osmosis."

Evidence now available indicates that tapeworms obtain many substances from the milieu exterieur by a process which cannot

be attributed solely to simple diffusion. The entry rate of certain monosaccharide sugars (Pfifer, 1960 a,b and c; Read, 1961), amino acids (Read, et al., 1960, a, b, 1962, 1963) certain purines, pyrimidines and their ribosides (MacInnis et al., 1965) is not a linear function of the external concentration but instead follows adsorption of Michaelis-Menten saturation kinetics. Additionally, and perhaps more significant, is that the movement of these substances can occur against a chemical concentration gradient, is contingent upon metabolic energy resources and is competitively inhibited by chemically similar compounds. Thus, it would appear that in tapeworms the acquisition of a number of compounds is a mediated process which meets the criteria set forth by Crane (1960) and Wilson (1962) for active transport.

Classical notions of the structure and nature of the tapeworm cuticle are inconsistent with any speculation that it functions as a physiologically active, absorptive surface. As a result, perhaps, several investigators (Schiefferdecker, 1974; Pratt, 1909; Young, 1908; Minckert, 1905; et al.) have been prone to attribute any directed movement of absorbed materials to so-called "resorpbierende", "nutritive" or "food-gathering" cells and the like residing primarily in the subcuticulum.

Light microscopy alone has failed to elucidate satisfactorily the functional aspects of the surfaces of tapeworms. Electron microscopic studies to date have been only preliminary in nature, though micrographs published by Read (1955), Kent (1957),

Threadgold (1962) and Rothman (1963) suggest the presence of mitochondrion-like structures in the cuticle of certain tapeworm species, as well as surface specializations which might be interpreted as concerned with absorptive function. On the basis of these studies, Rothman (1963) applied the term "tegument" to the surface investment of cestodes, presumably to differentiate it from cuticles (sensu stricto) devoid of such organelles. However, in a subsequent publication (Read, Rothman and Simmons, 1963) this investigator continued to regard the cestode integument as acellular. On the other hand, Threadgold interpreted the "tegument" as representing the apical portion of the subcuticular cells. Despite comments to the contrary, (Threadgold, 1962; Vickerman, 1963), it is this writer's opinion that the presence of mitochondria in the tapeworm cuticle, as depicted in previously published studies (ob. cit.), does not in itself provide convincing evidence for the cellular nature of this structure. Mitochondria, or remnants thereof, might equally appear in an extracellular cuticle as the result of apocrine secretory activity of the subcuticular cells.

Various roles for the tapeworm cuticle and/or elements of the subcuticle in certain physiological interactions at the host-parasite interface have been implied, but in spite of the foregoing investigations, the structure of the cestode integument and its relationships remain enigmatic. An integrated study employing techniques of light and electron microscopy, cytochemistry and autoradiography plus ancillary biochemical methods was therefore

undertaken and included the following general aims: (1) a structural definition of the cestode surface and associated components, and (2) an assessment of the structure and properties so resolved as fitted for particular physiological functions.

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II.

MATERIALS AND METHODS

A. Biological:

Unless otherwise specified, the Hymenolepis diminuta used in this study were obtained from 10-day old laboratory infections of Sprague-Dawley albino rats, maintained as described previously (Read, et al., 1963). The rats were killed by a sharp blow on the head and the worms flushed from the excised gut in the 25 mM tris-(hydroxymethyl) amino methane-maleate buffered Krebs-Ringer saline (KRT) of Read, et al. (1963). Specimens of Lacistorhynchus tenuis and Calliobothrium verticillatum were collected from naturally infected dogfishes (Mustelus canis) at Woods Hole, Massachusetts.

B. Electron microscopy:

Fixation of diced strobila or whole worms was carried out in ice cold 1% osmium tetroxide buffered at pH 7.4 according to Millonig (1961), 2% acrolein or 3% glutaraldehyde in a 0.06 or 0.09 M monobasic sodium phosphate-sodium hydroxide buffer at pH 7.2 to 7.4, for 60 to 90 minutes. Aldehyde-fixed tissues were washed thoroughly in Millonig's (1961) phosphate buffer and subsequently postfixed 90 minutes in osmium-phosphate. Tissues were rapidly dehydrated in ascending concentrations of cold ethanol (to 95%), then allowed to reach room temperature. Upon completion of dehydration in 100% ethanol and propylene oxide, the tissues were embedded in epon (Luft, 1961), eponaraldite (Mohlenhauer, 1964) or a mixture

of Dow epoxy resins according to the method of Lockwood (1964). Sections were cut on a Sorvall MT-1 or MT-2 ultramicrotome. Those displaying a light gold or silver interference color were collected onto copper grids and treated with aqueous uranyl acetate (Watson, 1958), lead citrate (Reynolds, 1963), or a combination thereof (5 minutes in uranyl acetate followed by 5 minutes in lead citrate with brief washing in carbonate-free glass distilled water). This material was examined in an RCA EMU 3F electron microscope operated at 50 KV.

C. Light microscopy and histochemistry:

Thick (0.5 to 1.0 μ) sectioned epoxy embedded tissues were mounted on glass microslides and stained with methylene blue and azure II, after the method of Richardson, et al. (1960), or a modified periodic acid-Schiff (PAS) technique. According to the latter, mounted sections were subjected to mild oxidation in 1% aqueous periodic acid at room temperature for 10 minutes, rinsed in distilled water, then incubated 30 minutes in the Schiff's reagent. The latter was prepared by adding 34 cc of 7.4% sulfurous acid (reagent strength) to 250 cc of 0.2% aqueous basic fuchsin. This solution was decolorized with norit prior to use. Some sections were counterstained with methylene blue and azure II (Richardson, et al., 1960) after applying the PAS reaction. Other epoxy embedded sections were stained by the Feulgen reaction for DNA. Mounted 1 μ sections were rinsed briefly in cold 1 N HCl, then incubated 35 minutes at 60 C in 1 N HCl. Following rinses in cold

1 N HCl and distilled water, the slides were placed in the fuchsin-sulfurous acid Schiff's reagent for 3 hours. Some of the latter were lightly counterstained with methylene-blue and azure II.

Tissue was also fixed in cold phosphate buffered 10% formalin (pH 7.2) 30 minutes to overnight, rinsed briefly in buffer, and quick frozen on dry ice or by quenching in cold (-78 C) acetone. Sections 6 to 10 μ thick were collected on a Harris microtome cryostat at - 20 C. Mounted sections were stained by the PAS reaction as described above, except that periodate oxidation was held to 5 minutes; certain of the latter were first incubated 60 minutes at 37 C in media containing 1% malt diastase (Calbiochem), 0.1% emulsin (Sigma) in 0.05 M phthalate buffer (pH 5.4), incubation in diastase followed by incubation in emulsin and vice/versa, or distilled water alone. Other cryostat sections were stained with Alcian blue at pH 3.6 and 2.0, 0.1% aqueous toluidine blue or 0.01% azure A (in 30% ethanol) at pH 4.0, 3.0 or 1.5 (Mowry, 1963; Barka and Anderson, 1963). In each case, the pH was adjusted with dilute acetic acid.

Fresh frozen sections were incubated for periods up to 60 minutes duration at 37 C in one of the following media: pronase (Calbiochem), 30 mg/50 ml 0.05 M tris-maleate (pH 8.3); trypsin (Nutritional Biochemical Corp.), 0.5% in 0.05 tris-maleate (pH 7.6); pepsin (Nutritional Biochemical Corp.), 0.5% in 0.1 M glycine-HCl (pH 2.0); papain (Difco) 0.5% in 0.05 M tris-maleate (pH 6.8), containing 1 mM cysteine and 1 mM ethylenediamine tetracetic acid (EDTA). Other sections were incubated in the respective buffers

alone. Following the incubation period, the slides were rinsed briefly in cold 10% neutral formalin, then either stained lightly with dilute methylene blue and mounted in glycerine, or examined directly.

D. Autoradiography:

1. General:

All experiments utilized 10-day old H. diminuta removed from hosts allowed to feed ad libitum until sacrifice. Unless specified otherwise, all worms were preincubated 30 minutes in beakers of KRT at 37 C in a shaking water bath. Incubations of individual worms were carried out at 37 C in stoppered 25 ml Erlenmeyer flasks charged with CO₂- N₂ (15:85) and containing 2 ml of the particular tris-maleate buffered medium (see below). Final pH of all media ranged from pH 6.8 to 7.2. Following the incubation period, the worms were removed from the flasks and plunged into cold fixative. Tissue destined for embedding in epoxy resins was extracted with several changes of 70% ethanol overnight at room temperature before dehydration was completed in ethanol and propylene oxide.

Sections mounted on glass microslides were dipped in 100% ethanol, allowed to air dry, coated with a liquid photographic emulsion (Kodak NTB-2 at 40 C), and stored in dessicant-containing, light-proof slide boxes for an appropriate exposure period (see below). The autoradiographs were developed in Kodak "Dektol" for 2 minutes, rinsed in a dilute acetic acid "stop", fixed, and cleared for 10 minutes in hypo. Subsequently, some slides were briefly

placed in 0.02 N sodium hydroxide to remove the gelatin (after the method of Revel and Hay, 1961) and stained with methylene blue and azure II (Richardson, et al., 1960).

Grain counts were made with the aid of an ocular reticule mounted in a 12X periplanatic eyepiece in conjunction with a Leitz "Ortholux" fitted with a 100X planapochromat objective. Background radiation was determined by counting several standard fields immediately adjacent to the tissue sections, or by counting the number of exposed grains over non-radioactive sections prepared in the same manner as the autoradiographs.

2. Incorporation of H_3 - proline and H_3 -leucine:

Following preincubation for 30 minutes in KRT, individual worms were transferred to the KRT- CO_2 incubation medium containing 15 μC of the tritium label (either L-proline 3-4 H_3 , with a specific activity of 5.0 curies/millimole, obtained from New England Nuclear Corporation, or L-leucine- H_3 , specific activity 0.5 curies/millimole, from Schwartz Biochemicals). At intervals of 5, 30 and 60 minutes worms were removed from the flasks and fixed in cold phosphate-buffered osmium tetroxide. In other experiments, worms were incubated 5 minutes in the tritium-containing medium (hereafter referred to as a 5 minute "pulse") then transferred to a KRT- CO_2 medium containing the corresponding non-labeled amino acid at a 1 mM concentration for periods of 30, 60 and 120 minutes (hereafter referred to as a "cold chase"). In certain cases, the preincubation and incubation media also contained 50 $\mu g/ml$ puromycin

dihydrochloride (Nutritional Biochemicals Corporation). All tissues were embedded in epon-araldite (after the method of Mollenhauer, 1964) and sectioned at 0.5 to 1.0 μ on a Sorvall ultramicrotome.

3. Incorporation of H_3 -glucose and H_3 -galactose:

After 30 minutes preincubation in beakers of KRT, individual worms were transferred to the KRT-CO₂ mixture containing 25 μ C D-glucose-6- H_3 (68.8 mc/mM, New England Nuclear Corporation) or 25 μ C D-galactose-1- H_3 (50.0 mc/mM, New England Nuclear Corporation) for periods of 5, 30 and 60 minutes. For a second group of worms, the preincubation and incubation media also contained puromycin (50 μ g/ml). At the indicated time intervals, worms were removed from the flasks and fixed in phosphate buffered acrolein (90 minutes), osmium tetroxide (90 minutes) or 10% formalin (overnight). Tissues fixed in acrolein were routinely post fixed 90 minutes in cold phosphate-buffered osmium. Acrolein and osmium fixed tissues were subsequently embedded in epon-araldite and sectioned at 0.5 to 1.0 μ . The formalin fixed tissues were frozen in acetone at -78 C and sectioned at 6 to 10 μ on a Harris microtome cryostat. Certain of these sections were incubated 60 minutes in 1% malt diastase (Calbiochem) or distilled water at 37 C. In addition, sections from worms exposed to H_3 -galactose were also incubated in 0.1% emulsin (Sigma) (in a 0.05 M phthalate buffer at pH 5.4), emulsin followed by diastase and vice/versa. Most of the cryostat and epoxy embedded

sections were stained with the PAS reaction before applying the photographic emulsion. Exposures were carried out in refrigerated, dessicated, light-proof slide boxes for 1 to 4 weeks.

4. Incorporation of S-35 sulfate:

Following 30 minutes preincubation in KRT, worms were incubated under CO₂- N₂ in a medium of KRT containing 10 μ C S-35 sodium sulfate (specific activity approximately 20 μ C/mM, obtained from New England Nuclear Corporation) for periods of 5, 60 and 120 minutes. Worms were fixed in phosphate buffered acrolein or osmium tetroxide and embedded in epon-araldite. Some of the 1 μ sections were stained by the PAS reaction prior to coating with the photographic emulsion. The autoradiographs were allowed to expose for periods up to 3 months before developing.

5. Incorporation of H₃-thymidine:

After preincubation for 30 minutes in KRT, individual worms were exposed to 16.5 μ C thymidine-methyl H₃ (2 curies/mM, New England Nuclear Corporation) for periods of 60 and 120 minutes. Worms were fixed in cold phosphate buffered osmium tetroxide for 90 minutes or 10% formalin for 30 minutes. Osmium fixed tissues were subsequently embedded in epon-araldite. Formalin fixed tissues were quick frozen on dry ice then sectioned at 6 to 10 μ in a Harris microtome cryostat. The sections were mounted on albuminized microslides and incubated 2 hours at 37 C in 0.1% DNase (Sigma) in 0.003 M magnesium sulfate (pH 6.8), 0.2% RNase (Sigma) in distilled water (pH 6.4), or in distilled water. All sections were

subsequently extracted 10 minutes in cold (4 C) 5% trichloroacetic acid. Autoradiographs were exposed for 3 to 7 days. Some of the plastic embedded sections were subsequently stained with methylene blue and azure II by the method described previously.

E. Chemical determinations:

The effect of puromycin on net glycogen synthesis was measured in worms taken from hosts starved 24 hours before sacrifice. These worms (5 per sample) were preincubated 30 minutes in KRT with or without puromycin (50 μ g/ml) at 37 C, then under CO₂-N₂ (15:85) in 10 ml of tris-maleate buffered saline (final pH 7.2) containing 10 mM glucose $\pm$ puromycin (50 μ g/ml). After 60 minutes, the worms were removed from the glucose containing media, blotted on filter paper, weighed, and placed in 70% ethanol. Five samples were removed from each of the preincubating media after 30 minutes immersion, weighed and also placed in ethanol. Glycogen was precipitated with ethanol from 30% potassium hydroxide digests of the worm carcasses, redissolved in 10 ml distilled water and determined as glucose equivalents by the anthrone method of Seifter, et al. (1950).

Following the above protocol, worms were also incubated under CO₂-N₂ in media containing 0.5 μ C uniformly labeled C₁₄-glucose in 1 mM non-labeled carrier, with or without puromycin (50 μ g/ml) for 60 minutes. Wet weights were recorded, and the tissue extracted overnight in 70% ethanol. The extracted worms

were homogenized in 10 ml of 70% ethanol per sample (5 worms each). Homogenates were extracted overnight with 2 changes of ether-ethanol (3:1), then with hot (80 C) 10% trichloroacetic acid for 10 minutes followed by 2 10-minute washes with trichloroacetic acid at room temperature. The preparation was then centrifuged for 30 minutes at 2,000 rpm and the pellet redissolved in 0.1 ml of 60% potassium hydroxide. The resulting suspension was then neutralized with 0.14 ml 60% perchloric acid then diluted to 2 ml with distilled water. One-half ml aliquots of each sample were pipetted onto shallow aluminum planchets, dried under infrared lamps and the radioactivity determined with a Nuclear Chicago gas flow Geiger Muller detector. Total protein in a second aliquot was determined by the method of Lowry et al. (1951).

III.

RESULTSA. General histology of the cestode integument:

Three primary zones may be distinguished in a cross section of the tapeworm proglottid. The most peripheral, forming a continuous layer around the circumference of the proglottid, corresponds to the cuticle of classical descriptions. The subcuticular zone, demarcated from the cuticle by a prominent basement layer, consists mainly of elongated cells oriented perpendicularly to the cuticle, circular, longitudinal and dorso-ventrally directed muscle fibers. The medullary zone contains the major organ systems of the tapeworm, including the excretory, nervous and reproductive structures, or in the immature proglottid, the somatic and germinative anlagen, and may be set off from the subcuticular zone by large longitudinal muscle fibers.

At the level of light microscopy (Figures 5 and 6), the free surface of the cuticle appears as a fringe of fine filamentous processes. This brush border is equivalent to the comidial layer described by Minckert (1905) and others in previous studies of the tapeworm surface. The cuticular matrix contains small granules which seem to be more numerous along the basal surface. Immediately beneath the subcuticular basement layer is a thin layer of muscle fibers. Constituting the predominant cell type of the subcuticular zone are somewhat flask-shaped cells whose long axes are oriented at right angles to the cuticle. These cells possess a prominent

basal, ovoid nucleus containing one or more nucleoli. Branched, tapering apical processes of these cells appear to project to the cuticular basement layer. The subcuticular cells do not form a continuous epithelium, but occur either singly or clustered in irregular trabecular arrays. Interdispersed between the definitive subcuticular cells are dorso-ventrally directed muscle fibers, extensions of medullary parenchymal cells, and amoeboid-appearing basophilic cells with a large nucleoplasm: cytoplasm ratio. The latter are especially numerous in the subcuticular and medullary zones of immature proglottids.

The topochemical properties determined for the integument of Hymenolepis diminuta are summarized in Table 1, and are in general agreement with the observations of other investigators who have studied the histochemistry of this and related tapeworm species (Monne, 1959; Hedrick and Daugherty, 1957; Waitz, 1963; Waitz and Schardein, 1964; Bogitsh, 1963). The staining qualities of the brush border, including (1) intense, diastase-resistant reactivity with Schiff's reagent following periodate oxidation, (2) orthochromatic or slight beta metachromatic azurophilia, and (3) the binding of thiazin or Alcian dyes at moderately low pH, meet Spicer's (1963) criteria for the presence of weakly acidic mucopolysaccharides. The lack of affinity for toluidine blue, azure A and Alcian blue at a pH less than 3.0 suggests that the basophilia of the cuticle may be due primarily to carboxyl groups rather than sulfuric acid residues which remain dissociated at much higher hydrogen ion concentrations. Esterification of sulfate

ions in the cestode integument could not be demonstrated autoradiographically after incubating worms for periods of 5, 60 and 120 minutes in KRT-CO₂ containing S35-labeled sodium sulfate, though under similar conditions there is considerable polysaccharide and protein synthesis in this tissue (Lumsden, 1965 a, b; this paper, Sections J and K). The properties of the cuticular matrix and subcuticular cells described in Table 1 can be attributed to the presence of neutral mucopolysaccharides or acid mucopolysaccharides whose otherwise potentially ionizable carboxylic, sulfonic or phosphoric groups are masked.

Evidence for the role of protein in the structure of the cuticular matrix was obtained from observations of the action of proteolytic enzymes on the tapeworm surface. Unfixed, cryostat sections of H. diminuta were mounted on microslides and incubated in buffered enzyme solutions or the buffers alone, and then examined microscopically. The results of these experiments are summarized in Table 2.

The differential activity of pepsin and trypsin on the tapeworm surface suggests a predominance of acidic proteins in the cuticular matrix. As reviewed by Bovey and Yanari (1960), pepsin preferentially hydrolyzes peptide bonds between dicarboxylic and aromatic amino acids, while the specificity of trypsin to "basic" peptide bonds (arginine-lysine) is marked (Desnuelle, 1960). Papain exhibits fairly broad substrate specificity (Dekker, et al., 1949) as does Streptomyces pronase; as shown by Nomoto, et al., (1960) the latter is capable of hydrolyzing a variety of peptide

bonds to the extent that the majority of amino acids constituting a given peptide chain may be liberated. The disintegrating effect observed in the glycine-HCl buffer may be due to activation of endogenous autolytic enzymes at low pH.

At the level of electron microscopy (Figures 7 and 8), the cuticle of all tapeworm species examined appears as a continuous layer whose free surface is projected as a series of tightly spaced finger-like processes morphologically resembling the microvilli comprising the brush border of numerous vertebrate and invertebrate epithelial cells. Vesicles and granules of variable size and consistency fill the moderately dense ground substance of the cuticular matrix. In aldehyde-fixed tissues, the microvilli frequently appear longitudinally striated due to the presence of fine tubules or filaments which extend the length of the microvillus. Mitochondria, whose size and abundance varied with the species of tapeworm examined, are present at all levels of the cuticle, but were most frequently observed in the proximal third of this structure, often adjacent to the basal membrane. Nuclei and lateral membranous partitions were not observed in the cuticular zone of the tapeworms utilized in this study.

The subcuticular basement layer (Figures 7 and 13) rests atop a narrow band of non-striated circular muscle fibers, which in turn lie superficial to bundles of smooth muscle fibers oriented parallel to the longitudinal axis of the proglottid (Figures 7 and 16). The basement layer may reach a thickness of 1.5 microns, and consists of a loosely packed lamina of fine, cross striated fibrils

TABLE 1

SOME TOPOCHEMICAL PROPERTIES OF THE CESTODE
 INTEGUMENT. THE DESIGNATIONS +, ++, AND +++ INDICATE
 A POSITIVE REACTION AND ITS RELATIVE INTENSITY, THE DESIGNATION -,
 A NEGATIVE REACTION

Reaction	Preparation	Brush border	Matrix	Subcut. cells
PAS	1	+++	+++	+++
Diastase, PAS	1	+++	+++	++
Emulsin, PAS	1	+++	+++	+++
Diastase, emulsin, PAS	1	+++	+++	++
Emulsin, diastase, PAS	1	+++	+++	++
PAS	2,3	+++	+/-	-
Toluidine blue, azure A, pH 4.0	1	+++	+++	+++
Toluidine blue, azure A, pH 3.0	1	++	+/-	-
Toluidine blue, azure A, pH 1.5	1	-	-	-
Alcian blue, pH 3.6	1	+	+	+
Alcian blue, pH 2.0	1	-	-	-
S-35 sulfate incorporation	3	-	-	-

1 - phosphate buffered formalin fixation, cryostat sections.

2 - phosphate buffered osmium fixation, epon embedded sections.

3 - phosphate buffered acrolein fixation followed by post-fixation in osmium-phosphate, epon embedded sections.

TABLE 2

THE EFFECT OF SOME HYDROLYTIC ENZYMES
ON THE STRUCTURAL INTEGRITY OF THE TAPEWORM CUTICLE.
A DISINTEGRATING EFFECT IS INDICATED BY THE DESIGNATIONS
+, ++, OR +++, A LACK OF SAME BY THE DESIGNATION --.

Medium	Effect
Pronase, 0.05 M tris-maleate, pH 8.3	+++
0.05 M tris-maleate, pH 8.3	-
Trypsin, 0.05 M tris-maleate, pH 7.6	+/-
0.05 M tris-maleate, pH 7.6	-
Pepsin, 0.1 M glycine-HCl, pH 2.0	+++
0.1 M glycine-HCl, pH 2.0	++
Papain, 5 mM cysteine, 1 mM EDTA, 0.05 M tris-maleate, pH 6.8	+++
5 mM cysteine, 1 mM EDTA, 0.05 M tris-maleate, pH 6.8	-
Diastase, H ₂ O	-
Emulsin, 0.05 pthalate, pH 5.4	-
Ribonuclease, H ₂ O, NaOH, pH 6.4	-

branching inwardly from the basement layer as part of the interstitial subcuticular and medullary stroma. At intervals, the basement membrane is perforated by tendrillar processes of the underlying subcuticular cells which appear to fuse with the basal cuticular membrane.

The elongate, flask-shaped subcuticular cells possess branching apical processes which extend between the bands of subcuticular musculature and basement layer to the base of the cuticle; at these points of abutment, the basal cuticular and subcuticular cell membranes are continuous (Figure 13), and the cytoplasm of the subcuticular cell merges with the cuticular matrix. Numerous mitochondria and vesicles are present in the apical processes of the subcuticular cells (Figures 23-25). The basal perinuclear cytoplasm (Figure 17) contains extensive, often dilated cisterne of granular endoplasmic reticulum and prominent complexes of smooth-surfaced Golgi membranes with associated vesicles. Electron opaque, osmiophilic inclusions of variable size, probably lipid droplets, occur in the perinuclear cytoplasm of these cells. Numerous dense granules approximately 150-200 A in diameter appear in the cytoplasm arranged in small packets or spiral configurations and presumably represent polyribosomes.

Interspersed among the subcuticular cells are processes of parenchymal cells residing primarily in the medullary tissues of the proglottid. These processes, which are packed with large rosettes of glycogen granules, project apicad, terminating at the

level of the subcuticular basement layer (Figures 5 and 23).

Rounded cells with large nuclei and cytoplasm dominated by aggregates of unattached ribonucleoprotein particles occur in the subcuticular zone near its junction with the medulla (Figures 46 and 47). Profiles of endoplasmic reticulum are sparse, appearing as dilated vesicular cisternae studded with ribosomes or tightly coiled whorls of granular membranes (Figures 48 and 49). The Golgi region is weakly developed and, where discernable, consists of a few vesicles or lamellae of smooth surfaced saccules. These cells closely resemble those comprising the medullary anlagen and are most frequently observed in the subcuticular and peripheral medullary tissues of proglottids lying at the anterior or mid-level of the strobila.

The morphology of the integument remains relatively constant over the entire length of the strobila. The numerical density of microvilli and cuticular mitochondria appears to be somewhat greater in the more anterior (or immature) proglottids. The cuticle of the scolex does not differ appreciably from that of the strobila, except in containing relatively fewer mitochondria and slightly shorter, more widely spaced microvilli. On the other hand, the acetabula, or suckers, in the anterior scolex of H. diminuta represent highly differentiated areas of the integument (Figures 31 and 32). In these structures, the musculature is well developed, and obviously homologous to the three layers observed in other regions of the integument. The transverse

bundles constitute the bulk of the acetabular tissue. The cuticle lining the acetabular crypts (Figure 31) is much thinner than that at the surface of the scolex (5.0 microns vs. 0.75 microns) and bears a reduced number of short (0.25 microns) microvilli. Numerous small mitochondria line the basal membrane of the acetabular cuticle. The basement layer is quite thin and amorphous, apparently lacking the fibrillar components characteristic of the subcuticular regions of the integument. The subcuticular cells of the scolex surface and acetabula are similar in their fine structure and relationship to the cuticular layer to those of the strobilar integument.

B. Fine structure of the apical and basal cuticular surfaces:

The microvilli lining the free surface of the cestode integument (Figures 7-12) are elongate cylindrical projections sharply attenuated at their distal extremities. The conical tip of each microvillus contains an extremely electron dense area immediately beneath the limiting membrane. The dimensions of the microvilli are subject to considerable interspecific variation; in H. diminuta the microvilli of the strobilar integument average 750 μ long by 160 μ wide at their bases, while in Lacistorhynchus and Calliobothrium the cuticular microvilli reach a length of over 2.0 microns. The medullary core of each microvillus is continuous with the cuticular matrix.

In tissues preserved with glutaraldehyde or acrolein and postfixed in osmic acid, numerous tubular or filamentous elements

can just be discerned in the periphery of each microvillus (Figures 11 and 12); the plasma membrane of the microvillus is separated from the zone of filaments by a distance of approximately 50 A. Each tubule, or filament, measures approximately 70 A in diameter, possessing an inner core 30 to 50 A thick. These structures appear to extend the length of the microvillus, continuing for an undetermined distance into the cuticular matrix. Much finer filaments, only poorly resolved in the present micrographs, appear to reside in the medullary portion of each microvillus. Short tubular profiles 175 to 250 A (Figure 14) in diameter are scattered throughout the cuticular matrix. Near the basal membrane, these tubules appear to be more strongly oriented, their longitudinal axes generally extending in a plane parallel to the transverse axis of the integument. These tubular structures appearing in the microvilli and cuticular matrix were only poorly retained, or not at all, in tissue fixed in osmium tetroxide alone.

The apical cuticular surface (Figures 9-12) is delineated by a continuous trilaminate membrane 85 to 100 A thick, consisting of two parallel opaque components separated by a less dense intermediate layer 30 to 35 A wide. In aldehyde-fixed material subsequently treated with osmic acid and stained with uranium and lead salts, the triple layered pattern of the microvillar surface is geometrically asymmetric, the thickness of the peripheral opaque layer measuring 25 to 35 A, and the opaque layer at the cytoplasmic surface 20 to 25 A. A fine, amorphous coating of low density can occasionally be seen applied to the outer surface of the unit

membrane.

The basal cuticular membrane (Figure 13) is a triple-layered structure measuring approximately 80 A thick, and follows a relatively straight course along the surface of the basement layer. Where the apical process of an underlying subcuticular cell meets the basal surface of the cuticle, there is a fusion of the plasma membranes (Figure 13) and the cytoplasm of the subcuticular cell and the cuticular matrix are continuous. Between the zones of subcuticular cell attachment, the basal membrane is frequently reflected as a small hillock approximately 200 μ in diameter (Figure 13); here, the basal membrane is markedly thickened (to 150 A) and coated with extremely fine filaments which radiate laterally and apically into the cuticular matrix. The crypt of each hillock is filled with a moderately dense, amorphous material which also lines the outer face of the remainder of the basal membrane. These specializations of the basal membrane most likely represent adhesive plaques or half desmosomes which provide for the secure attachment of the cuticle to the basement layer. Very rarely, in H. diminuta, the basal membrane may be thrown into a major cylindrical evagination approximately 0.75 microns in diameter enclosing a finely fibrillar matrix continuous with the basement layer (Figure 15). At these sites, the apical portion of the cuticle is reflected inwardly and approximates the projections of the basement layer, though the latter have never been observed in direct contact with the apical membrane or opening

onto the external surface. The nature or significance of these basal evaginations has not been ascertained.

C. The subcuticular basement layer:

The subcuticular basement layer (Figure 13) is a relatively thick (1.5 microns) heterogenous, extracellular zone lying between the basal cuticular membrane and sarcolemma of the circular subcuticular musculature. It is composed chiefly of fibrils measuring approximately 125 A in diameter, which are arranged at right angles to one another and loosely embedded in a hyaline matrix. In aldehyde-fixed tissues, fine vertical striations with a period of approximately 50 A can be resolved along the length of individual fibrils. Immediately apicad to the fibrillar zone is a thin (50 m μ) amorphous layer of increased density which parallels the course of the basal cuticular membrane. Morphologically, this layer closely resembles the "basement membrane" underlying epithelia in general. In such cases, the "basement membrane" is demonstrably rich in mucoproteins, and is considered to possess adhesive properties.

D. Mitochondria:

Mitochondria are relatively numerous in the cuticular matrix of Hymenolepis and Lacistorhynchus but are only occasionally encountered in the cuticle of Calliobothrium. In all three species, the cuticular mitochondria are chiefly confined to the medio-basal region, where, especially in Lacistorhynchus, they may be aligned in multiple tiers (Figure 8). The cuticular mitochondria of

Lacistorhynchus are sometimes branched; in section, the average dimensions of an ovoid profile are 1.2 by 0.6 microns, in contrast to the smaller (0.3 to 0.4 microns) cuticular mitochondria of Hymenolepis and Calliobothrium. The cuticular mitochondria are composed of a smooth outer membrane separated by a narrow, uniform space from a second inner membrane. The latter is plicated, forming the thin villiform cristae mitochondriales. These structures, which are characteristically sparse in the cuticular mitochondria of all three species examined, extend for variable distances into the inner matrix, their length seldom exceeding half the transverse diameter of the mitochondrion. The parallel membranes of a given crista are frequently thrown into somewhat regularly occurring angulations or undulations (Figures 8, 26, 50). A similar "zig-zag" configuration has been described for the cristae of several vertebrate and invertebrate mitochondria by Revel, et al. (1963) and Slautterback (1965). In tissues fixed in acrolein or glutaraldehyde, the mitochondrial matrix appears slightly less dense than the ground substance of the surrounding cuticular matrix, and usually contains a few very small granules, probably precipitates of calcium phosphate. The mitochondria of the juxtannuclear and apical cytoplasm of the definitive subcuticular cells do not differ morphologically from those of the cuticle. By contrast, the mitochondria occurring in the glycogen-filled medullary cells and their apical subcuticular extensions are larger, and contain relatively longer and more numerous cristae.

E. The subcuticular cell nucleus:

The interphase nucleus of the definitive subcuticular cell (Figure 18) is typically located toward the basal pole, and tends to have a somewhat irregular outline. In H. diminuta, the diameter of the nucleus ranges between 3.0 and 4.5 microns, and in Lacistorhynchus, 3.0 to 6.0 microns. The nucleus is enveloped by a double membrane, whose inner and outer components are separated by a perinuclear space averaging 125 A wide. At intervals about the circumference of the nuclear envelope, the inner and outer membranes fuse, forming a pore approximately 600 A in diameter (Figure 18). What appears to be a thin membrane, roughly half the thickness of the nuclear envelope, can be seen interdicting the fenestral aperture. These "septa" may in reality represent images of the lower halves of the nuclear pores, in which event the nuclear pores would be without true structural interception. The outer nuclear membrane is studded with dense 150 A particles, presumably ribosomes. Cisternae of granular endoplasmic reticulum are occasionally seen in direct continuity with the outer nuclear membrane (Figure 18).

The subcuticular cell nucleus contains one or more prominent nucleoli (Figure 18). These morphologically heterogeneous structures consist of a finely textured, amorphous, electron opaque central area surrounded by numerous granules of uniform size (150 A in diameter) and density which comprise the bulk of the nucleolar mass. These cortical components closely resemble

the cytoplasmic ribosomes, whose synthesis is believed to occur within the nucleolus (see review of Stevens, 1964). The sparse chromatin of the subcuticular cell nucleus appears mainly as peripheral condensations immediately adjacent to the inner nuclear membrane.

F. The endoplasmic reticulum of the definitive subcuticular cell:

The basal, para- and much of the supranuclear cytoplasm of the definitive subcuticular cell is dominated by elongate, branching profiles of the endoplasmic reticulum (Figures 17, 18, 23) whose outer surfaces are studded with closely spaced granules measuring 150 A in diameter. The cisternae are most often arranged in loosely parallel arrays which are generally directed along the longitudinal axis of the cell, though never attaining the degree of orientation observed, for example, in the exocrine cells of mammalian pancreas. Circular or oblong, rough surfaced profiles with an average diameter of 675 m μ are encountered with high frequency, suggesting that much of the endoplasmic reticulum is disposed as a tubular network. Particularly in the subcuticular cells of the more anterior, or immature, proglottids, the cisternae may be greatly dilated, reaching a diameter of more than 1.5 microns (Figures 17 and 18). Such expanded regions of the endoplasmic reticulum are often encountered directly adjacent to the Golgi apparatus and are continuous with the more lamellar or tubular granular reticular profiles. In aldehyde fixed tissues,

the lumen of the endoplasmic reticulum and the perinuclear space of the nuclear envelope are filled with an amorphous material of low to moderate density (Figures 17 and 18). Occasionally, direct continuity between the membranes of the endoplasmic reticulum and the outer membrane of the nuclear envelope is observed (Figure 18). The intercisternal ground substance of the subcuticular cell contains numerous 150 to 200 A particles, often grouped into rosettes, spiral or circular configurations. These granules, like those attached to the membranes of the endoplasmic reticulum and outer nuclear membrane, have a high affinity for aqueous uranyl acetate and therefore are believed to represent polyribosomes.

G. Golgi apparatus and vesicles:

The Golgi complex (Figures 17, 19-22) of the definitive subcuticular cell is located in the juxtannuclear cytoplasm, and is typically subdivided into a number of discrete zones. These subdivisions of the Golgi apparatus consist of several smooth-surfaced, flattened, parallel sacs (Figure 19) surrounded by numerous vesicles of varying dimensions, and frequently appear in intimate association with the ribosome-studded cisternae of the endoplasmic reticulum. Immediately adjacent to the Golgi zone, small outpocketings of agranular regions of the endoplasmic reticulum have been observed (Figure 21). The evaginations of the cisternal membranes appear thickened and coated with a layer of extremely fine bristles approximately 200 A long. Vesicles

appearing in the Golgi zone range from 40 to 85 A in diameter and typically possess a thickened inner cortex (300 to 400 A) surrounded by a thinner, denser limiting membrane from which radiate filaments (200 A long) closely resembling those coating the evaginated tips of cisternal membrane (Figures 19-22).

Mitochondria are usually concentrated about the periphery of the Golgi zones (Figure 17). Coated vesicles occur throughout the supranuclear cytoplasm (Figures 23-25) and are especially numerous in the apical processes leading to the base of the cuticle. Here also, elongate mitochondria are characteristically present in close approximation to the vesicular aggregates. Spherical, membrane-bound vesicles of variable size (60 to 125 μ in diameter) and consistency fill the cuticular matrix (Figures 8 and 26). Some of these cuticular vesicles, particularly those located in the basal region, display three distinct layers morphologically similar to those comprising the vesicles of the subcuticular Golgi zones and apical cytoplasm, while others appear devoid of the bristle coat. The density of the cuticular vesicles varies according to the distance from the basal cuticular membrane (Figure 8) suggesting a marked concentration, perhaps via dehydration, of vesicular contents with increasing proximity to the apical membrane.

H. Other cytoplasmic inclusions:

The glycogen of the tapeworm integument is contained within the apical processes of the deeper lying medullary parenchymal cells and subcuticular musculature. Little, if any,

of this polysaccharide appears in the definitive subcuticular cells of the strobila or the cuticular matrix, though the subcuticular cells of the acetabular integument may contain significant quantities of glycogen particles. At intermediate magnifications, glycogen in the periphery of the muscle fibers appears chiefly as single, roughly spherical granules measuring 20 to 40 μ in diameter (Figures 16, 31-33), while that occurring in the medullary parenchymal cells is typically organized as 60 to 200 μ rosettes comprised of smaller granules approximately 20 to 30 μ in diameter (Figures 32, 33 and 47). Intermediates between the typical compact rosettes and single granules were often observed in parenchymal cell processes immediately adjacent to the muscle fibers or the definitive subcuticular cells (Figure 33). Here, varying numbers of 20 to 40 μ granules were frequently assembled as short, linear aggregates rather than occurring singly or as components of larger rosettes. These structures closely resemble polyribosomes, and are distinguishable from clusters of ribonucleoprotein particles mainly by their size and lack of affinity for aqueous uranyl acetate. The greatest amounts of glycogen in the tapeworm proglottid are concentrated in the more centrally located medullary parenchymal cells, occurring predominantly in the rosette configuration, though single granules or granules associated in smaller clusters are also encountered.

Ovoid or more irregular, homogeneously dense osmiophilic inclusions measuring up to 2.5 microns in diameter are frequently

encountered in the juxtannuclear cytoplasm of the definitive subcuticular cells and the medullary parenchymal cell processes (Figures 19, 23, 47). These structures, which probably represent lipid droplets, are particularly numerous in the subcuticular and medullary cells of mature proglottids.

I. Origin and differentiation of the subcuticular cells:

Histological comparison of various levels of the tapeworm strobila reveals that the relative number of subcuticular cells per unit area in the integument of the mature proglottids does not differ appreciably from the density of subcuticular cells occurring in the anterior segments, nor is there a significant difference in cell size. Thus, with growth of the proglottid, the number of subcuticular cells must increase either by multiplication in situ or through the addition of new cells from non-integumentary sites of proliferation.

As it is generally accepted that DNA is replicated in the interphase nucleus just prior to the onset of mitosis, DNase labile nuclear incorporation of tritiated thymidine was used as an index of mitotic activity in the tissues of H. diminuta. In autoradiographs prepared from worms incubated 60 or 120 minutes in KRT containing tritiated thymidine, the nuclei of cells constituting the medullary anlagen, the germinal epithelia of the reproductive organs, and the amoeboid-like cells occupying the periphery of the medullary and subcuticular zones were intensely labeled (Figures 51-54). The nuclei of the definitive subcuticular cells failed to

incorporate tritium during the course of these experiments (Figure 54), though radioactivity was detected in the cytoplasm of these cells and in the cuticular matrix. Incubation of formalin-fixed, cryostat sections with DNase prior to application of the photographic emulsion reduced all nuclear labeling to background levels, while similar treatment with RNase was without effect. It can be concluded from these observations that intranuclear radioactivity following exposure to tritiated thymidine represents newly synthesized DNA, and that cells so labeled are undergoing mitotic division. Conversely, the apparent lack of nuclear DNA synthesis in the definitive subcuticular cells at all levels of the strobila is taken as evidence for the absence of mitotic activity among members of this particular cell population. Radioactivity appearing in the subcuticular cell cytoplasm and the cuticular matrix was unaffected by both nucleases. It is suggested that this labeling represents incorporation of metabolites derived from the degradation of tritiated thymidine into non-nucleic acid components.

Electron microscope study of thin sections taken at various levels of the strobila strongly suggest that the definitive subcuticular cells are derived from embryonic stem cells originating in the medullary tissues of the proglottid. Cells comprising the central, medullary anlagen of immature proglottids as well as those forming small groups in the medullary and basal subcuticular zones throughout the strobila possess a fine structure characteristic of non-differentiated, mitotically proliferating

cells (Figures 46 and 47). The cytoplasm is filled with numerous clusters of polyribosomes, while the endoplasmic reticulum is represented only by the nuclear envelope or occasional, irregularly shaped, ribosome covered vesicular cisternae. The large, central, somewhat eccentric nucleus contains scattered clumps of chromatin, and is surrounded by a nuclear envelope with many adherent ribonucleoprotein particles. A few ovoid mitochondria with sparse, villiform cristae are present in the peripheral cytoplasm. A Golgi region is not apparent, nor are such inclusions as glycogen or lipid droplets.

Solitary cells, frequently dispersed among the definitive subcuticular cells attached at the base of the cuticle, differ from the preceeding primarily in the appearance of the endoplasmic reticulum . In such cells, tightly spiraled whorls of ergastoplasmic membranes occur in cytoplasm otherwise dominated by clusters of free ribosomes (Figures 48 and 49). Occasional tubular profiles of granular endoplasmic reticulum, which in some cases are continuous with the lamellar, concentric arrays of membranes, have also been observed in these cells. In yet more differentiated cells (Figure 50), the scattered circular cisternae and whorled configurations are superseded by predominately tubular or lamellar ribosome-studded profiles. Smooth-surfaced saccules and vesicles of the Golgi apparatus are also distinguishable in the juxtannuclear area. These cells have assumed a more elongate

outline, with the apical poles directed toward the base of the cuticle, and most likely constitute precursors to the definitive subcuticular cells joined to the basal cuticular membrane.

The fine structure of the definitive subcuticular cell is consistent with a function in the synthesis and subsequent export of protein. The fusion of the open necks of the apical processes with the base of the cuticle would facilitate direct exchange of materials between the subcuticular cytoplasm and cuticular matrix. Moreover, though rich in structural and enzymic proteins, as revealed histochemically, the cuticle contains none of the structures usually associated with protein synthesis, such as ribosomes.

In an attempt to elucidate further the functional relationships of the subcuticular cells to the tapeworm surface, experiments designed to follow the cytological course of incorporation and ultimate fate of precursors to specific macromolecules involved in the structure of the integument were performed.

J. Amino acid incorporation:

Studies on the uptake and subsequent metabolism of amino acids in H. diminuta (Graff, et al., 1965; Kilejian, personal communication) indicate that much of the exogenous proline absorbed over a period of two hours is incorporated directly into protein; following incubation of worms in C_{14} -labeled proline negligible radioactivity appears in glycogen (the major polysaccharide fraction of H. diminuta), lipids or nucleic acids.

These studies also show that in two hour incubations, absorbed proline not incorporated into peptides is neither metabolized to any significant extent but remains in the free amino acid pool. Therefore, the tritiated isotope of this imino acid is an excellent agent with which to identify autoradiographically cytological sites of protein synthesis.

A general survey of the time course of incorporation of label in the cortical tissues of H. diminuta was conducted at the level of light microscopy. Tapeworms were incubated in KRT-CO₂ containing trace quantities of tritiated proline with a high specific activity for periods of 1, 5, 30 and 60 minutes. The worms were then fixed, dehydrated, embedded in epon and the autoradiographs prepared of the sectioned tissues. The results of these experiments are summarized in the following paragraphs and in Figure 2.

1. One minute incubations:

In some cases, a small number of exposed silver grains were randomly scattered across the proglottid; statistically, such counts were not significantly greater than background. In view of the high solubility of proline in aqueous media and even absolute alcohol, most of the free proline, as well as other free amino acids and low molecular weight peptides should have been extracted during fixation and preparation of the tissue for plastic embedding. However, it seems possible that the use of osmic acid as a fixative might result in some non-specific binding of

certain free amino acids (Caro and Palade, 1964). From the recent literature on protein synthesis, it would appear that the polymerization of amino acids into polypeptides occurs rapidly (see reviews of Zamecnik, 1960, and Fruton, 1963). Siekevitz and Palade (1960) were able to detect considerable radioactivity in alpha-chymotrypsin isolated from guinea pig pancreas one minute after the administration of C_{14} -leucine, while Warshawsky et al. (1963) could demonstrate autoradiographically the incorporation of tritiated leucine in mouse pancreas two minutes after injection of the labeled amino acid. In the present study, the use of thin (0.5 to 1.0 micron) sections may have precluded reliable detection of the small amounts of non-extractable peptides synthesized during the sixty seconds incubation with the labeled imino acid.

2. Five minute incubations:

Cortical labeling was concentrated in the cytoplasm of the subcuticular cells. The few grains appearing over the nuclei and cuticular matrix could be attributed to background radiation. A higher frequency of exposed grains was apparent in the basal portion of these cells. Radioactivity in the medullary tissues was confined to occasional spindle-shaped basophilic cells situated between the parenchymal cells, the vitellaria, the epithelium of the excretory ducts, and, in immature proglottids, the cells of the germinative and somatic anlagen. Labeling in the cortical regions of the proglottid was generally more intense in autoradiographs of tissue from the anterior region of the strobila.

This might have been due to (1) differential rates of amino acid absorption along the strobila, (2) a higher rate of protein synthesis in the subcuticular cells of the immature proglottids, or (3) a combination of these alternatives.

3. Thirty minute incubations:

Radioactivity in the subcuticular cells was intensified. Labeling of the cuticular matrix was apparent.

4. Sixty minute incubations:

Cuticular radioactivity was markedly increased from that observed at 30 minutes. Elevation in subcuticular labeling was also apparent, but did not follow a concomitant linear progression from 30 minute levels (Figure 2).

The delayed appearance of radioactivity in the cuticle could be explained as reflecting either a difference in synthetic rate between the subcuticular cells and the cuticle, or a translocation of material from subcuticular sites of precursor incorporation to the cuticular matrix. To resolve this question, tapeworms were incubated five minutes in KRT-CO₂ containing trace quantities of tritiated proline with a high specific activity (pulse incubation) then either fixed immediately or transferred to a medium containing 1 mM non-labeled proline for periods of 30, 60 and 120 minutes (chase incubation). The results of these experiments are summarized in the following paragraphs and in Figure 1.

5. Five minute pulse in tritiated proline, no chase
(Figure 27). See paragraph 2 above.

6. Five minute pulse in tritiated proline followed by a 30 minute chase with non-labeled proline:

Most of the exposed silver grains were still localized over the subcuticular cells. However, while the label appeared primarily in the basal region of these cells following five minutes incubation in tritiated proline, after a 30 minute chase with non-labeled proline radioactivity was concentrated in the supranuclear cytoplasm.

7. Five minute pulse in tritiated proline followed by a 60 minute pulse with non-labeled proline (Figure 29):

The relative distribution of exposed silver grains over the cuticle and subcuticular cells was essentially equivalent. Radioactivity in the subcuticular cells was frequently concentrated in the apical cytoplasm.

8. Five minute pulse in tritiated proline followed by a 120 minute chase with non-labeled proline (Figure 30):

Over 66% of the radioactivity in the cortical tissues was localized over the cuticular matrix. Most of the radioactivity remaining in the definitive subcuticular cells was restricted to the apical cytoplasm.

9. Effect of puromycin on amino acid incorporation:

The work of Yarmolinsky and de la Haba (1959) and many subsequent studies indicate that puromycin is a potent and specific inhibitor of protein synthesis. There is now considerable evidence that puromycin substitutes directly for aminoacyl-sRNA as an

acceptor of the carboxyl-activated peptide bound to the ribosome-template RNA complex, resulting in the release of incomplete peptide chains containing puromycin as the N-terminal component (Allen and Zamecknik, 1962; Nathans, 1964). To substantiate the identity of the tritium labeled material in the cestode integument following incubation in tritiated amino acids, the effect of puromycin on the incorporation of such radioactivity was ascertained. Tapeworms were preincubated 30 minutes in KRT-CO₂ containing 50 µg/ml puromycin dihydrochloride, then transferred to media containing puromycin and tritiated proline, tritiated leucine or uniformly labeled C₁₄-glucose. After five minutes in the tritiated amino acid-puromycin mixture, the worms were fixed and sectioned for autoradiography. Worms placed in the C₁₄-glucose-puromycin medium were homogenized in 70% ethanol after 60 minutes incubation, and radioactivity in the isolated protein fraction determined instrumentally. Controls were incubated in media lacking puromycin.

As seen in the tissue section autoradiographs (Figure 28), in the presence of puromycin subcuticular incorporation of tritiated proline and leucine was reduced by 79% and 71% respectively, while the specific activity of the total protein fraction isolated from worms incubated in uniformly labeled C₁₄-glucose and puromycin was reduced by 73%. In H. diminuta, there is some conversion of alpha-keto acids derived from the degradation of glucose to the corresponding amino acids, and subsequent incorpor-

ation of a portion of the latter (mainly alanine) into protein (Graff et al., personal communication). It has been demonstrated elsewhere (Lumsden, 1965a; this paper, Section K), that puromycin has no effect on the incorporation of exogenous glucose and galactose into polysaccharides. Thus, puromycin appears to selectively block the incorporation of pool amino acids, derived either from endogenous metabolism or absorbed directly from the incubation medium, into polypeptides. It seems warranted to conclude, therefore, that labeling of subcuticular cells following short term incubation in puromycin-free media containing tritiated proline is probably indicative of protein synthetic activity.

Histochemical studies have indicated significant quantities of polysaccharide in the cestode integument (Table 1). In an attempt to study cytologically the synthesis of these macromolecules, the incorporation of probable precursor monosaccharides was followed in tissue section autoradiographs.

K. The incorporation of monosaccharides:

1. The effect of puromycin on polysaccharide synthesis:

Metabolic studies on the distribution of radioactive carbon in H. diminuta following short term exposure to uniformly labeled C₁₄- glucose have indicated that most of the 70% ethanol insoluble radioactivity is in polysaccharide (Graff, et al., personal communication). However, in the present investigation it was important for interpretation of the tissue section auto-

radiographs that radioactivity in polysaccharide be differentiated from any possible labeling of tissue proteins; in H. diminuta some pyruvate from the degradation of glucose is converted to alanine (Graff et al., personal communication) and the incorporation of amino acids into protein occurs at very high levels in certain tapeworm cell types (Lumsden, 1965 b; this paper, Section J). As shown in the previous section and Lumsden (1965 b), puromycin is a potent inhibitor of amino acid incorporation in tapeworms. Therefore, its effect on polysaccharide synthesis was assayed biochemically in order to evaluate its possible use in differentiating between protein and polysaccharide synthesis in subsequent autoradiographic studies.

As indicated in Table 3, puromycin at concentrations of approximately 1 mM appeared to have little effect on net glycogen accumulation in H. diminuta during short term exposures in vitro, though under similar conditions the incorporation of radioactive proline, leucine and amino acids derived from glucose metabolites into protein is inhibited up to 70 or 80% (Lumsden, 1965 b; this paper, Section J).

2. Histological distribution of tritiated glucose incorporation:

Following five minutes incubation in radioactive glucose, labeling in tissue section autoradiographs was slight and essentially restricted to the medullary musculature. After 30 minutes (Figures 34 and 38), radioactivity in the spermatozoa, subcuticular and medullary musculature was particularly intense.

Labeling in the subcuticular tissues (other than the musculature) was moderate and slightly greater than in the medullary parenchymal cells, while the number of exposed silver grains appearing over the cuticle was barely above background. In the 60 minute samples (Figures 35 and 37), general parenchymal incorporation of radioactivity was substantially increased, though the musculature remained more heavily labeled. However, labeling in the latter tissue was markedly reduced from that seen at 30 minutes. Radioactivity in the subcuticular cells and cuticular matrix was substantially increased from 30 minute levels. In view of the high glucose absorption rate determined by Phifer (1960) for H. diminuta, most of the glucose in the medium is probably absorbed during the first 30 minutes incorporation, and, as seen in the autoradiographs, preferentially incorporated into the muscle tissues and spermatozoa. The sharp decline in grain counts over the muscle tissue between 30 and 60 minutes can be accounted for by dilution of the radioactive glucose with endogenous glucose (from stored polysaccharide) as muscle glycogen is degraded and resynthesized.

Thirty minutes equilibration in puromycin-KRT followed by incubation in a medium containing puromycin and radioactive glucose had no apparent effect on the distribution or magnitude of incorporation of the tritium label in the preferentially labeled spermatozoa, subcuticular and medullary musculature, and integumentary tissues (Figures 39-41), though grain counts of

TABLE 3

NET ACCUMULATION OF GLYCOGEN
BY HYMENOLEPIS DIMINUTA FOLLOWING 24 HOURS
HOST STARVATION (EACH VALUE REPRESENTS THE
MEAN OF 5 SAMPLES)

Group	μg glucose-glycogen/mg tissue wet weight
30 minutes preincubation in KRT only	3.2
+ 60 minutes incubation in 10 mM glucose-KRT	11.4
30 minutes preincubation in KRT + 50 $\mu\text{g}/\text{ml}$ puromycin	2.8
+ 60 minutes incubation in 10 mM glucose-KRT + 50 $\mu\text{g}/\text{ml}$ puromycin	10.2

the medullary parenchymal tissues averaged approximately 15% less in worms exposed to puromycin. These observations are consistent with the lack of an effect of puromycin on net polysaccharide synthesis in this cestode (Table 3) and indicate that at least in the musculature, integument and reproductive tissues, radioactive precursors administered as tritiated glucose were incorporated mainly into non-protein macromolecules.

Activity in the musculature, spermatozoa and medullary parenchymal cells was significantly diminished in cryostat sections digested with diastase prior to the preparation of the autoradiographs as compared with alternate control sections placed only in distilled water, indicating that most of the incorporated radioactivity in these particular tissues was initially in the glycogen fraction. On the other hand, diastase removed only 30% of the total incorporated radioactivity from the subcuticle (exclusive of the musculature) and had no effect on the amount of radioactivity demonstrable autoradiographically remaining in the cuticular matrix.

3. Incorporation of galactose (Figures 3,4, 42-45):

In contradistinction to the distribution of exposed silver grains seen in autoradiographs after short term incubations in tritiated glucose, the largest portion of incorporated radioactivity following comparable exposures to tritiated galactose was localized in the integumentary tissues. Little incorporated radioactivity could be detected in tissue sections after five minutes incubation, though after 30 minutes, tritium was concentrated

in the subcuticular cells and spermatozoa, and labeling of the medullary parenchymal cells and cuticular matrix was significant. In the 60 minute samples, radioactivity incorporated by the medullary parenchymal and subcuticular cells had increased by 30 and 13 percent respectively, while cuticular labeling was nearly doubled. Much of the latter appeared to be associated with the region of the brush border. By 60 minutes, labeling of the musculature had risen substantially, the grain counts averaging 58% above 30 minute levels. As was the case after exposure to tritiated glucose, most of the radioactivity incorporated by the musculature, medullary parenchymal cells and spermatozoa following incubation of worms in tritiated galactose could be removed with diastase, while only 30% of the label incorporated by the subcuticular tissues (exclusive of the subcuticular musculature) proved diastase-labile. Radioactivity incorporated into the cuticular matrix was insensitive to diastase hydrolysis (Figure 45). Treatment of sections with emulsin (a beta-glucosidase) either before or after diastase or alone, had no effect on the distribution of label in the autoradiographs. Puromycin had no apparent effect on the integumentary incorporation of tritium administered as H_3 -galactose in H. diminuta.

It can be inferred from the observations summarized in Sections J and K that, in H. diminuta, the subcuticular cells play an active role in the elaboration of macromolecules. Amino acids are readily incorporated by these cells, and a significant portion of the newly synthesized material is rapidly secreted into

the cuticular matrix. At least some of the radioactivity remaining in the subcuticular tissues after the two hour chase with non-labeled proline probably represents labeling of structural proteins. This would be the case particularly with the embryonic or differentiating subcuticular cells described in Section I., and it is not unlikely that there is some turnover of structural proteins in the definitive subcuticular cells as well during the course of these experiments. Further, it seems probable that despite dilution and exchange with non-labeled proline molecules during the subsequent incubation in 1 mM proline, a certain amount of tritiated proline absorbed during the initial five minute incubation remains in the free pool and is continually incorporated by the subcuticular cells during the chase phase.

The incorporation of glucose into macromolecules in H. diminuta involves primarily the synthesis of glycogen. The turnover of this polysaccharide appears to be highest in the muscle tissue, though as demonstrated histochemically (Hedrick and Daugherty, 1957; Waitz and Schardein, 1964; Cheng and Dyckman, 1964) and by electron microscopy (Lumsden, 1965 a), the medullary parenchymal tissues appear to contain the major glycogen deposits of the proglottid. The lag in incorporation of tritium by the musculature after incubation of the worms in tritiated galactose (contra. tritiated glucose incubations) is probably due to prerequisite epimerization of the galactose to glucose before label from the latter can appear in glycogen. Significant incorporation

of monosaccharides into non-glycogen polysaccharides is apparent in the integument, though on the basis of present evidence it cannot be determined whether such activity is restricted to the definitive subcuticular cells, as seems to be true in the case of protein synthesis, or whether some synthesis of these macromolecules also occurs directly in the cuticular matrix.

DISCUSSION

Though lacking nuclei and lateral membranous partitions, the adult tapeworm cuticle is delineated by triple-layered plasma membranes, contains normal appearing mitochondria and other characteristic inclusions of typical cytoplasmic ground substances and should therefore be regarded as a cellular structure. Rosario (1962) and Burton (1964) stated that the surface membrane of Hymenolepis spp. and the trematode Haematoloechus medioplexus, respectively, appeared much thicker than a "typical" plasma membrane. These investigators' published micrographs are of relatively low magnification and therefore do not resolve the fine structure of these surfaces. However, in some of Burton's (1964) pictures of Haematoloechus, a dense, amorphous material appears to form an irregular coating over the free surface of the cuticle. It seems likely, as suggested by Burton (1964), that the surface of the integument of Haematoloechus is represented by a plasma membrane of ordinary dimensions plus an extraneous coat. Björkman and Thorsell (1964) reported that the surface of the liver fluke Fasciola hepatica was lined by a plasma membrane 100 A thick. In the present study, the dimensions of the unit membrane delineating the apical cuticular surface of H. diminuta (85 to 100 A thick) agree closely with those reported for the plasma membrane at the free surface of numerous epithelial cells, which is consistently 15-35 A thicker than other cytoplasmic membranes (Sjöstrand, 1963).

Like the surface membranes of many vertebrate cell types, (Sjöstrand, 1959, 1963 a and b; Elfvin, 1961), the apical membrane is also geometrically asymmetric, but in the tapeworm it is the outer rather than the cytoplasmic leaflet which appears to be the thicker component. "Reversed asymmetry" is also evident in the micrographs published by Cleveland and Grimstone (1964) of the surface membrane of the protozoan Mixotricha paradoxa and in the apical membrane of keratinized mammalian epidermal cells (Matoltsy and Parakkal, 1965). It is this writer's opinion that such discrepancies in the fine structure of unit membranes should be interpreted with extreme caution as they may well be due to differential staining affinities, fixation artifact, or a failure to resolve the exact limits of the external calyx and the unit membrane proper.

The thin, amorphous coating irregularly adhering to the free surface of the apical cuticular membrane probably corresponds to the PAS-positive material visualized at the level of light microscopy. Considerable interest has been generated recently in the possible physiological significance of the "glycocalyx", a mucoprotein coating all cell membranes, but variously prevalent depending on cell type. Katchalsky (1964) has reviewed the properties of biological polyelectrolytes, emphasizing the role macromolecular polyanions, such as polysaccharides and proteins, might play in the organization of the cell surface. Philpott and Copeland (1963) and Philpott (1964) suggested a direct functional

relationship between electrolyte transport and the acid mucopolysaccharides at the surface of various osmoregulatory cells. The osmoregulatory ability of tapeworms, as investigated thus far (Read et al., 1959), seems severely limited, though movement of certain electrolytes, notably sodium and potassium ions, appears to be intimately associated with the mediated transport of monosaccharides in Hymenolepis, Lacistorhynchus and Calliobothrium (Fisher, 1965).

The tapeworm cuticle is morphologically well suited for an absorptive function. The free surface area is considerable, due to the presence of numerous, closely packed microvilli, thus providing an expanded interface for the exchange of material between the tapeworm and its microenvironment. The basal pole does not reveal any particular modifications for increased surface area, though the proximal distribution of mitochondria would be of significance in energy-requiring physiological events at the basal cuticular membrane.

The cuticular microvilli bear a striking resemblance to those of mammalian absorptive mucosal cells. McNabb and Sandborn (1964) have recently described a core of filamentous elements, each approximately 60 A in diameter, extending vertically from the apex of the microvillus into the apical cytoplasm of the duodenal epithelial cell. Like the 70 A tubules or filaments forming the core of the cestode microvillus, the filaments in the intestinal microvilli terminate apically in an accumulation of dense, amorphous material adjacent to the inner leaflet of the

plasma membrane. Other investigators (eg: Palay and Karlin, 1959; Millington and Finean, 1962; Trier, 1963) noted similar striations or filaments in microvilli from various regions of the mammalian intestine, which were regarded as extensions of the terminal web. Cores measuring 700 A in diameter isolated from fractionated hamster microvilli by Overton, et al. (1964) have been described in negatively stained preparations as "covered with 40 to 60 A particles which....tend to be aligned in parallel rows". The latter elements probably represent the more peripheral filaments of the cores described by McNabb and Sandborn (1964) in sectioned material. One can only speculate at this point as to the role of these structures in either mammalian or cestode microvilli, but presumably they might be of significance as cytoskeletal elements in determining the form and position of the microvillus. These filaments are distinguishable from more ubiquitously occurring microtubules primarily by size. The latter have been broadly defined by Maser and Philpott (1964) as cytoplasmic cylindrical structures ranging in diameter from 150 to 300 A after standard fixation and embedding procedures. Structures identifiable on this basis as microtubules have been observed in the cytoplasm of mammalian mucosal cells (Behnke, 1964; Sandborn, et al., 1964) and the cestode cuticle (this paper). Functionally, microtubules appear to be intimately associated with cytological movement or maintenance of structural asymmetry, occurring, for example, as the tubular components of cilia, flagella, the mitotic spindle, the marginal bands of nucleated vertebrate erythrocytes and

and the cortices of plant cells (Slautterback, 1963; Ledbetter and Porter, 1963; de-The', 1964; Maser and Philpott, 1964). Slautterback (1963), on strictly morphological grounds, has suggested that certain microtubules may function in the transport of water and low molecular weight solutes. In the cestode integument, microtubules might be instrumental in establishing a structural framework of suitable stiffness or elasticity.

Certain observations reported in previous studies of tapeworm fine structure should be re-examined in light of the present investigation. It should be pointed out that consistently good fixation of tapeworm tissue is, at best, very difficult to achieve, even with the new aldehyde preservatives, and that the shortcomings of earlier investigations merely reflect the limitations of the techniques available at the time these studies were undertaken. Rothman's (1963) "subcuticular pore canals" which extended "from the inner border of the cuticle through the subcuticle" probably correspond to the apical processes of the definitive subcuticular cells. The vacuolated structures appearing in Rothman's micrographs of the cuticular matrix, described by the author as "pore canals" or, alternatively, pinocytotic "globules", are probably equivalent to the cuticular vesicles demonstrated in the present study. The majority of these probably represent secretory vesicles arising from the subcuticular cytoplasm. Attempts to demonstrate pinocytosis in the cuticle of H. diminuta using colloidal gold as a tracer have been unsuccessful (Lumsden, unpublished). Rothman's statements that, in the scolex, "A subcuticle is absent..." and "Except for the fibrillar reticulum, the packets of granules and the muscle bundles, the scolex appears void" are obviously based on a severe fixation artifact. Threadgold (1962) described the cuticle of Dipylidium caninum as "interrupted by membranes very similar to

those occurring at the interfaces of cells...", though these are not demonstrated satisfactorily in the published micrographs. Further, the highly vacuolated appearance of the tissue depicted is indicative of very poor fixation, and therefore demands extremely cautious interpretation. Threadgold (1962) stated that such membranes "are usually indistinct and discontinuous" and could not be followed throughout the thickness of the cuticular matrix. The "light cells" with "long pseudopodial-like appendages" described by Threadgold (1962) in the subcuticle and medullary zone of Dipylidium may represent parenchymal cells from which glycogen was extracted during preparation of the tissue; the "dark cells" said to be connected to the cuticle would be equivalent to the subcuticular cells described in the present study.

A polydisperse glycogen fraction has been recently isolated by Orrell, Bueding and Reissig (1964) from Hymenolepis diminuta. Two components, one with a sedimentation coefficient between 100 S and 500 S (average 200 S) and a second between 700 S and 1500 S (average 1100 S) were separated by differential zone centrifugation after mild cold water extraction of the homogenized tissues (Bueding and Orrell, 1961, 1964). Following short term incubation of worms in C₁₄-labeled glucose, the specific activity of the lower molecular weight fraction was found to be twice that of the heavier component, indicating more rapid synthesis of the lighter material (Colucci et al., 1964; Orrell et al., 1964). These authors have suggested that the heavier component might arise through polymeriza-

tion of newly synthesized and preformed lower molecular weight glycogen subunits. Similar heterogeneity has been obtained with glycogens from Ascaris lumbricoides and Fasciola hepatica (Bueding and Orrell, 1961; Orrell et al., 1964). Electron microscopy of the water-extracted Ascaris polysaccharide fraction revealed that the glycogen was organized as aggregates of smaller granules; the frequency in estimated number of subunits per aggregate corresponded roughly with the distribution of molecular weights calculated from ultracentrifuge patterns (Orrell et al., 1964).

Based on electron microscopic examination of negatively stained rat liver glycogen fractions, Drochmans (1962) suggested that glycogen particles as they occurred intracellularly possessed a two-stage structure. The macromolecular organization of filamentous gamma subunits, measuring 3 μ in diameter and 20 μ long, was thought to constitute the basic cytological unit, the beta particle, which in the microscope usually appear as rough surfaced, single granules 150 to 400 A in diameter. In such tissues as mammalian liver, the beta particles could then be reproduced and joined as components of a larger unit, the alpha particle, whose morphology is that of a complex of numerous granular subunits, apparently closely packed beta particles, with an aggregate diameter reaching several thousand millimicrons. Within any given species, the appearance of the glycogen deposits (as alpha or beta particles) seems to be characteristic for a particular cell type, though as suggested by Revel (1964), the alpha and beta configura-

tion may represent two extremes of a graded spectrum.

The pleomorphism of glycogen structure seen in thin sectioned tissues of H. diminuta, L. tenuis and C. verticillatum can be directly correlated with the heterogeneity of the glycogen fraction isolated from Hymenolepis by Orrell et al. (1964). Identity of the beta particles and the 200 S component is supported by the following evidence: (1) higher specific activity of the lower molecular weight component following incubation of worms in uniformly labeled C₁₄-glucose is paralleled by preferential incorporation of tritiated glucose into glycogen by the musculature and spermatozoa, which contain glycogen primarily in the beta form; (2) a particle size of 150 to 300 A estimated from the electron micrographs of Orrell et al. (1964) for the smallest granules in an analogous polydisperse negatively stained glycogen fraction from Ascaris compares closely with the size of the beta particles (200-400 A) seen in the electron micrographs of sectioned tapeworm tissues. The larger upper size range of the latter may be due in part to heavy metal staining of the thin sections. Correspondence between the 1100 S component and the intracellular alpha particles can be inferred from similar observations of morphology and rate of labeling with radioactive precursors.

Glycogen in the beta or single granule form is frequently associated with cells thought to possess continual high energy requirements and, consequently, elevated carbohydrate metabolism. Such include those of vertebrate kidney, striated and cardiac muscle

(Fawcett and Selby, 1958; Revel et al., 1961; Franzini-Armstrong and Porter, 1964; Jamieson and Palade, 1964) and the cells comprising various osmoregulatory "salt glands" (Philpott and Copeland, 1963; Philpott, personal communication). The alpha form predominates in cells concerned primarily with carbohydrate storage, as in mammalian liver, chick "glycogen body", and the brown adipose cells of foetal mice (Revel et al., 1961). Sheldon et al. (1962) described abnormal intranuclear sequestering of beta glycogen in biopsied hepatic cells from patients with glycogen storage disease. In such cases, glycogen can be synthesized but not degraded, presumably because of a deficiency in one or more critical glycogenolytic enzymes. The cytoplasm of these cells (glucose-6-phosphatase deficient) was typically packed with large alpha glycogen particles. Bueding et al. (1964) reported differences in sedimentation coefficient distributions between glycogen fractions from normal and glycogenotic tissues. Marked polydispersity and an increase in molecular weight of the heavier hepatic fraction characterized samples from Type I (glucose-6-phosphatase deficient), Type III (amylo-1, 6-glucosidase deficient) or Type IV (low phosphorylase) glycogenoses, while samples from patients with the Type II syndrome (high muscle glycogen content, acid maltase deficiency) were composed primarily of glycogen particles less than 500 S.

The disparate distribution of glycogen labeling seen in the tapeworm tissue section autoradiographs following short term

incubations in radioactive glucose reflects differences in rate of glycogen turnover, which in turn is a function of polysaccharide utilization as well as net synthesis and deposition. Glycogen turnover in H. diminuta appears to be highest in the musculature, which contains glycogen primarily in the beta form. On the other hand, the alpha, or aggregate structure was characteristic of glycogen-containing cells with lower polysaccharide turnover (as inferred from the autoradiographs) and therefore may represent a more stable form of depot carbohydrate in tapeworm tissues. Evidence that the distribution of incorporated label is not due simply to graded diffusion of radioactive glucose through the tissues is the intense labeling seen over the deep medullary muscle fibers and sperm cells located in the central region of the proglottid simultaneous with heavy labeling of the more peripheral subcuticular musculature.

Colucci et al. (1964) observed that upon starvation of rat hosts for periods up to 48 hours, reduction in total helminth glycogen was accompanied by a shift in the mean sedimentation coefficient of the heavy component toward that of the lower molecular weight fraction. When glucose was supplied to these worms in vitro or in vivo (by refeeding the hosts), the molecular weight of the heavy component progressively increased toward its original value. The importance of host dietary carbohydrates in maintaining proper levels of metabolizable sugars in H. diminuta has been clearly shown by the studies of Read and co-workers (for review see Read, 1959).

Thus, decreased aggregate size in the heavy glycogen component concomitant with a decline in the nutritional status of the organism may be interpreted as reflecting sequential depolymerization of alpha glycogen through a series of intermediates comprised of increasingly fewer subunits. It is significant in this regard that little change occurred in the density or relative concentration of the lighter glycogen fraction during starvation (Orrell et al., 1964). This would be expected if (1) the lighter species represented a key metabolic source of glucose in cells of relatively high activity, such as cestode muscle, (2) whose polysaccharide levels were maintained in the absence of sufficient amounts of exogenous glycogenic compounds by incorporation of hexoses available from the degradation of depot polysaccharides. Upon reinstating an adequate monosaccharide pool in these cells through addition of glucose to the medium, glycogen resynthesized in the medullary parenchyma might be subsequently converted to the alpha structure, which would account for the increase in molecular weight of this portion of the total glycogen fraction with refeeding. Mobilization of the carbohydrate reserves of the tapeworm proglottid would then appear to involve (1) breakdown of alpha glycogen in parenchymal cells into lower molecular weight polymers, the beta particles; (2) disjunction of the beta configuration; (3) hydrolysis of glycogen to polyhexose oligosaccharides and free glucose; and (4) transport of glucose to cells of high metabolic activity, such as muscle or the definitive subcuticular cells.

One might speculate that the alpha configuration imparts

metabolic stability to the component glycogen molecules by sterically hindering enzymic access to the internal glycosidic bonds. The nature of the forces maintaining the integrity of the alpha particle is presently unknown, though as suggested by Krebs (in Orrell et al., 1964), it does not seem unlikely that aggregation and dissociation of the subunits might involve enzyme mediated formation and cleavage of chemical bonds.

While anucleate in the strict sense, the coenocytic cuticular cytoplasm is potentially under the influence of nuclei in the underlying subcuticular cells, joined without structural interception to the basal cuticular membrane. Though structurally quite disparate, the subcuticular cells and the cuticle thus constitute an integrated syncytium, the latter component being highly specialized for surface absorption, the former for biosynthetic and secretory activities. Observations of the sequential labeling of the integument following the administration of radioactive amino acids have provided direct evidence for the role of the definitive subcuticular cells in protein synthesis and its subsequent export to the cuticular matrix. Additionally, the fine structure of these cells is consistent with the elaboration of a proteinaceous secretory product.

Amino acid incorporated into proteins or peptides destined for secretion are believed to be polymerized on ribosomal templates preferentially associated with the membranes of the endoplasmic reticulum. Evidence for this contention has been

derived mainly from numerous studies of the fine structure of cells otherwise demonstrably engaged in protein synthesis and export, such as mammalian pancreatic acinar cells, plasma cells, fibroblasts, etc., which are characterized by a highly developed granular endoplasmic reticulum (for examples see Porter, 1961; Emmelot and Beneditti, 1961; Haguenu and Hollman, 1961; Jacob and Jurand, 1963; Tandler, 1962). More direct support for this hypothesis has come from studies combining techniques of electron microscopy and biochemistry (Siekevitz and Palade, 1960) or autoradiography (eg: Caro and Palade, 1961, 1964; Revel and Hey, 1963). By similar methods, the proteinaceous secretory product has been traced from the cisternae of the endoplasmic reticulum to the Golgi apparatus, where ostensibly it is packaged into smooth membrane bound vesicles for subsequent movement to the surface of the cell and expulsion (Greene, et al., 1963; Wershawsky, et al., 1963; Caro and Palade, 1964). Recent studies (Goodman and Lane, 1964; Fewer et al., 1964) have implicated the Golgi membranes as possible assembly points for certain export carbohydrates, such as the polyhexose sulfate esters of mucopolysaccharides.

A similar pathway of protein synthesis and intracytoplasmic movement in the subcuticular cell can be inferred from the present study of the cestode integument. While the lumen of the tubular granular endoplasmic reticulum in the subcuticular cells is usually of narrow caliber, greatly dilated cisternae adjacent to the Golgi apparatus were frequently encountered in cells demonstrably engaged in high levels of amino acid incorporation. It is tempting to

speculate that the amorphous material filling these cisternae represents accumulated protein awaiting transfer to the Golgi region. In the immediate vicinity of the Golgi zone, agranular outpocketings of the cisternal membranes, differentiated morphologically from the rest of the reticular membranes, were observed. Such outpocketings are characteristically thickened and coated with a layer of fine filaments. Vesicles concentrated in the adjoining Golgi region also possess a thickened bristled coat. Previous studies by Hirsch (1961), Zeigel and Dalton (1962), Essner and Novikoff (1962), Wissig (1963) and others have indicated that the majority of vesicles associated with the Golgi zone of protein-secreting cells probably arise from the endoplasmic reticulum. It seems justified at this point to identify most of the coated vesicles appearing in the subcuticular Golgi zone with the transport of protein from the granular cisternae of the endoplasmic reticulum. Roth and Porter (1964) have described bristle-coated vesicles derived from surface pits believed to be engaged in the uptake of protein at the cell surface. These and similar observations (Goldberg and Green, 1964; Jamieson and Palade, 1964) suggest that coated vesicles may be generally indicative of quantum intracellular protein transport.

Further elaboration of the secretory product may occur in the Golgi apparatus. The exact role of this organelle is yet uncertain, though mediating fusion of smaller vesicles, concentration of the secretory product, synthesis and/or conjugation of non-

protein components (such as polysaccharides) with the primarily proteinaceous material derived from the endoplasmic reticulum are but some of the functions which have been attributed to the Golgi membranes of secretory cells . The majority of the vesicles occurring in the apical cytoplasm and cuticular matrix, while slightly larger and denser, are probably Golgi derivatives. The absence of a filamentous coat on most of the cuticular vesicles is consistent with the observation of Roth and Porter (1964) that once-coated vesicles lose their bristles to form dense surfaced spheres of similar size as they migrate further from the site of origin. The presence of mitochondria in the periphery of the Golgi area and proximal to the aggregates of vesicles in the apical cytoplasm is suggestive of an energy requirement associated with the movement of these vesicles through the cytoplasm. Possible activity in this regard might include the addition or elimination of specific vesicular contents, as the density of the vesicle matrix increases with greater distances from the Golgi zone.

Autoradiographic and histochemical studies indicate that the subcuticular cells are also active in the synthesis of non-glycogen polysaccharides. Since the incorporation of monosaccharide precursors continues in the presence of puromycin, it is concluded that this incorporation is essentially independent of de novo protein synthesis, at least for periods of relatively short duration. These observations do not preclude the possibility of ultimate association between protein and polysaccharide as conjugated

macromolecules in the cestode integument. There is some evidence (Richmond, 1963) that the biosynthesis of mucoprotein complexes may involve completion of polysaccharide units prior to their attachment to the polypeptide, though Molnar et al. (1964) reported that puromycin prevented the incorporation of glucosamine into mammalian plasma glycoproteins.

While ribosomes associated with the endoplasmic reticulum appear to be involved chiefly with the synthesis of proteins destined for secretion, ribonucleoprotein particles occurring free in the cytoplasm are usually identified with the synthesis of intracellular proteins (Porter, 1961; Hay, 1962; Warner et al., 1962; Wettstein, et al., 1963). Accordingly, rapidly proliferating cells, engaged in the synthesis of quantities of structural proteins, are characterized by numerous free polyribosomes and a poorly developed endoplasmic reticulum. Clusters of cells having a similar fine structure are frequently encountered in the periphery of the medullary and subcuticular zones. During short term in vitro exposure to tritiated thymidine, the nuclei of these cells readily incorporate the tritium label into DNase-labile macromolecules, suggesting high levels of DNA replication and resultant mitotic activity among members of this cell population. On the other hand, there is no evidence indicating that the highly differentiated subcuticular cells attached to the base of the cuticle undergo mitotic division, though the total number of such cells obviously increases with the growth of the proglottid.

Cells with a fine structure intermediate between the

definitive subcuticular cells and the undifferentiated cells in the medullary tissues occur in the subcuticular zone, particularly of immature proglottids. Characteristic of many of these cells are tight concentric whorls of ergastoplasm in cytoplasm otherwise dominated by free ribosomes. Similar configurations have been described by Herman and Fitzgerald (1962) in regenerating pancreatic acinar cells, in the differentiating follicular cells of Drosophila ovaries by King (1960) and Waddington (1962), urodele notochord cells (Waddington and Perry, 1962), and in guinea pig oocytes by Adams and Hertig (1964). Weiss (1953) and others have suggested that such elaborate loops of granular membranes in differentiating cells constitute cytoplasmic foci of ER biosynthesis. Watson (1959) has illustrated granular, membranous aggregates of the same general nature, which, in his case, were on the surface of the nuclear envelope. Continuity of the granular endoplasmic reticulum and nuclear envelope appears to be of general occurrence in many cell types (see, for examples, Porter, 1957; Porter and Machado, 1960; Palade, 1956; Waddington, 1962), including the definitive subcuticular cells of tapeworms, and has led to the rather generally accepted hypothesis that the granular ER is produced from the nuclear envelope. Comparable concentric arrays of ergastoplasm have been attributed in other cases to degenerative processes (Fawcett and Ito, 1958; Zelander et al., 1964; Waddington and Okada, 1960). The possibility that the localized accumulation of granular membranes seen in certain tapeworm subcuticular cells are the result of some atretic

phenomenon cannot be absolutely discounted. However, the persistence of these configurations in a large number of similar cell types argues strongly for the proposition that the presence of closely packed arrays of granular cisternae may well be a normal manifestation of development in the tapeworm subcuticular cell. Concomitant with the appearance of more elongate granular profiles is the presence of cytoplasmic smooth surfaced vesicular aggregates reminiscent of those seen in the Golgi regions of the definitive subcuticular cells. These graded variations of cytoplasmic fine structure would be consistent with a reorientation of cell metabolism from the synthesis of proteins for internal consumption to protein synthesis for export.

Results of the present study suggest that histogenesis of the adult cestode integument involves the addition of subcuticular cells from medullary sites of proliferation, not too unlike the additive multiplication and cytomorphosis of cells in many stratified epithelia. Addition of biomass to the growing cuticle as the proglottid increases in size can be attributed largely to the biosynthetic and secretory activities of these attached subcuticular elements. Published studies of the fine structure of larval tapeworms are presently limited to the rather low resolution micrographs by Siddiqui (1963) of Taenia taeniformis cysticerci. However, from these and some preliminary electron microscope observations by Lumsden (unpublished) of Prochristianella penneri pleuroceroids and H. diminuta

cysticercoids, it would appear that the integument of infective cestode larvae is organized in much the same fashion as the adult worm.

Previous investigations of histogenesis in adult cestodes have dealt mainly with the reproductive organs, and few have attempted to elucidate the precise cellular origins of the tissue components. Prenant (1922) recognized the existence of two primordial cell types in the medullary zone of H. diminuta and Bothriocephalus microcephalus: "cellules fixes", which supposedly gave rise to the majority of somatic parenchymal tissues, and gametogenic "cellules libres". Similarly, Wisniewski (1930) described so-called "somatic-generative" and "gonial" cells, initially assembled as a central medullary "anlagen", in caryophyllaeid cestodes of the genus Archigetes. Contrary to the existing view that pre-existing cell masses formed the sex ducts and germinal epithelium in situ (Young, 1913; et al.), Wisniewski (1930) attributed the organization of the gonads and accessory ducts to derivatives of these two primordial cell types dispersed from the central parenchyma. Further precedent for the possible role of cellular migration in the growth of the cestode integument can be found in the excellent light microscope observations recorded by Douglas (1961) on morphogenic movements of reproductive tissue progenitors in certain nematotaeniid tapeworms. Douglas (1961) proposed that, in addition to histogenesis of the reproductive structures, "...nearly all the cellular components of

mature and gravid proglottids appear to be derived from a single streak of cells located immediately posterior to the scolex--the primary anlage!

Like the cestode integument, the cuticle of digenetic trematodes has been revealed as a cellular syncytium (Threadgold, 1963; Bjorkman and Thorsell, 1964; Burton, 1964). As demonstrated by Bjorkman and Thorsell (1964), the anucleate cuticle of the liver fluke Fasciola hepatica is lined apically by a plasma membrane, contains mitochondria and vesicular profiles interpreted by these authors as "an inconspicuous endoplasmic reticulum". Burton (1964) suggested that the subcuticular cells of Haematoloechus medioplexus are engaged in the synthesis and secretion of "dense bodies" which appear in the cuticular matrix. Surface microvilli, which appear to be a general feature of the cestode integument, are absent among the trematode species examined to date.

In contradistinction to parasitic platyhelminths, free living flatworms are covered by a more conventional, non-syncytial epidermis. As seen in electron micrographs published by Klima (1961), the epidermis of several species of triclad turbellarians (Planaria, Dendrocoelium) is comprised of a simple ciliated columnar epithelium resting on a fibrillar basement layer. The epidermis of the acoel Convoluta convoluta, as described by Pedersen (1964), is also composed of clearly delineated ciliated cells. However, the basal, nucleus-containing portion is sunk slightly into the region circumscribed by the subepidermal

musculature. The apical cytoplasm of the epidermal cells contain varying numbers of mitochondria, a prominent Golgi apparatus, occasional profiles of granular endoplasmic reticulum, and numerous vacuoles ranging from 300 A to 0.6 μ in diameter. Convoluta, like other acoels and cestodes, lacks a gut, though possesses a small ventral pit or "mouth" lined with an infolded epidermis which stretches into the interior of the animal for a short distance. Some of the vacuoles appearing in Pedersen's (1964) micrographs of the epidermal cells are partially filled with a heterogenous granular matrix, and many are in contact with the free surface, suggestive of phagocytic activity. A few microvilli are dispersed between the cilia at the apical surface. In its fine structure, the integument of Convoluta represents an intergrade between the typical columnar epithelium comprising the epidermis of planarians and the highly modified syncytial surface of trematodes and cestodes. There is evidence (C. P. Read, personal communication) that, like tapeworms, Convoluta absorbs amino acids through the surface integument.

The derivation of the adult cestode integument will probably be resolved only by studying the fine structure of developing postembryonic larval forms. However, some speculation regarding the ontogeny of the cestode surface, based on the structure of the adult integument, appears warranted at the present time.

It seems quite probable that the cestode integument differentiates from an epithelium similar to that seen in the acoel

turbellarian Convoluta, the underlying perinuclear cytoplasm becoming differentiated for protein synthesis, the apical cytoplasm becoming specialized for transmembrane absorption. As strobilization ensues, with the concomitant need for rapid growth of all portions of the proglottid, more subcuticular elements are simply added from a pool of undifferentiated cells to the base of the already differentiated apical cytoplasm. It should be pointed out that among the platyhelminths, cestodes have no parallel in magnitude of somatic growth, and the secondary attachment of cellular elements to the expanding syncytium would be expedient and mechanically more feasible than the alternative replication and subsequent polar differentiation of discrete epidermal units in situ. The syncytial arrangement of the apical cytoplasm would in turn facilitate this additive type of increase in the underlying attached subcuticular cell population. Consistent with this hypothesis are Young's (1935) observations on the comparative histology of several groups of platyhelminths. Young (1935) described a "progressing degeneration" of the surface epithelium in certain turbellarians, prompting him to state:

"The observations suggest rather strongly that the cuticle of trematodes and cestodes has developed by condensation and loss of (certain)...cellular structures, with the resultant absence of a true epithelium in the former worms."

Comparison of the fine structure of the cestode integument with similar studies of other flatworms, while few, suggests

a graded spectrum of organizational variations reflecting particular biological adaptations manifest in each group. Among the turbellarians, the ciliated integument serves primarily as an organ of locomotion, consistent with the free living existence of the majority of these forms throughout the life history. On the other hand, the advent of endoparasitism has obviated the need for an elaborate surface locomotor apparatus among adult cestodes and digenetic trematodes, though it has imposed stringent requirements for a greater degree of physiological integration with the environment. Accordingly, in these parasitic forms, the integument has become variously modified for roles primarily related to the interchange of materials with the host organism.

Accompanying the development of parasitism among platyhelminths has been increased complexity of the life cycle. All digenetic trematodes and cestodes, with the possible exception of Hymenolepis nana, require at least one intermediate host, of a species other than the definitive host, for larval development. Frequently, two or more obligatory plus optional transport or paratenic hosts constitute elements in the life cycles of these organisms. In lieu of more efficient means of enhancing the probability of contact between a given parasitic larva and its requisite host, the reproductive potential of parasitic flatworms has undergone tremendous augmentation relative to that of contemporary free living species. The digenetic trematodes have

developed the phenomenon of polyembryony, while in the majority of cestode life histories there is no multiplication of larval stages, but a metameric repetition of the adult reproductive organs. That strobilization has been of considerable selective advantage during the evolutionary history of cestodes is evidenced by the overwhelming success of these organisms as endoparasites, in spite of a life cycle so hazardous that, as estimated by Stunkard (1962), only one individual in a million ever completes it.

Proglottidization, which may continue for the life of apolytic adult tapeworms, doubtlessly imposes stringent nutritional requirements for the maintenance of a high rate of growth and differentiation. Concomitant with the development of strobilization, the acquisition of an efficient surface absorptive mechanism would have been of considerable adaptive significance in satisfying the intensified requirements for assimilation of potential metabolites. It is suggested that physiological and morphological modifications of the integument for membrane absorption constituted a major somatic contribution making possible the advent of strobilization.

The biosynthetic capacity of adult tapeworms is indeed impressive, and attests to the efficiency of body surface alimentation in acquiring sufficient quantities of energy and utilizable carbon sources. Taenia saginata may reach a length of ten meters and produce as many as 400,000 eggs per day; Diphyllobothrium latum

grows approximately two centimeters per day and over a lifespan of ten years will produce some seven kilometers of proglottids and two billion eggs (Stunkard, 1962). Twenty-four hours after excystment in its definitive host, Hymenolepis diminuta enters an exponential phase of growth which may continue for as long as fourteen days, at which time apolysis of gravid segments begins; strobilization is initiated by four days postinfection, reaching a level of 600-700 proglottids at the eighth day (Roberts, 1961). The importance of exogenous, actively absorbed nutritional factors, especially host dietary carbohydrates, in growth and development has been clearly demonstrated by the work of Read and co-workers (for summary, see Read, 1959).

The digenetic trematodes, on the other hand, probably having arisen independently from more rhabdocoel-like progenitors, have retained the alimentary tube. Adult trematodes lack the degree of surface specialization expressed among cestodes and are in the main primarily histophagous. Body size seldom exceeds 10 millimeters (though some of the Didymozoidae may reach a length of several centimeters), and there is but one set of reproductive organs per worm. The reproductive potential of adult Digenea is modest in contrast to the cestodes, being manifest instead in the asexual propagation (by polyembryony) of the precercarial larval stages (Cort et al., 1954). It is pertinent to the foregoing discussion to note that, like adult cestodes, the trematode sporocyst lacks a gut and must rely on surface absorption for

assimilation of nutrients, which, in view of the amount of synthetic activity accompanying larval proliferation, must assume considerable proportions.

SUMMARY

The host-parasite interface, in the case of cestodes, is defined morphologically as a cell membrane circumscribing a complex coenocytic integument composed of a peripheral cuticle, underlying muscular and secretory cells. Studies with the electron microscope reveal the cuticle of certain cyclophyllidean, tetraphyllidean and trypanorhynch cestodes (Hymenolepis diminuta, Calliobothrium verticillatum and Lacistorhynchus tenuis) as an anucleate syncytium, whose apical and basal surfaces are defined by triple-layered plasma membranes. The former is projected as a series of closely spaced microvilli, each comprised of a core of fine tubules or filaments approximately 70 A in diameter and a limiting plasma membrane 85 to 100 A thick. The cuticular matrix is filled with vesicles of varying density, contains mitochondria and other characteristic inclusions of typical cytoplasmic ground substance. The basal cuticular membrane is closely applied to the underlying extracellular basement layer by half-desmosomes. Subcuticular cells, continuous via apical cytoplasmic extensions with the cuticular cytoplasm, possess morphological specializations consistent with synthesis and elaboration of a protein secretory product. Such specializations include a well developed granular endoplasmic reticulum and "coated" vesicles, apparently derived from the cisternae of the ER, associated with a prominent Golgi apparatus. Similar vesicles occur amid aggregates

of mitochondria in the apical cell processes and the cuticular matrix.

Incorporation of tritiated proline and leucine, as seen in tissue section autoradiographs, was used to indicate sites of protein synthesis in 10-day-old H. diminuta. When worms were incubated 5 minutes under CO₂-N₂ in a tris-maleate buffered Krebs's Ringer saline containing the tritiated amino acid, incorporated radioactivity in the proglottid cortex was restricted to the subcuticular cells. Following a 2 hour chase with non-labeled proline or leucine, over 66% of the cortical label appeared in the cuticular matrix. Puromycin inhibited subcuticular incorporation of proline and leucine by 79% and 71% respectively, lowered the specific activity of the chemically isolated protein fraction following incubation in U-C₁₄-glucose by 73%, but had no effect on polysaccharide synthesis. Studies with tritiated glucose and galactose suggest that the subcuticular cells, and perhaps the cuticle, are also engaged in the synthesis of non-glycogen polysaccharides.

Electron microscopy of thin sectioned tapeworm tissues reveals polymorphism of glycogen deposits consistent with previous observations of molecular weight heterogeneity of the polysaccharide fraction isolated from H. diminuta and other helminths. Two types of glycogen particles are distinguishable morphologically:

- (1) single granules (beta particles) 200-400 A in diameter and
- (2) rosettes (alpha particles) comprised of varying numbers of 200 A particles, with an aggregate diameter of 600 to 2000 A.

Mixtures of these particles occur in certain parenchymal cells, though the alpha particles tend to predominate in cells storing large quantities of polysaccharide while the beta particles are characteristic of the musculature and spermatozoa. Tissue section autoradiography following in vitro administration of H₃-glucose indicates a high rate of glycogen turnover in tissues containing this polysaccharide primarily in the beta form compared with those containing a preponderance of alpha glycogen. It is suggested from these studies that the alpha configuration constitutes a reserve depot carbohydrate in cestode tissues, while the beta particle represents a source of more readily mobilizable hexose.

Evidence is presented for the derivation of some subcuticular cells from medullary centers of mitotic proliferation; the stem cells appear to migrate peripherally, undergoing differentiation and subsequent attachment to the basal cuticular surface. Expansion of the integument in a rapidly growing proglottid is visualized as the product of additive cytological multiplication and biosynthesis of macromolecular constituents of the cuticular matrix by the subcuticular cells.

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VII.

PLATES

FIGURE 1.

Distribution of incorporated radioactivity in the integument of H. diminuta following exposure to H_3 -proline for 5 minutes and subsequent incubation in non-labeled proline (1 mM) for 0, 30, 60 and 120 minutes. Stippled columns = cuticular zone; clear columns = subcuticular zone. Values expressed are mean percentages of total integumentary activity and were calculated from grain counts of 100 standard fields.

FIGURE 2.

Time course of incorporation of H_3 -proline in the integument of H. diminuta. Stippled columns = cuticular zone; clear columns = subcuticular zone. Values expressed are means of grain counts of 100 standard fields.

1

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FIGURE 3.

The incorporation of H_3 -glucose and H_3 -galactose by various tissues of *H. diminuta*, expressed as grain counts per standard field (each point represents the mean of 100 determinations). P, medullary parenchymal cells; M, musculature; SC, subcuticular cells; C, cuticle. Stippled columns = 30 minute incubations; clear columns = 60 minute incubations.

FIGURE 4.

Distribution of radioactivity in autoradiographs prepared from sections treated with diastase prior to applying the photographic emulsion. Worms (*H. diminuta*) were incubated 60 minutes in KRT containing H_3 -glucose or H_3 -galactose. Values expressed are percentages of grain counts of control (non-digested) sections, and were calculated from counts of 100 standard fields. C, cuticle; S, subcuticular cells; P, medullary parenchymal cells; M, musculature.

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FIGURE 5.

Photomicrograph of the integument of H. diminuta. C, cuticular zone; S, subcuticular cell. The very dark areas between the subcuticular cells are glycogen deposits which have been stained by the PAS reaction. Osmic acid fixation, section poststained with methylene blue and azure II following PAS reaction. X 2,000.

FIGURE 6.

Photomicrograph of the integument of H. diminuta.
Note the darkly staining granules in the cuticular zone (C), especially at the basal surface.
S, subcuticular cell; B, basement layer. Osmic acid fixation, methylene blue-azure II stain.
X 3,000.

6

FIGURE 7.

Electron micrograph of the integument of *H. diminuta*. Mv, microvilli; M, mitochondria; B, basement layer; C, circular subcuticular musculature; L, longitudinal subcuticular musculature; S, apical process of a subcuticular cell. The latter extend between the muscle fibers, perforate the basement layer and are continuous with the basal cuticular membrane (arrow). Acrolein-osmium fixation, section stained with uranyl acetate and lead citrate. X 22,900.

FIGURE 8.

Electron micrograph of the integument of Lacistorhynchus tenuis. Note the basal distribution of the mitochondria. The remaining cytoplasm is filled with rounded vesicles of varying consistency; the densest are generally located near the apical surface. Osmic acid fixation, stained with uranyl acetate and lead citrate. X 29,200.

FIGURES 9 and 10.

Longitudinal section through the microvilli of H. diminuta. The delimiting, triple-layered plasma membrane is clearly demonstrated at the arrows. The outer dense component appears thicker than the inner dense component. Acrolein-osmium fixation, uranyl acetate stain. Fig. 9, X 131,600; Fig. 10, X 134,400.

FIGURES 11 and 12.

Cross section through the microvilli of H. diminuta. Note the assymetry of the plasma membrane and the fine, amorphous material adhering to the surface of the microvilli. Numerous tubules or filaments can be discerned just beneath the plasma membrane (arrows). Fine filamentous structures also appear in the central region of each microvillus. Acrolein-osmium fixation, uranyl acetate-lead citrate stain. X 144,000.

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FIGURE 13.

Electron micrograph of the cuticular basement layer of H. diminuta. Most of this structure is formed by loosely arranged, finely striated fibrils; cross sections of these fibrils are demonstrated at the black arrow. The basal cuticular membrane is shown at the white arrow; a half-desmosome appears immediately to the right of this indicator, and another is present at the junction of a subcuticular cell (S) and the basal cuticular surface. Note the moderately dense, amorphous material lining the mesial surface of the basal cuticular membrane. Acrolein-osmium fixation, uranyl acetate-lead citrate stain. X 39,500.

FIGURE 14.

Electron micrograph of the basal third of the cuticle of H. diminuta; numerous tubular profiles 200 to 300 Å in diameter can be seen at the arrows, extending in a plane roughly paralleling the transverse axis of the underlying basement layer. Acrolein-osmium fixation, stained with uranyl acetate and lead citrate. X 41,000.

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FIGURE 15.

An evagination of the basal cuticular membrane is depicted at E. These structures are encountered with very low frequency, and their significance is, at present, not understood. None have been seen opening onto the apical surface, though this might be suggested by their orientation. H. diminuta. Acrolein-osmium fixation, uranyl acetate-lead citrate stain. X 23,700.

FIGURE 16.

Electron micrograph of the circular (C) and longitudinal (L) subcuticular musculature of H. diminuta. Numerous beta glycogen granules (b) are scattered between the myofibrils and along the periphery of the muscle cells. Typical alpha glycogen particles (a) are present in processes of medullary parenchymal cells which extend between the subcuticular cells and muscle fibers into the cortical regions of the proglottid. Alpha particles are only occasionally seen in the muscle cells (arrows). BL, reticular fibers of the cuticular basement layer; M, mitochondrion. Acrolein-osmium fixation, uranyl acetate, lead citrate stain. X 32,400.

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FIGURE 17.

Electron micrograph of the basal, nucleus (N)-containing portion of a subcuticular cell (*H. diminuta*) in the anterior strobila. The perinuclear cytoplasm is filled with granule-studded profiles of the endoplasmic reticulum (ER). Adjacent to the Golgi zones (G) the ER is commonly dilated. Note the material of moderate density within the cisternae. Acrolein-osmium fixation, uranyl acetate-lead citrate stain. X 29,300.

FIGURE 18.

Details of the nucleus and perinuclear cytoplasm of a subcuticular cell (*H. diminuta*) are depicted in this micrograph. Continuity between the granular endoplasmic reticulum (ER) and the ribosome-studded nuclear envelope is shown at the white arrow. A nuclear pore is indicated at the black arrow. The numerous dense granules occurring in small clusters throughout the cytoplasm are probably polyribosomes. Note the heterogenous appearance of the prominent nucleolus (Nu). Acrolein-osmium fixation, stained with uranyl acetate and lead citrate. X 37,900.

FIGURES 19 to 22.

Details of the Golgi apparatus in the definitive subcuticular cell. Osmic acid fixation, uranyl acetate-lead citrate stain.

FIGURE 19.

The Golgi zone consists of laminated stacks of smooth surfaced saccules (G) and vesicles. The latter typically possess a thickened coating, as shown at the arrow. The dense material at L is probably a lipid droplet. The cytoplasm adjacent to the Golgi zone contains numerous profiles of granular endoplasmic reticulum and unattached ribosomes. X 33,970.

FIGURE 20.

A coated vesicle is indicated at the arrow. X 40,300.

FIGURE 21.

The endoplasmic reticulum adjacent to the Golgi zone appears to vesiculate (black arrow); here the membranes are thickened (white arrow) in a manner similar to that of the coated Golgi vesicles. X 39,500.

FIGURE 22.

Coated vesicles in the Golgi zone. X 39,500.

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FIGURE 23.

Vesicles occur throughout the supranuclear cytoplasm (arrows) of the definitive subcuticular cell, often in association with mitochondria. A multivesiculate body appears at V. The dense material (L) appearing in the adjacent medullary cell process is probably a lipid droplet. Note alpha glycogen particles (a) in an adjacent medullary cell process. N, nucleus; G, Golgi. L. tenuis. Osmic acid fixation, stained with uranyl acetate and lead citrate. X 29,200.

FIGURE 24.

Mitochondria and vesicles (arrows) in the apical process of a subcuticular cell. (H. diminuta). Note alpha glycogen particles (a) in adjacent parenchymal cells. Osmic acid fixation, lead citrate stain. X 27,900.

FIGURE 25.

A coated vesicle (arrow) in the apical cytoplasm of a definitive subcuticular cell. L. tenuis. Osmic acid fixation, uranyl acetate-lead citrate stain. X 51,700.

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FIGURE 26.

Vesicles in the cuticular matrix of L. tenuis.
Osmic acid fixation, stained with uranyl
acetate and lead citrate. X 27,650.

FIGURE 27.

Autoradiograph prepared from a worm (H. diminuta) incubated 5 minutes in tritiated proline then fixed in osmic acid. Note concentration of exposed silver grains over the subcuticular cells. Methylene blue-azure II stain. X 2,000.

FIGURE 28.

Autoradiograph prepared from worms treated with puromycin and given a 5 minute pulse of tritiated proline. Incorporation of the tritium label is inhibited up to 80% of control values (compare with Figure 27). Osmic acid fixation, stained with methylene blue and azure II. X 2,000.

FIGURE 29.

Tissue section autoradiograph from a worm given a 5 minute pulse of H₃-proline, then transferred to a medium containing non-labeled proline at a concentration of 1 mM for 60 minutes prior to fixation in osmic acid. Radioactivity is evenly distributed between the subcuticular cells and cuticular matrix. Methylene blue-azure II stain, X 2,000.

FIGURE 30.

Autoradiograph of tissue from a worm given a 5 minute pulse of H₃-proline followed by a 2 hour "chase" with non-labeled proline. Over 66% of the cortical radioactivity is concentrated in the cuticular matrix. Osmic acid fixation, stained with methylene-blue and azure II. X 2,000.

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FIGURE 31.

Electron micrograph of the apical portion of an acetabulum in the scolex of H. diminuta. The surface of the cuticle is lined with short microvilli (V); a few small mitochondria (M) are present along the basal surface. The basement layer (BL) is composed chiefly of a moderately dense, non-fibrillar amorphous substance (compare with Figure 13). Note marked development of the transverse muscle fibers (T). C, circular subcuticular musculature; L, longitudinal subcuticular musculature. Beta glycogen particles (b) are present in the acetabular musculature; a mixture of alpha (a) and beta granules appear in the interfibrillar parenchymal cells. The nucleus of a subcuticular cell appears at N. Glutaraldehyde-osmium fixation, uranyl acetate-lead citrate stain. X 23,700.

FIGURE 32.

Alpha and beta glycogen particles in the
acetabular tissues of H. diminuta.
Glutaraldehyde-osmium fixation, uranyl
acetate-lead citrate stain. X 26,000.

FIGURE 33.

Beta glycogen particles (b) predominate in the medullary muscle fibers shown in the upper right quadrant of this micrograph. A mixture of alpha and beta glycogen particles fill the parenchymal cells (P) surrounding a flame cell (FC) which has been sectioned slightly tangentially at the level of the ciliary tuft. Many of the beta particles in the parenchymal cells appear closely approximated, forming short linear arrays. The peripheral, alternating rod-like structures surrounding the closely packed, hexagonally shaped cilia of the flame cell are interconnected (arrows); in longitudinal sections, these structures have been seen to extend from the level of the basal bodies to the junction of the flame cell with its collecting duct. H. diminuta. Glutaraldehyde fixation, uranyl acetate-lead citrate stain. X 27,840.

FIGURE 34.

An autoradiograph prepared from H. diminuta incubated 30 minutes in KRT-tritiated glucose. Note concentration of exposed silver grains over the medullary musculature (arrows). S, subcuticular cells. Osmic acid fixation, PAS-methylene blue and azure II staining. X 1240.

FIGURE 35.

An autoradiograph of H. diminuta incubated 60 minutes in KRT-tritiated glucose. While radioactivity is still concentrated in the medullary musculature (M), it is markedly reduced from 30 minute levels (compare with Figure 7). Osmic acid fixation, PAS-methylene blue and azure II. X 1240.

FIGURE 36.

An autoradiograph prepared from H. diminuta incubated 60 minutes in H_3 -glucose; the section was treated with diastase prior to application of the photographic emulsion. Note paucity of radioactivity in the medullary muscle fibers (M). Formalin fixation, cryostat section, PAS. X 1080.

FIGURE 37.

Distribution of incorporated tritium in the integument of H. diminuta following 60 minutes exposure to H_3 -glucose. Osmic acid fixation, PAS-methylene blue and azure II staining. X 1900.

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FIGURE 38.

Autoradiograph of a section taken from the anterior strobila of H. diminuta following 30 minutes incubation in KRT-tritiated glucose. Note concentration of radioactivity in the subcuticular (SM) and medullary (MM) musculature. V, excretory vessel. Acrolein-osmium fixation, PAS. X 1200.

FIGURE 39.

Autoradiograph of a section taken from the anterior strobila of H. diminuta following 30 minutes incubation in KRT-tritiated glucose + 50 µg/ml puromycin dihydrochloride. Distribution and magnitude of the incorporated radioactivity does not differ significantly from controls (Fig. 38). Acrolein-osmium fixation, PAS. X 1200.

FIGURES 40 and 41.

Distribution of radioactivity in the integument of H. diminuta following 60 minutes incubation in KRT-tritiated glucose + 50 µg/ml puromycin dihydrochloride. Note position of label in cuticle (Fig. 40) and subcuticular cell (Fig. 41, arrow). Osmic acid fixation, PAS-methylene blue-azure II staining. X 2,000.

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FIGURES 42 - 45.

Incorporation of tritium by H. diminuta following 60 minutes incubation in H₃-galactose.

FIGURE 42.

Note concentration of label in cuticle and subcuticular cells. Osmic acid fixation, methylene blue and azure II staining. X 1690.

FIGURE 43.

Note paucity of activity in medullary musculature relative to subcuticular incorporation at left. Compare with Figure 35 (glucose-H₃ incorporation). Osmic acid fixation, methylene blue-azure II stain. X 1690.

FIGURE 44.

Autoradiograph of a worm treated with puromycin prior to and during incubation in H₃-galactose. Note heavy concentrations of label in the cuticle and subcuticle. Puromycin does not appear to inhibit the incorporation of this monosaccharide into the integument. Osmic acid fixation, methylene blue-azure II staining. X 1690.

FIGURE 45.

Cryostat section treated 60 minutes with diastase followed by 60 minutes treatment with emulsin prior to application of the photographic emulsion. Note concentration of activity remaining in the cuticle and subcuticular tissues. Formalin fixation, PAS. X 770.

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FIGURE 46.

Cell from the peripheral medullary tissues of H. diminuta. Note numerous polyribosomes (arrows) in cytoplasm. Acrolein-osmium fixation, uranyl acetate-lead citrate stain. X 30,000.

FIGURE 47.

Micrograph of a region of the medullary zone of L. tenuis, just beneath the subcuticle. A cell whose cytoplasm is filled with free ribosomes (R) is shown in the right center. Portions of a medullary parenchymal cell (P) filled with alpha glycogen granules and a subcuticular cell (S) are shown to the left of this cell. Osmic acid fixation, uranyl acetate, lead citrate stain. X 19,750.

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FIGURES 48 and 49.

Portions of some cells seen in the subcuticular zone of *L. tenuis*. In Figure 48, the cisternae of the granular ER assume a concentric configuration; cross sections of tubular cisternae and free ribosomes fill the rest of the cytoplasm. In Figure 49, a more loosely coiled array of granular membranes is demonstrated, which are continuous with a more extended cisterna (arrow). A vesicular profile appears at ER. The remaining cytoplasm is dominated by unattached ribosomes. Osmic acid fixation, uranyl acetate stain.
X 56,600.

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FIGURE 50.

Portion of a cell observed in the subcuticular zone of L. tenuis. The upper left corner projects toward the cuticular zone (not shown in this micrograph). Note the concentric layers of granular ER in combination with more elongated, dilated profiles. Golgi-like vesicles are present at V. A medullary parenchymal cell is present at P. Osmic acid fixation, uranyl acetate, lead citrate stain. X 30,000.

FIGURE 51.

Autoradiograph of a section through the anterior strobila of *H. diminuta*; the worm was incubated 120 minutes in KRT-tritiated thymidine before fixation in osmic acid. Note labeling of nuclei in cells of the medullary anlage (arrow) as well as cells in the medullary periphery. Methylene blue-azure II stain, X 550.

FIGURE 52.

Autoradiograph of a section from a mature proglottid (*H. diminuta*) after incubation for 120 minutes in tritiated thymidine. Note labeled nuclei of cells in peripheral medullary zone (arrows). D and V indicate dorsal and ventral excretory vessels respectively. L, lipid droplets. Osmic acid fixation, methylene blue-azure II staining. X 870.

FIGURE 53.

Distribution of exposed silver grains over cells of the medullary anlage (arrows) following 120 minutes incubation in tritiated thymidine H. diminuta). Portions of the subcuticular zone are shown at S. Osmic acid fixation, methylene blue-azure II stain. X 2000.

FIGURE 54.

A section through the integumentary region of a mature proglottid after 120 minutes incubation in H_3 -thymidine. Note absence of label in nuclei of subcuticular cells (asterisk); radioactivity has been incorporated into the cytoplasm of these cells and the cuticular matrix. The nucleus of certain unattached cells in the basal subcuticular zone is intensely labeled (arrows). The structures at L are lipid droplets. Osmic acid fixation, methylene blue-azure II staining. X 1490.

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